

2013-01-01

Influences Of Ecological Light Pollution On Advertisement Calls Of *Spea multiplicata* (Amphibia: Anura: Scaphiopodidae) In Rural And Urban Populations In The Northern Chihuahuan Desert And An Evaluation Of Hybrid *S. Bombifrons* X *S. Multiplicata* Calls

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INFLUENCES OF ECOLOGICAL LIGHT POLLUTION ON
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by

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THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences

THE UNIVERSITY OF TEXAS AT EL PASO

December 2013

ACKNOWLEDGEMENTS

I would like to thank my graduate committee without which this research would not have been possible: Dr. Jerry Johnson who took me on as graduate student, and supplied me with equipment and guidance, Dr. Carl Lieb for helping me with localities, assistance with specimens, and patience, and Dr. Eric Hagedorn for help with basic statistics and someone to reminisce with about how much we miss Wisconsin.

Other faculty members that I would like to thank are Dr. Michael Moody who allowed me use of his lab and equipment, and advice. Ana Betancourt in the UTEP DNA Analysis Core Facility (funded by NIMHD grant G12MD007592) sequenced samples for this study. Funding by the department of Biological Sciences and College of Sciences for conference travel.

Frank Portillo who answered numerous questions about software and DNA protocols, Fernie Medina for her assistance with fieldwork and her enthusiasm. I cannot thank Chris Anderson enough for his assistance in the field, and his continuous encouragement throughout this process. I am thankful for my family for their continued encouragement and support.

ABSTRACT

Spea is a genus of toad-like, arid adapted frogs distributed throughout much of the western U.S. and northern Mexico. Two species, (*S. bombifrons* and *S. multiplicata*) are syntopic throughout most of the northern Chihuahuan Desert, a region that is experiencing rapid urbanization. For this study, I examined 936 male advertisement calls from urban and rural populations of *S. multiplicata*, and a rural population of *S. bombifrons* in west Texas and south-central New Mexico. Advertisement calls from urban and rural *S. multiplicata* were compared against light level to assess the potential influence ecological light pollution plays in sexual selection at urban breeding sites. Univariate statistical analyses and principal components analysis indicated that urban frogs call at significantly different rates and frequencies after the effects of light level and water temperature are removed. Rural spadefoots call at a wider breadth of frequencies and call more slowly than urban populations. Additionally, hybrid (*S. bombifrons* x *multiplicata*) advertisement calls were identified at one rural breeding site. One suspected hybrid and three specimens from each parent species were vouchered for hybrid verification. Analysis of the mitochondrial gene *cyt b* recovered the suspected hybrid female parentage as *S. bombifrons*. Whereas, analysis of the nuclear gene *RAG1* indicated that the hybrid sequence is heterozygous at 55% of variable sites. Univariate analysis of hybrid advertisement calls indicate that call interval, calls per minute, and pulses per second are intermediate between *S. bombifrons* and *S. multiplicata*.

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INTRODUCTION

Introduction to the Family Scaphiopodidae

The North American spadefoots (Scaphiopodidae Cope, 1865) consist of two genera: *Scaphiopus* (Southern Spadefoots – *S. couchii*, *S. holbrookii*, and *S. hurterii*) and *Spea* (Western Spadefoots – *S. bombifrons*, *S. hammondi*, *S. intermontana*, and *S. multiplicata*). The family is distributed throughout much of North America (Fig. 1.), from southcentral and southwestern Canada through much of the U. S. and south into central Mexico. They are absent in the Pacific Northwest, upper Midwest, and northern New England in the U. S., and on Mexico's Sierra Madre Occidental (Conant and Collins, 1998). Adult North American spadefoots range in size from 50 to 91 mm snout-vent length (SVL) (Stebbins, 2003; Vitt and Caldwell, 2009) and are described as toad-like in appearance. Commonly called spadefoots or spadefoot toads, this idiomatic name comes from the keratinous tubercle (spade) found on the outside edge of each hind foot. All species are fossorial in nature, using their spades to shuffle backwards into the soil (Vitt and Caldwell, 2009). Bragg (1945), described spadefoots occurring in arid climates as having a xeric breeding pattern, during which time emergence is triggered by rainfall, as opposed to a more mesic pattern in other areas that take place during particular seasons. Spadefoots living in the southwestern U. S. are xeric adapted, and are well known for their explosive breeding strategies (Bragg. 1945). Males call for females at ephemeral pools during the first major rainstorm of the summer monsoon season (Degenhardt et al., 1996), with reproductive and foraging behaviors usually being completed within a few days following the first substantial rainfall of the season (Vitt and Caldwell, 2009). Bragg (1965) produced a monograph describing

much of what was known at that time about spadefoot toads. A summary of the natural history of two native western spadefoots (*Spea bombifrons* and *S. multiplicata*) inhabiting the Chihuahuan Desert near El Paso, Texas, is presented below.

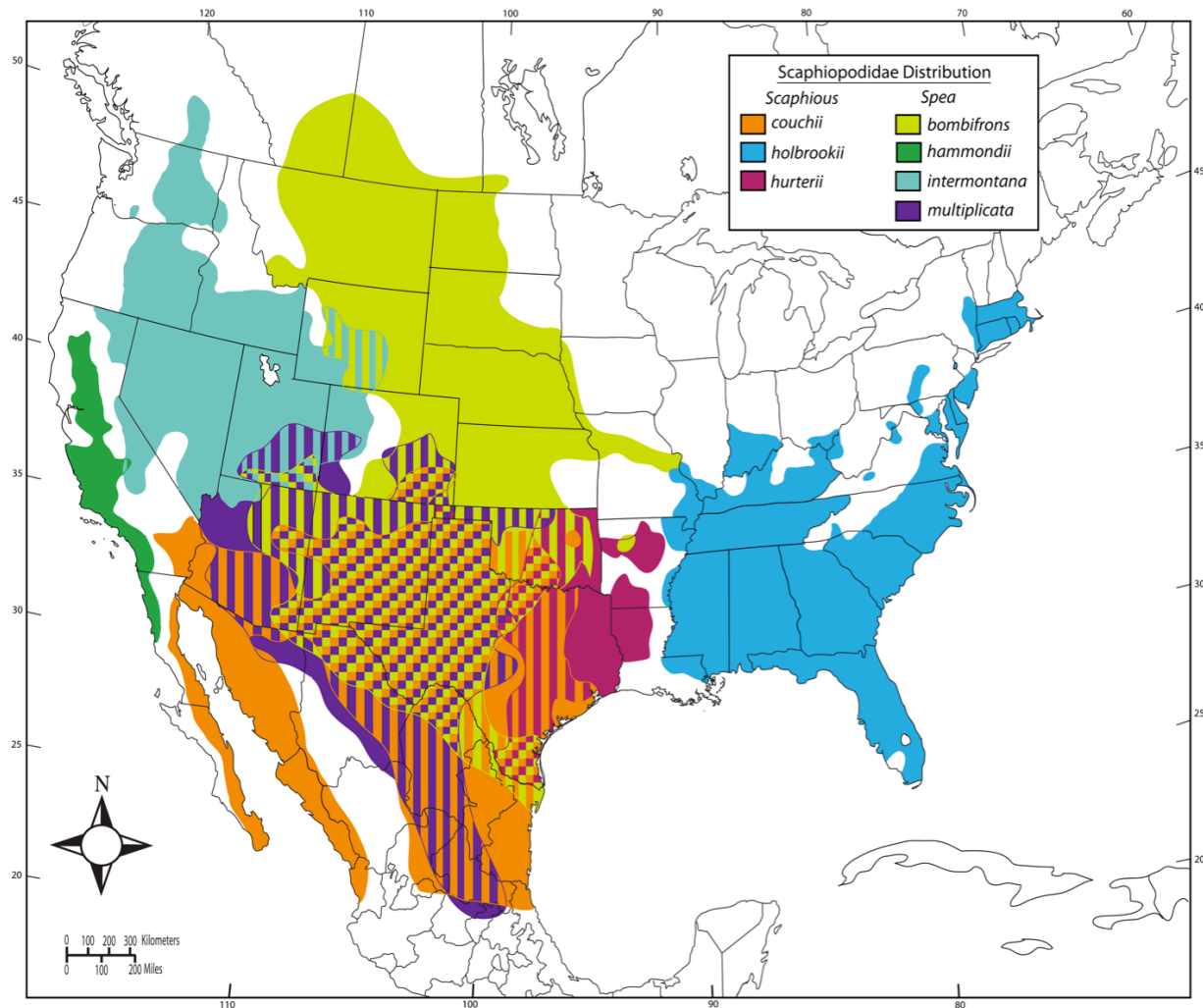


Fig. 1. Geographic distribution of the family Scaphiropodidae depicting individual species distributions and areas of overlap.

