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Growth and Development of Field Caught Blanchard's Cricket Frogs (*Acris blanchardi*)

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Abstract.*—Blanchard's Cricket Frogs (*Acris blanchardi*) live about 1 y and undergo development quickly. In addition to relatively quick metamorphosis, as seen in past outdoor studies, active spermatogenesis in juvenile males can be observed as early as 60 d after metamorphosis, and female development (i.e., ovary and oviduct) is notably progressed prior to overwintering. We assessed life-history traits of field caught *A. blanchardi* over three time points and three Oklahoma locations. We also compared the development of wild *A. blanchardi* from one location to findings from previous studies using the same site. We captured cohorts of wild *A. blanchardi* in the fall (i.e., prior to overwintering), spring (i.e., first observation of emergence), and summer (i.e., first observation of active breeding). Besides general growth and development, we assessed gonadal maturation via histopathology. Ovary, oviduct, and testis development significantly varied among seasons; however, variation in testis development among seasons was less pronounced than ovary and oviduct development. Significant among-pond variation was limited to oviduct development. An assessment of field captured *A. blanchardi* from two previous years indicated significant year-to-year variation in ovary development but not oviduct or testis development. Despite observed differences in gonad development, variations were often predictable and could be potentially attributed to environmental factors (e.g., water temperature). Nonetheless, the results of our study provide specifics of *A. blanchardi* development that may be useful for future studies that wish to use this native species as an amphibian research model.

Key Words.—amphibians; life history; North American native frog; reproductive development

Introduction

Amphibian development is complex, and a recent estimate is that for 33–44% of extant amphibian species, larvae occur in aquatic environments where exposure to chemical contamination can potentially occur (Liedtke et al. 2022). To better evaluate the exposure of amphibians to environmental contamination, it is important to understand the range of natural variability in development. The rate of larval development and time to sexual maturity can vary tremendously according to species (Ogielska and Kotusz 2004; Piprek et al. 2012). 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 (Denver 1997; Szekely et al. 2017). 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 (Ogielska and Kotusz 2004; Storrs and Semlitsch 2008; Piprek et al. 2012). For example, while the Criolla (*Leptodactylus latrans*) and Skipper Frog (*Euphlyctis cyanophlytis*) reach reproductive maturity within a year, other species such as the Common Frog (*Rana temporaria*) and Tropical Clawed Frog (*Xenopus tropicalis*) do not reach reproductive maturity until 2–3 y after metamorphosis (Augert and Joly 1992; Olmstead et al. 2009; Phuge and Gramapurohit 2013; Lopez et al. 2017). These protracted development times place constraints on the utility of some amphibian species as models for assessing reproductive development and sexual maturity endpoints. Thus, species that can develop from egg to sexual maturity in the shortest interval are efficient candidate models for assessing a multitude of developmental endpoints following contaminant exposure at any point in that developmental timeline.

The Blanchard's Cricket Frog (*Acris blanchardi)* is a small hylid species widely distributed across much of the central U.S. The species is relatively

