THE EFFECTS OF ESTRADIOL AND ATRAZINE ON *ACRIS BLANCHARDI* DEVELOPMENT

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THE EFFECTS OF ESTRADIOL AND ATRAZINE ON

ACRIS BLANCHARDI DEVELOPMENT

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Title of Study: THE EFFECTS OF ESTRADIOL AND ATRAZINE ON *ACRIS BLANCHARDI* DEVELOPMENT

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Abstract: Developmental toxicology studies commonly use amphibian models to detect effects of endocrine disrupting compounds (EDCs). Many of these studies however utilize model species not native to North America. For example, the African clawed frog (*Xenopus laevis*) is most often used for EDC studies. While extremely useful due to its sensitivity to contaminants and established life history, it likely does not accurately describe North American native amphibian responses to EDC exposure. While native species have been used, there is high degree of species sensitivity and illustrates a need for a broader range of native species models. Furthermore, the rate of development in the amphibian species used can influence the detectability of EDC related effects. For instance, common practice is euthanasia of animals at, or immediately after, metamorphosis. However, many of these species do not reach sexual maturity until at least a year after metamorphosis. When euthanized too early, there is the potential of misidentifying or missing EDC related effects due to lack of maturity or undifferentiation of organs such as gonads. Due to these reasons, we proposed the use of a native species, *Acris blanchardi¸* for future developmental assays. The rapid development of this species makes it a promising model as EDC related effects can be detected even prior to overwintering in southern populations (i.e., Oklahoma). To support our claims of rapid development, the first chapter first chapter here outlines a brief life history assessment by comparing wild animals collected over three time points: fall, spring, and summer. We saw that juvenile males were already actively producing mature spermatozoa. Females already possessed diplotene and pre-vitellogenic oocytes prior to overwintering. Following life history assessment, chapters 3 and 4 outline outdoor exposure experiments using 17β-estradiol (E2) and atrazine. Neither EDC influenced time to metamorphosis, recovery, or sex ratio. Estradiol effects were limited to a slight feminization of males in the form of oviduct development and depressed ovary and oviduct development in females. Atrazine had no effect on male development and limited effect on female body and gonad size in an algae supplemented group. For the final chapter, we performed a laboratory study where we exposed *A. blanchardi* larvae to E2 throughout development and with the removal of environmental cues. Male testis development was depressed in both E2 concentrations.

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CHAPTER I

Introduction

Endocrine disruption, while not a new concept, is a relatively newer term in the scope of the history of ecotoxicology (Matthiessen 2003; Folmar 1994). Endocrine disrupting compounds (EDCs) are synthetic or naturally occurring substances that disrupt hormone expression (Hampl et al. 2016). When introduced to a developing organism, they have the ability to influence body growth (e.g., mass), gonadal maturity, or reproductive capabilities (Hoffman and Kloas 2012; Saaristo et al. 2018; Gray et al. 1998). Commonly used EDCs are natural steroids (e.g., estradiol), synthetic steroids (e.g., diethylstilbestrol), and pesticides (e.g., atrazine). The degree of sensitivity to compounds however is variable between phyla or even species.

Amphibians are often used as model organisms for EDC and developmental studies. While a number of traits make them useful models for assessing effects of contaminant exposure, there can be a relatively high degree of species sensitivity. Indeed, the amphibian phyla contains a large and diverse group of species with a range of different morphologies, physiologies, and life histories (Burggren and Warburton 2007). These differences often leads to variation in species specific responses to EDCs. For example, concentrations of 17α-ethynylestradiol (EE2) that have been reported to sex reverse *X. laevis* had little to no effect on species such as *Hyla arborea* and *Bufo viridis* (Tamschick et al. 2016).

In addition to species sensitivity, it is important to use appropriate amphibian models relative to native EDC exposure. For instance, the majority of developmental EDC studies utilize species such as *X. laevis.* While useful due to its sensitivity and established life history, this African species is not appropriate when assessing North American native amphibian responses to contaminant exposure. Furthermore, as previously noted, there is a high degree of species-specific sensitivity even when assessing native amphibian responses to EDC exposure (Storrs and Semlitsch 2008).

Development rate or maturity can also not only influence sensitivity but detectability of EDC related effects. For instance, Storrs and Semlitsch (2008) suggests that frogs with shorter larval periods like *Hyla versicolor* are more susceptible to EEDC induced prolonged time to metamorphosis compared to species with longer larval periods (i.e. *Rana sphenocephala*). Additionally, collection and subsequent of amphibians prior to sufficient maturation may lead to misidentification or missed EDC influenced effects, especially in the gonads. For example, increased occurrences of intersexuality were reported in a study performed by Carr et al. (2003) in which *X. laevis* were exposed to high concentrations of atrazine. However, a re-examination of histologic sections could not confirm these results and found they more closely resembled undifferentiated gonads (Kloas et al. 2009).

With the factors addressed above, I sought to introduce a relatively unused native amphibian model for future developmental studies. Blanchard's cricket frog (*Acris blanchardi)* is a common species native to North America and distributed throughout the Midwest and into southern regions including Oklahoma and Texas (Burdick and Swanson 2010; McCallum et al. 2011). This native model is a short-lived species that matures, breeds, and perishes within a year of metamorphosis. The use of this species for EDC studies not only provides an additional needed native model but also

allows for fast assessment of EDC related effects of body and gonad maturity compared to other species. While sensitivity and testability of *A. blanchardi* to EDC exposure is relatively untested, in terms of developmental assessment, a few studies have demonstrated potential sensitivity to feminizing contaminants (Hoskins et al. 2018). However, these results are inconsistent with subsequent studies using the same or similar concentrations of the same contaminant (Hoskins et al. 2019).

Results

In the following chapters I assessed development of *Acris blanchardi* from the wild and post-exposure to EDCs 17β-estradiol (E2) and atrazine. In chapter two, I present a brief life history study in which I examined wild *A. blanchardi* development over three time points (fall, spring, summer). My results illustrate a species capable of rapid and continued body and gonadal growth. Both male and female body size continued to increase between fall and spring collections, indicating continued growth during overwintering. The majority of juvenile male *A. blanchardi* collected in the fall had mature testis and were actively producing spermatozoa. Ovaries of fall collected females were typically pre-vitellogenic but already beginning nuclear resorption in preparation and vitellogenesis the following summer and breeding season. As oviduct development is dependent on estrogen release form ovaries, oviduct formation was the least developed between the three gonadal endpoints. However, all gonads continued to mature overwinter and into summer collections where the majority of animals had fully mature gonads. Similarly, body size (body mass and snout-vent length) also continued to increase overwinter. The results of this brief assessment indicates that male *A. blanchardi* are capable of reaching sexual maturity before overwintering in the year they hatched. Furthermore, it will be possible to detect EDC related effects in juvenile males. While less developed

than males, gonads of females are capable of reaching a degree of maturity in which EDC effects may be detected.

In chapter 3, I assessed *A. blanchardi* sensitivity to E2 concentrations based on those reported to induce complete or partial feminization in other amphibian species such as *X. laevis* and *Rana pipiens*. In an outdoor system, I exposed *A. blanchardi* larvae from Gosner stage 26 through metamorphosis before allowing an additional 60 days of maturity. After analysis, I found that E2 had no effect on sex ratio, time to metamorphosis, recovery (i.e., survival), or body morphometrics of either sex. However, female gonad development (ovary and oviduct) was significantly depressed by E2 exposure. Furthermore, male feminization in the form of oviduct formation, while not significant, was greatest in the highest E2 concentration (2.3 µg/L). However, testis development was not influenced by E2 concentrations, even in males with oviduct development. My results suggest that *A. blanchardi* is relatively insensitive to E2 concentrations reported to cause significant feminization (e.g., sex reversal) in other amphibian species.

In chapter four, I investigated *A. blanchardi* sensitivity the herbicide atrazine at concentrations reported to influence development in *A. blanchardi* and other amphibians. Using the same outdoor system as used in chapter three, I exposed *A. blanchardi* larvae to environmentally relevant atrazine concentrations (0 – 50 µg/L) in addition to positive E2 controls (2.3 and 25 µg/L). Sixty days after metamorphosis, I found that neither atrazine or E2 influenced time to metamorphosis, recovery, or sex ratio. Females from an algae supplemented atrazine group (50*s* µg/L) were significantly smaller (body mass and snout-vent length) and had smaller ovaries compared to controls. These effects were limited to the algae supplemented group and were not observed in the non-algae supplemented 50 µg/L group. Additionally, neither ovary or oviduct development was

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Finally, in chapter five, I attempted to assess *A. blanchardi* sensitivity in an indoor system and without environmental influence. Similar to outdoor experiments, I exposed *A. blanchardi* larvae to E2 (2.3 or 25 µg/L) through metamorphosis. Days for maturity were modified to 35 days instead of 60 due to fear of loss. Thirty-five days after metamorphosis, E2 did not influence time to metamorphosis, recovery, or sex ratio. Estradiol treatment did not influence female body or gonadal development. Male body and gonad size was not influenced by E2. However, testis development of E2 treated males was significantly depressed compared to controls. Despite the slight decrease in testis maturity, the results of this study indicate that even in an indoor system *A. blanchardi* are relatively insensitive to E2 exposure.

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CHAPTER II

Growth and development of wild *Acris blanchardi*

Abstract

The Blanchard's cricket frog (*Acris blanchardi*) has been reported to develop at a rapid rate compared to many amphibians. In addition to relatively quick metamorphosis (i.e., <60 days), as seen in past outdoor studies, active spermatogenesis in juvenile males can be found as early as 60 days after metamorphosis. Additionally, female development (i.e., ovary and oviduct), is notably progressed prior to overwintering. With the data collected from past studies, we sought to assess and describe life history traits of wild caught *A. blanchardi* over three time points and from three Oklahoma locations. Cohorts of wild *A. blanchardi* were captured in the fall (i.e., prior to overwintering), spring (i.e., first sight of emergence), and summer (i.e., first sight of active breeding). In addition to general body development including body mass and snout-vent length, we assessed gonadal maturation via histopathology and using the USEPA *Larval Amphibian Growth and Development Assay* (LAGDA). From these animals and time points, we determined that testis development (i.e., spermatogenesis) was relatively consistent across time points with most males possessing stage 5 testis, even as juveniles. Comparatively, ovary development of juvenile females was not as progressed. The majority of fall females possessed ovaries that were scored within 0.5 -1 stage of mature development. Furthermore, oviduct development was typically at least 2 stages from full maturity. Despite depressed development in juvenile females,

as compared to male development, we believe that this rapidly developing species is a promising model for developmental studies.

Introduction

Toxicology studies are often focused on the effects of contaminants on reproductive development. Amphibians are good models for assessing effects on development as they can be readily exposed and monitored from eggs through adults. However, the rate of larval development and time to sexual maturity can vary tremendously among species. Indeed, larval periods among species range from days to years and may be influenced not only by genetics but environmental factors such as temperature, water depth, food availability, and density, among others. Further, post-metamorphic development also varies among species, with maturity and full reproductive ability potentially attained within the first season by some species, or years after metamorphosis by others. For example, while species such as *Leptodactylus latrans* and *Euphlyctis cyanophlytis* reach reproductive maturity within a year other common species such as *Rana temporaria* and *Xenopus tropicalis* do not reach reproductive maturity until 2 or 3 years after metamorphosis (Lopez et al. 2017; Phuge and Gramapurohit 2013; Augert and Joly 1992; Olmstead et al. 2009). These protracted development times place constraints on the utility of some amphibian species as models for assessing reproductive development and sexual maturity endpoints. Further, maintaining amphibians after metamorphosis in the laboratory or in semi-field conditions for extended periods of time is costly and often subject to continued mortality due to any number of factors. Thus, species that can develop from egg to sexual maturity in the

shortest interval are good candidate models for assessing the multitude of developmental endpoints following exposure at any point in that developmental timeline.

Blanchard's cricket frog (*Acris blanchardi)* is a small, hylid species widely distributed across much of North America. The species is relatively short-lived, completing metamorphosis and maturing in the fall of its hatching year before overwintering (Burdic and Swanson 2010; McCallum et al. 2011; Windle et al. 2020). Past studies have reported progressed sexual maturity (i.e., active spermatogenesis and oogenesis) in *A. blanchardi* within ~60 days of metamorphosis (Windle et al. 2020; 2021). Its ubiquitous distribution, ability to be cultured in the laboratory, and its rapid development have likely played a role in its attractiveness as a research model. Indeed, it has been used in toxicology studies over the past several years to evaluate multiple endpoints including somatic growth, gonadal development, and detectability of feminization (Hoskins and Boone 2018; Hoskins et al. 2019; Windle et al. 2021; Windle et al. 2022). We have used this species successfully in outdoor mesocosms to evaluate responses of sexual development following larval exposure to estradiol and atrazine (Windle et al. 2021; Windle et al. 2022). Despite the recent research *A. blanchardi,* the development rate has note been thoroughly examined, especially in wild species and through the entire year-long development from egg to breeding adult. Better understanding of development is necessary for this species to be widely useful as a model for amphibian development.

This study was designed to assess and describe the progression of development for *A. blanchardi*. Specifically, our goal is to provide additional information on body growth and gross and histopathological gonadal development for *A. blandchardi* cohorts from three distinct populations in Oklahoma.

Methods

Over three time points, metamorphosed *A. blanchardi* were collected from three locations. All ponds surrounded Payne County, Oklahoma (36.1450º N, 97.0068º W). Fall metamorphs were collected in September 2021 and representative of 2021 hatched individuals. Spring collection (April 1 – 20, 2022) began at the first sighting of adult *A. blanchardi* and before active chorusing. Finally, summer collection (May 15 – 19, 2022) began at first sighting of active amplexus and chorusing. Frogs were collected from all locations throughout collection periods. For example, frogs were collected from Wilson's three times over the spring collection period. Captured frogs were euthanized via immersion in 5% buffered MS-222. Body mass (BM) and snout-vent length (SVL) were collected from euthanized animals before histopathology preparation.

To assess gonad maturation, animals were examined by a board certified histopathologist at Experimental Pathology Laboratories. Histological procedures and gonad staging was based on guidelines from Larval Amphibian Growth and Development Assay (LAGDA; EPA 2015; Table 1). In brief, gonad development (ovary and testis) staged on a scale of 1-5, female oviducts were staged using a 1-5 scale, and male oviducts a 1-4. Two species-specific modifications to USEPA 2015 include an additional ovarian stage of 4.5 and oviduct stage of 5 (female only). Stage 4.5 ovaries were added to detect subtle developmental stages in the form of notable nuclear resorption and increased eosinophilic ooplasm. Stage 5 female oviducts contained large basophilic glands distended by mucoid material.

Results

Body morphometrics for both sexes were notably different across seasons and locations (Table 1 and 2). Snout-vent length of females collected in the summer was 1.5x that of fall females. Body mass of summer females was over 3x that of fall females (Figure 1). Similarly, SVL of summer males was approximately 1.5x that of fall males. Male BM of summer males was over 2x that of fall males (Figure 2).

As juvenile metamorphs, fall ovary development is typically within a single stage of full maturity. For all locations, 50 – 67% of females possessed stage 4 ovaries (Table 3). Immediately after emergence, most spring females possessed ovaries within half a stage of fall animals (4 vs 4.5). During active breeding and within a few weeks of emergence, all but 11% of summer females had fully mature ovaries (Table 3). Female oviduct development was notably lower between time points, compared to ovary development. For example, 71-83% of fall females possessed stage 3 oviducts while the remaining oviducts were stage 2 (Table 4). Most females did not reach full oviduct development (Stage 5) until summer collection. In one location (i.e., Kyle), the majority of the collected females had stage 4 oviducts but post-ovulatory ovaries in addition to newly matured oocytes.

Testis development did not notably differ between seasons. For all locations but one (i.e., Kyle), testis development was limited to stage 4 and 5 across all seasons (Table 5). All but 8% of summer males from Kyle's possessed stage 5 testis. Male oviduct development was limited to stage 1 for all locations and seasons.

Discussion

We examined gonadal and gross development of wild *A. blanchardi* over three timepoints to assess gonadal maturity and development and the utility of this species for developmental studies. *Acris blanchardi* has been reported to develop relatively quickly after metamorphosis, continuing to mature through the winter in preparation of breeding season the following spring and summer (McCallum et al. 2011; McCallum 2003).

As previously reported, females in the fall were substantially developed as the majority of fall females were scored with ovaries within 0.5 – 1 stage of full maturation (i.e., all oocytes vitellogenic). These results are similar to those of control females used in past outdoor mesocosm studies. Indeed, in those studies 60 days after metamorphosis over 80% of pooled control females were scored with stage 4.5 ovaries (Windle et al. 2021; 2022). Comparatively, only ~40% of pooled fall wild females were scored with stage 4.5 ovaries. The exact age of wild females was not known however and wild caught females may have been collected less than 60 days after their respective metamorphosis. Regardless, between experimental and wild females, *A. blanchardi* ovary development was notably progressed in comparison to other amphibian species. While few studies have used the same scoring guidelines used here (i.e., LAGDA), one study reported ovary development of *X. tropicalis* females 2 months after metamorphosis was limited to stage one and two (Svanholm et al. 2021). Furthermore, while alternative scoring methods were used, ovaries of native amphibian species *Rana cyanophlyctis* and *Rana pipiens* primarily contained immature oocytes even at 1-2 years of age (Kulkarni and Pancharatna 1996; Force 1933).

Female oviduct development was less progressed in comparison to ovary development. While the majority of ovaries from wild and experimental animals were within a stage of full

maturation, oviducts of juvenile females were primarily 2 stages from full maturation. Furthermore, 24% of wild fall females had stage 2 oviducts, a stage not seen in control experimental animals (Windle et al. 2021; 2022). Decreased oviduct maturation is however to be expected due to decreased need for mature oviducts until ovaries have reached full maturity and are ready for oviposition. As with ovary maturation, oviduct maturation continued through overwintering with a movement of 59% of oviducts reaching stage 3 by spring. Oviduct development of other amphibian species has not been well studied/examined. However, as oviduct development is correlated to ovary development and resulting estradiol levels, it is to be expected that oviduct development of species with reduced ovary maturation will be reduced compared to *A. blanchardi* (Dubowsky and Smalley 1993). Interestingly, most females collected from Kyle pond in the summer were scored with stage 4 oviducts. However, these same females were the only ones to possess post-ovulatory follicles in addition to fully mature oocytes. This is to be expected though as a mucoid layer from the oviducts is used to coat passing eggs during oviposit (Altig and McDiarmid 2007; Olmstead et al. 2009).

Male development, primarily testis development (i.e., spermatogenesis), was relatively consistent across timepoints. For instance, 83-85% of fall males from Wilson and Tietz ponds had stage 5 testis and moved to 100% by summer collection. A slight dip to 70% was observed in spring caught males from Tietz males. Interestingly, while the majority of males collected from Kyle pond (92%) reached full maturity by the summer, there was a noticeable hindered development in fall and spring males compared to males from the other ponds. These inconsistencies between ponds may be due to an environmental influence such as low food availability, water temperature, or decreased water levels. However, a study performed by

Gordon et al. (2016) suggests *A. blanchardi* development is not overtly sensitive to pond drying. Furthermore, water temperatures from Kyle pond were consistent with those from Tietz's whose males reached stage 5 testis maturity by summer. At this time, we are unsure why this slight developmental reduction in testis development was observed at Kyle's. Overall, testis development of wild caught males is congruent with experimental data collected between two studies (Windle et al. 2021; 2022). In these studies, 100% of control males at 60 days post-metamorphosis were actively producing spermatozoa and scored with stage 5 testes. Similar to female development, few studies have examined testicular development with the same guidelines. However, a number of species appear to begin active spermatogenesis within the same time frame examined. For instance, species such as *X. tropicalis* produce mature spermatozoa in 5-8 weeks after metamorphosis (Svanholm et al. 2021). Yet reduced development has been reported in a few native species (Chavadej et al. 2000; Lopez et al. 2017). For instance, *Rana catesbeiana* and *L. latrans* have been reported to not begin full production of spermatozoa until 7 months to a year after metamorphosis (Chavadej et al. 2000; Lopez et al. 2017).

Environmental factors (e.g., waterbody, water temperature, etc.) can influence *A. blanchardi* development. For instance, the Wilson pond was a spring fed pond that received a consistent water supply despite little rainfall during collection times. Conversely, the Tietz and Kyle ponds were strictly runoff and more prone to evaporation. Furthermore, water temperature from the Wilson pond was at times a few degrees (≤2ºC) lower than the water from the other two ponds. With these factors we would expect a consistent degree of decreased development in both males and females. However, while females appeared to oviposit earlier at Kyle pond (rain-

filled), male development was the greatest at Wilson's. Furthermore, male development was the least progressed at Kyle's.