The Plains Spadefoot, *Spea bombifrons* (Fig. 2), is grayish or brownish in color with an overall trace of green and darker brown or gray dorsal markings (Wright and Wright, 1949; Degenhardt et al., 1996). The skin is mostly smooth with small red or yellow tubercles. Four light-colored irregular stripes running along the dorsum are usually present. The eyes are

distinctly large, with vertical ellipse-shaped pupils and a bony boss (absent in the other two Chihuahuan Desert spadefoots) protruding between the eyes; the eyelids are wider than the space between them (Degenhardt, et al., 1996; Stebbins, 2003). The dark keratinized spade is wedge-shaped. Adults range in size between 38 to 57 mm SVL, and male calls were described as brief hoarse sounding bleats lasting 1/2–3/4 of a second (Conant and Collins, 1998; Degenhardt et al., 1996).



Fig. 2. *Spea bombifrons* photographed in Doña Ana County, New Mexico, following a significant summer rainfall event.

Spea bombifrons is distributed from southcentral Canada (Alberta, Saskatchewan, and Manitoba), south through central plains of the U. S. to extreme northern Mexico; a few disjunct populations occur east and southeast of its normal geographic range (Conant and Collins, 1998) (Fig. 1). According to Stebbins (2003), the species occupies a variety of habitats including plains, mixed-grass prairies, farmlands, and sagebrush and desert grasslands with loose sand or

gravel soils conducive for burrowing. They generally avoid river bottoms and wooded areas (Degenhardt et al., 1996).

Studies examining the stomach contents of *S. bombifrons* have found that diet varies depending on prey availability. Anderson et al. (1999) examined the stomach contents of 24 males from playa wetlands in northwestern Texas and eastern New Mexico over two field seasons. The diet consisted of 12 invertebrate taxa. Coleopterans were the most frequently consumed group of invertebrates (63%), primarily species within the family Carabidae (64%). A study by Whitaker Jr. et al. (1977) found diets of adults collected in Yuma County, Colorado, consisting of three major food types: adult and larval moths (over 60%); carabid ground beetles (17%); and other ground-dwelling arthropods (23%). The diet seems to be dependent on nocturnal invertebrates detected while foraging (Anderson et al., 1999).

Like other spadefoots, *S. bombifrons* burrows underground and stays dormant for most of the year, or longer depending on the localized nature of the monsoonal rainstorms. They emerge during periods of substantial summer rains, which normally occur from May through August, to briefly breed and forage for food (Wright and Wright, 1949). Between 90 and 100% of breeding activity transpires the first night after a significant rainfall event, although some males have been known to remain for an additional night (Woodward, 1984, Degenhardt et al., 1996). Males are typically observed calling from the edge of a pond or freely floating in the pool (Woodward, 1987); amplexus is inguinal (Wright and Wright, 1949). A study by Woodward (1987) found that females laid clutches averaging over 1,600 eggs, although clutch sizes could vary by over 500 eggs; Wright and Wright (1949) reported that egg masses were normally attached to submerged plants. At the optimal temperature of 30° C, eggs developed and hatched in 20 hours (Justus et al., 1977; Degenhardt et al., 1996). To complete metamorphosis, tadpoles required 15–

19 days (Voss, 1961; Justus et al., 1977), but a mesocosm experiment by Pfennig and Simovich (2002) showed tadpoles needed 27–28 days to metamorphose. Newly metamorphosed tadpoles ranged from 18 to 22 mm SVL (Degenhardt et al., 1996).

Spea multiplicata, the New Mexico Spadefoot, is brown or grey overall, with smaller speckled dark spots and red protruding tubercles over the dorsal region (Fig. 3). It resembles *S. bombifrons* in coloration, but lacks the intraorbital boss. The pupil is vertical, with a multi-colored iris, mainly reddish-gold. The spades on the hind feet are wedge-shaped and undersized, similar to those on *S. bombifrons*. Adults range from 38 to 63 mm SVL (Stebbins, 2003). Its mating call is unique among the spadefoots, resembling the sound of a fingernail running along the teeth of a comb; each metallic-sounding trill lasts 0.75 to 1.5 seconds (Degenhardt et al., 1996; Conant and Collins, 1998).



Fig. 3. Adult male *Spea multiplicata* photographed on a road soon after emerging from the ground following a significant rainfall event in El Paso County, Texas.

Spea multiplicata ranges from Oklahoma, south throughout central Texas, New Mexico, and Arizona to central Mexico (Fig. 1). It inhabits a variety of environments, from sagebrush

flats, shrublands, short-grass plains, playas, alkali flats, river valleys, and agricultural fields, but they generally avoid more xeric desert environments (Degenhardt et al., 1996; Conant and Collins, 1998).

Food habits of *S. multiplicata* are varied; Whitaker Jr. et al. (1977) found that the diets of 295 individuals from Utah consisted of larval moths (ca. 35% of food load) and 73 other species of invertebrates. Dimmitt and Ruibal (1980a) examined the stomach contents of 25 individuals captured in southeastern Arizona, and found that termites and beetles constituted 72% and 22% of the total stomach contents, respectively. Another diet study by Anderson et al. (1999), surveyed 38 samples from northwestern Texas and eastern New Mexico and found 20 different invertebrates species, with ground beetles (Carabidae) being consumed most often (68% of the total diet). This was possibly due to the beetle's nocturnal habits that matched the spadefoot's foraging period. It is interesting that the species seems to have a wide dietary breadth even though they represent the smallest spadefoot found within their geographic range (Anderson et al., 1999).

Spea multiplicata has natural history behaviors similar to other spadefoots. They lie dormant in underground burrows until the summer monsoon season begins. They also tend to be secretive in nature, often hiding in burrows after emergence until seeking out playas and other low-lying areas that fill with rainwater. Emergence is significantly correlated with rainfall, although low pitched sound of thunder and raindrops hitting the soil were reported to be a main cue for emergence, along with a requirement of soil temperatures being above 25° C (Dimmitt and Ruibal, 1980b). Breeding usually occurs in mid or late July, although it may be postponed until later if drought conditions persist. Breeding occurs over an average of 1.6 days, with males calling while floating in the water (Sullivan and Sullivan, 1985; Degenhardt et al., 1996); non-

calling males have been observed actively seeking females near calling males (Sullivan and Sullivan, 1985). Egg masses are cylindrical with an average clutch size of about 1,000 +/- 500 eggs (Wright and Wright, 1949), and like other spadefoots, egg masses are attached to submerged vegetation (Wright and Wright, 1949; Degenhardt et al., 1996). Eggs can hatch in as little as two days; tadpole development is complete in approximately 21 days (Degenhardt et al., 1996).

*Hybridization between *Spea bombifrons* and *S. multiplicata**

According to Mayr (1963), hybridization is defined as “the crossing of individuals belonging to two unlike natural populations that have secondarily come into contact.” Hybridization is not an uncommon occurrence in nature (Liou and Pierce, 1994); of all plants and animals, between 10-30% consistently hybridize (Abbott et al., 2013).

Hybrid offspring of pure-species parents either have relatively fit genotypes with unique genetic variation, or more often, are less fit than the parent species (Grant, 1963; Dobzhansky, 1970; Barton and Hewitt, 1985; Arnold and Hodges, 1995). For instance, a hybrid may have advantageous characteristics as young, and then have low fitness values or be sterile as adults (Pfennig and Simovich, 2002).