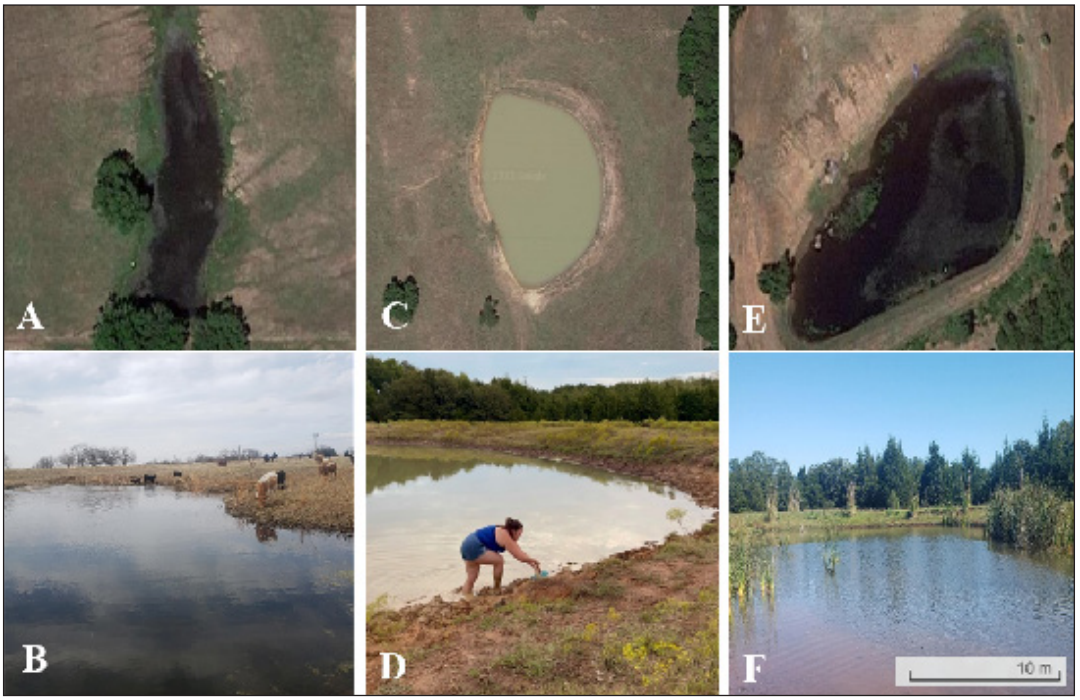

Figure 1. Aerial and ground level views of (A, B) Wilson Pond, (C, D) Tietz Pond, and (E, F) Kyle Pond used for field collection of metamorphosed Blanchard's Cricket Frogs (*Acris blanchardi*), Payne County, Oklahoma, USA. Roadways and identifiable coordinates have been removed for the sake of pond owner privacy. (A, C, D, from Google Images and B, D, F photographed Shauni Windle).

short-lived, completing metamorphosis and maturing in the fall of its hatching year before overwintering (Burdick and Swanson 2010; McCallum et al. 2011). Past studies have reported sexual maturity (i.e., active spermatogenesis and oogenesis) in *A. blanchardi* within 60 d of metamorphosis (Windle et al. 2021, 2022). The distribution of this species in much of the southern and midwestern U.S., its ability to be cultured in the laboratory, and its rapid development has likely played a role in its attractiveness as an ecotoxicology research model. In addition to attempts to understand its decline in other areas of North America, the native status and rapid development of *A. blanchardi* has led to its use in recent toxicology studies examining contaminant effects on growth, gonadal development, and feminization (Hoskins and Boone 2018; Hoskins et al. 2019; Windle et al. 2021, 2022).

We have used this species successfully in outdoor mesocosms to evaluate sexual development following larval exposure to estradiol and atrazine (Windle et al. 2021, 2022). With some exceptions, the progression of *A. blanchardi* development has not been thoroughly examined, especially in the natural environment and throughout the annual cycle from egg to breeding adult. McCallum et al. (2011) provides the most extensive examination of body growth and gonad development of *A. blanchardi* museum specimens. Gonadal development in these specimens from Arkansas and Missouri (USA) demonstrated year-round presence of sperm in males that varied by month and ovarian development that peaked in spring/summer of the year following metamorphosis.

We designed this study to describe the progression of *A. blanchardi* development among locations, seasons, and years in Oklahoma, USA. Specifically, we aimed to provide data for body growth rates and gonadal development of *A. blanchardi* cohorts across three seasons and three locations to evaluate growth and sexual development up to the time of breeding. In addition, we compared sexual development across three consecutive years for one study site.

Materials and Methods

Study area.—We selected three ponds (Wilson, Tietz, and Kyle) in Payne County, Oklahoma, USA, based on surrounding land use and availability from landowners. We selected ponds in watersheds with limited proximal urban or row crop agriculture land use to reduce the influence of intense anthropogenic pollution. We chose ponds for which the watershed was either pasture with low density cattle production (Wilson and Tietz) or low density residential with

Table 1. Table 1. Pond characteristics, mean (± standard error), and spot measurements for water temperature in Payne County, Oklahoma, USA, where Blanchard's Cricket Frog (*Acris blanchardi*) were collected. The number of temperature measurements at a pond was one, except for two measurements in Fall and three in Spring at Wilson Pond and two measurements in Spring at Kyle Pond.