The rapid development of male *A. blanchardi,* as observed in both wild and experimental animals, makes this species an ideal model for toxicology and developmental studies. Indeed, prior to overwintering, juvenile *A. blanchardi* are producing sperm at relatively the same degree as active breeding adults in the summer. Although juvenile female development, is not as progressed in comparison to males, ovaries of juvenile females are notably more mature than those of other amphibian species at similar ages. Thus, *A. blanchardi* is a useful species when shorter tests are needed. However, females may not be appropriate for assessing effects on full maturation immediately after metamorphosis. Oocyte development, and resulting oviduct development, likely requires greater energy expenditure than spermatogenesis as is true across many taxa (Bowerman et al. 2016; Hayward and Gillooly 2011). Nevertheless, the progressed ovary development observed in this and past studies do still demonstrate the potential to assess developmental effects even 2 months after metamorphosis. Moreover, development in mesocosms appears to be at a very similar rate to wild species suggesting that mesocosm testing designs will mirror real world environmental scenarios.

Tables and Figures

Table 1. Gonad histopathology definitions (USEPA 2015).

Tissue	Stage	Diagnostic Criteria
Testis	**1**	Undifferentiated gonad.
	2	Individual primary spermatogonia and undifferentiated somatic cells populate the medullary region.
	3	Seminiferous tubules with primary spermatogonia and cysts of secondary spermatogonia.
	4	Primary spermatocytes with rete testis formation; may have occasional spermatocysts that contain round or elongated spermatids.
	5	All stages of spermatogenesis evident.
Ovary	**1**	Undifferentiated gonad.
	2	Gonad identifiable as an ovary based on the presence of a discontinuously open lumen lined with epithelial cells; germ cells within the cortex consist of primary oogonia, cysts of primary mitotic oogonia, secondary oogonia, and very early meiotic oocytes.
	3	First appearance of diplotene oocytes in cortex; the most prevalent germ cell types at this stage are cysts of secondary oogonia and cysts of leptotene-pachytene primary meiocytes.
	4	Pre-vitellogenic (Dumont Stage I) diplotene oocytes are the most prevalent germ cell type observed by area and absolute cell counts; the central lumen is proportionately smaller while the whole ovary grows greatly in size and volume due to the growth of the oocytes; cysts in earlier stages of oogenesis become fewer in number and are located along the periphery of ovary.
	4.5	Ovary contains several large oocytes in which the germinal vesicle (nucleus) is beginning to deteriorate. Nuclear deterioration is characterized by increased irregularity in the contour of the nuclear envelope, nuclear blebbing and/or fragmentation, and scattering of the perinucleoli. In addition, the ooplasm of the enlarged oocytes is often slightly more eosinophilic than that of its smaller, less mature cohorts, and faint alveolar spaces are often evident near the cell periphery.
	5	Ovary consists almost entirely of vitellogenic oocytes (Dumont Stage IV); previtellogenic diplotene oocytes can be found along the periphery of the ovary and germ patches of primary and secondary oogonia are difficult to locate.
Oviduct (Müllerian Duct)	**1**	Oviduct consists of a fibrous tag attached to the suspensory ligament or is missing entirely.
	2	Oviduct has a lumen lined by a single layer of epithelial cells.
	3	Oviduct lined by multiple layers of epithelial cells, that form frond-like internal projections.
	4	Oviduct lined by basophilic glands.
	5*	Basophilic glands distended with mucoid material

Summary of diagnostic criteria for gonad histopathologic assessment. Staging based on criteria established in EPA 2015.

Table 2. Mean (±SE) snout-vent length (SVL; mm) and body mass (BM; mg) of female metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, winter) and three locations.

Pond	Season	n	SVL	BM
Wilson	**Fall**	7	19 (0.9)	509 (29)
	Spring	10	22 (0.7)	907 (79)
	Summer	10	27 (0.7)	1415 (93)
Tietz	**Fall**	7	17 (0.6)	315 (41)
	Spring	8	20 (1.0)	591 (104)
	Summer	9	27 (1.1)	1251 (151)
Kyle	**Fall**	9	17 (0.7)	390 (41)
	Spring	10	19 (0.9)	507 (59)
	Summer	10	29 (0.7)	1729 (86)

Table 3. Mean (±SE) snout-vent length (SVL; mm) and body mass (BM; mg) of male metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, winter) and three locations.

Pond	Season	n	SVL	BM
Wilson	**Fall**	12	18 (0.5)	442 (29)
	Spring	12	21 (0.6)	794 (88)
	Summer	10	24 (0.7)	1000 (51)
Tietz	**Fall**	13	16 (0.4)	309 (18)
	Spring	9	18 (0.5)	419 (40)
	Summer	10	24 (0.8)	939 (55)
Kyle	**Fall**	11	17 (0.6)	342 (30)
	Spring	15	18 (0.9)	424 (59)
	Summer	13	23 (0.4)	918 (30)

Table 4. Ovary development stage (%) of female metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, summer) and three locations. Ovary staging graded based on the following criteria (USEPA 2015): 1 – undifferentiated gonad, 2 – germ cells consist of primary oogonia, secondary oogonia, and very early meiotic oocytes, 3 – first appearance of diplotene oocytes in addition to mostly secondary oogonia, 4 – pre-vitellogenic diplotene oocytes primary germ cell, 4.5 – germinal vesicle beginning to deteriorate and ooplasm becomes eosinophilic, 5 – ovary consists entirely of vitellogenic oocytes. Stage 4.5 is an intermediate stage and a species-specific modification of USEPA 2015. This modification allows for detection of subtle developmental changes.

Pond	Season	n	Stage 2/3	Stage 4	Stage 4.5	Stage 5
Wilson	**Fall**	8	0	50	50	0
	Spring	9	0	0	78	22
	Summer	10	0	0	11	89
Tietz	**Fall**	6	0	67	33	0
	Spring	8	0	37	38	25
	Summer	9	0	0	0	100
Kyle	**Fall**	8	0	50	50	0
	Spring	10	0	10	90	0
	Summer	10	0	0	0	100

Table 5. Oviduct development stage (%) of female metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, summer) and three locations. Oviduct staging based on the following criteria (USEPA 2015): 1 – missing entirely or consisting only of small fibrous tag, 2 – oviduct lumen lined by single layer of epithelial cells, 3 – oviduct lumen lined by multiple layers of epithelial cells, 4 – oviduct lined by basophilic glands, 5 – basophilic glands distended with mucoid material. Stage 5 oviduct staging is an additional stage and a species-specific modification to USEPA 2015.

Pond	Season	n	Stage 2	Stage 3	Stage 4	Stage 5
Wilson	**Fall**	8	25	75	0	0
	Spring	9	0	22	67	11
	Summer	10	0	11	0	89
Tietz	**Fall**	6	17	83	0	0
	Spring	8	0	75	0	25
	Summer	9	0	0	0	100
Kyle	**Fall**	8	29	71	0	0
	Spring	10	0	80	20	0
	Summer	10	0	0	70	30

Table 6. Testis development (%) of male metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, summer) and three locations. Staging based on the following criteria (USEPA 2015): 1 – undifferentiated gonad, 2 – individual primary spermatogonia and undifferentiated somatic cells, 3 – seminiferous tubules with primary spermatogonia and cysts of secondary spermatogonia, 4 – primary spermatocytes with rete testis formation with occasional spermatids, 5 – all stages of spermatogenesis evident.

Pond	Season	n	Stage 1/2	Stage 3	Stage 4	Stage 5
Wilson	**Fall**	12	0	0	17	83
	Spring	13	0	0	14	85
	Summer	9	0	0	0	100
Tietz	**Fall**	13	0	0	15	85
	Spring	13	0	0	30	70
	Summer	9	0	0	0	100
Kyle	**Fall**	11	0	36	18	45
	Spring	15	0	0	40	60
	Summer	13	0	0	8	92

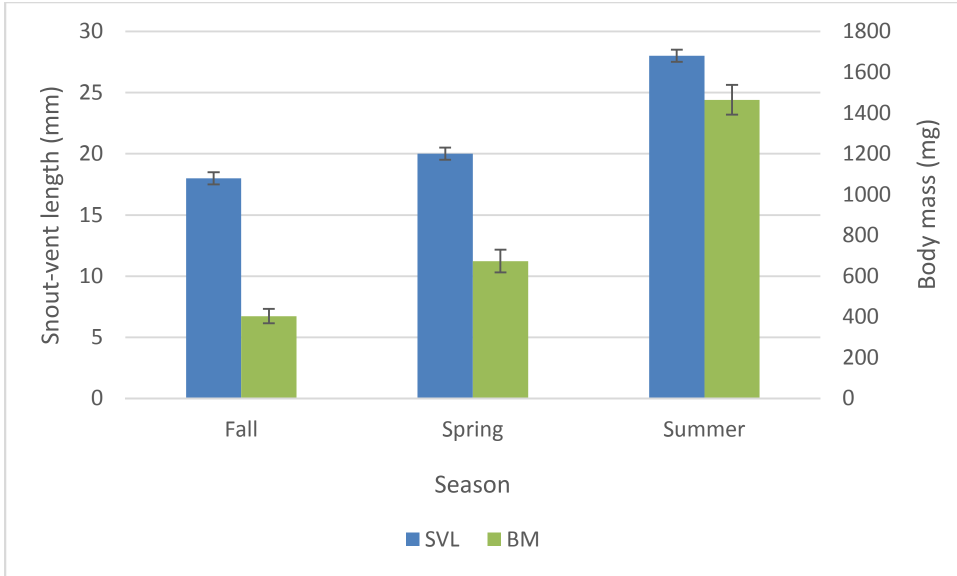

Figure 1. Mean (±SE) snout-vent length (SVL; mm) and body mass (BM; mg) of female metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, winter) and three locations. Fall n = 28; Spring n = 23; Summer n = 28.

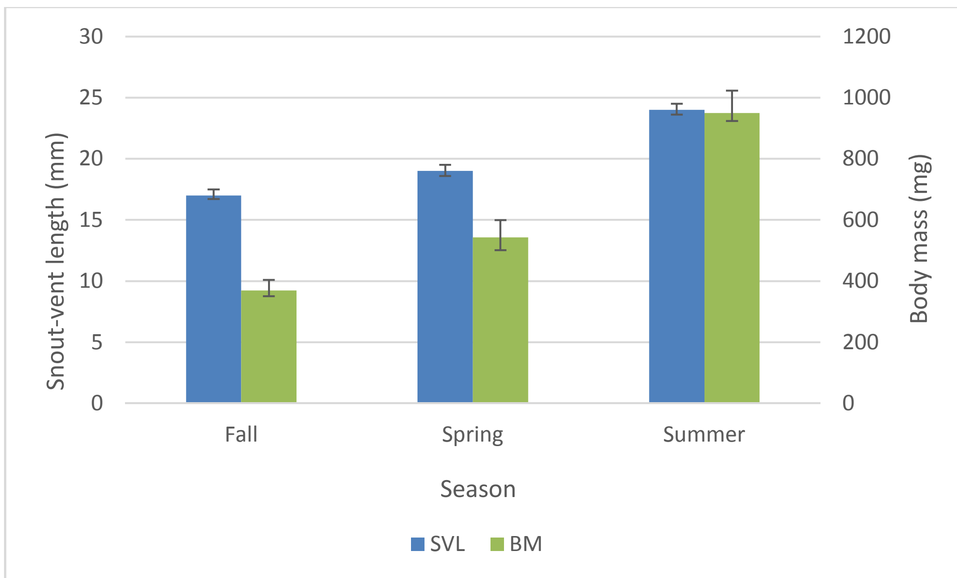

Figure 2. Mean (±SE) snout-vent length (SVL; mm) and body mass (BM; mg) of male metamorphosed *Acris blanchardi* across three time points (i.e., fall, spring, winter) and three locations. Fall n = 36; Spring n = 37; Summer n = 32.

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CHAPTER III

EVALUATING A DEVELOPMENTAL ENDOCRINE TOXICITY ASSAY FOR BLANCHARD'S CRICKET FROG (*ACRIS BLANCHARDI*) IN OUTDOOR ENCLOSURES

Abstract

A developmental toxicity testing design was evaluated for larval and post-metamorphic Blanchard's cricket frogs (*Acris blanchardi*) raised in outdoor enclosures. Larvae were chronically exposed to 17β-estradiol (0.0 - 2.3 µg/L E2) from free swimming (Gosner stage 26) until metamorphosis. Juvenile frogs were allowed to mature within the enclosures for 60 days to assess effects of larval exposure on development, including body mass, snout-vent length (SVL), sex ratio, gonad size, and gonadal histopathology. Forty-eight percent of the initial 600 animals were recovered at the end of the study. Recovery was not influenced by E2 exposure, but larval losses were negatively impacted by unusually high spring rain events that flooded some larval tanks, and heat-related mortality of late-stage larvae during summer. All surviving larvae completed metamorphosis within an average of 47 days. Overall, E2 exposure did not influence sex ratio, or the body mass, SVL, or gonad size of either males or females. Development of testes was not influenced by E2 exposure, but oviduct development in males was 4.5-fold greater in the highest treatment. Oviduct and ovary development in females exposed to the two highest E2 treatments were half that of control females. Although not treatment related and despite ad-lib feeding, variation in terminal body mass and SVL within enclosures was pronounced, with minimum – maximum differences ranging from 207 to 1442 mg for body mass and 1mm to 15

mm for SVL. This design allowed us to assess the effects of larval exposure to a contaminant on post-metamorphic development of a native amphibian in a semi-realistic field environment. With modifications to decrease flooding or overheating, this enclosure design and species is a good test system for assessing contaminant effects on development of an amphibian from early larval stages through reproductive maturity.

Introduction

Amphibians are useful models for assessing effects of exposure to environmental contaminants and indeed have been used to examine toxicity on several aspects of development, survival, reproduction, and behavior (Hopkins 2007; Kloas and Lutz 2006; Burggren and Warburton 2007). Yet, most studies focus on larval stages, to a lesser extent post-metamorphic stages, and only a few examine toxicity across both life stages (Carr et al. 2003, Hoskins et al. 2018, Hoskins et al. 2019). Further, although most amphibians share some fundamental traits (e.g., both aquatic and terrestrial life stages) that lend to their usefulness as a model species they are nonetheless a diverse group of over 8,000 species with a range of morphology, physiology, and life history attributes that undoubtedly play a role in the observed differences in species-specific responses to chemical exposure (Burgrren and Warburton 2007). As an example, exposure to the same concentrations of 17α-ethynylestradiol (EE2), a known endocrine disruption compound (EDC), resulted in significantly more sex-reversed females in the well-established lab model, *Xenopus laevis,* than in *Hyla arborea* and *Bufo viridis* (Tamschick et al. 2016). Additional evidence of differences in species sensitivities includes copper sensitivity between *Lithobates sphenocephalus* and *Anaxyrus terrestris* (Lance et al. 2012; Lance et al. 2013). Survival at copper concentrations of 150 µg/L found a notable difference between the two species with 10% survival

for *A. terrestris* 40% survival for *L. sphenocephalus* . Evidence of further differences in sensitivity can be found for a number of model and non-model amphibians exposed to different contaminants (Harris et al. 2000; Howe et al. 2004; Wacksman et al. 2006). Thus, there is a need to develop amphibian models germane to species likely to experience exposure under real field conditions, particularly protocols that allow for long-term development as part of the assessment.

Blanchard's cricket frog (*Acris blanchardi*) is a common species across the Great Plains and Eastern United States. *A. blanchardi* are semi-aquatic hylids that breed throughout spring and summer in urban and rural ponds, depressional wetlands, ditches, and slow rivers (Burdick and Swanson 2010; McCallum et al. 2011). Their semi-ubiquitous distribution and association with urban and rural environments facilitates their potential for exposure to a variety of environmental contaminants. *A. blanchardi* are typically smaller and quicker to reproductive maturity than most other amphibian species within the same ecological community. Indeed, cricket frogs will produce male offspring that can develop large spermatic cysts capable of reproduction within 60 days post-metamorphosis (McCallum et al. 2011). Further, male *A. blanchardi* do not appear to have seasonal reproductive senescence as reported by McCallum et al. (2011) who noted maintenance of sperm structures throughout the entirety of the year in specimens collected in multiple North American states including Arkansas, Missouri, Nebraska, and South Dakota.

Acris blanchardi and *A. crepitans* have been evaluated for susceptibility to various environmental contaminants in a few prior studies (Reeder et al. 1998; Reeder et al. 2005; Hoskins et al. 2017; Krynak et al. 2017; Ade et al. 2010). Feminization of *A. blanchardi* following atrazine exposure was evaluated in several recent studies with mixed results (Hoskins and Boone 2018; Hoskins et al. 2019). For example, Hoskins and Boone (2018) reported that no

testicular ova (TO) were found in *A. blanchardi* frogs exposed to environmentally relevant concentrations of atrazine (0.1-10 μg/L) from Gosner 25 staged larvae until metamorphosis, or Gosner 46. In a subsequent study however, TO development was seen in *A. blanchardi* exposed to the same concentrations and for the same length of time (Hoskins et al. 2019). Differences in TO prevalence between the two studies may be attributed to differences in testing methods, as tadpoles in the first study were exposed in beakers with complete water and atrazine renewals every 48 hours, compared to a single atrazine spike at the initiation of exposure in outdoor enclosures in the latter study. In addition to gonad development, both above mentioned studies also examined potential effects of atrazine on size at metamorphosis. Both studies found no atrazine induced effect on mass. Further, increased levels of TO were observed for museum specimens of *A. crepitans* collected from highly urbanized areas contaminated with PAHs and PCBs (Reeder et al. 2005). Mortality of *A. crepitans* and *A. blanchardi* post contaminant exposure has also been examined in the past. For instance, Krynak et al. (2017) reported *A. blanchardi* larvae (11 – 18 days post fertilization) exposed to the herbicide Rodeo™ (active ingredient glyphosate) experienced a 37% decrease in survival when exposed to 2.5 mg/L compared to controls. Additionally, Ade et al. (2010) reported a significant decrease in survival to metamorphosis when *A. crepitans* larvae were exposed to the contaminant imidacloprid from Gosner stage 25 until metamorphosis. Interestingly, mass was influenced within this study and noted an increase in mass (mg) with increasing imidacloprid concentrations.

Designing specific test systems for any species can often be a challenge, and this can be especially true for amphibians that require accommodations for aquatic and terrestrial life stages if the objective is to encompass the entirety of developmental range from oviposition to sexual

maturity. Although laboratory test systems afford more control over test conditions, outdoor enclosures or mesocosms are not devoid of such controls and yet have the benefit of a level of realism lost in the laboratory (Caquet et al. 1996; Boone and James 2005). This realism may include cues that influence development and allows for light and drying cycles and temperature fluctuation that may be important for health of and proper development (Szekely et al. 2017; Denver 1997; Laurila et al. 2001). Thus, developmental toxicity protocols that allow for a native frog to be tested from larval through advanced stages post-metamorphosis greatly aides our ability to test effects from potential developmental toxicants.

This study was designed to evaluate the use of an outdoor enclosure system (also referred to as terrariums or cosms) and *A. blanchardi* as a combined model test system for assessing potential chemical induced effects on frog development. We used 17β-estradiol (E2) as a model EDC to evaluate the effects of larval exposure on the growth and sexual development of mature frogs based on known effectiveness for other amphibian species (Kloas et al. 2009; Lutz et al. 2006; Piprek et al. 2012) . Processes sensitive to endocrine disruption include reproduction, growth, behavior, and abnormal gonadal development (Hu et al. 2008; Hoffmann and Kloas 2012; Hoskins et al. 2017; Saaristo et al. 2018; Gray et al. 1998), and EDCs can modify hormones in ways that interfere with processes mediated by endogenous steroids (e.g. gonad development; Kiyama and Wada-Kiyama 2015; Hampl et al. 2016). E2 and testosterone are often used as positive controls to induce estrogenic or androgenic effects in comparative studies of synthetic EDCs (Massari et al. 2010; Sharma and Patino 2010; Kloas et al. 2009, Pickford et al. 2003).