Spea bombifrons and *S. multiplicata* are sympatric throughout much of the southwestern U. S. and northern Mexico (Stebbins, 2003) (Fig. 4). A phylogenetic analysis of 33 individuals in the genus *Spea* by Wiens and Titus (1991) indicated that *S. bombifrons* and *S. multiplicata* are the most distantly related species. Because spadefoot toad breeding events are entirely tied to seasonal rainfall, explosive breeding congregations of anurans often consist of a mixture of several species, including related congeneric species within *Spea* (Simovich, 1994; pers. observ.

by author). Syntopic breeding spadefoot communities often demonstrate necessary characteristics of species reinforcement (e.g., low hybrid fitness relative to parents, and character displacement in male and female mating behaviors), however in certain environmental conditions this normally unfavorable act can be advantageous (Wiens and Titus, 1991; Pfennig and Simovich, 2002; Reyer, 2008).

In a controlled mate choice study conducted by Pfennig and Simovich (2002) the hybrid offspring between female *S. multiplicata* and male *S. bombifrons* were 11% larger than pure *S. multiplicata* offspring, but had a lower survival rate and took longer to develop. If *S. multiplicata* x *bombifrons* hybrids did survive metamorphosis, males were sterile, and females were found to be less fecund. However, hybridization events between female *Spea bombifrons* and male *S. multiplicata* were not necessarily selected against in certain conditions. Those crosses produced *S. bombifrons* x *multiplicata* offspring that metamorphosed faster than *S. bombifrons* offspring, but were smaller and had a 4% lower survival rate. Consequently, Pfennig and Simovich (2002) found an inverse relationship between frequency of hybrids and pond size, where smaller pools contained more hybrids. There was also a significant relationship between number of heterospecific mating events and male density in the pond, and female *S. bombifrons* are more likely to mate with male *S. multiplicata* if conspecific males were rare (Wirtz, 1999; Malmos et al., 2001).

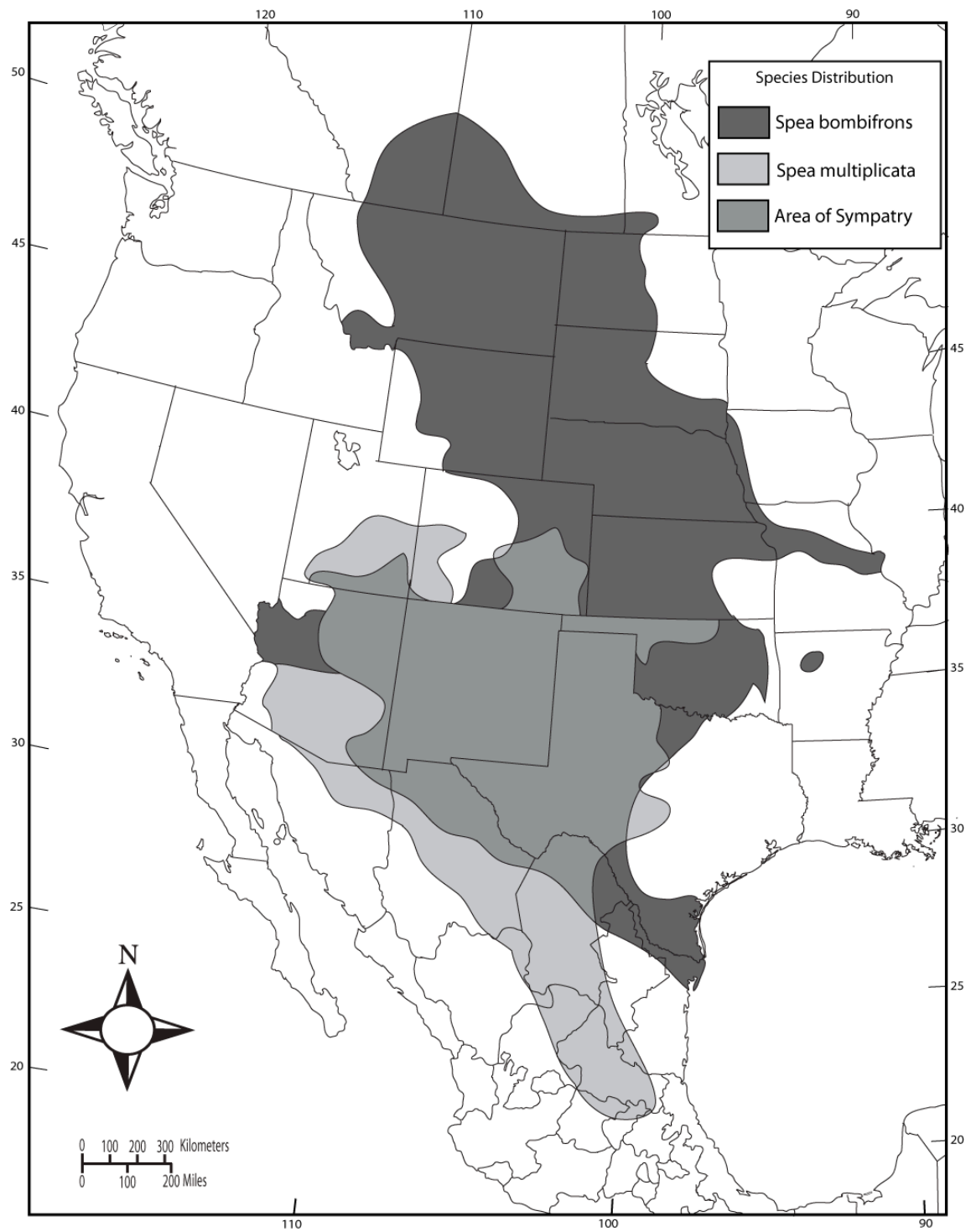


Fig. 4. Geographic distribution of *Spea multiplicata* and *S. bombifrons*, and area of sympatry.

Ecological light pollution

Over the past 60 years, substantial urban expansion and development have significantly increased the artificial light illumination of the night sky (Navara and Nelson, 2007) on a global scale (Smith, 2008). Ecological light pollution, which differs from astronomical light pollution, alters the natural light regimes in ecosystems, and circadian rhythms of affected individuals and populations; astronomical light pollution hinders the view of the night sky (Longcore and Rich, 2004).

There are several sources of ecological light pollution, including sky glow from distant cities, shielded illumination from street lighting, and brightly lighted fishing vessels and oil platforms miles off-shore. Many effects of ecological light pollution are unknown and there is a pressing need to examine ecological consequences of artificial lighting, especially at night. Artificial lighting from urban areas is often brighter than the natural sunset, and is known to alter behaviors of many species (Navara and Nelson, 2007). The specific effects of ecological light pollution vary among species; for some, the light can be disorienting, attractive, or repulsive, and can disrupt communication, migration patterns, reproduction, foraging, give misguided cues of daylight length, and it can even be fatal (Longcore and Rich, 2004; Navara and Nelson, 2007; Smith, 2008). Hölker et al. (2010) even suggested that light pollution is a threat to global biodiversity, as 30% of all vertebrates are nocturnal and thus have adapted senses for living in darkness. Furthermore, nocturnality may have played an important role in the evolution of many species.

Artificial night lighting has been shown to have various detrimental effects on specific groups of wildlife (e.g., Buchanan, 1993; Salmon et al., 1995; Baker and Richardson, 2006; Kenpenaers et al., 2010; and Longcore, 2010). Natural lighting has been shown to temporally

structure communities by effecting activity patterns and circadian rhythms (Rotics and Kronfeld-Schor, 2011). A microcosm experiment by Rotics and Kronfeld-Schor (2011) looked at the effects of artificial night lighting on two species of spiny mice (*Acomys* sp.) in a desert community. The authors examined the Golden Spiny Mouse (*Acomys russatus*), a diurnal species and the Common Spiny Mouse (*A. cahirinus*), a nocturnal species. They found *A. cahirinus* reduced their activity period and foraging behaviors in response to increased night lighting. It was hypothesized that an increased perceived predation risk was the probable cause for the reduction in activity. Bird et al. (2004) observed similar effects in the nocturnal Santa Rosa Beach Mouse (*Peromyscus polionotus leucocephalus*). In that case, two types of long wavelength lights were tested: low-pressure sodium vapor lights and incandescent yellow bug lights, both of which are used along Florida's coastlines to reduce disorientation effects on sea turtles; the effects of those lights on other animals had not yet been tested. They found that *P. p. leucocephalus* reduced patch use near both types of lights and collected fewer seeds near sodium vapor lights. The mice were choosing areas without lights to forage and spent less time feeding in artificially lit areas, likely due to a higher perceived predation risk.