				Water Temperature (˚C)		
Pond	Size (m^2)	Water source	Surrounding watershed	Fall	Spring	Summer
Wilson	1,124	Spring-fed	Pasture	24 (0.0)	21 (1.0)	24
Tietz	3,158	Run-off	Pasture	25	27	27
Kyle	2,450	Run-off	Low density residential	28	27 (0.7)	28

each house on > 0.4 ha plot (Kyle; Fig. 1, Table 1). Minimum distance between ponds was 2 km, which increased the likelihood that we sourced specimens from distinct populations (Gray 1983; Badje et al. 2021). All ponds had fish populations dominated by sunfish (*Lepomis* spp.) and bass (*Micropterus* spp.).

Field and histological methods.—To quantify morphological and reproductive development across seasons and locations, we collected 8–10 each of male and female metamorphosed *A. blanchardi* by hand. We collected frogs from each pond and across three time periods (i.e., seasons: fall and the following spring and summer). Fall sample collections occurred from 15–23 September 2021 and we assumed the animals hatched from eggs laid in 2021. We collected spring frogs from 1–20 April 2022 beginning with the first sighting of adult *A blanchardi*, but before the start of active chorusing. We collected the last group of frogs (i.e., summer) from 15–19 May 2022 beginning with the first sighting of active amplexus. Collections occurred multiple times throughout each collection period.

In addition to collections specifically for this study, we used prior published data from Kyle Pond collections for year-to-year comparisons (Windle et al. 2021, 2022). We had collected frogs from Kyle Pond during the fall of previous years to compare development of field captured and captive reared *A. blanchardi.* Capture methods varied slightly between years but were similar to those used in this study, including techniques and time of the year. For example, the sample size for this study was 19 metamorphs in the Kyle Pond fall collection but 20–30 in previous years.

We recorded water temperature at the beginning of each collection event (Table 1). Additionally, we calculated the means of daily minimum, maximum, and mean air temperature for the months of collection, covering the period of Fall 2021 to Spring 2022 (Table 2). We used data from Oklahoma Mesonet (https://www.mesonet.org/).

We euthanized frogs post-collection by immersing them in 5% buffered MS-222, measured body mass (BM; ± 1 mg) and snout-vent length (SVL; ± 1 mm), and prepared them for histopathology. Histology preparation included exposing the kidney-gonad complex (KGC) after removal of head, limbs, and viscera. We then submerged remaining tissue, including KGC, in Bouin's fixative (Cat.No.:50-320-01; Thermo Fisher, Waltham, Massachusetts, USA) for 24 h. We then rinsed fixed tissue with 70% ethanol and submerged in 10% formalin for a minimum of 48 h prior to shipment for histopathology. A board-certified Veterinary Pathologist (Experimental Pathology Laboratories, Sterling, Virginia, USA) scored histologic sections of gonad for maturation while blinded to collection site and seasons. Histological procedures and gonad staging were based on guideline methodology adapted from the *Xenopus laevis*-based Larval Amphibian Growth and Development Assay (LAGDA; Environmental Protection Agency [EPA] 2015; see Appendix Table). In brief, we staged gonad development (ovary and testis) and Müllerian duct (oviduct) development using scales of 1 to 5. For each tissue type, a higher score was indicative of increased maturity. Two species-specific modifications to EPA (2015) included the addition of ovarian Stage of 4.5 and the addition of oviduct Stage 5, neither of which had been described for the African Clawed Frog (*X. laevis*; Windle et al. 2021). Ovarian Stage 4.5 is characterized by oocytes that had nuclear resorption and increased eosinophilic ooplasm as compared to

Table 2. Mean air temperature (± standard error) across seasons (i.e., fall, spring, summer) in Payne County, Oklahoma, USA. Daily high, low, and mean temperatures found on Oklahoma Mesonet (https://www.mesonet.org/) from months when collections occurred. The values represented by n are the number of days in the month used to calculate the corresponding temperatures.