Methods

Adult and larval collection

Amplexed *A. blanchardi* pairs and/or single females paired with multiple calling males were collected from two adjacent non-agricultural ponds in Payne County, Oklahoma. Land-use around the ponds was primarily wooded with mowed grass related to low intensity residential development near the shore of the ponds. Breeding frogs were collected starting May 14, 2019 and continued until June 13, 2019 to provide a sufficient number of fertilized egg masses. Individual pairs and groups were briefly housed in aerated plastic containers with de-chlorinated water from the lab. Pairs were then left at the collection site overnight. Containers were checked the morning following collections and any eggs present were taken to the lab and allowed to hatch. Date of first observed development and hatching was recorded. Larval development was observed as early as 24 hours after oviposition and hatching occurred as early as 72 hours after oviposit. Tadpoles were raised to stage 26 (Gosner 1960) within the respective chambers in which they hatched, at which point they were combined into one container and randomly sorted before being transferred into individual beakers in groups of 20 for placement into outdoor enclosures. Enclosures were set up west of Stillwater on Oklahoma State University property. Treatment with E2 began immediately after placing tadpoles into outdoor enclosures. First treatments began on May 23, 2019, with the last treatment administered on July 22, 2019.

Males that successfully fertilized eggs, plus an additional 10 males collected from the same pond as breeding pairs, were euthanized and processed for histopathology to provide

background information and familiarize the pathologist with the gonadal structures of this species.

Additional *A. blanchardi* (*n* = 38) were collected in late September from the same collection ponds for comparison of their sex ratios and gonadal abnormalities to our control frogs from the enclosures. All 38 frogs were Gosner stage 45 or greater and assumed to be young of the year based on time of collection (McCallum et al. 2011).

Experimental exposures

Outdoor enclosures (d = 122 cm; h = 61 cm; *n*=30) consisted of open-bottomed circular steel enclosures set a minimum of 7 cm in the ground (Figure 1). Stainless steel tanks (volume = 25 L, depth = 21 cm) were buried in the ground inside each enclosure and filled with 20L of filtered water. Tadpoles were reared and exposed in this tank. Tadpole density (20 tadpoles/20 L) in each tank was based on recommendations by the United States Environmental Protection Agency amphibian guidelines (US EPA 2009). To provide the terrestrial aspect of this design, natural vegetation was allowed to grow inside each enclosure and was trimmed to the height of the enclosure (61 cm) to ensure vegetation was controlled and for ease of access for feeding and water exchanges. Finally, woven mesh netting (0.76 mm x 0.76 mm) plant insect barriers (AgFabric®; 122 cm x 140 cm) were modified and fixed to the top edge of enclosure with 12 mm diameter polyurethane tubing to contain animals as well as limit wild animal interaction. A shade structure with an 60% UV filtration shade cloth was erected over all units (Figure 1). Light intensity and temperature under the shade structure and within selected enclosures (ambient temperature in and out of the water) were monitored continually throughout the study using data

loggers (ONSET HOBO Pendant®). Loggers were randomly assigned and moved to new enclosures each week.

Experimental design consisted of five replicate enclosures for each of the six treatments (0.0, 0.015, 0.053, 0.18, 0.64, 2.3 µg/L E2) with 20 tadpoles to an enclosure (n=600). Our highest concentration (2.3 µg/L) was included in an attempt to cause complete feminization of cricket frogs and was based on similar E2 concentrations found to induce near complete feminization of male *Xenopus* (Lutz et al. 2008, Kloas et al. 2009). From the highest concentration, a decreasing gradient was established to illicit partial feminization. Static renewal water exchanges were performed every 3-4 days and each consisted of a 70% exchange with fresh water containing respective test solutions. Methanol (0.04 ml) was used as a carrier and was added to the control treatment. Prior to storing in 227 L polyethylene tanks, water from an onsite spigot was filtered through an iSpring 2-Stage Heavy Duty Whole House Water Filtration System® equipped with a single sediment filter followed by 3 carbon filters. Stainless steel containers dedicated to individual treatments were used for water exchanges. Water was added slowly to each to tank to decrease trauma to tadpoles. Larvae were supplemented with 175 mg-350 mg (amounts were increased in accordance with growth) of a ground mixture of TetraMin® and SeraMicron® spirulina every other day. Tadpoles were counted to the extent possible during every second water exchange (i.e. once per week). Treatments were discontinued on an individual unit basis when at least 75% of the tadpoles reached Gosner Stage 45 or greater. Once metamorphs were detected, calcium dusted pin-head crickets (3mm) were provided ad libitum. Remaining tadpoles were continually fed with the TetraMin® mixture until all tadpoles had metamorphosed. Following termination of exposure, tanks in each enclosure were maintained using water without

E2 added and following the same water exchange schedule. Plain wood "paint" sticks (0.5 cm x 3 cm x 10 cm) were placed in tanks to provide a platform for metamorphs to rest or use as a means of escape from the tanks.

Analytical chemistry

Source water was monitored for atrazine, desethylatrazine, deisopropylatrazine, bisphenol a, and select phthalates four times during the study to confirm that the source water was not contaminated. To measure exposure to estrogens, E2 and estrone, water samples were collected before and after renewals from a single enclosure from each treatment each week for the first 6 weeks, resulting in six before and after samples collected for each treatment. Analysis was conducted on unfiltered samples. Additionally, due to concerns that partitioning to algae and particulates in the water may limit E2 bioavailability, four samples were obtained from a single enclosure at the highest concentration prior to exchange. One sample was analyzed without filtering and three samples were extracted after filtering out algae with 0.45 micron glass microfiber filters. The filter for each sample was also extracted with ethyl acetate to evaluate how much E2 and estrone accumulated on the algae and other particulate material in the system.

Metamorph collection and histology

All frogs within a replicate were euthanized 60 days after termination of chemical exposure (based on average date for that replicate). Frogs from a given enclosure were placed together in a plastic tank with ~1 cm of water prior to being euthanized in 5% buffered MS-222. Death was confirmed by lack of a heartbeat and response to touch. Snout-vent length ($\pm$1mm; SVL), body mass ($\pm$1mg; BM), and a photo of the throat patch were collected immediately after

death. Frogs were dissected at the pectoral girdle to prepare gonads for fixation without their complete removal from the body cavity. In brief, the ventral skin and musculature were removed and intestines cut away to leave the kidney-gonadal complex. Further, the head was removed and placed in a labeled specimen tube to be frozen (-40°C) for later analysis. Finally, hind limbs were removed and discarded and the remaining carcass with the gonads was placed in Bouin's solution (Thermo Fisher Scientific Cat.No.:50-320-01) for 24 hours, after which it was rinsed with 70% ethanol and stored in 10% formalin. Fixed tissues were marked with unique identification numbers and assumed sex based on gonadal morphology before being sent to Experimental Pathology Laboratories (EPL) in Sterling, Virginia for histological processing and evaluation. For shipment, the fixed tissues were wrapped in formalin-soaked tissue paper and double bagged in marked (i.e., identification, presumed sex, and date euthanized) Ziplock® bags. Prior to shipment, gonads and a reference stage micrometer were photographed in order to measure the length and width of gonads to the nearest 1.0 mm (Figure 2).

Endpoints

In addition to SVL and BM described above, we quantified time to metamorphosis (time when treatment was terminated for each enclosure), recovery (percentage of the initial 20 individuals recovered at 60 days post metamorphosis) and sex ratio (based on gross morphology of gonads). Time to metamorphosis for a given enclosure was based on when 75% of the tadpoles reached Gosner Stage 45. Recovery is based on total losses from all causes (e.g., flooding of tanks, cannibalism, unknown) and thus not necessarily attributable to E2 exposure.

Size of female (area) and male (volume) gonads was estimated from their length and width measurements using the following formulas. Area of each ovary was estimated based on the ellipse calculation ($ab\pi; a = \frac{length}{2}, b = \frac{width}{2}$) and then averaged for each individual. Similarly, the volume of each testis was estimated based on ellipsoid volume calculation ($\frac{4}{3}abc; a = \frac{length}{2}, b = \frac{width}{2}, c = \frac{width}{2}$) and then averaged for each individual. To avoid possible damage to the testes, the second width measurement was not measured but assumed the same as the first. Weights of gonads were not collected due to their small size and the need to avoid any damage prior to histopathological analysis.

At EPL, the mid-sections of frog carcasses were processed routinely for paraffin embedding, and oriented for sectioning in the transverse plane. Decalcification of the spine was not required. At microtomy, three sections (each approximately 4-6 microns thick) were acquired through the proximal, middle, and distal gonad regions, respectively. Such sections automatically contained bilateral portions of kidney, and bilateral Müllerian ducts (referred to as "oviducts" throughout the remainder of this report for consistency). All sections were mounted on glass slides, stained with hematoxylin and eosin (H&E), and covered with glass cover slips using a commercial mounting medium. For histopathologic examination, gonads were assessed for signs of feminization including but not limited to the occurrence of intersex, mixed sex, and testicular oocytes (Table 1). Intersex was defined as the occurrence of both sex tissues within a single organism and testicular oocytes are the occurrence of oocytes within a testis. These definitions were identified and established by Lutz et al. 2008 in an effort to standardize EDC experimentation within amphibians. Pathological findings were graded for severity according to the following scale: grade 1 = minimum, grade 2 = mild, grade 3 = moderate, grade 4 = severe.

Gonad (testis and ovary) development was staged on a scale of 1-5, while oviducts were staged using 1-4 scale. Staging of gonads and gonadal ducts were based on the prescribed criteria in USEPA 2014 which was designed for the assessment of gonadal development in juvenile *Xenopus laevis* frogs. Stage 1 oviducts were characterized by the complete lack of an oviduct remnant, or a fibrous tag that marked the site of the atrophied oviduct. As compared to a Stage 1 oviduct, a Stage 2 oviduct had a visible lumen that was lined by a single layer of flattened to cuboidal epithelial cells. Oviducts were scored as Stage 2 if these criteria were met either bilaterally or unilaterally. A Stage 3 oviduct had feather-like epithelial fronds and folds that projected into the lumen, while Stage 4 oviducts exhibited substantial mucous glandular development and/or increased length and tortuosity, the latter of which was evident unilaterally or bilaterally as three or more elongated profiles of oviduct.

One novel adaptation of the US EPA (2014) ovarian staging system was the incorporation of a Stage 4.5 score, which was created in order to facilitate the detection of potential subtle developmental changes in the ovaries of cricket frogs. Stage 4.5 ovaries generally contained several large oocytes in which the germinal vesicle (nucleus) was beginning to deteriorate, and in addition, the ooplasm of the enlarged oocytes tended to be slightly more eosinophilic than that of its smaller, less mature cohorts, and faint alveolar spaces were often evident near the cell periphery. None of the examined ovaries was mature enough to receive a Stage score of 5, in which vitellogenic oocytes predominate.

Statistical analysis

Average ovary area, testis volume, BM (male and female separately), and SVL (male and female separately) of individual frogs were averaged by tank and then analyzed for E2 treatment

effects using one-way analysis of variance blocked by replicate (Proc GLM, SAS version 9.4, (SAS Institute, Inc., Cary, NC, USA). Sex ratios, ovary developmental score, and oviduct scores for females, and presence of oviducts in males were analyzed for E2 treatment effects using Chi-square (χ^2). Due to a low number of observed gonad abnormalities, data from all replicates were pooled prior to Chi-square analysis (Zar 1998). Collected females were only shown to possess oviducts at stages 3 and 4. Furthermore, due to low numbers with male oviduct staging, analysis was performed based on either no oviduct presence or positive oviduct presence. For example, two individuals were staged with stage 3 oviduct presence and were grouped with stage 2. Furthermore, ovary development was staged as either 3, 4, or 4.5. Due to a low number of observations (6) within stage 3 which would violate assumptions of analysis, only observations from 4 and 4.5 were used for Chi-square analysis (Zar 1998). Recovery was identified as the percentage of tadpoles to reach metamorphosis and collected at time of experimental termination and was calculated by dividing the total sum of collected individuals from each experimental group by the initial populations (i.e., 120).

Results

Experimental exposures

Water concentrations of E2 were relatively close to nominal ranging from 90-115% of expected after renewal and 33-73% before renewal (Table 2). Estrone was commonly found in the system but was always lower than E2. Thus, E2 was likely the primary driver for estrogenicity given its higher potency. Filtering of samples only slightly reduced the E2 concentration with 72±25% of expected in the filtered water and 18±6% of expected concentration recovered from

the filter. Extraction efficiency from the particulate in the filter was not measured so those values should be considered semi-quantitative. The source water had concentrations of plasticizers above the quantitation limits in each measurement. However, bisphenol A never exceeded 200 ng/L, sum phthalates never exceeded 150 ng/L, and atrazine and metabolites were not detected (<10 ng/L). All values are well below those known or suspected to cause estrogenicity in aquatic organisms.

Recovery and sex ratios

Recovery of frogs 60 days post metamorphosis was not influenced (p=0.4) by E2 treatment (Table 3). Overall, 48% of the initial total population of 600 were recovered at the end of the trial, ranging from 36% recovery for the initial control population to 61% for the 0.64 E2 dose group (Table 3). There was a significant difference in recovery (p<0.001) between replicates. Days to metamorphosis was essentially equal across treatment groups, ranging from an average of 60 to 66 days (Table 3). Sex ratios (percent females) ranged from 44 to 57% and were not significantly influenced by E2 treatment ($\chi^2 = 4.34$, df = 5, $p = 0.47$). However, the highest female sex ratios were found in the two highest E2 treatments (Table 3). Additionally, 40% of the 38 wild cricket frogs captured in late September (presumed to be young of the year) were female, which is similar to the percentage of female control frogs (40%).

Gross morphology

BM and SVL of males (p=0.9; p=0.8 respectively) and females (p = 0.3; p = 0.2, respectively) did not differ among E2 treatments (Table 4). There was no significant difference in BM or SVL between replicates for female frogs (p=1.0; p=0.2) but there was for males (p=0.002;

p=0.001) due to decreased mass and length in replicate 4 males. On average, males from replicates 1, 2, 3, and 5 were 262 mg heavier and 2 mm longer than replicate 4 males. Overall, females were, on average, 260 mg heavier and 2 mm longer than males (Table 4). Further, we observed notable variation in BM and SVL within individual enclosures. For example, at 60 days post metamorphosis, minimum and maximum BM of frogs could differ by as much as 1442mg or as little as 207 mg. Similar variation was observed for SVL differences between the largest and smallest frog which ranged from 1mm to 15mm.

Ovary area (p=0.1) and testis volume (p=0.7) did not vary significantly among E2 treatments (Table 5). There was no significant difference between replicates for either ovary area (p=0.4) or testis volume (p=0.3). Average area of ovaries ranged from 5.4 to 7.2 mm^2 with no discernable pattern among treatments. Average testis volume ranged 1.0-1.2 mm^3. Four males and one female either had only one gonad or one discernable gonad, but these occurrences were scattered with no discernable relation to E2 concentrations. One male from the September source pond collection had a single testis.

Histopathology

Oviduct development in male cricket frogs was not significantly influenced by E2 exposure (χ^2 = 10.60, df = 5, p = 0.06; Figure 3). However, the proportions of male frogs with oviducts were 2.5-fold and 4.5-fold greater when exposed to 0.64 and 2.3 µg/L, respectively, compared to frogs in the control group. Percentages of males with oviduct development considerably increased between the low-medial concentrations (0.0-0.18 µg/L; 10-17% respectively) and the two highest concentrations (0.64 and 2.3; 27 and 43% respectively).

Similarly, oviduct development was hindered in female frogs exposed to increasing concentrations of E2, with the percentage of females exhibiting stage 4 oviduct development in the two highest E2 groups running about half that of the control group ($\chi^2 = 12.66$, df = 5, $p = 0.03$; Figure 4). Testis development was not influenced by E2 exposure and all testes from all treatment groups were scored as stage 5. Lastly, ovary development was significantly influenced by increasing E2 concentrations ($\chi^2 = 14.14$, df = 5, $p = 0.01$). The percentage of females exposed to 0.64 and 2.3 µg/L exhibiting stage 4.5 ovary development decreased to approximately half the percentage of control females (Figure 5).

Discussion

Reliability of the outdoor enclosure testing approach

Using outdoor enclosures provided an opportunity to assess development within a more environmentally relevant context. Animals were exposed to variable environmental conditions (e.g., weather, light intensity, temperature) with a seasonal pattern similar to those within wild populations. Additionally, while primary food sources were supplemented throughout aquatic and terrestrial life stages, and had to be given the length of the trial, secondary food sources such as naturally occurring algal growth and insects provide a level of environmental realism. Conditions within the enclosures were favorable for development with all surviving tadpoles completing metamorphosis. In a previous laboratory study, 23% of *Acris blanchardi* larvae appeared developmentally stalled and failed to begin metamorphosis (Gosner stage 42) within 60 days (Hoskins et al. 2018). On average, our frogs completed metamorphosis within 47 days which is

similar to the 42 to 44 days observed for *A. blanchardi* control larvae raised in outdoor mesocosms (Youngquiest et al. 2015; Gordon et al. 2016).

Our overall recovery of frogs from the original sample size of 600 was around 50%, and although less than expected in this test system, it was not completely dissimilar to other studies with wild species. Granted, we do not expect the high recovery values achievable in laboratory settings with model species such *X. laevis*, where overall survival (i.e. recovery) often exceeds 90% (Kloas et al. 2009; Carr et al. 2003; Lutz et al. 2008). However, in comparative studies with the same or similar species as ours, recovery is often between 60 to 70%, although these studies do not track recovery to 60 days post metamorphosis (Hoskins et al. 2018; Krynak et al. 2017). For example, 66% of the starting population of *A. blanchardi* exposed to various concentrations of atrazine in a laboratory setting survived until termination of the experiment, about 60 days from study initiation, although there was no evidence of atrazine related mortality (Hoskins et al. 2018). Recovery of various species of frogs raised as controls in outdoor enclosures is typically between 60-80% (Langlois et al. 2010, Youngquiest et al. 2015; Melvin and Houlahan 2012). Recovery values from the present study were greatly influenced by an unusual number of intense spring rainfall events that flooded some tanks, coupled with overheating of some enclosures later in the summer. Overall, total rainfall for Stillwater, Oklahoma in 2019 was 64% above average, with the most significant rain events occurring in late May, overlapping with the larval stages of replicates 1 and 2. Indeed, recoveries for replicates 1 and 2 averaged 35%, downwardly influencing overall recovery. Once precipitation events diminished, recoveries in replicates 3 - 5 improved to 57% overall. We expect that with an improved design that affords protection from intense weather events, recovery in enclosures could be generally improved to 60-70%, similar to

that found in other studies with native frogs and better than what is often reported for *in situ* amphibian survival, which is typically reported as approximately 10% under natural field conditions (Melvin and Houlahan 2012).

Sensitivity of cricket frogs to exogenous E2

Overall, *A. blanchardi* does not appear to be comparably sensitive to exogenous E2 exposure relative to other tested species, especially *X. laevis*. The highest E2 concentration used in this study (2.3 µg/L) resulted in 54% females which did not differ significantly from the 44% we observed for controls. In contrast, Lutz et al. (2008) reported an increase from 50 to 92% female *X. laevis* exposed to 1.5 µg/L E2, and Wolf et al. (2010) reported an increase in phenotypic females, mixed sex, and other abnormalities in *X. laevis* exposed to 0.2 µg/L E2. These and other studies demonstrate the sensitivity of *X. laevis* feminization to E2, but few studies exist for other anuran species (Pickford et al. 2003, Lutz et al. 2008). Indeed, the sensitivity of other species of frogs to E2 is less understood and results from available studies demonstrate variation in response. For example, *Rana pipiens* tadpoles exposed to 1 µg/L E2 resulted in 72% females, 12 percentage points higher than controls, with remaining individuals either intersexed or mixed (Mackenzie et al. 2003). Similarly, Tamschick et al. (2016) observed sex reversal in *X. laevis, H. arborea,* and *B. viridis* exposed to 17α-ethinylestradiol (EE2; 50, 500, 5,000 ng/L) albeit at varying levels of sensitivity. At the lowest EE2 concentration of 50 ng/L, over 31% of male *X. laevis* were sex-reversed but no sex reversal was seen at this concentration in *H. arborea* and *B. viridis.* Additional variation in species sensitivity was seen between *H. arborea* and *B. viridis* where 5,000 ng/L EE2 induced sex-reversal in 15 and 33% of male frogs,

respectively. These studies demonstrate a level of diversity in response among species and exposure timing that highlight the difficulty in comparing study results.

Variation of BM and SVL between enclosures, males and females, and even within enclosures was not entirely surprising. Females within the present study were on average heavier and larger than males regardless of treatment. Larger females are typical of *A. blanchardi*, particularly as they continue to grow and emerge the spring after hatching (McCallum et al. 2011). Additionally, genetic differences within replicates and enclosures may also lend to size variation, especially with mixed clutches (Warne et al. 2013). While competition could also be a reason for BM and SVL variation, *ad-lib* feeding likely keeps competition to a minimum. Finally, simple variation within single clutches is possible (Ficetola and De Bernardi 2009). For example, Ficetola and De Bernardi (2009) noted *Rana latastei* siblings possessed, on average, a difference of over 4% in SVL and over 5% in body width.