Experiments on bats and artificial night lighting have also shown detrimental effects on behavior, including delayed nocturnal emergence and reduced foraging activity (Boldogh et al., 2007; Stone et al., 2009). Boldogh et al. (2007) compared emergence activity of house-dwelling bat colonies roosting in illuminated and non-illuminated buildings, and found that bats emerged from roosts within 30 minutes following dusk when no lights were installed as opposed to a severely reduced number of emerging bats and a timing delay in others living in illuminated buildings. That delay meant bats in illuminated buildings missed a larger quantity of flying insects. Additionally, parturition in bats living in illuminated buildings occurred later and growth

rate of neonatal bat timing was reduced. An additional study by Stone et al. (2009) surveyed night time foraging routes of a population of Lesser Horseshoe Bats (*Rhinolophus hipposideros*) with and without street lighting, and found the majority chose alternative routes, thereby avoiding street lighting. These alternate routes may be more energetically costly for bats, provided fewer food resources, increased predation risks, or exposure to the elements, all of which could result in lower population size.

Birds living in artificially illuminated areas tended to initiate morning songs earlier than historical data had shown (Miller, 2006; Kempenaers et al., 2010; Longcore, 2010). Miller's (2006) study compared historical data of the onset of dawn songs by the American Robin (*Turdus migratorius*) and found that robins began singing earlier in brightly lit areas compared to historical data and that cloud cover amplified artificial brightness, thereby initiating songs even earlier. Furthermore, Kempenaers et al. (2010) observed that yearling male Blue Tits (*Cyanistes caeruleus*) were as successful at extra-pair copulations as mature males when breeding with females nesting on forest edges exposed to night-lights. An early dawn song is a signal of male quality so the fittest males will sing earlier, but if inferior males are living in illuminated areas, an early morning male song could be a dishonest signal of fitness (Longcore, 2010).

Another group of animals that have been greatly affected by artificial night lighting are sea turtles. Hatchling sea turtles naturally orient themselves toward the illuminated sea and the lower seaward horizon (Proffitt et al., 1986; Salmon et al. 1995) during a behavior called seafinding (Karnad et al, 2009). Conflicting signals caused by bright urban lights can cause hatchlings to crawl towards the bright urban lights, usually onto roadways, or crawl in non-directional patterns (Salmon et al, 1995; Harewood and Horricks, 2008). In a study by Karnad et al. (2009) hatchlings of the Olive Ridley Sea Turtle (*Lepidochelys olivacea*) were placed in

arenas with an experimental light source and found significant orientation towards higher light wavelengths and brighter lights. Coastal urban development has introduced these types of lights, which can result in extended disorientation leading to dehydration and exhaustion (Harewood and Horrocks, 2008) and decreased survival rates (Karnad et al., 2009).

To date, no studies have investigated the effects of urban lighting on wild populations of North American spadefoots. Some anurans do display positive phototactic behaviors by congregating under lights to feed on insects (Perry et al., 2008), and it is well known that bufonid toads forage under outdoor house and street lights during summer months (J. D. Johnson, pers. observ.). This practice, which may be beneficial to the toads energy-wise, can be deadly due to incidental mortality caused by passing vehicles, opportunistic predators, or direct killing by humans.

Many anurans are nocturnal, with eyes adapted to darkness and are active at very low light intensities comparable to moonlight illumination (Buchanan, 1993). Additionally, the pupil of the anuran eye constricts very slowly in response to bright lights, most likely resulting in visual impairment (Cornell and Hailman, 1984; Buchanan, 1993). It was believed that artificial lighting did not usually affect anuran behavior, although Buchanan (1993) observed modifications in foraging behavior in captive *Hyla chrysoscelis* exposed to artificial lights. They were observed under four increasing light conditions in a laboratory setting where light intensity ranged from 0.003 lux (similar to bright moonlight) to 12.0 lux with high intensity white light; infrared light was the control. The frogs were significantly less successful at foraging under all lighting conditions and when subjected to quick increases in light intensity. A study by Baker and Richardson (2006) examined movement and calling patterns of male Green Frogs (*Lithobates clamitans*) and found that when exposed to artificial light from a flashlight, frogs did

not call as often and produced fewer multi-note calls than did frogs calling under natural lighting. Thus, urban lighting conditions may adversely affect anuran advertisement call behavior, possibly resulting in negative consequences for population and community ecology (Baker and Richardson, 2006).

The study presented herein will have two major points of focus. First, the differences of call characteristics of *S. multiplicata* in terms of ecological light pollution at sites within and surrounding highly urbanized El Paso, Texas, and rural parts of southern New Mexico. The second focus will be on call characteristics of hybrids generated from crosses between *S. bombifrons* and *S. multiplicata* in southern New Mexico.

MATERIALS AND METHODS

Study areas

Actively calling choruses of spadefoots were recorded at breeding sites, mostly ephemeral, lentic water bodies within or near the city of El Paso, Texas, where they had been previously observed. Sites were classified as urban or rural based on the proximity to and the extent of urban development and ecological light pollution. The species, *Spea multiplicata* and *S. bombifrons*, surveyed in this study are indigenous to El Paso County, Texas, and adjacent areas of Doña Ana County in southern New Mexico.

Both historical and recent data have shown that the city of El Paso and surrounding urban areas have undergone growth in both human population and geographic space. Historical records indicate the city of El Paso had a population of 736 in 1880 and had grown to 649,121 in 2010 (U.S. Census Bureau, 2013). Data collected in 1850 recorded El Paso County as having a population of 200 (El Paso-Juarez, 2006), but recent data shows the population of El Paso County at 800,647 in 2010 (U.S. Census Bureau, 2013). Contemporary data indicated a 17.8% increase in population of El Paso County during the years 2000 through 2010. Because of the close proximity of El Paso to Ciudad Juárez, Chihuahua, México, populations were first combined and documented in 1900 at 33,098; in 1996 the combined population had grown to 2,210,772, representing an astounding growth increase of over 6,000% in less than 100 years (El Paso-Juarez Regional, 2006). The urban area of El Paso has sprawled with increasing population pressures. In 1950, the city of El Paso measured 75 square km². In the past 49 years the city has increased to 642 km², an increase of more than 850% (“El Paso-Juarez Regional,” 2006). This

rapid population growth and expansion of the urban area has resulted in additional artificial lights flooding the city and adjacent natural areas, which may have resulted in abnormal behavioral effects on wildlife (e.g., Buchanan, 1993; Salmon et al., 1995; Baker and Richardson, 2006; Kenpenaers et al., 2010; and Longcore, 2010). The glow of El Paso/Ciudad Juárez can be seen at night from as far away as Indio Mountains Research Station, located about 220 km to the southwest in southeastern Hudspeth County, Texas (J. D. Johnson, pers. observ.).

Rural sites adjacent to El Paso in Doña Ana County, New Mexico, were surveyed for spadefoot toads. Historic U. S. Census data indicates that Doña Ana County had a population of 10,187 in 1900, and had grown to 209,233 by 2010. Las Cruces is the largest city in Doña Ana County, accounting for roughly 45% of its population of 97,618 in 2010 (U. S. Census Bureau, 2013). All of southcentral New Mexico has undergone considerable population growth, although 75% of Doña Ana County is owned by the federal government and 4,451 km² is undeveloped Bureau of Land Management land (Doña Ana County's Public Lands, 2012).

Eleven areas were chosen as possible field sites based on records in the UTEP Biodiversity Collections and anecdotes by UTEP professors, students, and local naturalists. Two accessible urban sites in El Paso and two rural sites in southern New Mexico were used for this study because they received enough rainfall, during rainy seasons of 2012 and 2013 (July through July), to induce toad egress and subsequent calling for mates.

Urban site 1 was Eastwood (Album) Park (Fig. 5) at the intersection of Wedgewood Drive and Album Avenue (31.78354°N, 106.34564°W; 1214 m elevation). Habitat within the park was a grassy, manicured lawn with some shade trees. During data collecting, rainwater along with irrigation water from the sprinkler system gathered at the center of the park, creating

a pool approximately 1 m deep at the center. Lights from the adjacent baseball field flooded onto the pool until approximately 22:00 h.