		Air Temperature (°C)		
Season	n	High	Low	Mean
Fall 2021	31	25.1 (0.75)	10.3 (0.82)	17.6 (0.72)
Spring 2022	30	23.7 (0.89)	8.5 (1.22)	16.3 (0.85)
Summer 2022	30	26.8 (1.08)	15.8 (1.06)	21.3 (1.00)

Stage 4 oocytes. We added this stage to facilitate the detection of subtle developmental stage changes. Stage 5 oviducts contained large basophilic glands distended by mucoid material.

Statistical analyses.—We analyzed BM and SVL separately for males and females using Two-way Analysis of variance (ANOVA), with pond and season as main factors (R Core Team 2024). Male frog mass was not normally distributed, and we log-transformed to meet normality assumptions. All other data were normally distributed and homoscedastic according to Shapiro-Wilk and Levene's tests ($P > 0.05$). We followed ANOVA with protected Tukey HSD comparisons to determine specific differences among ponds and seasons, alpha = 0.05. We analyzed frequency data across ovary, oviduct, and testis developmental stages among ponds and seasons using Fisher's Exact Test. In addition, we analyzed frequency data for ovary, oviduct, and testis developmental stages across years 2019–2021 for Kyle Pond.

Results

In the timeframe of the study, we caught 61 frogs from Wilson Pond, 56 frogs from Tietz Pond, and 66 frogs from Kyle Pond. We used data from an additional 56 frogs collected in previous years from Kyle Pond. Snout-vent length for females (Table 3). did not differ among ponds ($F_{2,70} = 3.024$ $P = 0.055$) but did differ for males ($F_{2,96} = 3.203$, $P = 0.016$; Table 3). In the latter case, males from Wilson Pond were about 10% larger than those from either Tietz or Kyle Ponds (Tukey HSD, $P < 0.001$). Irrespective of ponds, SVL for females and males differed among all three seasons (Female: $F_{2,76} = 111.1$, $P < 0.001$; Male: $F_{2,102} = 88.29$, $P < 0.001$). In both cases, SVL values were lowest in the fall, followed by spring and then summer where SVL values ranged from 40–70% larger than SVL of frogs collected in the preceding fall (Tukey HSD, $P < 0.001$).

Body mass of females ($F_{2,70} = 4.941$, $P = 0.001$) and males ($F_{4,96} = 4.239$, $P < 0.001$; Table 3) significantly differed among ponds. Body mass of Wilson females was about 30% larger than for those from Tietz Pond, which was significant (Tukey HSD, $P = 0.007$). Body mass for females from Kyle Pond did not differ significantly from either Wilson or Tietz ponds (Tukey HSD, $P = 0.173$, 0.336). Similarly, BM of Wilson males was about 30% larger than those from both Tietz and Kyle ponds, and also was significant (Tukey HSD, $P < 0.001$). Seasonal BM, like SVL, significantly differed for females and males among all three seasons (Female: $F_{2,76} = 86.56$, $P < 0.001$; Male: $F_{2,102} = 88.89$, $P < 0.001$). Female frogs collected in the summer were on average 75% heavier than animals collected in the spring, which were 50% heavier than those from the preceding fall, and these differences were significant (Tukey HSD, $P < 0.001$). Similarly, summer collected males were 120% heavier than spring collected frogs, which were on average 60% heavier than fall animals, and the differences were significant (Tukey HSD, $P < 0.001$).

Oviduct significantly varied between ponds in both spring and summer collections (Fisher's Exact Test, $P = 0.006$, 0.001) with development evenly distributed across stages for Wilson Pond, but not for Tietz and Kyle ponds (Table 4). Conversely, ovary (Fisher's Exact Test, $P > 0.05$; Table 4) and testis (Fisher's Exact Test, $P > 0.05$; Table 5) development did not

Table 3. Mean (± standard error) snout-vent length (SVL) and body mass (BM) of female and male metamorphosed Blanchard's Cricket Frogs (*Acris blanchardi*) for fall, spring, and winter and at three locations in Payne County, Oklahoma, USA. Significant differences of SVL by season were Fall < Spring < Summer. Significant differences of BM by ponds were limited to Wilson > Tietz whereas, seasonal differences were Fall < Spring < Summer. The number of frogs measured is given by n.