Ovary and oviduct development in females were negatively influenced by E2 exposure, a result observed in previous studies (Piprek et al. 2012; Storrs and Semlitsch 2008). This result appears species-specific and influenced by the timing of E2 exposure relative to larval developmental stage. For instance, inhibition of ovary development was observed in *Bombina bombina* and *Bombina variegate* when treated with E2 immediately after hatching or at the beginning of gonadal differentiation (Gosner stage 33). However, gonad development was not influenced in *B. bombina* and *B. variegate* tadpoles exposed to E2 at a later time in development (Gosner stage 40). Assuming gonad differentiation with Blanchard's cricket frogs begins at a similar time to that of *B. bombina* or *B. variegate* (Gosner stage 33), it is possible that the initiation of E2 treatment within our Gosner stage 26 tadpoles may have inhibited ovary and

oviduct development. The mechanism behind the reduction in female oviduct and ovary development is unclear but could be due to negative feedback inhibition (Kloas and Lutz 2002; Storrs and Semlitsch 2008).

To our knowledge, there have been no studies that explore the susceptibility of *A. blanchardi* to exogenous exposure of sex steroids such as (E2). However, based on the findings of other studies examining larval E2 exposure, we hypothesized E2 would induce changes in gonad development or sexual determination, primarily in the form of feminization of male gonads (Mackenzie et al. 2003; Pickford et al. 2003; Lutz et al. 2008). Our results however of E2 effects, or the lack thereof, suggest that despite the necessity and disadvantage of pooling data for statistical analyses, it is clear that E2 had limited effect on sexual development of *A. blanchardi* in the test system evaluated here. Further, it is likely that larval *A. blanchardi* are generally not sensitive to exogenous exposure to E2 regardless of the test system, although it would be prudent to examine E2 sensitivity under various other exposure scenarios (ex. laboratory settings, different developmental stages, etc.). Regardless, our results call into question the utility of using E2 as a positive control for this species in studies of developmental toxicity at the concentrations evaluated here.

Reliability of exposure in the enclosure

Measured E2 concentrations of the water during larval exposure were close to nominal with reductions of 30-70% prior to renewal. Moreover, filtering water prior to analysis suggested little E2 was bound to phytoplankton (72% recovery in water). Despite some losses and variability, the exposure was adequately maintained in the enclosure. Thus, the lack of sensitivity

to E2 is unlikely due to lack of exposure. Losses of E2 concentrations prior to renewal is likely due to some adsorption of E2 to suspended particulate matter and algae that accumulated on the bottom and sides of the tank. Inconsistency between replicates is likely due to variability in algal growth and collection of particulates during sampling, which was included in the whole water extraction used for analysis. Given the hydrophobicity of E2 (Log Kow = 4.01; Ujang and Henze 2004) some sorption was expected.

Maintaining consistent exposure is more challenging in an outdoor enclosure compared to a laboratory-based design due to the inability to have a flow-through system, increased likelihood of chemical partitioning due to increased organic material in the system, and increased biological activity that may decrease the half-life in water. This effect is stronger for more hydrophobic compounds, and measured concentrations and frequent exchanges are necessary. There is also the potential for dilution of the exposure during rain events. In this study, any relevant dilution from rain events would have resulted in reduced exposure concentrations for less than 24h, as exposure water was renewed after each rain event. Using this study design, it is important to consider compound-specific approaches to maintaining concentrations in the outdoor enclosure.

Conclusion

The use of this outdoor enclosure design and cricket frogs allowed for the study of contaminant effects on development of a native amphibian from an early larval stage through sexual maturity. Outdoor housing allows frogs to develop under realistic conditions that include environmental cues important for development of many amphibians such as photoperiod,

temperature, etc. (Edge et al. 2016; Laurila et al. 2001). Additionally, the test design of this study allowed us to monitor tadpole development through metamorphosis and easily collect metamorphs at the end of our experiment. However, potential hazards for outdoor housing such as flooding need to be considered in developing the testing enclosures. Even with hazard avoidance, method designs should take into account the potential for lower recovery of organisms as compared to laboratory studies with model organisms. But recoveries can be achieved that are similar to, or exceed, those observed *in situ*.

Additionally, the results of this study suggest cricket frogs have lower susceptibility to E2 exposure than some other species and only small developmental effects were noted. Furthermore, high variation of body size within our populations could not be attributed to E2 and were likely due to natural variation (Warne et al. 2013). In practical terms, E2 at tested concentrations is not a good positive control for developmental effects in this system. The surprisingly limited effects of E2 needs further investigation both in terms of a possible lack of sensitivity of cricket frogs to EDCs and mechanisms of sexual differentiation in the species which are not well understood.

Acknowledgements

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Tables and Figures

Figure 1**.** Upper left: Mesocosm field site with 60% UV filtration shade cloth. Upper right: Single mesocosm fixed with mesh netting to keep out predators in addition to keeping metamorphosed frogs contained. Lower left: Steel container from a mesocosm containing tadpoles. Green coloration due to algal growth. Lower right: Field site in which adult frogs were collected.

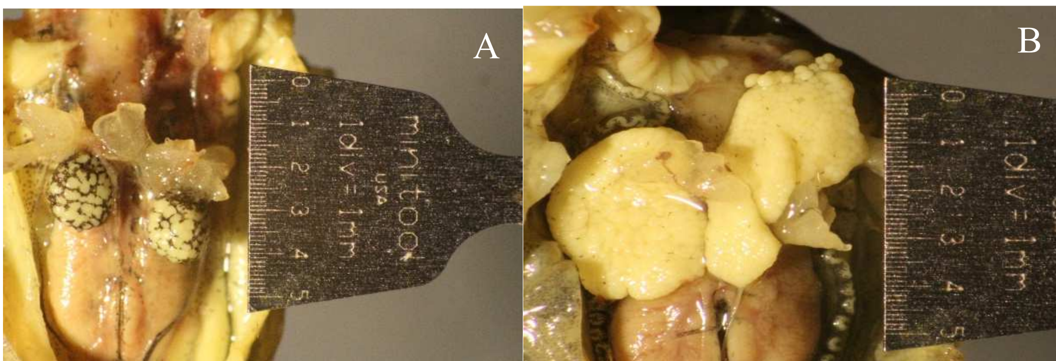

Figure 2. Exposed gonads of male (A; 0.015 µg/L) and female (B;2.3 µg/L) E2 treated *Acris blanchardi*. Yellow coloration due to Bouin's fixative.

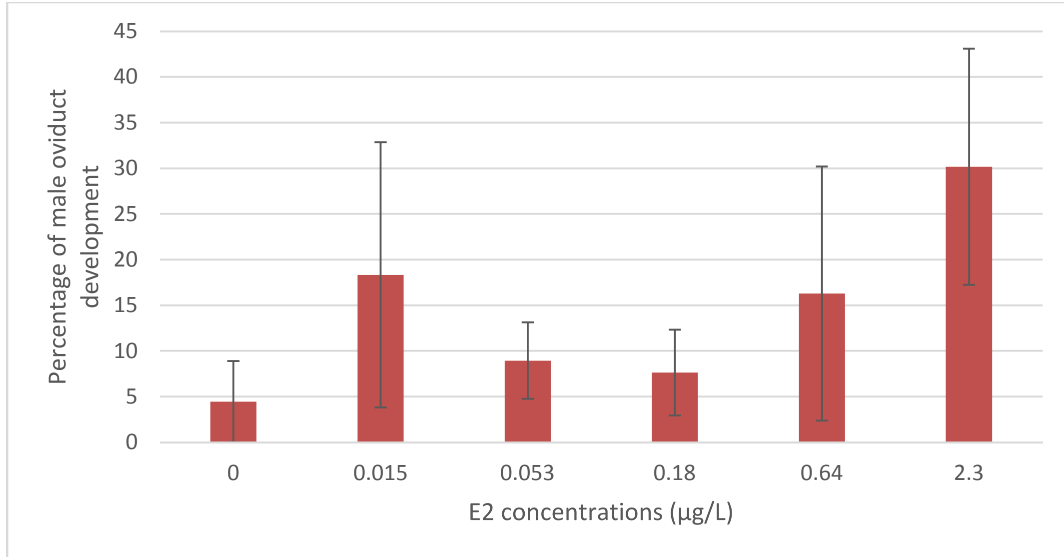

Figure 3. Mean percentage±SE of juvenile male *Acris blanchardi* frogs with oviduct development (Stage 2 or 3) at approximately 60 days post-metamorphosis. Data from replicates (n=5) averaged for respective treatment groups. Frog larvae were exposed to estradiol (E2) from Gosner stage 26 until metamorphosis (Gosner stage 45).

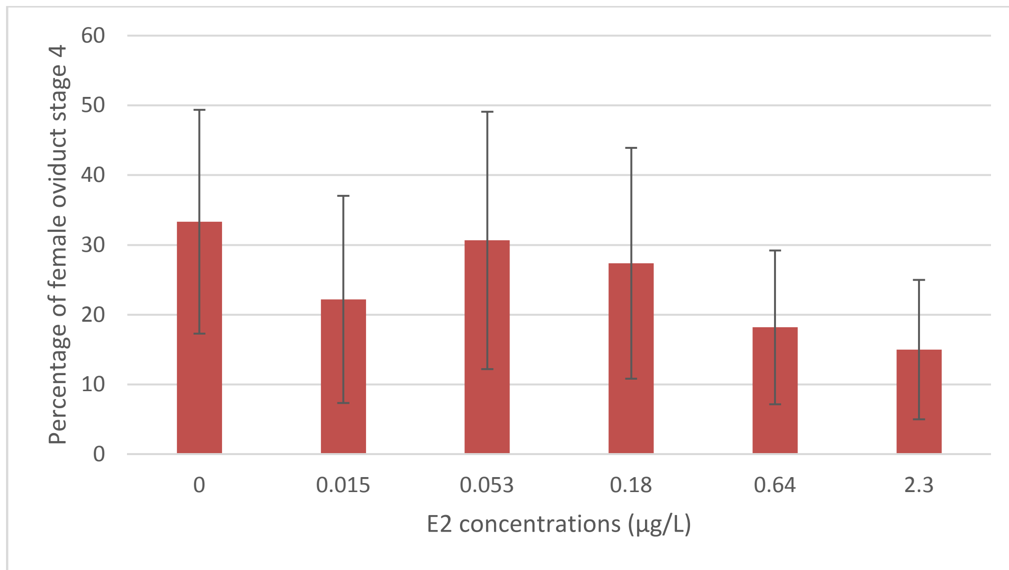

Figure 4**.** Mean percentage±SE of juvenile female *Acris blanchardi* frogs with oviduct development staged at 4 at 60 days post-metamorphosis. Data from replicates (n=5 for all treatments except for 0.18 which possesses an n=4) averaged for respective treatment groups. Frog larvae were exposed to estradiol (E2) from Gosner stage 26 until metamorphosis (Gosner stage 45).

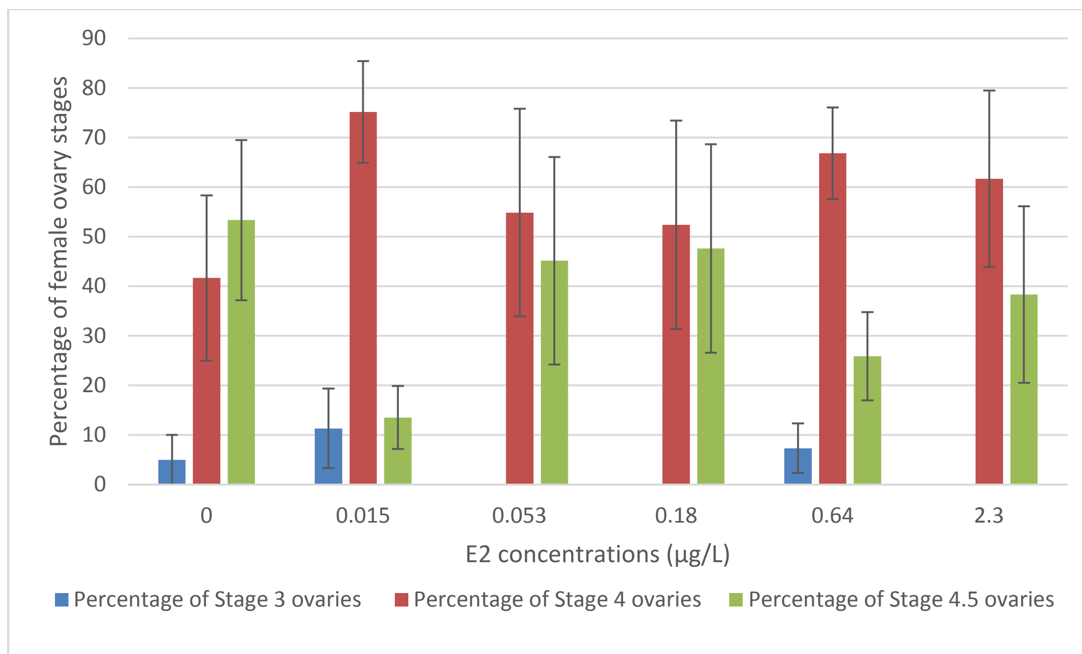

Figure 5. Mean percentage±SE of juvenile female *Acris blanchardi* frogs with respective ovary development stages (3, 4, or 4.5) at 60 days post-metamorphosis. Data from replicates (n=5 for all treatments except for 0.18 which possesses an n=4) averaged for respective treatment groups. Frog larvae were exposed to estradiol (E2) from Gosner stage 26 until metamorphosis (Gosner stage 45).

Table 1. Definitions for amphibian gonadal abnormalities established by Lutz et al. 2008.

Feature	**Definition**
Intersex	Ovarian and testicular tissue present as separate structures (not in the same gonad)
Mixed Sex	Ovarian and testicular tissue present in same gonad
Testicular Oocytes	Oocytes within testicle(s)

Table 2. Water concentrations in each treatment before and after water exchanges (n=6). E2 = 17β-estradiol, % indicates percent of nominal.

			0 ng/L	15 ng/L	53 ng/L	180 ng/L	640 ng/L	2300 ng/L
Before Exchange	E2	ng/L	<5 (0)	17 (6)	61 (13)	208 (64)	578 (232)	2100 (1200)
		%	NA	112 (41)	115 (24)	116 (36)	90 (36)	91 (53)
	Estrone	ng/L	<5 (0)	<5 (0)	<5 (0)	18 (17)	58 (43)	242 (202)
After Exchange	E2	ng/L	<5 (0)	9.8) (6.5)	39 (19)	82 (27)	207 (67)	941 (323)
		%	NA	65 (43)	73 (35)	46 (15)	33 (10)	41 (14)
	Estrone	ng/L	<5 (0)	<5 (0)	5 (9)	44 (46)	266 (220)	602 (349)

Table 3. Recovery of *Acris blanchardi* frogs among treatments, days to metamorphosis, and percentage of juvenile cricket frogs designated as female. Recovery was defined as live collection upon termination of study approximately 60 days after metamorphosis. Blanchard's cricket frogs were designated as females based on gross gonadal morphology. Wild group includes specimens collected from the original collection pond during late September of the same year as this mesocosm study and thus represents the same cohort of frogs as used in the mesocosms.

Estradiol (μg/L)	Starting number	Recovered (%)	Days to metamorphosis	Percentage female (%)
0.0	100	36	66	44
0.015	100	49	62	49
0.053	100	54	61	46
0.18	100	38	63	40
0.64	100	61	60	57
2.3	100	50	63	54
Total (mean±SE)	600	48±3.9	-	49
Wild	38	-	-	40

Table 4. Mean body mass±SE (mg) and average SVL±SE (mm) of juvenile *Acris blanchardi* frogs at approximately 60-days post-metamorphosis (*n*=5). Cricket frogs larvae were exposed to estradiol from Gosner stage 26 until metamorphosis (G. Stage 45). Body mass and SVL measurements from frogs from the same mesocosm were averaged to then find averaged body mass and SVL of respective E2 treatment groups.

Estradiol (µg/L)	Male body mass (mg)	Male SVL (mm)	Female body mass (mg)	Female SVL (mm)
0.0	1086±112	22±0.8	1418±76	24±0.3
0.015	978±48	21±0.6	1016±94	21±0.9
0.053	988±73	22±0.3	1318±104	24±0.8
0.18	1029±83	21±0.6	1076±298	19±4.8
0.64	969±33	22±0.5	1165±83	23±0.7
2.3	958±103	21±0.9	1212±128	23±0.9
F-value	0.3	0.5	1.3	1.9
p-value	0.9	0.8	0.3	0.1

Table 5. Mean (SE) gonad length (mm), width (mm), and area/volume (mm^2/mm^3) of female and male *Acris blanchardi* frogs at approximately 60 days post-metamorphosis. Frog larvae were exposed to estradiol from Gosner stage 26 until metamorphosis (G. Stage 45). Ovary area based on ellipse calculation (length/2*width/2*π). Testis volume based on ellipsoid volume calculation (length/2*width/2*width/2*π4/3).

Estradiol (µg/L)	n	Ovary length (mm)	Ovary width (mm)	Ovary area (mm^2)	Testis length (mm)	Testis width (mm)	Testis volume (mm^3)
0.0	5	3.9(0.4)	2.4(0.1)	7.1(0.5)	1.6(0.07)	1.1(0.03)	1.1(0.1)
0.015	5	3.4(0.1)	2.0(0.1)	5.4(0.4)	1.5(0.04)	1.1(0.02)	1.0(0.1)
0.053	5	3.8(0.1)	2.1(0.5)	7.2(0.4)	1.5(0.05)	1.1(0.05)	1.0(0.1)
0.18	5	3.9(0.3)	2.3(0.1)	6.6(0.5)	1.5(0.05)	1.1(0.03)	1.1(0.1)
0.64	5	3.9(0.2)	2.3(0.1)	7.2(0.6)	1.6(0.04)	1.2(0.03)	1.2(0.1)
2.3	5	3.7(0.1)	2.2(0.1)	6.4(0.5)	1.6(0.06)	1.1(0.05)	1.1(0.1)

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CHAPTER IV

ATRAZINE AND ESTRADIOL EFFECTS ON DEVELOPMENT OF *ACRIS BLANCHARDI* (BLANCHARD'S CRICKET FROG) EXPOSED IN OUTDOOR ENCLOSURES

Abstract

The herbicide atrazine has been proposed as a potential endocrine disrupting compound (EDC) for amphibians. Using atrazine concentrations below or at those typically found in surface waters (0.5, 5.0, 50 µg/L), we exposed *Acris blanchardi* (Blanchard's cricket frog) larvae throughout development until metamorphosis (i.e., Gosner stages 26 – 45). An additional 50µg/L treatment (50*s* µg/L) was utilized where supplemented algae was added to control for indirect atrazine effects from reduced food sources. In addition to atrazine, experimental groups also included a negative control and two positive controls, 17β-estradiol (E2) at 2.3 and 25 µg/L. At 60 days post metamorphosis, *A. blanchardi* metamorphs were euthanized for analysis of gross and histopathological development. Atrazine did not significantly influence mortality (mean recovery of 54% across treatments), sex ratio, body mass (BM), snout-vent length (SVL), gonad size, nor gonad development of *A. blanchardi*. Females exposed to 50*s* µg/L atrazine had 29% less mass, were 10% shorter, and had a 29% lower mean ovary area (mm^2) as compared to negative controls, suggesting algae enrichment had a significant negative effect. Males exposed to estradiol (25 µg/L) showed an increased level of oviduct development. Ovary area was also significantly influenced by estradiol treatment at 2.3 and 25 µg/L. Overall, estradiol had much

less effect than predicted based on other model species (e.g., *Xenopus laevis*). Development of *Acris blanchardi*, overall, was not affected by long-term exposure to environmentally relevant concentrations of atrazine. However, this species also was largely insensitive to exogenous estradiol in this test system

Introduction

Endocrine disrupting compounds (EDCs) may induce effects that include, but are not limited to, effects on body morphology, survival, reproductive development, and phenotypic expression of sex (Coster and van Larebeke 2012). Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) has been suggested as an EDC, specifically via estrogenic disruption (Hayes et al. 2010.; Russart and Rehn 2016; Brodeur et al. 2013), though results are highly inconsistent (Kloas et el. 2009; Van Der Kraak et al. 2014; Hanson et al. 2019).