Urban site 2 was a large water retention area on the northeast side of Travis White Park (Fig. 5) (31.750263°N, 106.333564°W; 1195 m elevation). The water retention area filled with approximately 1 m of rainwater at the deepest point and perimeter of the pool was stippled with various shrubs and grasses. The water retention area was bordered by residential housing along the northwest, northeast, and southeastern sides. Lights from residential housing and Travis White Park continually illuminated the water retention area.

Rural site 1 (Berino Tank; Fig. 5) was a shallow earthen livestock pond located ca. 3.5 km (straight line) northeast of Berino, New Mexico (32.10009°N, 106.566928°W; 1253 m elevation). Berino Tank was surrounded by a few Honey Mesquite trees (*Prosopis glandulosa*), had a sandy substrate, and was surrounded by Chihuahuan Desert scrub habitat. The tank and area surrounding the tank showed evidence of heavy cattle grazing, but had no urban growth and very low nocturnal light levels.

Rural site 2 (Beech Tank; Fig. 5) consists of an earthen livestock pond located ca. 0.5 km north of New Mexico Highway 404 in Doña Ana County, New Mexico (32.01836°N, 106.51713°W; 1344 m elevation). Honey Mesquite trees (*Prosopis glandulosa*), and Calabazilla (*Cucurbita foetidissima*) were growing near the tank, which was surrounded by Chihuahuan Desert scrub and grasses. This tank had no urban growth, very low nocturnal light, and evidence of moderate cattle grazing.

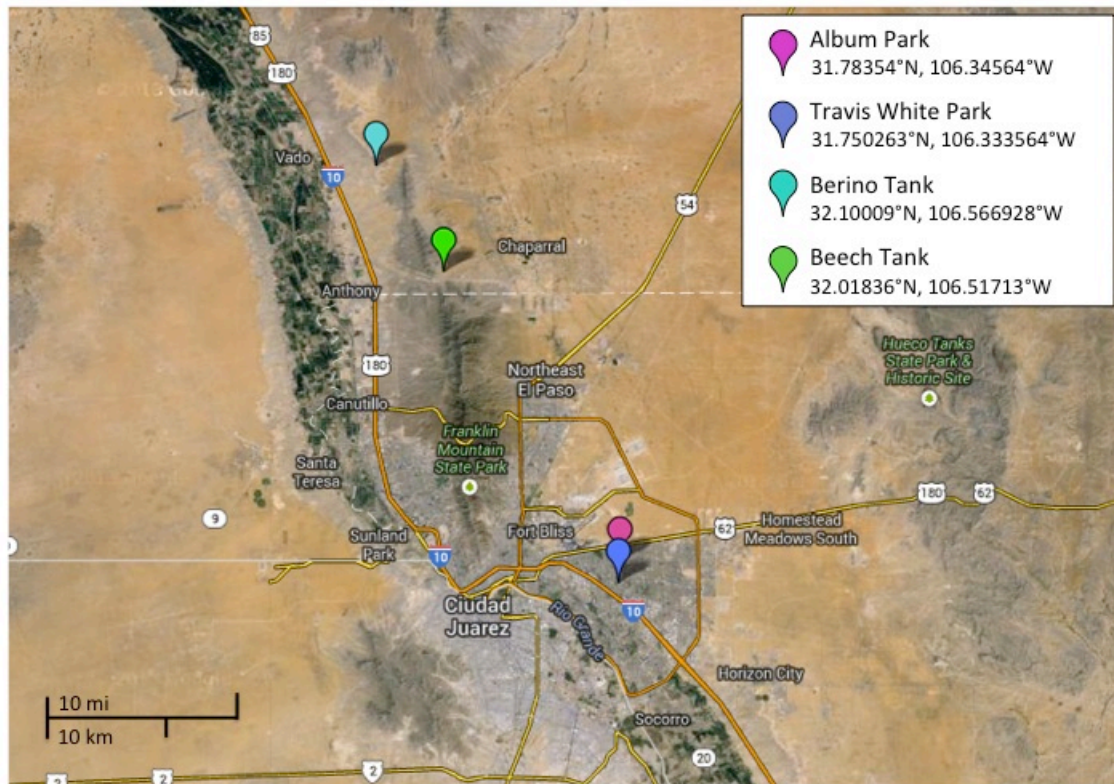


Fig. 5. Map of urban and rural field sites located in El Paso County, Texas, and adjacent Doña Ana County, New Mexico.

Data collection

This study was conducted between 4 July 2012 and 25 July 2013. Breeding congregations of spadefoot toads were located by driving to areas of localized rainstorm activity that contained natural or man-made water retention features (e.g., tanks, dams, or water retention ponds). Once a site was reached, the area was assessed to determine if there was sufficient rainfall to elicit egress and advertisement calls, by locating and listening for a breeding chorus. Actively calling males were found using flashlights and headlamps if ambient light was too dim to locate them. Toads were approached, by wading into the pool until the focal toad was within 2 m; if scared away another was sought. If the focal toad ceased calling while being approached but did not

flee, a pre-recorded call of a conspecific was played to elicit a continuation of the calling behavior. One recording was made per toad. Flashlights were turned off several seconds prior to recording sessions. Calls were recorded using a Sony ICDAX412 digital voice recorder; all were recorded in MP3 format for ease of use in later analysis. A Samson Dynamic SCR10S microphone was connected to the digital voice recorder, and attached to a 2 m long boom used to facilitate recording toads as close to the animal as possible. The number of calls recorded, were generally between the ranges suggested by Heyer et al. (1994), although due to the willingness of each toad to call or not, recordings ranged from two to 39 calls in a single recording.

Individuals were captured immediately after recording was completed and placed into resealable plastic zippered storage bags until processing. Toads were processed individually and the following pair of body size measurements were recorded: snout-vent-length (SVL) to the nearest 0.1 mm using dial calipers, and mass to the nearest 0.1 g using a digital scale. Body condition was determined using the residual index ($\log \text{ mass} / \log \text{ SVL}$), as recommended by Bancilia et al. (2010) for use with amphibians. Each frog was positively identified to species, noting color pattern and presence or absence of an intraorbital boss. Body size data, call recording information, and notes for each frog were recorded on a data sheet at the time of capture. Not all frogs recorded were captured for body size measurements because some escaped and could not be relocated. All frogs captured were held for the duration of data collecting and were then released back into the pool after recording activity was completed for that evening.

Environmental data and start time were recorded on arrival at each site. A digital thermometer (Timex® TX5170) was used to record temperature and humidity on arrival and every hour until data collection was completed. Ambient light level was measured in visual magnitudes per arcsecond done by pointing the meter (Unihedron® Sky Quality Meter) directly

overheard and pressed the power button until a reading was given. A handheld GPS (Garmin®) device was used to record latitude/ longitude and elevation at each site. Dissolved oxygen and water temperature was measured using Extech Exstik® II, and pH was measured using Oakton EcoTestr® at each pool prior to the start of call recordings. Dissolved oxygen and pH meters were cleaned with distilled water before and after use.

Specimens were collected under New Mexico Department of Game and Fish permit #1778 and UTEP IACUC protocol #A-201004-1, and then transported to UTEP for processing. Dr. Carl S. Lieb carried out euthanization and preservation procedures under approved IACUC protocol #A-614. Blood and tissue samples were preserved in 95% ethanol, and seven whole specimens were fixed in 10% buffered formalin and placed in 70% ethanol for long-term storage in the UTEP Biodiversity Collections (UTEP #20764–70).