Pond	Season	n	SVL (mm)	BM (g)
Females				
Wilson	Fall	7	18.7 (0.89)	0.5 (0.03)
	Spring	10	22.1 (0.69	0.9 (0.08)
	Summer	10	27.1 (0.67)	1.4 (0.09)
Tietz	Fall	7	16.6 (0.65)	0.3 (0.04)
	Spring	8	19.8 (1.01)	0.6 (0.10)
	Summer	9	27.3 (1.07)	1.3 (0.15)
Kyle	Fall	9	17.3 (0.75)	0.4 (0.04)
	Spring	10	18.8 (0.85)	0.5 (0.06)
	Summer	10	29.1 (0.70)	1.7 (0.09)
Males				
Wilson	Fall	12	18.3 (0.59)	0.4 (0.03)
	Spring	12	21.4 (0.65)	0.8 (0.09)
	Summer	10	24.2 (0.66)	1.0 (0.05)
Tietz	Fall	13	16.5 (0.37)	0.3 (0.02)
	Spring	9	17.7 (0.53)	0.4 (0.04)
	Summer	10	24.1 (0.75)	0.9 (0.06)
Kyle	Fall	11	16.5 (0.59)	0.3 (0.03)
	Spring	15	17.5 (0.37)	0.4 (0.06)
	Summer	13	23.0 (0.43)	0.9 (0.03)

differ significantly among ponds. Seasonal effects were significant for all three tissues (Fisher's Exact Tests, Ovary $P < 0.001$; Oviduct $P < 0.001$; Testis $P = 0.003$; Tables 4–5). In all cases, there was a seasonal shift in gonad developmental stage as animals moved from the preceding fall to full sexual maturity in the following summer. This transition to sexual maturity for males started in fall but then appears to pause through the following spring after which virtually all males attain full gonad development by summer. Females on the other hand exhibited a more gradual transition in ovary and oviduct development as they matured from fall through the next summer, with virtually no individuals reaching Stage 5 ovaries or oviducts until summer.

Gonad development of animals collected in the fall and from Kyle Pond varied between years. Ovary development varied significantly across years at Kyle Pond (Fisher's Exact Test, $P = 0.003$) with a shift from more Stage 3 ovaries in 2019 to more stage 4.5 ovaries in 2020 and 2021 (Table 6). Conversely, neither testis (Fisher's Exact Test, $P = 0.295$)) or oviduct (Fisher's Exact Test, $P = 0.135$) development varied significantly among years. Oviduct data existed for only Stage 2 and 3 and thus comparisons across time are tenuous, but generally the distribution did not follow any clear pattern. Testis development was generally weighted toward Stage 5 in 2019 and 2021, with a relatively even distribution among stages in 2020, but again these changes were not significant.

Table 4. Oviduct and ovary development stage (%) of female metamorphosed Blanchard's Cricket Frog (*Acris blanchardi*) for fall, spring, and summer and at three locations in Payne County, Oklahoma, USA. Oviduct and ovary staging based on the following criteria (EPA 2015). Stage 5 oviduct staging is an additional stage and a species-specific modification to EPA (2015). The number of frogs examined is given by n.

Pond	Season	n	Stage 2	Stage 3	Stage 4	Stage 5
Oviduct						
Wilson	Fall	8	25.0	75.0	0	0
	Spring	9	0	22.2	66.7	11.1
	Summer	10	0	0	10.0	90.0
Tietz	Fall	6	16.7	83.3	0	0
	Spring	8	0	75.0	0	25.0
	Summer	9	0	11.1	0	88.9
Kyle	Fall	8	28.6	71.4	0	0
	Spring	10	0	80.0	20.0	0
	Summer	10	0	0	70.0	30.0
Ovary			Stage 2/3	Stage 4	Stage 4.5	Stage 5
Wilson	Fall	8	0	50.0	50.0	0
	Spring	9	0	0	77.8	22.2
	Summer	10	0	0	0	100.0
Tietz	Fall	6	0	66.7	33.3	0
	Spring	8	0	37.0	38.0	25.0
	Summer	9	0	0	11.1	88.9
Kyle	Fall	8	0	50.0	50.0	0
	Spring	10	0	10.0	90.0	0
	Summer	10	0	0	0	100.0