Atrazine is used extensively to control broad-leaf weeds (Van Der Kraak et al. 2014). This compound has a low K_{oc} (40 – 394), moderately high-water solubility of 33 mg/L, and average half-life of 130 days indicating that it is a relatively mobile and a moderately persistent compound (Van Der Kraak et al. 2014; Brodeur et al. 2013, EPA 2016). Atrazine loss from application sites primarily involves microbial degradation, but runoff and leaching can also occur leading to movement into surface and ground water (EPA 2016).

Exposure to EDCs can lead to abnormal development (e.g., gonad, body, etc.) and feminization of exposed organisms (Huang et al. 2015). Although atrazine has been reported to have estrogenic activity, there is not consistent agreement as to the existence, magnitude, and extent of effects under varying exposure scenarios (Russart and Rhen 2016; Hayes et al. 2002;

Coady et al. 2004; Kloas et al. 2009; Van Der Kraak et al. 2014; Hanson et al. 2019). For example, hermaphroditism and skewed sex ratios of *Xenopus laevis* after larval exposure to atrazine have been reported at concentrations between 0.1-200 ppb when exposed throughout development (Hayes et al. 2002). Conversely, no effects on gonad development were observed in *X. laevis* following continuous exposure at 0.01, 0.1, 1, 25 and 100 µg/L (Kloas et al. 2009). Likewise, sex ratios and gonadal morphologies of *Rana clamitans* larvae were not affected by continuous exposure to atrazine concentrations of 10 and 25 µg/L (Coady et al. 2004). Further, observations of overall gross gonadal morphology (e.g., mixed sex, missing gonads, and size differences) and body morphology (e.g., snout-vent length and body mass) following atrazine exposure have shown mixed results. Carr et al. (2003) reported a 5% increase in intersex for *X. laevis* exposed to 25 µg/L; however, a subsequent review of the original histologic sections did not confirm microscopic evidence of intersex (Kloas et al. 2009). Additional studies in *X. laevis* and *R. clamitans* failed to demonstrate histopathological abnormalities in the gonads of atrazine-treated frogs (Kloas et al. 2009; Coady et al. 2004).

Amphibians are useful for assessing the potential effects of contaminant exposure and have been used extensively as physiological and behavioral research models (Hopkins 2007: Kloas and Lutz 2006). However, amphibians are a large and diverse group of species with a range of morphological, physiological, and life history traits which can lead to varying species-specific responses to EDCs (Burggren and Warburton 2007). For example, Tamschick et al. (2016) observed species-specific differences in responses among *X. laevis, H. arborea,* and *Bufo viridis* following larval exposure of 17α-ethinylestradiol (EE2). Moreover, while commonly used models such as *X. laevis* are useful due to the well-established understanding of their development and

physiology, the use of native amphibian models is comparatively sparse (Hogan et al. 2006; Mackenzie et al. 2003). In the present study, *Acris blanchardi*, a widespread native species of North America, was used to assess the potential for atrazine-mediated effects on morphological and reproductive development. *A. blanchardi* are semi-aquatic hylids that breed throughout spring and summer months in rural and urban water bodies (Burdic and Swanson; McCallum et al. 2011). Their ubiquitous distribution lends to their utility as a model for assessing exposure and effects of contaminants.

The objective of this study was to further assess the potential effects of atrazine on a native North American amphibian. Specifically, we evaluated the effect of atrazine exposure during the larval period on somatic morphology and gonadal development of post-metamorphic *A. blanchardi*. Exposure and development occurred in outdoor enclosures that allowed for environmental cues (i.e., temperature, light, and humidity fluctuations) that may influence normal development (Szekely et al. 2017; Denver 1997; Laurila et a. 2001; Windle et al. 2021). We used 17β-estradiol (E2) as an estrogenic EDC positive control and tested atrazine at various environmentally relevant concentrations (Hanson et al. 2019; Van Der Kraak et al. 2014). Estradiol concentrations were based on concentrations reported to cause feminization in *X. laevis* and other native amphibian species (Kloas and Lutz 2006; Lutz et al. 2008; Mackenzie et al. 2003; Coady et al. 2005).

Methods

Adult and larval collection

Acris blanchardi were collected as pairs during amplexus, and as single females that were later paired with multiple males. All frogs were collected from non-agricultural ponds in Payne County, Oklahoma (36.1450ºN, 97.0068ºW) from May 27 until June 1, 2020. Land area immediately surrounding the ponds were primarily wooded with mowed grass due to residential development. There was no agriculture in the watershed and previous analysis determined ponds were free of atrazine contamination (Windle et al. 2021). Larvae from successful oviposits were briefly held in the laboratory until Gosner stage 26 was reached. Detailed protocols regarding adult and larval collection established in Windle et al. (2021).

Gosner stage 26 (Gosner, 1960) larvae were combined into one container and haphazardly sorted into groups of 25 and then randomly assigned to outdoor enclosures at our field research facility. Treatment with estradiol and atrazine began immediately after larvae were placed into outdoor enclosures; between June 5, 2020 and June 11, 2020. Time between start dates was based on individual clutch collection and hatch dates to ensure larvae for each replicate began at Gosner stage 26.

Parental frogs were euthanized and submitted for histopathologic examination to provide a representative subsample of mature *A. blanchardi* for the year. An additional 25 individuals were collected September 2020 for histopathology to provide a representation of wild frogs hatched that year. Wild individuals were assumed to be from 2020 clutches due to their smaller body size and lack of mature characteristics such as male throat patches and complete gonad

development (determined via gross and histopathologic examination). All procedures were completed following approved Oklahoma State University IACUC protocols.

Experimental exposures

Details of the outdoor enclosure system used in this study are described in Windle et al. (2021). Briefly, the enclosure system consisted of a shade structure covering screen-enclosed metal enclosures which contained a water source for larval development and treatment exposure and a naturally vegetated terrestrial environment for metamorphs (Figure 1). An exception to the system described previously was the addition of 55% light filtration Farm Plastic® to provide shading and protection from heavy precipitation events, erected beneath the shade cloth (Figure 1).

Our experimental design consisted of five replicate enclosures for each of seven treatments consisting of a negative control, two positive estradiol controls (2.3 and 25 µg/L), and four atrazine treatments (0.5, 5.0, 50, 50*s* µg/L). Algae (taxonomy not verified) was supplemented into the fourth atrazine treatment (50*s*) to negate potential losses of food resources due to atrazine which can effectively reduce algae at the higher concentration tested. Algae for supplementation was grown from naturally occurring algal growth present in the polyethylene holding tanks above. Four 25-L steel tanks were filled with water from the above tanks before a single spike of nitrogen and phosphorous, 1 mmol and 0.1 mmol, respectively, was added to each algal grow tank to develop rigorous algal growth. Subsequently, TetraMin® was added at the same rate as tadpole tanks, which was expected to degrade and provide nutrients similar to those found in the exposure tanks. At the end of each water exchange, 400 ml was collected from these

tanks and added to 50*s* µg/L enclosures. Estradiol concentrations were based on concentrations previously used to feminize *X. laevis* and *A. blanchardi* (Lutz et al. 2008; Windle et al. 2021). Lutz et al. established an EC50 (increased female phenotype) of 0.12 ug/L E2. We established a dose-response relationship in Windle et al. 2021 based on this concentration. While not statistically significant, we found feminization in the form of male oviduct development at 2.3 ug/L. We based our E2 concentrations in the present study of 2.3 and 25 ug/L on these results. Atrazine concentrations were based on environmentally relevant concentrations (Hanson et al. 2019; Beaulieu et al. 2020) and concentrations reported to elicit feminization in *A. blanchardi* and *Rhinella arenarum* (Hoskins and Boone 2018; Brodeur et al. 2013). Methanol (0.04 mL, 0.0002% of exposure water) was used as a carrier for all treatments and added to the negative control. Spiking solutions were made using technical grade Atrazine (Syngenta Crop Protection, 97.5% purity) and high purity estradiol (Sigma, >98% purity). Further analytical details can be found in SI.

Metamorph collection and measurement endpoints

Treatments were discontinued once a minimum of 75% of the surviving larvae in an enclosure reached Gosner stage 45 at which point exchanges continued with only carbon filtered water. Typically, approximately 87+1.1% of unit were at GS 45 and the remaining animals would reach GS 45 by the next exchange. Furthermore, delayed, or failed metamorphosis by individuals in discontinued tanks was recorded. Following metamorphosis, animals were reared an additional 60 days to facilitate differentiation of gonads and to allow the animals to reach sexual maturity (McCallum et al. 2011). Frogs were collected 60 days post-metamorphosis (date based on average for each replicate), euthanized in 5% buffered MS-222 and then patted dry. Snout-vent

length (±1 mm; SVL) and body mass (±1 mg; BM) were recorded and the kidney-gonad complex was exposed, intact, in the body cavity after removal of the head, limbs, and viscera. Carcasses were immersed in Bouin's solution (Thermo Fisher Cat.No.:50-320-01) for 24 hours and then rinsed with 70% ethanol and immersed in 10% formalin until shipment to Experimental Pathology Laboratories (EPL), Inc., for histopathologic processing and evaluation. For shipment, frog carcasses were wrapped in formalin soaked tissues and marked with a unique identification number, assumed sex based on macroscopic gonad morphology, and date euthanized.

Time to metamorphosis (days), recovery (number of the initial 25 individuals recovered at 60 days post-metamorphosis), and sex ratio (based on gross morphology of gonads) were all quantified in addition to SVL and BM. The term "recovery" is used in place of survival because we cannot confirm that all losses were due to treatment as opposed to other unknown factors as would be expected for outdoor studies (e.g., predation). Gonad sizes were estimated by ellipse area ($ab\pi; a = \frac{length}{2}, b = \frac{width}{2}$) for ovaries and testes (Windle et al. 2021). Bilateral gonad measurements were averaged to provide a single measurement for each individual. To avoid possible damage to fixed testis tissue, the second width measurement was assumed equal to the first. This method was used in lieu of gonad weight to avoid damaging gonads prior to histopathological analysis.

Gonads and oviducts (Müllerian ducts) were evaluated by a pathologist (JCW) who is board-certified by the American College of Veterinary Pathologists, and has extensive experience in the examination of reproductive tissues from amphibians used in endocrine disruptor bioassays. These assessments were performed without awareness of the treatment group status of individual animals (i.e., blinded). Reproductive tracts were examined for microscopic

abnormalities, and scoring the of gonad and oviduct development was based on criteria outlined in EPA 2015 (Table 2) that were originally developed for assessing *X. laevis*. One modification created to account for species-specific differences in ovarian development was the addition of an intermediate stage 4.5, which allowed for a more granular assessment of ovarian development in cricket frogs.

Statistical analysis

Testis area, ovary area, BM, and SVL of individual frogs were averaged by enclosure, separated by sex, and analyzed using one-way analysis of variance blocked by replicate (Proc GLM Mixed, SAS Version 9.4), (SAS Institute, Inc., Cary, NC, USA). When significant, a Duncan's post-hoc was performed. Recovery, larval death, and time to metamorphosis were analyzed similarly without separation of sex. Sex ratio and gonad scoring via microscopic examination was analyzed using Chi-square (χ^2). Prior to analysis, we pooled stage 1 and 2 female oviducts, 3 and 4 female oviducts, and stage 2 and 3 male oviducts to avoid violations of chi square assumptions (Zar 2010).

Results

Experimental exposure and environmental control

Estradiol concentrations after water renewals ranged from 83 – 104% of nominal. Just before renewal (i.e., 3 – 4 days after the last water exchange) concentrations ranged from 27 – 30% of the initial nominal concentration (Table 1). Estrone, a metabolite of estradiol, was also detected in samples before and after renewal at higher concentrations than estradiol (Table 1). For example, mean estrone concentrations within both before and after 2.3 µg/L samples were 6.1

µg/L. Atrazine concentrations ranged from 90 - 92% following water renewals and 102-128% in samples prior to renewal (Table 1). Atrazine metabolites desethyl atrazine and deisopropyl atrazine were negligible to the total atrazine in the system. Dosing solutions of both atrazine and estradiol were within 10% of expected prior to and after completion of the study.

Mean air temperature at the field site but not under the shade cloth was 29°C while mean temperature under the shade cloth was 28°C. Mean air temperature within enclosures slightly varied but was relatively consistent, ranging from 27 - 28°C. Similarly, mean water temperature slightly varied but remained between 26 - 27ºC. Light exposure varied most notably between enclosures on the east and west side of the array and was especially reduced under the shade cloth. For example, within the same 2-week period, mean light intensity outside the shade cloth was 1357 lum/ft^2 compared to 327 lum/ft2 under the shade. Furthermore, the easternmost row of enclosures averaged 350 lum/ft^2 compared to the westernmost row at 199 lum/ft^2. Despite variations in light, algal growth appeared primarily dependent on treatment. Mean chlorophyll-a concentrations from controls and the lowest atrazine treatment (0.5 µg/L) ranged between 6389 – 7992 mg/L. Comparatively, atrazine-related algal loss occurred most notably in 50 and 50*s* µg/L units despite supplementation and resulted in mean concentrations of 3361 and 5658 µg/L respectively. Interestingly, the second highest mean chlorophyl-a concentration occurred in the 5 µg/L atrazine group at 7662 mg/L.

Recovery and sex ratio

Percent recovery of *A. blanchardi* at 60 days post-metamorphosis was not influenced by treatment (p = 0.29). Overall, 54% of the initial population was recovered with mean recovery by

treatment ranging from 45% to 65% (Table 3). Further assessment of recovery found approximately 60% of total deaths occurred during larval stages and was not influenced by treatment (p = 0.29). Similarly, sex ratio (percent female) was not influenced by treatment (χ^2 = 6.77; p = 0.34). Mean percent females ranged from 64% in the 5 µg/L atrazine group to 76% in the 25 µg/L E2 group (Table 3). Time to metamorphosis ranged from 31 to 35 days across treatments and was not influenced by treatment (p = 0.39; Table 3). There were no instances of delayed or failed development across all treatments. All animals reached metamorphosis by experiment termination.

Macroscopic somatic and gonadal morphology

Body mass and SVL of males were not influenced by estradiol or atrazine exposure (Table 4; p = 0.7 and p = 0.9, respectively). Conversely, BM and SVL of females treated with 50*s* µg/L atrazine were smaller than control females (Table 4; p = 0.02 and p = 0.5, respectively). Indeed, control females were over 29% heavier and 11% longer than females treated with 50*s* µg/L atrazine. Similarly, females treated with 5.0 and 50 µg/L atrazine were significantly larger (i.e., 31% heavier and 11% longer) than 50*s* µg/L atrazine treated females.

Ovary area was influenced by both estradiol treatments and 50*s* µg/L atrazine with a 23 – 27% decrease in size compared to mean control ovary area (Table 5; p = 0.02). Mean testis area ranged from 1.2 – 1.8 mm^2 and did not vary significantly among treatments (Table 5; p = 0.2).

Histopathology

Feminization in the form of male oviduct development was significantly influenced by the highest estradiol treatment (χ^2 = 41.6; p = <0.001; Figure 2). Mean oviduct development

(Stage 2 and 3 combined) for males in the 25 µg/L E2 group was 74%, a 9-fold increase over that for control males, and 5-fold greater than males exposed to 2.3 µg/L E2. Among males, stage 3 oviduct development was only observed in the 25 µg/L E2 treatment group. Ninety-one to 100% of males exposed to atrazine exhibited no oviduct development or oviduct remnants (both of these conditions were scored as stage 1 oviduct development) and were comparable to controls (92%).

Testis morphology and staging did not differ among treatments ($\chi^2 = 6.7$; $p = 0.35$). A low overall incidence of testicular oocytes (TO) occurred in the study (9 individuals or 6% total) but the prevalence and severity of this finding were comparable among the control and treated groups (Table 6; Figure 5). A single mixed sex individual (i.e., ovarian and testicular tissue in a single gonad) was observed in each estradiol treatment group.

Female oviduct development did not differ among treatments ($\chi^2 = 11.9$; $p = 0.06$; Figure 3). However, oviduct development did appear inhibited to an extent by 25 µg/L E2. While 100% of control females developed stage 3 or 4 oviducts, only 87% of the females exposed to 25 µg/L E2 exhibited similar degrees of development. Ovary development was inhibited by both estradiol treatments ($\chi^2 = 18.8$; $p = 0.005$; Figure 4) with 65 to 67% of females with stage 4.5 ovaries compared to 88% of control females. No atrazine treatment had an effect on ovary or oviduct development ($\chi^2 = 5.2$; $p = 0.3$; $\chi^2 = 2.8$; $p = 0.6$, respectively).

Discussion

We did not observe any consistent effects of atrazine exposure on *A. blanchardi* developmental rate, sex ratio, or body size, despite exposing larvae continuously from early development through complete metamorphosis. These results, like many before, are congruent

with some studies but not others. Langlois et al. (2010), Sullivan and Spence (2003), and Hoskins and Boone (2018) have shown sensitivity of *Rana pipiens, X. laevis,* and *A. blanchardi* to atrazine exposure under various testing conditions, and other authors have indicated effects on development rate, sex ratios, body size, and survival (Zaya et al. 2011, Hayes et al. 2010; Diana et al. 2000). Conversely, an equal number of studies demonstrate a lack of effect on these endpoints under various exposure conditions for several species including *X. laevis, R. pipiens, R. clamitans* (Coady et al. 2004; Allran and Karasov 2000; Kloas et al. 2009). Studies reporting effects on similar endpoints to ours have used atrazine concentrations comparable or higher than those tested here, and primarily with the model species *X. laevis*, in addition to others including *R. pipiens, Hyla versicolor,* and *A. blanchardi*. For instance, *H. versicolor* and *X. laevis* metamorphs exposed throughout early development to atrazine concentrations ranging from 200 to 2000 µg/L were significantly smaller in both SVL and BM (Diana et al. 2000; Sullivan and Spence 2003). However, atrazine concentrations used in these studies approach or surpass reported maximum environmental relevant concentrations (EPA 2016). Furthermore, and when the same species were exposed to comparable concentrations from the present study (e.g., 0.1 – 25 µg/L), no atrazine induced effects were observed for body size (Diana et al. 2000; Kloas et al. 2009; Carr et al. 2003). A notable difference between our study methodology and comparable studies is the timeframe during which we measured endpoints. It is common for anurans to be collected and euthanized immediately after tail resorption (Diana et al. 2000; Carr et al. 2003; Hoskins and Boone 2018). We allowed an additional 60 days of development to provide enough time for complete differentiation of gonads and for frogs to reach sexual maturity (McCallum et al. 2011). Our assumption is that development at a more mature stage should provide a better

indication of future reproduction success. However, this period may also allow for recovery and thus reduced observation of effects.

Atrazine related effects on developmental rates and sex ratios also vary among studies and species (Hoskins and Boone 2018; Langlois et al. 2010; Hayes et al. 2003; Coady et al. 2004). For instance, Brodeur et al. (2013) reported a significant decrease in time to metamorphosis in *Rhinella arenarum* exposed to a concentration similar to ours (10 µg/L). However, at the same concentration, but with different species, multiple studies have reported the opposite effect or no effect at all (Coady et al. 2004; Hoskins and Boone 2018). Similarly, at concentrations lower or similar to those used in this study, feminized sex ratios in species such as *R. pipiens, A. blanchardi*, and *X. laevis* have been reported (Langlois et al. 2010; Hoskins and Boone 2018; Hayes et al. 2010). However, a number of additional studies contradict these results with the same or similar concentrations and species (Coady et al. 2004; Kloas et al. 2009; Lent et al. 2018).

Some studies report reduced survival of amphibian larvae (e.g., *X. laevis, A. blanchardi, Pseudacris crucifer*) exposed to atrazine concentrations ranging from 3 to 500 µg/L (Storrs and Kiesecker 2004; Hoskins et al. 2019; Rimayi et al. 2018). We did not measure larval survival *per se* in our test system, but average recovery (a proxy for survival in this case) of the initial stock did not differ between control tanks and atrazine tanks. This measure is not true survival as related to treatment, given that other unknown factors (e.g., spider predation and flooding) contribute to losses in outdoor tanks but it is informative and suggests a lack of treatment-induced effects on survival. Furthermore, considering the nature of our experimental design (i.e., outdoor enclosures, prolonged time post-metamorphosis), our recovery (54%) was relatively comparable

albeit lower to similar studies (Hoskins and Boone 2018; Hoskins et al. 2019; Carr et al. 2003). Despite collection immediately after metamorphosis (i.e., GS 46), Hoskins and Boone (2018) reported about 50% overall survival of metamorphosed *A. blanchardi* exposed in the laboratory. A later outdoor study performed by Hoskins et al. (2019), reported 84% overall survival of metamorphosed and atrazine treated *A. blanchardi.* However, the authors' discarded experimental units with low recovery (i.e., less than 3 individuals in 4 of 31 enclosures). Inclusion of all replicates results in a calculated survival of 68%. Our lack of effect on survival is in agreement with other studies in which survival of species including *X. laevis, A. blanchardi, R. clamitans, R. pipiens,* and *H. versicolor* was not affected by atrazine concentrations ranging from 1 – 400 µg/L (Zaya et al. 2011; Hoskins and Boone 2018; Hoskins et al. 2019; Diana et al. 2000, Orton et al. 2006; Coady et al. 2004).