Laboratory protocol and DNA sequence analysis

DNA was extracted from seven individual spadefoots, three *Spea bombifrons*, three *S. multiplicata* and one suspected hybrid (Fig. 6). Genomic DNA was extracted from blood and tissue samples using IBI DNA Extraction Kit (Shelton Scientific, Peosta, Louisiana). Approximately 25 mg of tissue was removed for extraction and placed in a 1.5 ml microcentrifuge tube. Samples were soaked in sterile deionized water for 90 min at 0.2°C to allow ethanol to diffuse out of the tissue, and then allowed to dry at 0.2°C for at least one hour. Tissue samples were digested in a solution of 200 µl of buffer GT and 20 µl of Proteinase K and incubated at 60°C for 30 min, inverting every 5 min. Next, 200 µl of buffer GBT was added to each microcentrifuge tube. Each sample was then vortexed for 5 sec and allowed to incubate at 60°C for 20 min, after which, 200 µl of 100% ethanol was added and samples were then vortexed

again for 10 sec. The entire solution containing digested tissue was transferred into a spin column and centrifuged at 14,000-16,000 rpm for 2 min; solution that passed through the filter of the spin column was discarded. Next, 400 µl of Buffer W1 was added and samples were again centrifuged at 14,000-16,000 rpm for 30 sec. The fluid was discarded and 600 µl of Wash Buffer was added; samples were centrifuged at 14,000-16,000 rpm for 30 sec and the fluid was discarded, and centrifuged again for 3 min at 14,000-16,000 rpm. Finally, 100 µl of Elution Buffer, pre heated to 60°C, was added and samples were centrifuged at 14,000-16,000 rpm for 30 sec. One hundred µl of DNA fluid was deposited in pre-labeled microcentrifuge tubes following each repetition. The final solutions retained from the final step were used for Polymerase Chain Reaction (PCR).

One mitochondrial gene (mtDNA) sequence was amplified using primers SCB1-F; 5'-TCCCAACCCCATCTAACATC-3', and XCB2-R; 5'-GAGGGCTAAGATTAGGATGGATA-3'. Mitochondrial genes cytochrome *b* (cyt *b*), was selected because of its utility in previous studies that investigated systematics of the Scaphiropodidae (e.g., García-París, et al., 2003; Rice and Pfennig, 2008). Unlinked nuclear loci have an evolutionary history different from that of mitochondrial genes, in order to tell both sides of an evolutionary story both mitochondrial and nuclear genes must be used (Wiens et al. 2010). The nuclear gene recombination activating protein-1 (RAG1) was used because it was found to be informative at the species level in Scaphiropodidae (Roelants and Bossuyt, 2005). Amplification was completed using a denaturation temperature of 95°C (initial denaturation period of 2 min, subsequent steps were 35 sec in duration), annealing at 50°C for 35 sec, and extension at 72°C for 95 sec with 4 sec added to the extension per cycle for 32 (cyt *b*) and 34 cycles (RAG1). Amplicons from PCR were visualized with 1% agarose gel electrophoresis, and samples were purified following standard

protocols specified for EXOSAP (Affymetrix Santa Clara, California). Sequencing of forward and reverse strands of PCR products was executed with an ABI 3700xl capillary DNA sequencer at the UTEP DNA Core Facility. Chromatograph data obtained from sample contigs were interpreted and aligned in the program Sequencer version 4.9 (Gene Codes Corp. Ann Arbor, Michigan).

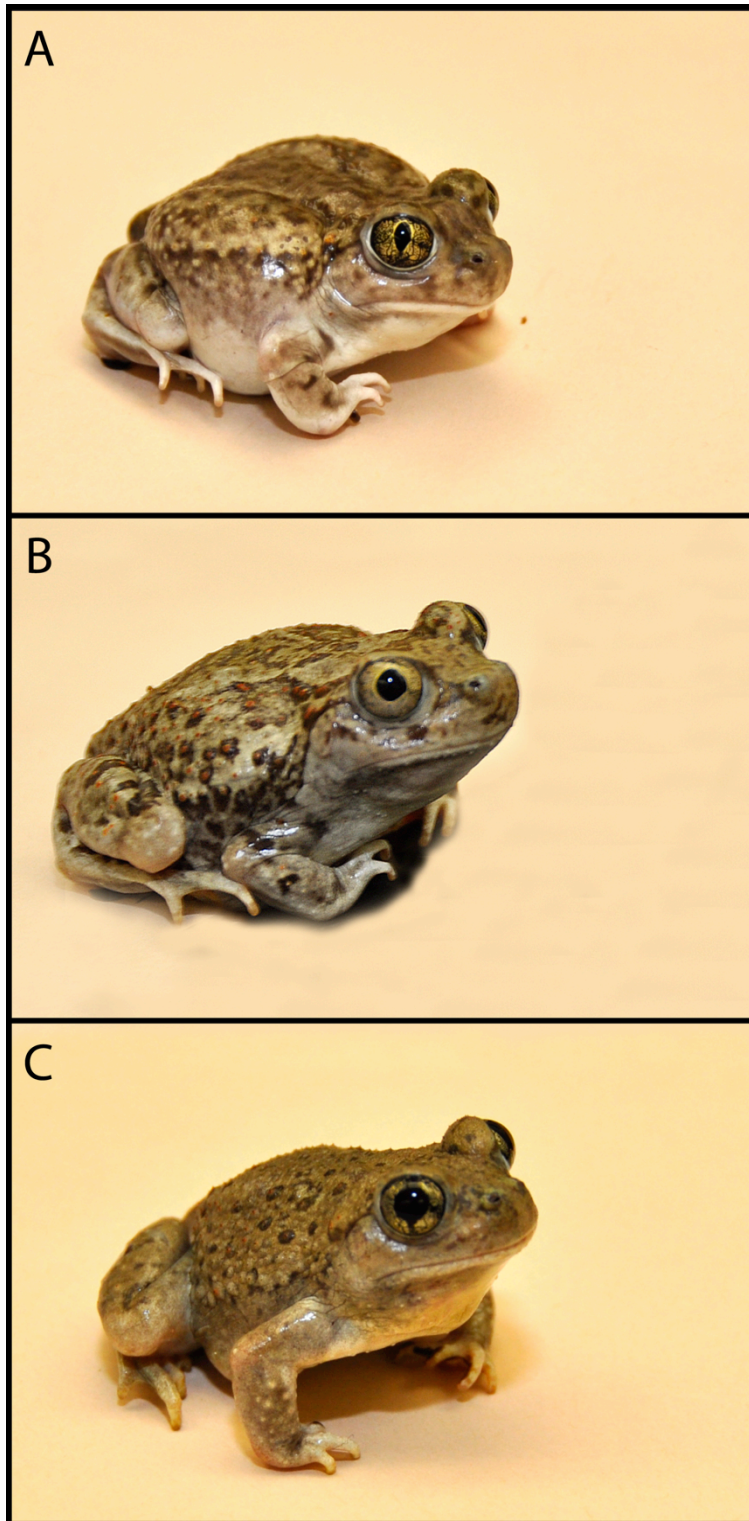


Fig. 6. Photographs of three spadefoot toads used for DNA analysis. A) *Spea bombifrons* (UTEP 20770), B) *S. bombifrons* x *S. multiplicata* hybrid (UTEP 20767), and C) *S. multiplicata* (UTEP 20764).

Mating call analyses

Advertisement call data was analyzed using Raven Pro Software version 1.4 (Cornell Lab of Ornithology, Ithaca, New York). Spectrograms were viewed in a Hann window with 50% overlap on a time grid and discrete Fourier transform (DFT) size of 256 samples on a frequency grid. Frogs call with consistent measurable auditory features (Cocroft and Ryan, 1995) and the following features were analyzed in this study.

The frog species in this study produce a series of distinct calls consisting of a series of rapid pulses followed by a brief pause and then another call (Fig. 7). The length of each call was measured from the start time of a call to the beginning of the following inter-call interval. Inter-call interval was the time measured between regular calls. Calls per minute were assessed as 60 divided by the sum of call interval and inter-call interval. Pulses per second were measured from one well-formed call recorded from each individual and assessed by dividing the number of pulses by the length of the call in seconds. The entirety of each call was measured for first quartile frequency measured in hertz (Hz). This frequency measurement divides the call into two breaks in time containing 25% and 75% of the energy of the total call. The summed energy has to surpass 25% of the total energy of the entire call measured. Third quartile frequency was also recorded in Hz for each call; this divides the call into two breaks in time containing 75% and 25% of the energy of the total call, with the summed energy of the call exceeding 75%. The peak or dominant frequency of each call was also measured; peak frequency is the frequency at which the greatest frequency occurs (Charif et al., 2010). Sound energy is derived from metabolic energy, and produced as a wave that moves through matter (air or water); it is perceived as sound

when the wave's pressure moves against the tympanum. Consequently, most frogs are not very efficient in this energy conversion (Wells, 2007).