Discussion

Amphibian development is sensitive to a variety of environmental factors including water temperature, food availability, density of conspecifics, predators, and other factors that are spatially and temporally variable (Seale 1982; Smaga et al. 2022). We evaluated variation in several measures of growth and development in Cricket Frogs as influenced by their pond of origin, season, and year. Generally, we observed an increase in body size and weight as frogs transitioned through winter and into the following summer breeding season, regardless of their pond of origin. Previous works indicate that *A. blanchardi* develop relatively quickly after metamorphosis and continue to mature through winter in preparation for breeding the following spring and summer (McCallum 2003; McCallum et al. 2011). Also, although individual ponds undoubtedly differ relative to characteristics relevant to frog growth, the pattern of growth over time should be predicably consistent (i.e., frogs gain more size and weight from fall

Table 5. Testis development (%) of male metamorphosed Blanchard's Cricket Frog (*Acris blanchardi*) across fall, spring, for summer and at three locations in Payne County, Oklahoma, USA. Staging was based on accepted criteria (EPA 2015). The number of frogs examined is given by n.

Pond	Season	n	Stage 1/2	Stage 3	Stage 4	Stage 5
Wilson	Fall	12	0	0	16.7	83.3
	Spring	13	0	0	15.4	84.6
	Summer	9	0	0	0	100.0
Tietz	Fall	13	0	0	15.4	84.6
	Spring	10	0	0	30.0	70.0
	Summer	10	0	0	0	100.0
Kyle	Fall	11	0	36.4	18.2	45.5
	Spring	15	0	0	40.0	60.0
	Summer	13	0	0	7.7	92.3

through the following summer), as observed in our study.

Unlike the overall pattern of growth, magnitude of growth will more likely differ among ponds due to one or more of the aforementioned environmental factors (McCallum and Trauth 2021). For example, frog larvae can develop at different rates and reach metamorphosis at different sizes due simply to water temperature, which could be locally influenced by multiple factors such as water source, aquatic vegetation, and surface area/depth of the water body (Nevo 1973; Bull and Hayes 2001; Bunnell and Ciraolo 2010; Perez et al. 2012). Cooler water temperatures can also result in slower developmental rates and larger body size at metamorphosis (Dastansara et al. 2017). We observed frogs from Wilson Pond that were larger and heavier than those from Kyle and Tietz ponds. Wilson is a spring-fed pond and average water temperature was 3°–4° C cooler than the other two ponds, which could at least partially explain observed differences in body size among ponds (Thompson and Popescu 2021).

We observed no significant differences in the variation of testes development among ponds, and although the same was true for ovary development, there was an obvious difference in seasonal distribution of ovary development between Wilson Pond and the other two collection sites. Congruent with their larger body size, ovaries of females collected from Wilson Pond were more mature compared to those from the other ponds; however, by summer, essentially all frogs from all locations had Stage 5 ovaries. This observation coincides with the greater SVL and BM of Wilson frogs and may be related to further carryover effects of the cooler larval environment. Indeed, gonad development is influenced primarily by genetic and hormonal pathways that are triggered by external environmental factors (Calatayud et al. 2018). Furthermore, gonad development is energetically demanding, and it may be sensitive to external factors such as temperature, hydrology, nutrition (Girish and Saidapur 2000; Yoneda and Wright 2005; McCoy et al. 2007). It is possible that the higher temperatures in Tietz and Kyle ponds led to more energy being allocated to body growth prior to winter, thereby compromising ovary development in some individuals. The noticeable lack of variation in testes development among ponds, however, may be due to less energy expenditure for sperm production versus oocyte development. Moreover, the observed degree of testes development is not entirely surprising considering male Cricket Frogs reach sexual maturity prior to winter and do not appear to lose their ability to produce sperm over winter (McCallum 2003; McCallum et al. 2011). This is a useful trait for Cricket Frog males that begin calling early in the spring as they arrive at ponds soon after emerging from hibernation, much earlier than females, and may occur within a few meters of the edge of the water (Green and Pauley 1987; McCallum and Trauth 2003a).