Undoubtedly, testing conditions and species plays a significant role in observed results. Our exposure concentrations ranged from 0.5 – 50 µg/L and were based on environmental relevance (Hanson et al. 2019) as well as concentrations that have elicited effects (Hoskins and Boone 2018; Brodeur et al. 2013). Nonetheless, our concentrations were similar or lower compared to many studies reporting effects on amphibian development (Langlois et al. 2010; Storrs and Kiesecker 2004; Diana et al. 2000; Tavera-Medoza et al. 2002a; Hayes et al. 2003). Exposure concentrations were consistent throughout the exposure period, ranging from an average of 90 – 128 percent of nominal; with little to no losses between exchanges, suggesting that larvae were continuously exposed to atrazine at prescribed concentrations. A slight increase in atrazine was noted between water exchanges, likely due to evaporation.

Hoskins and Boone (2018) and Hoskins et al. (2019) provide the only direct comparison with respect to *A. blanchardi.* In a controlled laboratory study, they observed increased female sex ratios in *A. blanchardi* exposed to 0.1 and 10 µg/L of atrazine, compared to controls (Hoskins and Boone 2018; Table 7). In brief, approximately 32% of control animals were female while approximately 60% in both aforementioned atrazine groups were female. Comparably, we did not find a significant change in sex ratio among metamorphs exposed as larvae to similar concentrations (0.5 and 5 µg/L) or up to 50 ug/L. Additionally, the sex ratio of a small subsample of wild juvenile *A. blanchardi* was comparable to all treated animals (Table 2). Variation in testing procedures may explain the lack of congruence between our studies, but the mechanism(s) responsible are unclear. For instance, *A. blanchardi* raised for this study were exposed to varying temperatures and humidity levels throughout the development and exposure period, while those exposed to atrazine by Hoskins and Boone (2018) were reared in the laboratory under presumably more stable conditions. While not a thoroughly studied or observed occurrence, Ruiz-Garcia et al. (2021) did note a few studies that observed skewed anuran sex ratios after rearing in extreme high or low temperatures. However, we cannot be certain of sex determination related to temperatures in our study as we are unable to genetically test our animals. An additional testing procedure difference between our study and those performed by Hoskins and Boone (2018) is the stage at which metamorphs were euthanized. As is common in anuran toxicology studies, Hoskins and Boone collected and euthanized *A. blanchardi* immediately after metamorphosis. Conversely, we allowed an additional 60 days post-metamorphosis. The additional maturation time may be a reason for our result differences but the magnitude of the maturation difference is unknown. Sex ratio was not assessed in Hoskins et al. 2019; however, similar to our results, both studies found

that atrazine concentrations equal to or less than 100 µg/L did not affect body size, developmental rate, or survival.

We did not observe significant effects of atrazine on morphological development including ovary size. However, ovary size and overall morphological development is not commonly influenced by atrazine exposure (Coady et al. 2004; Kloas et al. 2009). Testis size also was not significantly affected by atrazine exposure. Atrazine exposure reportedly induces negative effects on testes size, although transient increases in testes volume have also been observed (Tavera – Mendoza et al. 2002a; Victor – Costa et al. 2010; Rimayi et al. 2018). Mean testis area for our atrazine-exposed frogs was variable, but generally similar to controls.

In the current study, exceptions to the general lack of atrazine-associated effects on *A. blanchardi* development were the effects of 50 µg/L atrazine with supplemented algae on morphometrics, particularly for females (i.e., 50*s* treatment group). Effects of atrazine on herbivorous aquatic organisms are always subject to potential confounding effects of herbicidal activity. Indeed, studies are often designed specifically to examine indirect effects of atrazine and other contaminants on amphibians resulting from loss of food resources (Relyea et al. 2005). While an algae supplemented treatment groups was included to guard against such an event, weekly chlorophyll-a measurements indicate algal loss due to atrazine was not fully negated. Compared to controls and lower atrazine treatments, 50*s* chlorophyll-a measurements were on average 1398 mg/L lower. Comparatively however, 50*s* measurements were on average greater than non-supplemented 50 µg/L by 2296 mg/L. Regardless, the resulting morphometrics of 50*s* females including smaller body mass and ovaries compared to controls, was not expected. These effects were limited to 50*s* treated females however. We assume, but cannot confirm, that our

supplemented algae were similar to the algal communities in the exposure tanks. Different algae vary in nutritional quality and some have toxic properties (Pryor 2003; Kupferberg et al. 1994). However, the lack of effect on males would suggest that toxicity was not an issue. Female *A. blanchardi* are ultimately larger than males and grow faster (McCallum et al. 2011) and a reduction in nutrient quantity and/or quality could have disproportionate effects on female growth during early maturation (Schmidt et al. 2012; Scott and Fore 1995). Supplementation of algae could also have negatively affected nutrition by decreasing the relative proportion of tadpole food consumed in the supplemented tank. If algae were generally of lower nutritional quality than tadpole food, tadpoles in the supplemented atrazine treatment might have on average consumed fewer calories during development, resulting in lower body weight and size post-metamorphosis (Kupferberg 1997; Scott et al. 2007).

In addition to atrazine exposure, we exposed *A. blanchardi* larvae to two concentrations of estradiol (2.3 and 25 µg/L). Despite results from other studies, albeit with different species, (Kloas et al. 2009; Mackenzie et al. 2003; Langlois et al. 2010) in which these concentrations elicited effects on developmental rates and sex ratio, there were limited effects attributable to estradiol in our study. Indeed, in previous studies involving *X. laevis, Rana curtipes,* and *R. pipiens*, delayed development and increased female sex ratios were observed at concentrations similar to, or lower than, those of the present study (Mackenzie et al. 2003; Sharma and Patino 2010; Saidapur et al. 2001; Phuge and Gramapurohit 2015). For instance, concentrations 100 times less than the highest concentrations used in the current study have been shown to induce complete feminization of *X. laevis* (Kloas et al. 2009). Furthermore, while complete feminization or sex reversal of genetic males has been noted in several studies (Saidapur et al. 2001; Phuge and Gramapurohit 2015), feminization in our study was limited to increased oviduct development in

male frogs exposed to estradiol. In addition to developmental rate and sex ratio, a common endpoint for amphibian estradiol studies includes body size morphometrics (Pickford et al. 2003; Lutz et al. 2008; Langlois et al. 2010). Interestingly, morphometric responses to estradiol exposure appear mixed. For instance, *Euphlyctis cyanophlyctis, Rhinella arenarum,* and *X. laevis* larvae were significantly larger than controls when exposed to estradiol concentrations ranging from 0.2 - 100 µg/L (Phuge and Gramapurohit 2015; Lutz et al. 2008; Brodeur et al. 2013; Lent et al. 2018), whereas body sizes of estradiol-exposed *A. blanchardi* in the present study were decreased.

Estradiol significantly influenced both ovary size and morphological development, which were observed findings in comparable amphibian estradiol studies, but such effects appear to be species dependent (Windle et al. 2021; Piprek et al. 2012; Hayes and Mendez 1999). For instance, similar to our findings, ovaries of *E. cyanophlycti* treated with 10 and 100 µg/L were significantly smaller than control animals (Hayes and Mendez 1999). Conversely, *X. laevis* larvae treated with estradiol concentrations ranging from 0.2 – 6 µg/L exhibited larger ovaries compared to controls (Lutz et al. 2008). Histopathologic assessment methods differ between studies but overall remain similar with respect to oocyte maturation and lumen size (Wolf et al. 2010; Piprek et al. 2012). Similar to ovary size, ovary development varied between studies and at concentrations both lower and higher than ours (Piprek et al. 2012; Mackenzie et al. 2003). As an example, ovary development of *Bombina bombina* and *Bombina variegata* exposed to 100 µg/L estradiol was significantly inhibited, while ovary development of *X. laevis* and *Hyla arborea* exposed to the same concentration estradiol was not affected (Piprek et al. 2012). It may be possible increased estradiol exposure during development induced a negative feedback response or diminished

estradiol receptor activity during gonadal development resulting in smaller ovaries (Newbold et al. 2004). For instance, Newbold et al. (2004) noted decreased uterine weight and estrogen receptor levels in mice treated with the synthetic estrogen diethylstilbestrol as neonates (1-5 days post-parturition). Additionally, in the current study, mean testes area and development were not statistically affected by estradiol exposure. However, estradiol exposure typically decreases testes size as well as development (Piprek et al. 2012).

The limited sensitivity to exogenous estradiol in this species was noted in a previous study (Windle et al. 2021). Compared to that study, estradiol concentrations in the current experiment were higher by an order of magnitude, with limited additional effect on gonadal development. Differences in sensitivity compared to other studies could be related to variation in testing conditions and species sensitivity. The outdoor enclosures experienced algae growth and detritus accumulation, despite frequent water exchanges, and these factors may have impacted estradiol stability and bioavailability (Shi et al. 2010). However, despite some estradiol losses between exchanges, concentrations were maintained above 25% on average and returned to close to nominal concentrations at each exchange (Table 1). Thus, although there may have been minor temporary reductions in dose relative to nominal, the active concentration was still much higher than those reported to have a strong effect in previous studies (Kloas et al. 2009). Taken as a whole, these results suggest that *A. blanchardi* is not very susceptible to exogenous estradiol exposure. Timing of exposure could potentially contribute to the decreased sensitivity, as susceptibility windows have been demonstrated with other amphibian species (Piprek et al. 2012). However, exposure began very early in development (i.e., free swimming stage) so it is unlikely that this sensitivity window was missed. Furthermore, there is no known mechanism to suggest

that decreased uptake or increased metabolic degradation of estradiol is likely. However, further research would be needed to eliminate such mechanisms from consideration. Future studies with controlled laboratory exposure of *A. blanchardi* tadpoles is warranted to determine if any aspects of the field exposure might have reduced susceptibility to estradiol.

Conclusion

Under the testing conditions used in this study, we found no strong evidence that *A. blanchardi* development is sensitive to either atrazine or estradiol exposure throughout larval development. The low recovery of individuals, which is common for toxicity tests using this species, potentially reduces the ability to observe and quantify developmental effects that occur at a low frequency (Hoskins and Boone 2018; Hoskins et al. 2019). Regardless, our study would have identified any overt development effects occurring in a large percentage of an exposed population. Despite a trend toward decreased body size and feminization via oviduct development, *A. blanchardi* does not appear to be as sensitive to estradiol exposure as other amphibian species (Lutz et al. 2008; Mackenzie et al. 2003). Given the low sensitivity to estradiol, care should be taken in extrapolating these results to other species, such as *Rana*, where exposure to exogenous estrogens has been associated with demonstrable effects.

Acknowledgements

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Tables and Figures

Figure 1. *Top left:* Enclosure with metamorphosing *A. blanchardi. Top right:* Enclosure with juvenile *A. blanchardi. Bottom left* Outdoor enclosures covered by 60% UV filtered shade cloth and 55% light filtered Farm Plastic®. *Bottom right:* Juvenile *A. blanchardi.*

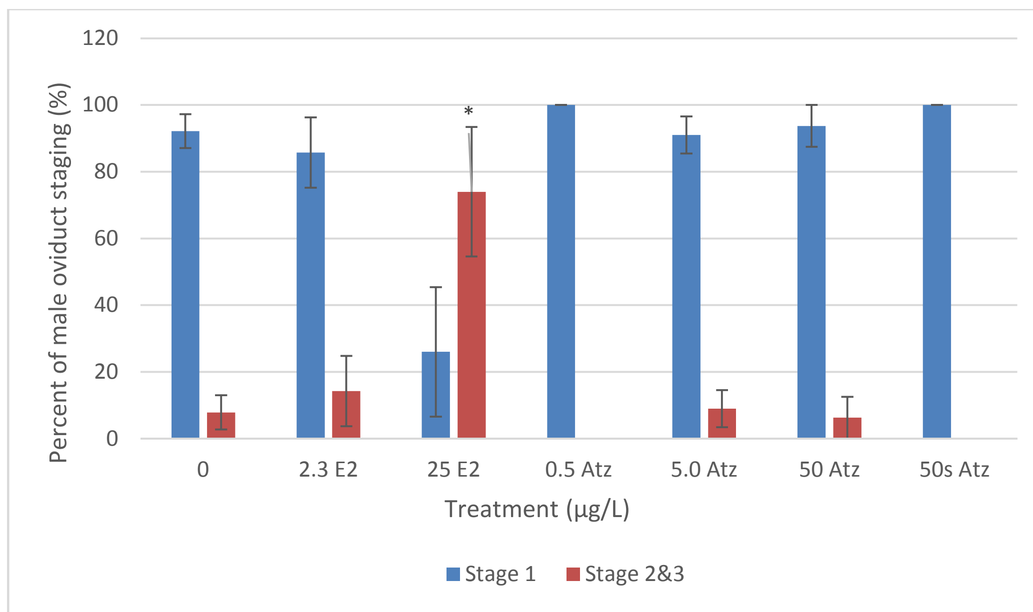

Figure 2. Mean percentage (±SE) of juvenile male *Acris blanchardi* with oviduct development at approximately 60 days post-metamorphosis (n = 5 replicates) following larval exposure to estradiol and atrazine. Algae supplemented atrazine treatment labeled as 50*s*. Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations. An asterisk (*) indicates treatment of significance.

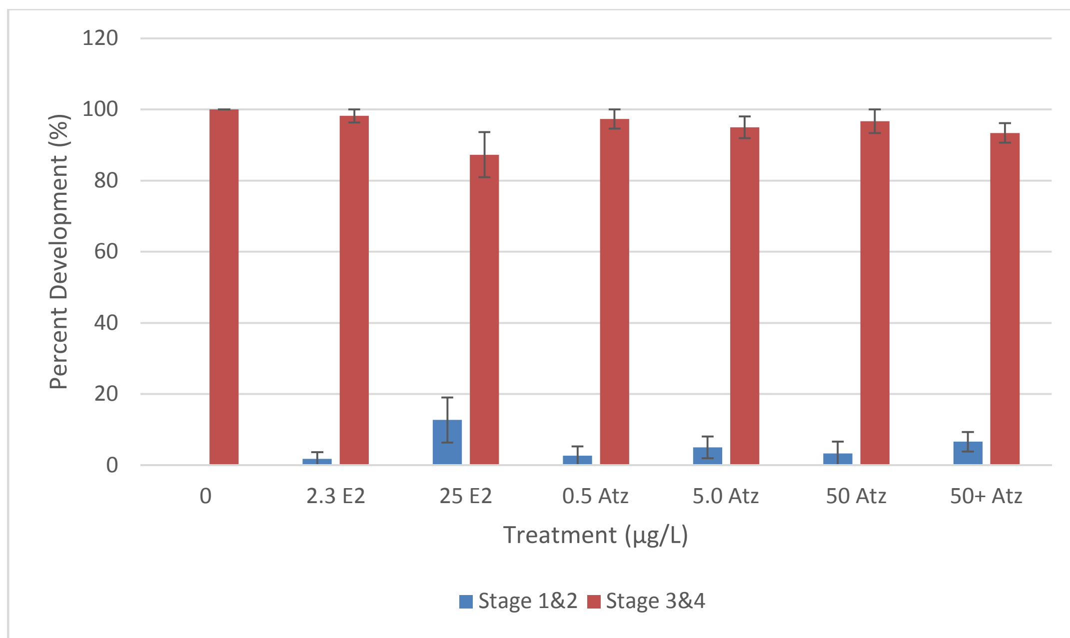

Figure 3. Mean percentage (±SE) of juvenile female *Acris blanchardi* oviduct development at approximately 60 days post-metamorphosis (*n* = 5 replicates) following larval exposure to estradiol and atrazine. Algae supplemented atrazine treatment labeled as 50*s*. Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations.

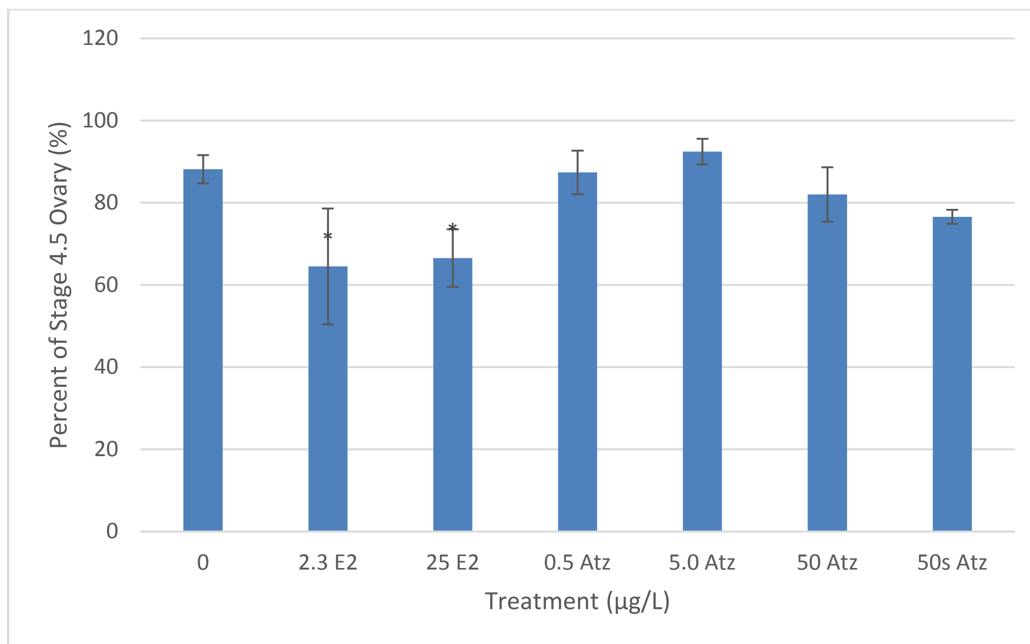

Figure 4. Mean percentage (±SE) of juvenile female *Acris blanchardi* with stage 4.5 ovaries at approximately 60 days post-metamorphosis (n = 5 replicates) following larval exposure to estradiol and atrazine. Algae supplemented atrazine treatment labeled as 50*s*. Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations. An asterisk (*) indicates treatment of significance.