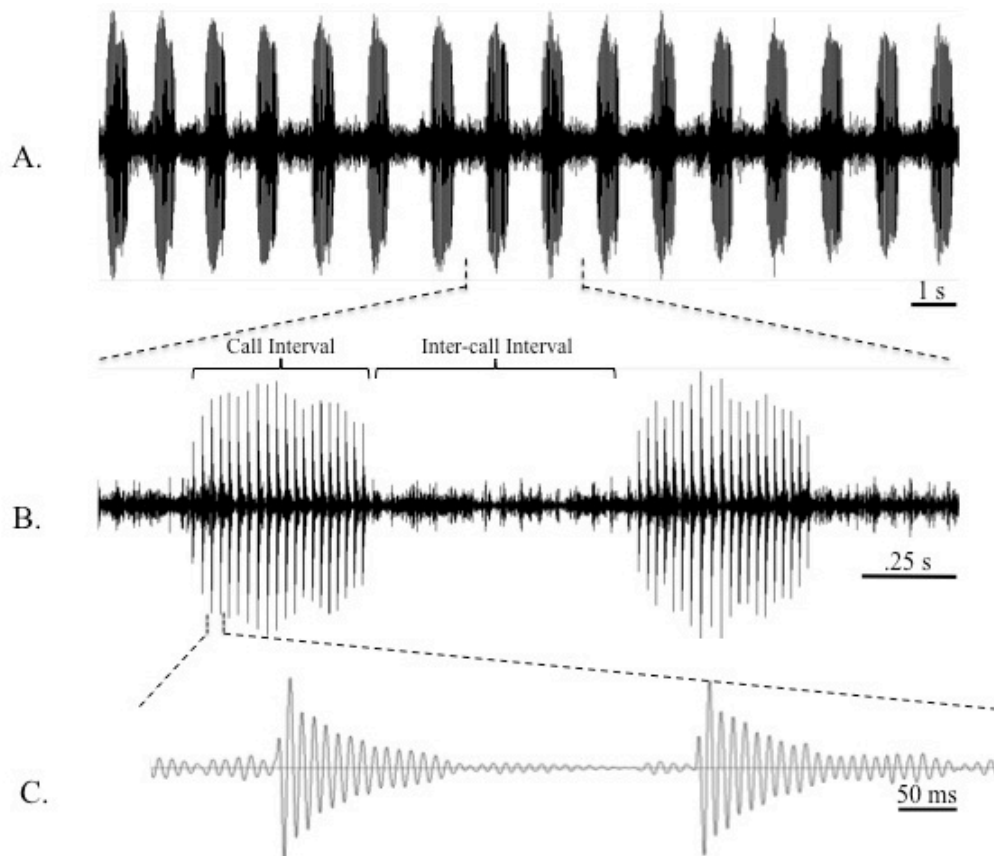


Fig. 7. Diagram of temporal and spectral call properties of a *S. bombifrons* x *S. multiplicata* hybrid. Waveform diagrams are from a single recording from a hybrid male. A. = 16 consecutive calls, B. = zoom view of two calls depicting 20 pulses each from call B., and C. = zoom view of two pulses from the call B.

Statistical analyses

Prior to statistical analyses, each individual and data associated with it was assigned to one of three species: *Spea bombifrons*, *S. multiplicata*, or *S. bombifrons* x *multiplicata* hybrid (for organizational purposes, hybrids were treated as a third species). Populations designated for

this study consist of *S. multiplicata* from Album Park (urban population), and Berino Tank and Beech Tank (rural populations). Comparisons between species consist of only rural *S. multiplicata*, *S. bombifrons*, and hybrids to eliminate the influence of confounding urban ecological variables. Call variables statistically analyzed consist of: call interval, inter-call interval, first quartile frequency, third quartile frequency, peak frequency and calls per minute. The alpha level of 0.05 was used to determine significance for the analyses of variance and covariance. Principal Component loadings greater than 0.32 or less than -0.32 are considered poor, greater than 0.45 or less than -0.45 are fair, greater than 0.55 or less than -.055 are good, greater than 0.63 or less than -0.63 are very good, and greater than 0.71 or less than -0.71 are excellent. These loadings are tied to the percentage of variance in a component, accounting for more than 10%, 20%, 30%, 40%, and 50%, respectively (Tabachnik and Fidell, 1989).

Univariate and multivariate statistics were analyzed using IBM SPSS version 20.0 (2011). Analysis of variance (ANOVA) was used to compare body condition and mass between populations. A Kruskal-Wallis test, the non-parametric equivalent to ANOVA, was used to compare SVL between populations, due to skewed data. Additionally, two linear regressions were constructed, one for log transformed mass regressed against SVL of rural and urban data, and another for SVL regressed against average peak call frequency of urban and rural *S. multiplicata*. An analysis of covariance (ANCOVA) was implemented to compare pulses per second between urban and rural populations to determine the effect of water temperature on calling rate; water temperature was the covariate. As water temperature has been shown to influence vocal characterizations (Parris et al., 2009) it was tested as a covariate. Similarly, ANCOVA was also used to test the effect of light pollution on call variables (call interval, inter-call interval, first quartile frequency, third quartile frequency, and peak frequency). A principal

component analysis (PCA) of log-transformed (base 10 log) call variables was run using the feature which allowed variables to be identified as either urban and rural populations in the component scatter plot using a correlation matrix, allowing for comparisons of the component loadings by population. Correlation matrices are recommended when variables with different units of measure are used (Noy-Meir et al., 1975; McGarigal et al., 2000). Log transformation of data is recommended when variables of different measurements are used in a PCA (McGarigal et al., 2000) therefore, all call data in PCA's were log-transformed.

Four separate ANOVA's compared log transformed call variables, pulses per second, body condition, mass, and SVL between each species. A PCA was run with log-transformed call variables from each species, using varimax rotation, with species distinguished by color code. Varimax rotation increases the differences between significant loadings and non-loading variables, and can help to better visualize data (McGarigal, et al., 2000).

RESULTS

A total of 73 spadefoot toads were used in this study, 17 *Spea bombifrons*, 51 *Spea multiplicata*, and five hybrids (*S. bombifrons* x *multiplicata*). Nine hundred thirty six calls were analyzed, 196 from *S. bombifrons*, 678 from *S. multiplicata*, and 62 from *S. bombifrons* x *multiplicata* hybrids. Fifteen *S. bombifrons* were recorded, captured, and measured, while the remaining two were recorded, but escaped capture before being measured; all 17 were found at the Berino Tank. Of the 51 *S. multiplicata*, 28 had calls recorded and were captured and measured, 22 were recorded but escaped, and one non-calling male was captured and measured. Twenty-one were found at urban site 1 (Album Park), while 19 were found at rural site 1 (Berino Tank), and 11 at rural site 2 (Beech Tank) (Tables 1, 2). Because *S. bombifrons* was found only at Berino Tank, they were excluded from analyses comparing urban and rural populations. Calling spadefoots were detected at urban site 2 (Travis White Park) on two occasions, but males in the pond became wary of the researcher and ceased calling, therefore no call data was collected from Travis White Park. Suspected hybridization between *S. bombifrons* and *S. multiplicata* was observed only at rural site 1 (Berino Tank).

Table 1. Descriptive statistics of morphometric data of *Spea multiplicata* grouped by urban and rural populations.

Population	Count (n)	Mean SVL (mm)	Mean Mass (g)	Mean Body Condition	Range SVL (mm)	Range Mass (g)	Mean Body Condition
Urban	12	43.9	10.4	0.613	38.0-47.8	6.4-13.6	0.509-0.676
Rural	17	44.6	10.3	0.613	33.3-48.1	4.4-21.04	0.423-0.759

Table 2. Descriptive statistics of morphometric data of *Spea bombifrons*, *S. multiplicata* and hybrids from rural sites.