Oviduct development, to some degree, also seemed to follow a pattern consistent with body size and ovary development with Wilson frogs exhibiting a quicker transition to late-stage oviducts. Again, however, by summer oviduct development was comparable for Wilson and Tietz, in contrast to frogs from Kyle Pond where most frogs had Stage 4 oviducts. Stage 5 oviducts are characterized by mucoidal material and preparation for oviposition (EPA 2015). During oviposition, these oviduct mucoidal layers are sloughed coating passing ova (Olmstead et al. 2009). This phenomenon would thus cause Stage 5 oviducts to revert to Stage 4, a likely scenario in this case for frogs from Kyle Pond. Indeed, ovaries from Kyle Pond frogs contained numerous post-ovulatory follicles and thus these frogs likely experienced a breeding event before those from either Wilson or Tietz ponds (Altig and McDiarmid 2007; Olmstead et al. 2009). This could be related to pond water temperature as warmer water is related to timing of oviposition (Wheeler et al. 2018). Notably, the water temperature of Kyle Pond was, on average, warmer than the other collection sites.

Table 6. Gonad development (percentage of field captured individuals) in male and female Blanchard's Cricket Frog (*Acris blanchardi*) collected over three years from Kyle Pond (Payne County, Oklahoma, USA) during the fall. Gonad staging was based on accepted criteria (EPA 2015).

Tissue Type	2019	2020	2021
Ovary			
Stage 3	69.2%	0	0
Stage 4	30.8%	91.7%	50.0%
Stage 4.5	0	8.3%	50.0%
Oviduct			
Stage 2	46.2%	75.0%	28.6%
Stage 3	53.8%	25.0%	71.4%
Testis			
Stage 3	11.1%	23.1%	36.4%
Stage 4	27.8%	46.2%	18.2%
Stage 5	61.1%	30.8%	45.5%

We expected the annual variation in development because conditions such as rainfall, temperature, and other variables can fluctuate tremendously from year to year; however, we observed significant differences only for ovary development. Environmental factors (e.g., pond drying) may be the cause for the observed variation between years (McCallum and Trauth 2021). For example, ovary development was the least progressed during the heaviest rain year (2019) compared to the driest year (2021). Gordon et al. (2016), however, noted that development including size at metamorphosis is not sensitive to events such as pond drying. This observation suggests that *A. blanchardi* gonad development may also not be sensitive to fluctuating pond levels between years. Additional reasons for observed inconsistencies in ovary development between years may be due to the age of collected *A. blanchardi*. Each yearly collection was performed between mid and late-September but exact breeding time and resulting hatching may have varied between years.

Knowledge of the natural history traits of an organism are important when formulating research questions and designing studies, yet we often have sparse information on traits for many species. Indeed, one could argue that there is little incentive to pursue studies geared toward collecting natural history data, especially for species deemed to be of low conservation importance (Stankey and Shindler 2006; Jaric et al. 2022). The impetus for this work stemmed from our efforts to examine effects of contaminants on amphibians and the desire for an amphibian model that would allow us to track development from eggs through reproductive maturity (Windle et al. 2021, 2022). The Cricket Frog we used generally fulfilled that need, although it is clear that over-wintering was required before all individuals reached full reproductive development and body size. Further, patterns of development were generally predictable regardless of collection site and year, except in some cases where local site nuances (e.g., water temperature) likely influenced the timing of some aspects of development. All gonads appeared normal with no intersex individuals. Intersex for this species and other amphibians have been reported in numerous studies and undoubtedly represent normal variation in development in many cases, although other causes have been suggested (e.g., McCallum and Trauth 2003b; Reeder et al. 2004). These data augment previous work (McCallum 2003; McCallum et al. 2011; Windle et al. 2021, 2022) and provide additional specifics on the development of a species found throughout much of the central U.S.

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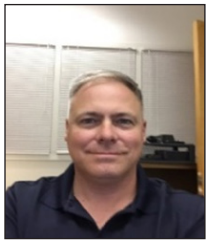

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Appendix Table. Gonad histopathology definitions (EPA 2015) and a summary of diagnostic criteria for gonad histopathologic assessment. Staging based on criteria established in EPA 2015. An asterisk (*) indicates a female specific guideline.

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