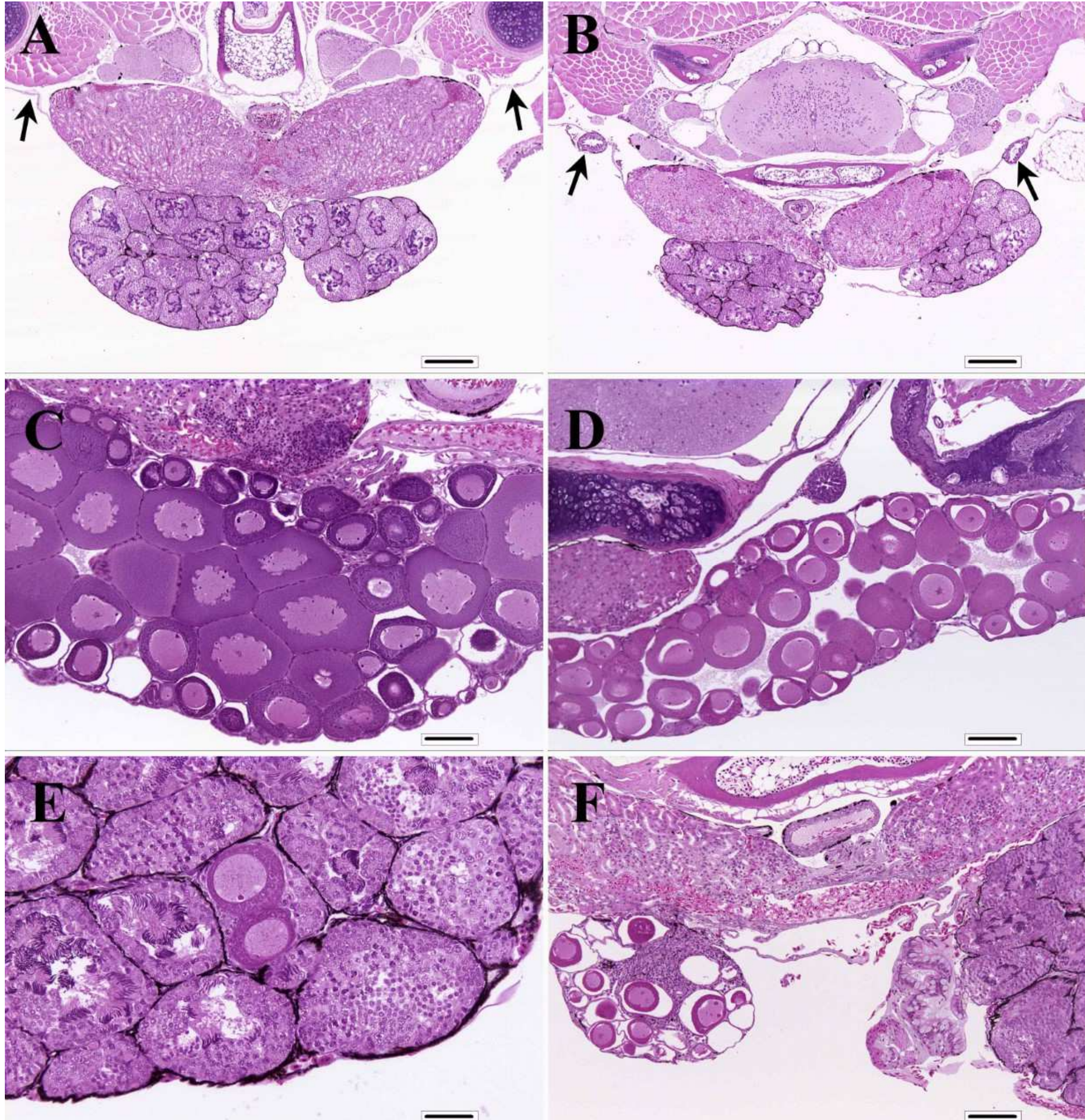

Figure 5. Estrogen-induced abnormal or delayed gonad development of juvenile Acris blanchardi at 60 days post-metamorphosis. Male feminization in the form of oviduct retention shown in image B compared to an unaffected male in image A. Decreased ovary stage in female A. blanchardi demonstrated in images C and D. Image C illustrates a stage 4.5 ovary while image D which illustrates a stage 4 ovary. Male feminization in the form of testicular oocytes or mixed sex (i.e., testicular and ovarian tissue in the same individual) illustrated in images E and F, respectively. Bar scaling: A and B = 250 microns; C, D, F = 100 microns; E = 50 microns.

Table 1. Water Analytical Values

Estradiol			Control	2.3	25		
E2	**After Renewal**	**µg/L**	<0.2	1.9 (0.7)	26 (6)		
		%	-	83 (25)	104 (24)		
	Before Renewal	**µg/L**	<0.2	0.69 (0.3)	6.8 (2.1)		
		%	-	30 (12)	27 (8.4)		
Estrone	**After Renewal**	**µg/L**	<0.2	6.1 (4.1)	33 (22)		
		%	-	-	-		
	Before Renewal	**µg/L**	<0.2	6.1 (7.4)	32 (24)		
		%					
Atrazine			**Control**	**0.5**	**5.0**	**50**	**50*s***
	After Renewal	**µg/L**	<0.05	0.45 (0.1)	5.0 (0.6)	45 (6)	46 (8)
		%	-	90 (0.2)	100 (12)	90 (12)	92 (16)
	Before Renewal	**µg/L**	<0.05	0.64 (0.1)	5.1 (0.6)	53 (7)	51 (5)
		%	-	128 (10)	102 (12)	106 (14)	102 (10)

Mean (±SD) water concentration of 17β-estradiol (E2), estrone, and atrazine in each enclosure before and after water renewal (n = 10). Water renewal or exchanges performed every 3 to 4 days in which 70% of water was removed and replaced with freshly spiked filtered water. Algae supplemented atrazine treatment labeled as 50*s*.

Table 2. Gonad histopathology definitions (USEPA 2015).

Tissue	Stage	Diagnostic Criteria
Testis	**1**	Undifferentiated gonad.
	2	Individual primary spermatogonia and undifferentiated somatic cells populate the medullary region.
	3	Seminiferous tubules with primary spermatogonia and cysts of secondary spermatogonia.
	4	Primary spermatocytes with rete testis formation; may have occasional spermatocysts that contain round or elongated spermatids.
	5	All stages of spermatogenesis evident.
Ovary	**1**	Undifferentiated gonad.
	2	Gonad identifiable as an ovary based on the presence of a discontinuously open lumen lined with epithelial cells; germ cells within the cortex consist of primary oogonia, cysts of primary mitotic oogonia, secondary oogonia, and very early meiotic oocytes.
	3	First appearance of diplotene oocytes in cortex; the most prevalent germ cell types at this stage are cysts of secondary oogonia and cysts of leptotene-pachytene primary meiocytes.
	4	Pre-vitellogenic (Dumont Stage I) diplotene oocytes are the most prevalent germ cell type observed by area and absolute cell counts; the central lumen is proportionately smaller while the whole ovary grows greatly in size and volume due to the growth of the oocytes; cysts in earlier stages of oogenesis become fewer in number and are located along the periphery of ovary.
	4.5	Ovary contains several large oocytes in which the germinal vesicle (nucleus) is beginning to deteriorate. Nuclear deterioration is characterized by increased irregularity in the contour of the nuclear envelope, nuclear blebbing and/or fragmentation, and scattering of the perinucleoli. In addition, the ooplasm of the enlarged oocytes is often slightly more eosinophilic than that of its smaller, less mature cohorts, and faint alveolar spaces are often evident near the cell periphery.
	5	Ovary consists almost entirely of vitellogenic oocytes (Dumont Stage IV); previtellogenic diplotene oocytes can be found along the periphery of the ovary and germ patches of primary and secondary oogonia are difficult to locate.
Oviduct (Müllerian Duct)	**1**	Oviduct consists of a fibrous tag attached to the suspensory ligament or is missing entirely.
	2	Oviduct has a lumen lined by a single layer of epithelial cells.
	3	Oviduct lined by multiple layers of epithelial cells, that form frond-like internal projections.
	4	Oviduct lined by basophilic glands.
	5*	Basophilic glands distended with mucoid material

Summary of diagnostic criteria for gonad histopathologic assessment. Staging based on criteria established in EPA 2015.

Table 3. Recovery, time to metamorphosis, and percent female

Treatment (µg/L)	Starting number	Recovered (%)	Time to metamorphosis (Days)	Percent female (%)
Control	125	48 (7)	33 (0.9)	65 (8)
Estradiol				
2.3	125	62 (5)	33 (0.8)	65 (10)
25.0	125	57 (5)	33 (1.0)	76 (8)
Atrazine				
0.5	125	50 (8)	31 (1.1)	75 (7)
5.0	125	50 (6)	33 (1.4)	64 (6)
50.0	125	45 (10)	35 (1.4)	72 (8)
Atrazine + Algae				
50.0*s*	125	65 (4)	35 (1.1)	69 (9)
Wild	25	-	-	52

Mean (±SE) recovery, time to metamorphosis, and percentage female of juvenile *Acris blanchardi.* Recovery was defined as live collection upon termination of study approximately 60 days after metamorphosis. Female designation based on gross gonadal morphology. Algae supplemented atrazine treatment labeled as 50*s*. Wild group includes specimens collected from the original collection pond during late September of the same year as this study and thus represents the same cohort of metamorphs as used in the enclosures. Treatments presented as nominal concentrations.

Table 4. Body morphometrics

Treatment (µg/L)	Male		Female	
	Body Mass (mg)	Snout-vent length (mm)	Body Mass (mg)	Snout-vent length (mm)
Control	904 (73)[a]	20 (0.9)[a]	1169 (80)[a]	23 (0.7)[a]
Estradiol				
2.3	771 (66)[a]	21 (0.6)[a]	930 (61)[ab]	22 (0.5)[ab]
25.0	796 (93)[a]	20 (1.0)[a]	973 (81)[ab]	22 (0.7)[ab]
Atrazine				
0.5	988 (98)[a]	21 (0.5)[a]	1178 (169)[ab]	23 (0.8)[ab]
5.0	833 (101)[a]	21 (1.4)[a]	1208 (44)[a]	23 (0.6)[a]
50.0	921 (152)[a]	21 (1.7)[a]	1204 (111)[a]	23 (0.9)[a]
Atrazine + Algae				
50.0*s*	839 (22)[a]	21 (0.3)[a]	836 (53)[b]	21 (0.5)[b]
F-value	0.6	0.4	3.0	2.4
p-value	0.7	0.9	0.02*	0.05*

Mean (±SE) body mass and snout-vent length of juvenile *Acris blanchardi* approximately 60-days post-metamorphosis (n=5) following larval exposure to estradiol and atrazine. Algae supplemented atrazine treatment labeled as 50*s*. Body mass and snout-vent length measurements from frogs from the same enclosure were averaged prior to calculating treatment averages. Means with the same letter (i.e., a, b, c) did not differ (p = 0.05). Treatments presented as nominal concentrations.

Table 5. Gonad Size

Treatment (µg/L)	n	Ovary area (mm^2)	Testis area (mm^2)
Control	5	7.6 (0.3)[a]	1.5 (0.1)[a]
Estradiol			
2.3 E2	5	5.7 (0.4)[bc]	1.2 (0.1)[a]
25 E2	5	5.5 (0.6)[c]	1.3 (0.2)[a]
Atrazine			
0.05	5	7.4 (1.1)[abc]	1.8 (0.1)[a]
5.0	5	7.1 (0.2)[ab]	1.5 (0.1)[a]
50.0	5	6.5 (0.2)[abc]	1.5 (0.2)[a]
Atrazine + Algae			
50.0*s*	5	5.5 (0.4)[c]	1.4 (0.1)[a]
F-value		3.1	1.5
p-value		0.02*	0.21

Mean (±SE) gonad size (ovary and testis area) of juvenile *Acris blanchardi* approximately 60-days post-metamorphosis (n = 5) following larval exposure to estradiol and atrazine. Algae supplemented atrazine treatment labeled as 50*s*. Ovary and testis area based on ellipse area calculation ($ab\pi; a = \frac{length}{2}, b = \frac{width}{2}$). Gonad measurements from frogs from the same enclosure were averaged prior to calculating treatment averages. Means with the same letter (i.e., a, b, c) did not differ (p = 0.05). Treatment concentrations presented are nominal concentrations.

Table 6. Testicular oocytes

Treatment (µg/L)	Number of Males	Grade Severity
Control	1	2
Estradiol		
2.3 E2	1	2
25 E2	2	2 & 3
Atrazine		
0.05	1	1
5.0	2	2
50.0	1	2
Atrazine + Algae		
50.0*s*	1	1

Number of juvenile male *Acris blanchardi* approximately 60 days post-metamorphosis with testicular oocytes (TO) and respective grades of severity following larval exposure to estradiol and atrazine. Algae supplemented atrazine treatment labeled as 50*s*. Treatment concentrations presented are nominal concentrations.

Table 7. Study Comparison of Percent Female

Source	**Treatment (µg/L)**	**Percent (%) Female**
Hoskins and Boone 2018	**Control**	30
	Atrazine	
	0.1	60*
	1	50*
	10	60*
Windle et al. 2021	**Control**	44
	Estradiol	
	0.015	49
	0.053	46
	0.18	40
	0.64	54
	2.3	54
	Wild	40

Percent female of juvenile *A. blanchardi* in comparable studies in which, *A. blanchardi* were exposed to either atrazine or estradiol throughout development. Hoskins and Boone (2018) data was based on pooled data while Windle et al. (2021) based on mean percentage across 5 replicates (n = 5). Values labeled with an asterisk (*) presented as approximate as they were collected from a figure rather than precise values. Wild individuals were juvenile *A. blanchardi* captured in the fall of the same year in which the Windle et al. (2021) study was performed.

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CHAPTER V

DEVELOPMENT OF *ACRIS BLANCHARDI* FOLLOWING 17β-ESTRADIOL EXPOSURE IN AN INDOOR SYSTEM

Abstract

In outdoor enclosure studies, *Acris blanchardi* has shown marginal response to 17β-estradiol (E2) despite using concentrations known to elicit complete feminization in other amphibian species. In this study, we examine the effects of E2 on A. blanchardi development in a controlled laboratory setting to factor out any unknown influences that might have masked effects in previous mesocosm studies. Larval frogs were exposed to the same concentrations we used in outdoor experiments (2.3 and 25 μg/L) and into feminization (e.g., sex ratio), we examined E2 influence on recovery, time to metamorphosis, body size (body mass and snout-vent length), gonad size, and gonad development. At 35 days post-metamorphosis, E2 did not influence mortality, sex ratio, time to metamorphosis, or body size. Ovary size of females treated with 25 μg/L E2 were on average 0.5 mm^2 smaller than ovaries of control animals. No further effects on ovary development were noted. Testis size was not influenced by E2 exposure, but testicular development (i.e., spermatogenesis) significantly decreased in males exposed to 25 μg/L. Feminization in the form of male oviduct development was not significantly influenced by E2 exposure. As observed in previous mesocosm studies, overall exogenous E2 had limited effect on *A. blanchardi* development compared to other anuran species (*e.g., X. laevis*).

Introduction

We previously reported on the design of a developmental toxicity assay using 17β-estradiol (E2) exposure on larval *Acris blanchardi* in outdoor enclosures (Windle et al. 2021; Windle et al. 2022). Despite the use of E2 concentrations shown to elicit complete or partial feminization of genetic males in other species including *Xenopus laevis, Rana curtipes,* and *Rana pipiens* (Lutz et al. 2008; Saidpur et al. 2001; Mackenzie et al. 2003), we found limited comparative effects. For example, although there was a significant increase in oviduct formation in male *A. blanchardi* treated with the 25 µg/L E2, the effect was minimal in comparison to the complete feminization or intersex development of species such as *X. laevis* at a 100x lower concentration (Lutz et al. 2008). Different factors could explain observed differences in sensitivity including the species used, when exposures occurred and for how long, and other experimental design nuances. Our previous studies utilizing outdoor enclosures benefit from a level of environmental realism lost in laboratory studies, yet likewise may suffer from a lack of control over factors that mask or enhance effects. Indeed, a notable difference between our study and those that report significant E2 influenced developmental changes is the chosen venue of exposure (Sharma et al. 2010; Saidapur et al. 2001; Oka et al. 2006) and the majority of the studies reporting E2 influence are completed in a controlled laboratory experiment. Divergence between the outcomes of laboratory and outdoor studies has been reported for other native amphibian species such as *Rana dalmatina* and *A. blanchardi* (Miko et al. 2015; Hoskins et al. 2018; Hoskins et al. 2019). For instance, mortality and time to metamorphosis of *R. dalmatina* exposed to varying concentrations of glyphosate were significantly greater when exposed in the laboratory rather than outdoor enclosures (Miko et al. 2015). Similarly, despite the use the same

concentrations of atrazine, Hoskins et al. (2018 and 2019) observed increased feminization post-laboratory exposure but not after exposure in outdoor enclosures.

Given these observations from other studies and the surprising lack of sensitivity we observed for A. blanchardi exposed to excessive levels of E2 during a very sensitive period of development in outdoor enclosures, we designed a replicate laboratory study to further assess *A. blanchardi* sensitivity to E2 exposure without the potential confounding effects of environmental factors experienced outside the laboratory.

Methods

Adult and larval collection

Collection of amplexed pairs of *A. blanchardi* began in late May 2021 and were collected as in previous years from ponds in Payne County, Oklahoma (36.1450ºN, 97.0068ºW) (Windle et al. 2021). Hatched larvae from two clutches (i.e., separate oviposits from different pairs) were reared to Gosner stage 26 and then haphazardly sorted into groups of 20. Sorted larvae were randomly assigned to treatment tanks in the laboratory. Treatment with estradiol began June 2, 2021 once larvae were placed in treatment tanks.

An additional 60 metamorphosed *A. blanchardi* were collected late September 2021 for histopathology. Histopathologic findings from these individuals were used for comparison between wild juvenile *A. blanchardi* and our treated metamorphs. All procedures were completed following approved Oklahoma State University IACUC protocols.

Experimental exposures

Treatments consisted of five replicates for each E2 treatment concentration (2.3 and 25 μg/L) in addition to a negative control. Larvae were treated in 25-L steel tanks containing 20-L of dechlorinated water. Throughout the experiment the room was maintained on a 12:12 hour light cycle and kept at 23±1ºC. Water temperature remained relatively consistent with room temperature and tank water heaters were used to maintain 23±1 ºC. to maintain water temperature around 23±1ºC. Seventy percent water exchanges were performed bi-weekly with freshly spiked water. Treatment was discontinued for individual animals once larvae reached GS 45 and were moved to terrestrial tanks lined with moistened autoclaved and coconut shell mix. Tadpoles were fed a mixture of TetraMin© and Spirulina and metamorphs were fed fruit flies ad libitum.

Analytical Chemistry

Weekly water samples were collected before and after each water exchange to measure water quality and estrogen (i.e., estrone and estradiol) concentrations, resulting in a total of 36 samples. In addition to analytical chemistry, tank water was assessed for water quality including ammonia, pH, ammonia, and conductivity.

Metamorph collection and measured endpoints

Metamorphs were reared for an additional 35 days post-metamorphosis before euthanasia via submersion in 5% buffered MS-222. Euthanized metamorphs were measured for body mass (±1 mg; BM) and snout-vent length (±1 mm; SVL) before dissection in preparation for histopathology. All viscera was removed to expose the kidney-gonad complex which was left in the body cavity and then submerged in Bouin's fixative (Thermo Fisher Scientific Cat.No.:50-

320-01). After 24 hours, fixed tissues were rinsed with 70% ethanol and moved to 10% buffered formalin. Fixed tissues were then sent to Experimental Pathology Laboratories for hisopathologic analysis. Prior to shipment, fixed gonads were photographed for later measurement. Gonad size (i.e., ovary and testis area) was based on the ellipse area ($ab\pi; a = \frac{length}{2}, b = \frac{width}{2}$. Histopathologic criteria for oviduct, ovary, and testis development were based on guidelines established by the US EPL (2016) for *X. laevis.* More specific details for gonad scoring can be found in Table 1. In brief, however, ovary development based on the degree of vitellogenic oocyte maturation while testis development based on degree of spermatogenesis.

Additional modification of our past experimental design includes modifying the endpoint time to metamorphosis (days). In the past, this endpoint was defined as the time when treatment was discontinued (i.e., 75% population reaches GS 45) for the entire unit. Due to the sporadic nature of metamorphosis, here we define time to metamorphosis as number of days between the beginning of treatment and when individual metamorphs were moved to terrestrial tanks. In addition to time to metamorphosis, recovery and sex ratio, based on gross gonad morphology, were quantified.

Statistical analysis

Body morphometric data including BM, SVL, and gonad size were analyzed using a one-way analysis of variance blocked by replicate (Proc GLM Mixed, SAS Version 9.4), (SAS Institute, Inc., Cary, NC, USA). These endpoints were separated by sex due to sex dependent size differences. Similarly, endpoints not influenced by sex including mean recovery and time to metamorphosis were analyzed using one-way analysis of variance blocked by replicate. Sex ratio

and histopathologic findings (i.e., ovary, testis, oviduct development) were analyzed via Chi-square (χ^2). Histopathologic scores were pooled in instances where one or only two individuals were scored with a particular grade. For example, one instance occurred where a female oviduct was scored as stage 1 compared to 2 or 3. This individual was grouped as a stage 2 for statistical analysis. Similarly, a single male was scored with a stage 3 oviduct rather than 1 or 2 and thus grouped in stage 2.

Results

Sex Ratio, recovery, time to metamorphosis

Recovery of *A. blanchardi* at 35 days post-metamorphosis was not influenced by E2 treatment (Table 2; p = 0.9). However, mortality was high for both larvae and metamorphs. On average, 48% of experimental animals reached metamorphosis and 25% of the original population survived to 35 days post-metamorphosis (Table 2). Sex ratio was not influenced by E2 treatment (χ^2 = 0.1; p = 0.5) as percent females ranged from 53% in the control group to 62% in the 25 µg/L E2 (Table 2). Time to metamorphosis did not differ between treatments and ranged between 51 and 56 days (p = 0.49). One control individual failed to develop by termination of experiment (100 days) and was omitted from the study.

Somatic and Gonadal Morphology

Estradiol did not influence BM or SVL for females (p = 0.54 and 0.67, respectively) or males (p = 0.88 and 0.58, respectively) (Table 3). However, ovary area was 0.5 mm^2 smaller for females exposed to 25 µg/L compared to control animals (Table 4; p = 0.02). Mean testis area did

not differ significantly among treatments, ranging from 0.5 mm^2 in controls to 0.3 mm^2 in 25 µg/L E2 treated males (Table 4; $p = 0.35$).

Histopathology

Male oviduct development (i.e., feminization) did not significantly vary among treatments (Figure 1; $\chi^2 = 4.0$; $p = 0.11$). Testicular development (i.e., spermatogenesis) was significantly influenced by E2 exposure (Figure 2; $\chi^2 = 7.6$; $p = 0.02$). Indeed, 30% of males treated with 25 µg/L had stage 5 testis compared to 85% of control males with stage 5 testis (Figure 1). One intersex individual (i.e., having both ovary and testis tissue) was observed in the 25 µg/L E2 treatment group.

Female oviduct development was not influenced by E2 treatment ($\chi^2 = 0.5$; $p = 0.77$).. Ovary development was variable among treatments, with 17 and 27% of females treated with 2.3 and 25 µg/L E2, respectively, scored with stage 4.5 ovaries. In comparison, 36% of control females were scored with stage 4.5 ovary development. However, these differences were not significant ($\chi^2 = 1.2$; $p = 0.55$; Figure 3).

Discussion

Estradiol did not significantly influence *A. blanchardi* sex ratio, recovery, time to metamorphosis, or body size. While these results are largely supported by our previous study conducted outdoors, contradicting results from similar studies suggests species-dependent sensitivity (Windle et al. 2021; Windle et al. 2022 Wolf et al. 2010, Mackenzie et al. 2003). For instance, complete and partial feminization has been reported for *X. laevis* and *R. pipiens* when exposed to concentrations well below those used in this study (Sharma and Patino 2010; Lutz et

al. 2008; Kloas et al. 2009; Mackenzie et al. 2003). Similar species sensitivity to estrogenic contaminants has been observed post exposure to17α-ethinylestradiol (EE2), albeit at relatively high concentrations (e.g., 50, 500, 5000 ng/L; Tamschick et al. 2016). For example, at 50 ng/L no sex reversal was observed in *Hyla arborea* and *Bombina viridis* while a 31% increase in male sex reversal was observed in *X. laevis.* Furthermore, observed feminization in our studies was limited to male oviduct development while compared to other studies that observed complete feminization of genetic males, intersex, and mixed sex (Mackenzie et al. 2003, Wolf et al. 2010).

Like E2-related feminization, body size morphometrics from previous studies are relatively inconsistent, including our own findings (Windle et al. 2022). However, the observed decreased body size observed in Windle et al. (2022) was limited to females exposed to the same concentrations used here (i.e., 2.3 and 25 μg/L). This sex-dependent effect does not appear to occur in other E2-treated amphibians (Phuge and Gramapurohit 2015; Lutz et al. 2008; Brodeur et al. 2013). Additionally, concentrations ranging from 0.2 – 100 μg/L resulted in a significant increase in body size in species including *Euphlyctis cyanophlyctis, Rhinella arenarum,* and *X. laevis* (Phuge and Gramapurohit 2015; Lutz et al. 2008; Brodeur et al. 2013; Lent et al. 2018).

We found ovary size to be significantly decreased in female *A. blanchardi* treated with 25 μg/L which is consistent with findings of our past outdoor study (Windle et al. 2022). However, as before, E2-related effects on gonad size appear species-dependent (Piprek et al. 2012; Hayes and Mendez 1999; Lutz et al. 2008). For instance, Lutz et al. (2008) reported increased ovary size in *X. laevis* when exposed to 0.2 – 6 μg/L E2 through larval development. Conversely, ovaries of *E. cyanophlycti* exposed to higher E2 concentrations (10 and 100 μg/L) were significantly smaller (Hayes and Mendez 1999). Ovary development of 25 μg/L E2 treated females was notably

decreased compared to controls and as with multiple endpoints in this study, decreased ovary development is consistent with our previous findings (Windle et al. 2021; Windle et al. in review). While assessed via different histopathologic methods, Piprek et al. (2012) observed decreased ovary development in *B. bombina* and *B. variegate* exposed to 100 µg/L E2 but not in *X. laevis* and *H. arborea* exposed to the same concentration. We have speculated in the past that decreased ovary size and development may be due to diminished estradiol receptor activity post-continuous larval exposure but have not yet confirmed this hypothesis (Newbold et al. 2004).

A novel finding in this experiment, in comparison to our outdoor studies, was decreased testicular development (i.e., spermatogenesis) in 25 µg/L treated males. Potential reasoning for this divergence from our past studies may be due to the time in which we collected metamorphs. In our outdoor studies, metamorphs were collected at 60 days post-metamorphosis while metamorphs in this study were collected at 35 days. In the 25 difference, it is possible that a level of spermatogenetic recovery occurred in the 2 month metamorphs. Indeed, in a similar study, decreased testicular development was observed in *B. bombina* and *B. variegate* metamorphs that were collected immediately after metamorphosis (Piprek et al. 2012). However, the concentration used by Piprek et al. (2012; 100 µg/L), was notably higher than concentrations used in this study. Conversely, Hu et al. (2008) reported increased testicular development in *X. laevis* after exposure to 1 µg/L E2. Regardless, a degree of spermatogenetic recovery has been observed in multiple fish species post-exposure to various contaminants (Lor et al. 2015; Kida et al. 2016). In future experiments, it may be beneficial to sacrifice subsets of animals at various timepoints post-exposure to better assess developmental recovery. Despite observed decreased testicular development, we did not observe E2-related effects on testicular size. Interestingly, the opposite

effect has been observed in *X. laevis* after exposure to E2 concentrations ranging from 0.015 – 1.5 μg/L (Lutz et al. 2008).

Conclusion

The overall results of this study, in addition to our past studies, demonstrate reduced E2 sensitivity regardless of exposure method (i.e., outdoor or laboratory). Indeed, *A. blanchardi* development does not appear sensitive to concentrations notably higher than those reported to elicit developmental effects in other amphibian species such as *X. laevis* (Lutz et al. 2009). A limitation we encountered with this species, primarily in the laboratory setting, was relatively high mortality of larvae. While we do not believe our low recovery significantly diminishes the importance or reliability of our results, increasing recovery is a goal for future experiments.

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Tables and Figures

Table 1. Gonad histopathology definitions (USEPA 2015).

Tissue	Stage	Diagnostic Criteria
Testis	**1**	Undifferentiated gonad.
	2	Individual primary spermatogonia and undifferentiated somatic cells populate the medullary region.
	3	Seminiferous tubules with primary spermatogonia and cysts of secondary spermatogonia.
	4	Primary spermatocytes with rete testis formation; may have occasional spermatocysts that contain round or elongated spermatids.
	5	All stages of spermatogenesis evident.
Ovary	**1**	Undifferentiated gonad.
	2	Gonad identifiable as an ovary based on the presence of a discontinuously open lumen lined with epithelial cells; germ cells within the cortex consist of primary oogonia, cysts of primary mitotic oogonia, secondary oogonia, and very early meiotic oocytes.
	3	First appearance of diplotene oocytes in cortex; the most prevalent germ cell types at this stage are cysts of secondary oogonia and cysts of leptotene-pachytene primary meiocytes.
	4	Pre-vitellogenic (Dumont Stage I) diplotene oocytes are the most prevalent germ cell type observed by area and absolute cell counts; the central lumen is proportionately smaller while the whole ovary grows greatly in size and volume due to the growth of the oocytes; cysts in earlier stages of oogenesis become fewer in number and are located along the periphery of ovary.
	4.5	Ovary contains several large oocytes in which the germinal vesicle (nucleus) is beginning to deteriorate. Nuclear deterioration is characterized by increased irregularity in the contour of the nuclear envelope, nuclear blebbing and/or fragmentation, and scattering of the perinucleoli. In addition, the ooplasm of the enlarged oocytes is often slightly more eosinophilic than that of its smaller, less mature cohorts, and faint alveolar spaces are often evident near the cell periphery.
	5	Ovary consists almost entirely of vitellogenic oocytes (Dumont Stage IV); previtellogenic diplotene oocytes can be found along the periphery of the ovary and germ patches of primary and secondary oogonia are difficult to locate.
Oviduct (Müllerian Duct)	**1**	Oviduct consists of a fibrous tag attached to the suspensory ligament or is missing entirely.
	2	Oviduct has a lumen lined by a single layer of epithelial cells.
	3	Oviduct lined by multiple layers of epithelial cells, that form frond-like internal projections.
	4	Oviduct lined by basophilic glands.
	5*	Basophilic glands distended with mucoid material

Summary of diagnostic criteria for gonad histopathologic assessment. Staging based on criteria established in EPA 2015.

Table 2. Mean (±SE) recovery, days to metamorphosis, and percentage female of juvenile *Acris blanchardi.* Recovery was defined as live collection upon termination of study approximately 35 days after metamorphosis. Female designation based on gross gonadal morphology. group includes specimens collected from the original collection pond during late September of the same year as this study and thus represents the same cohort of metamorphs as used in the enclosures. Treatments presented as nominal concentrations.

Treatment (µg/L)	Starting number	Recovered (%)	Days to metamorphosis	Percent female (%)
Control	100	27 (6)	33 (0.9)	53 (6)
Estradiol				
2.3	100	26 (9)	33 (0.8)	60 (11)
25.0	100	26 (8)	33 (1.0)	62 (11)
Wild	60	-	-	39

Table 3. Mean (±SE) body mass and snout-vent length of juvenile *Acris blanchardi* approximately 35 days post-metamorphosis following larval exposure to estradiol. An $n = 5$ used in analysis except for female controls, male 2.3, and male 25 groups in which an n of 4 was used due to a lack of individuals from one replicate. Body mass and snout-vent length measurements from frogs from the same enclosure were averaged prior to calculating treatment averages. Means with the same letter (i.e., a, b, c) did not differ ($p = 0.05$). Treatments presented as nominal concentrations.

Treatment (µg/L)	Male		Female	
	Body Mass (mg)	Snout-vent length (mm)	Body Mass (mg)	Snout-vent length (mm)
Control	224 (9)[a]	14 (0.3)[a]	262 (30)[a]	15 (0.6)[a]
Estradiol				
2.3	226 (34)[a]	14 (0.7)[a]	250 (24)[a]	15 (0.7)[a]
25.0	244 (26)[a]	14 (0.6)[a]	295 (33)[a]	14 (0.5)[a]
F-value	0.1	0.6	0.7	0.1
p-value	0.9	0.6	0.5	1.0

Table 4. Mean (±SE) gonad size (ovary and testis area) of juvenile *Acris blanchardi* approximately 35-days post-metamorphosis following larval exposure to estradiol. An $n = 5$ used in analysis except for female controls, male 2.3, and male 25 groups in which an n of 4 was used due to a lack of individuals from one replicate. Ovary and testis area based on ellipse area calculation ($ab\pi; a = \frac{length}{2}, b = \frac{width}{2}$). Gonad measurements from frogs from the same enclosure were averaged prior to calculating treatment averages. Means with the same letter (i.e., *a, b*) did not differ ($p = 0.05$). Treatment concentrations presented are nominal concentrations.

Treatment (µg/L)	Ovary area (mm^2)	Testis area (mm^2)
Control	1.9 (0.1)[a]	0.5 (0.1)[a]
Estradiol		
2.3 E2	1.7 (0.1)[ab]	0.4 (0.1)[a]
25 E2	1.4 (0.1)[b]	0.4 (0.1)[a]
F-value	4.3	1.1
p-value	0.02*	0.21

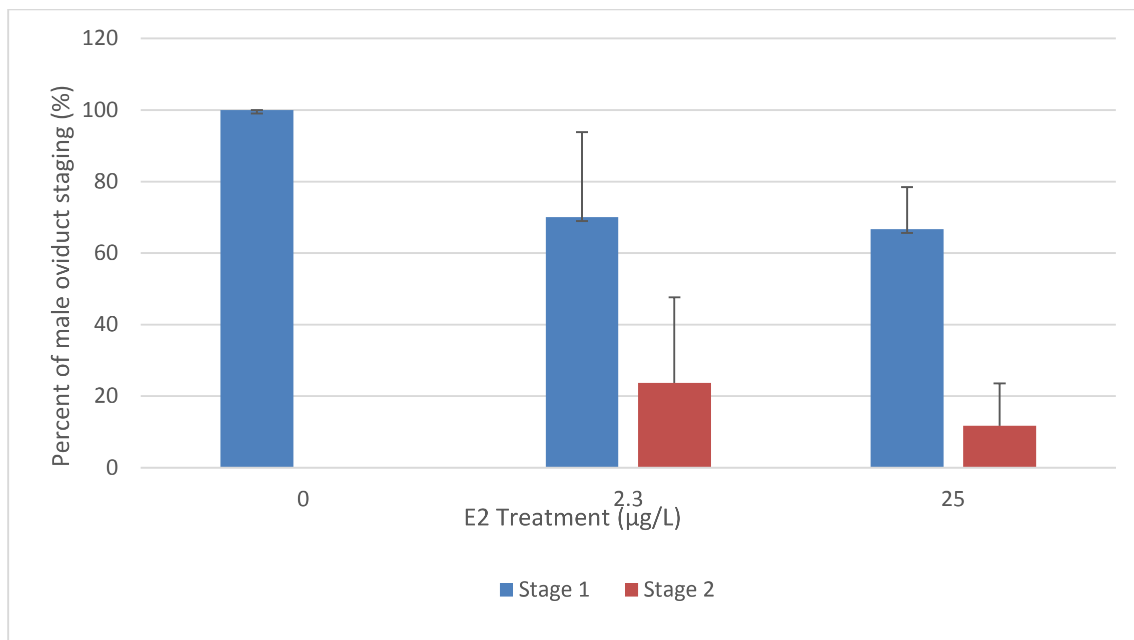

Figure 1. Mean percentage (±SE) of juvenile male *Acris blanchardi* with oviduct development at approximately 35 days post-metamorphosis (*n* = 5 replicates) following larval exposure to estradiol (E2). Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations.

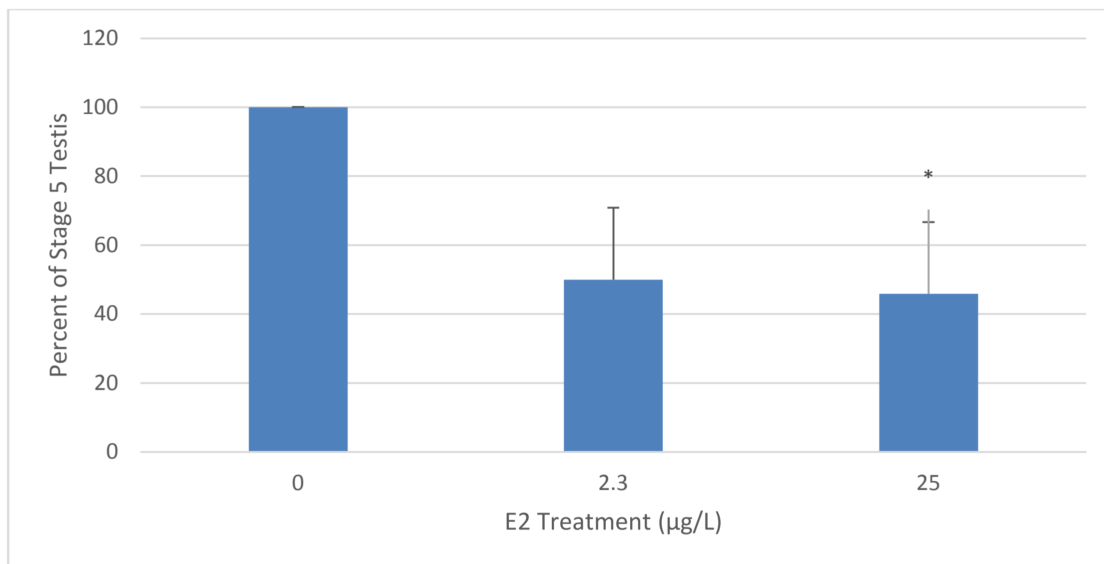

Figure 2. Mean percentage (±SE) of juvenile male *Acris blanchardi* with stage 5 testis at approximately 60 days post-metamorphosis (*n* = 5 replicates) following larval exposure to estradiol (E2). Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations. An asterisk (*) indicates treatment of significance.

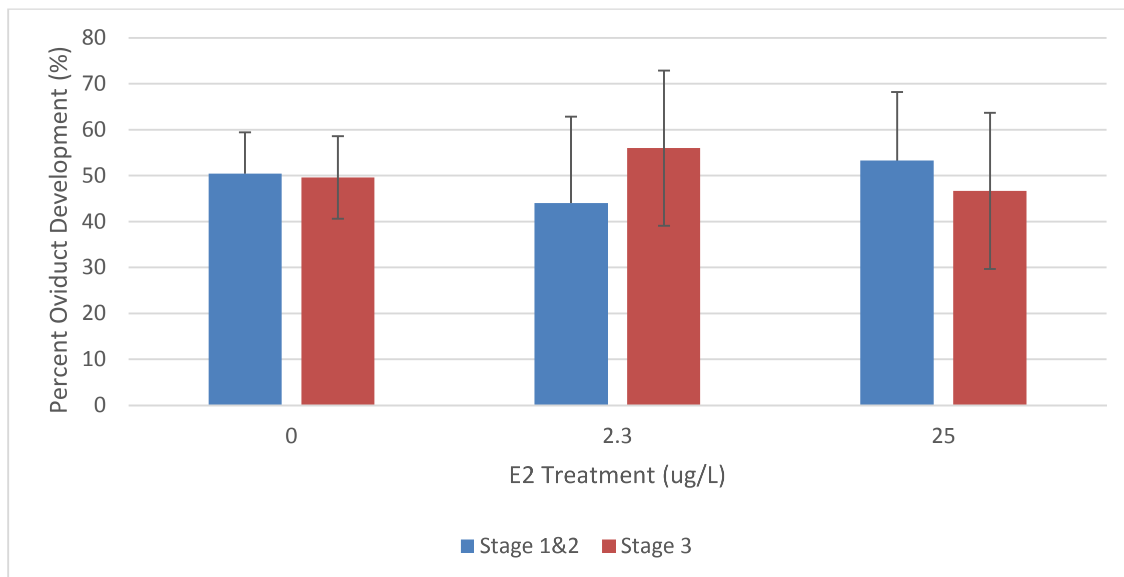

Figure 3. Mean percentage (±SE) of juvenile female *Acris blanchardi* oviduct development at approximately 60 days post-metamorphosis (*n* = 5 replicates) following larval exposure to estradiol (E2). Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations.

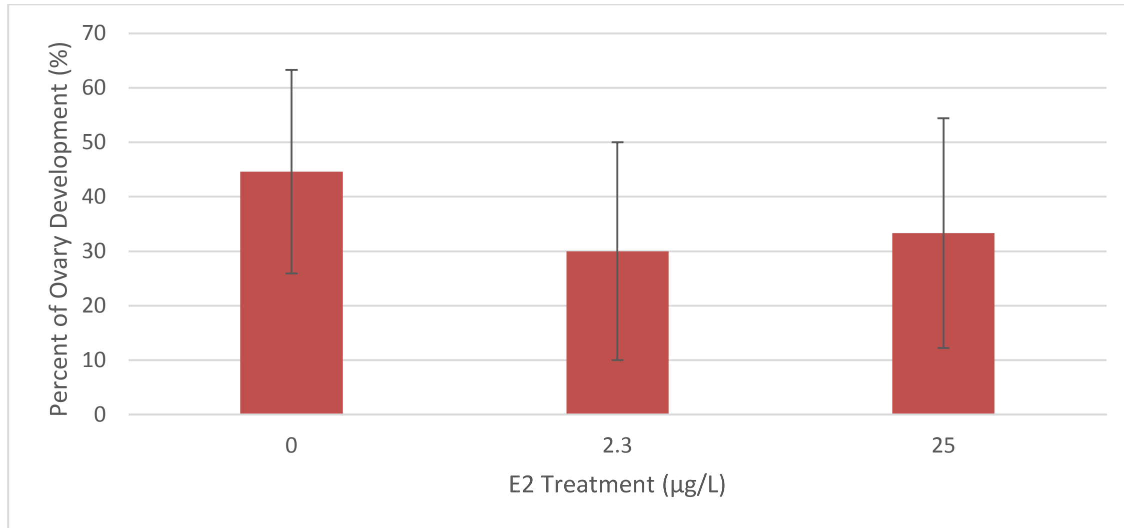

Figure 4. Mean percentage (±SE) of juvenile female *Acris blanchardi* with stage 4.5 ovaries at approximately 35 days post-metamorphosis (*n* = 5 replicates) following larval exposure to estradiol (E2). Data presented is averaged from replicates and respective treatment groups. Treatment concentrations presented are nominal concentrations.

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