Species	Count (n)	Mean SVL	Mean Mass	Mean BC	Range SVL	Range Mass	Range BC
<i>S. bombifrons</i>	15	48.5	14.56	0.687	41.3-53.3	11.22-17.6	0.63-0.736
<i>S. multiplicata</i>	29	44.29	10.35	0.605	33.3-55.5	4.4-21.04	0.423-0.678
Hybrid	3	50.22	14.99	0.691	48.2-53.3	13.86-16.37	0.678-0.703

Statistical analyses comparing rural and urban populations of Spea multiplicata

An ANOVA comparing urban and rural *Spea multiplicata* found that body condition and mass were not significantly different between populations. The same results were found using a Kruskal-Wallis test comparing SVL ($p < 0.05$) (Fig. 8; Table 1). A linear regression of log transformed mass and snout-vent length had an R^2 value of 0.823 (Fig. 9). A second linear regression of SVL and average call peak frequency of urban and rural *S. multiplicata* had an R^2 value of 0.422 (Fig. 10). An ANCOVA was run comparing calls per minute produced between urban and rural populations with water temperature as the covariate. The results indicated that water temperature does significantly influence the calling rate of urban and rural spadefoots ($p < 0.05$). However, when the effect of water temperature is removed, there remains a significant difference between calls per minute between urban ($\mu = 43.73$) and rural populations ($\mu = 33.72$) of *S. multiplicata* ($p < 0.05$). Therefore, urban populations were calling at a faster rate than rural populations (Fig. 11). Furthermore, an ANCOVA comparing all other call variables (call interval, inter-call interval, first quartile, third quartile frequency, and peak frequency) with light level as the covariate suggests that light significantly influences ($p < 0.05$) call interval, third quartile frequency, peak and frequency, but does not influence inter-call interval, and first

quartile frequency ($p < 0.05$). When the effect of light is removed, all call variables were significantly different between populations ($p < 0.05$) (Table 3). Rural populations produced significantly longer calls ($p < 0.05$), and a wider range of frequencies than urban populations (Fig. 11).

A PCA was run using log-transformed call variables (Fig. 12) using the feature which allows for variables to be identified by population in component scatter plots. This allows for comparisons of the component loading by population. The rescaled rotated component matrix showed principal component one loadings for log third quartile frequency and log peak frequency in the excellent range, and log first quartile frequency as fair. Component two showed log inter-call interval and log calls per minute had values in the excellent range. Lastly, component three loadings of log call interval were considered excellent (Table 4). Components one through three account for 96.8% of the variance (Table 5).

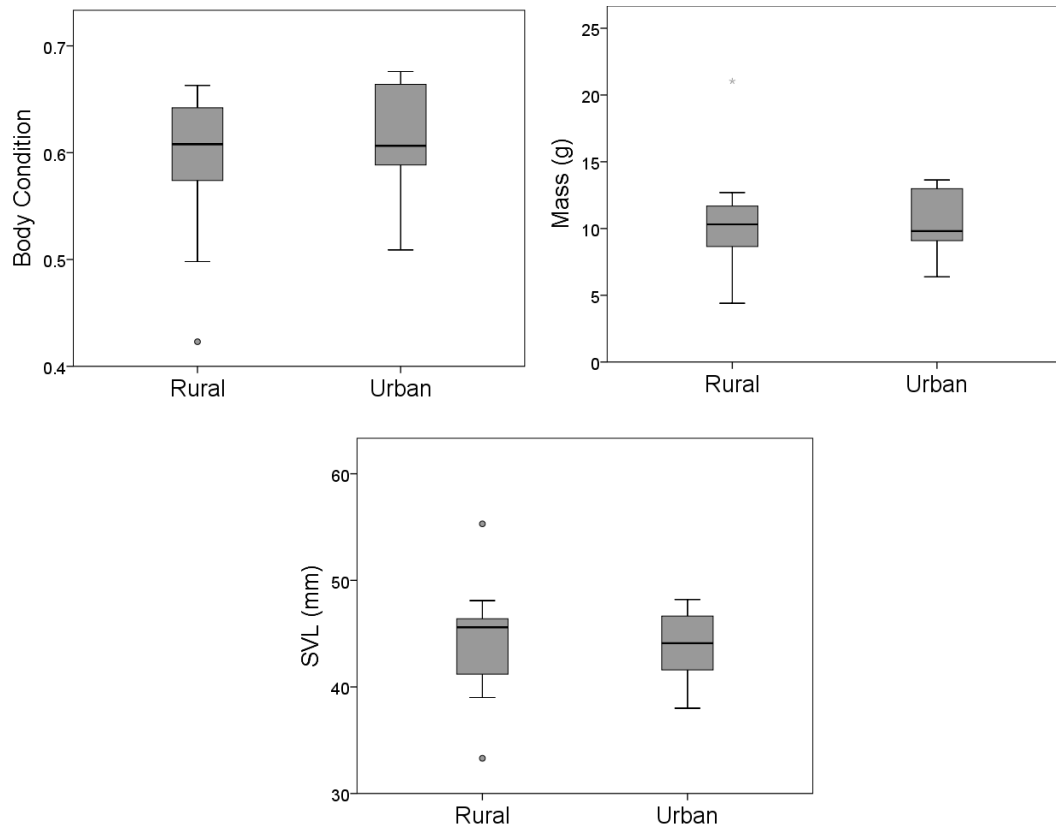


Fig. 8. Box and whisker plots depicting body conditions (measured as log SVL/log mass), mass (g), and SVL (mm) of all measured *Spea multiplicata* individuals grouped into rural and urban populations.

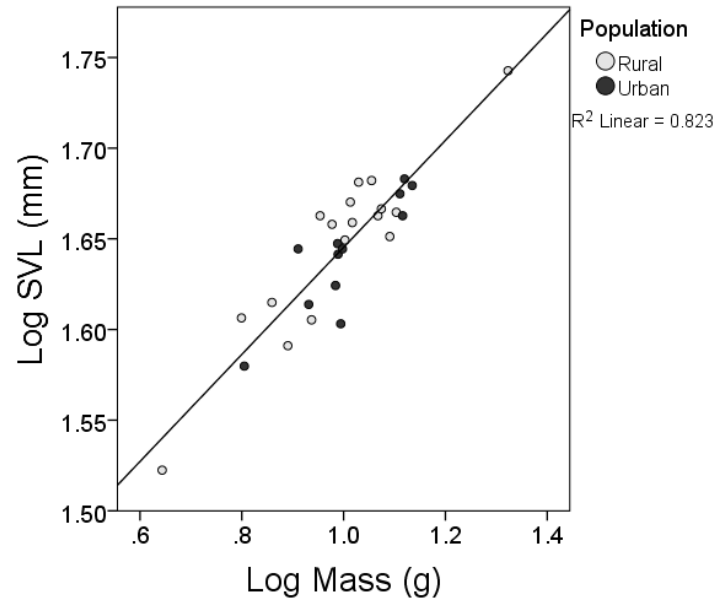


Fig. 9. A linear regression depicting log-transformed SVL (mm) regressed against log-transformed mass (g) from measured individual *Spea multiplicata* grouped into rural and urban populations. R^2 value equals 0.823.

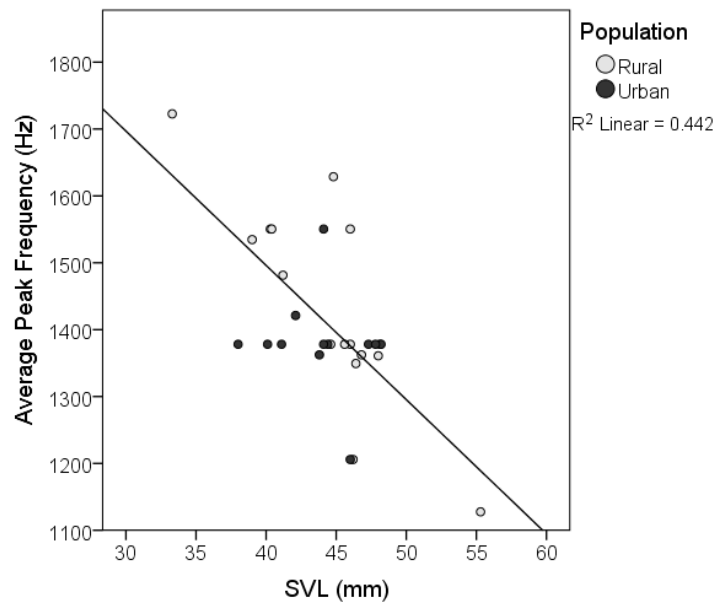


Fig. 10. A linear regression depicting the average peak frequencies (Hz) regressed against SVL (mm) from individual *Spea multiplicata* grouped into rural and urban populations. R^2 value equals 0.442.

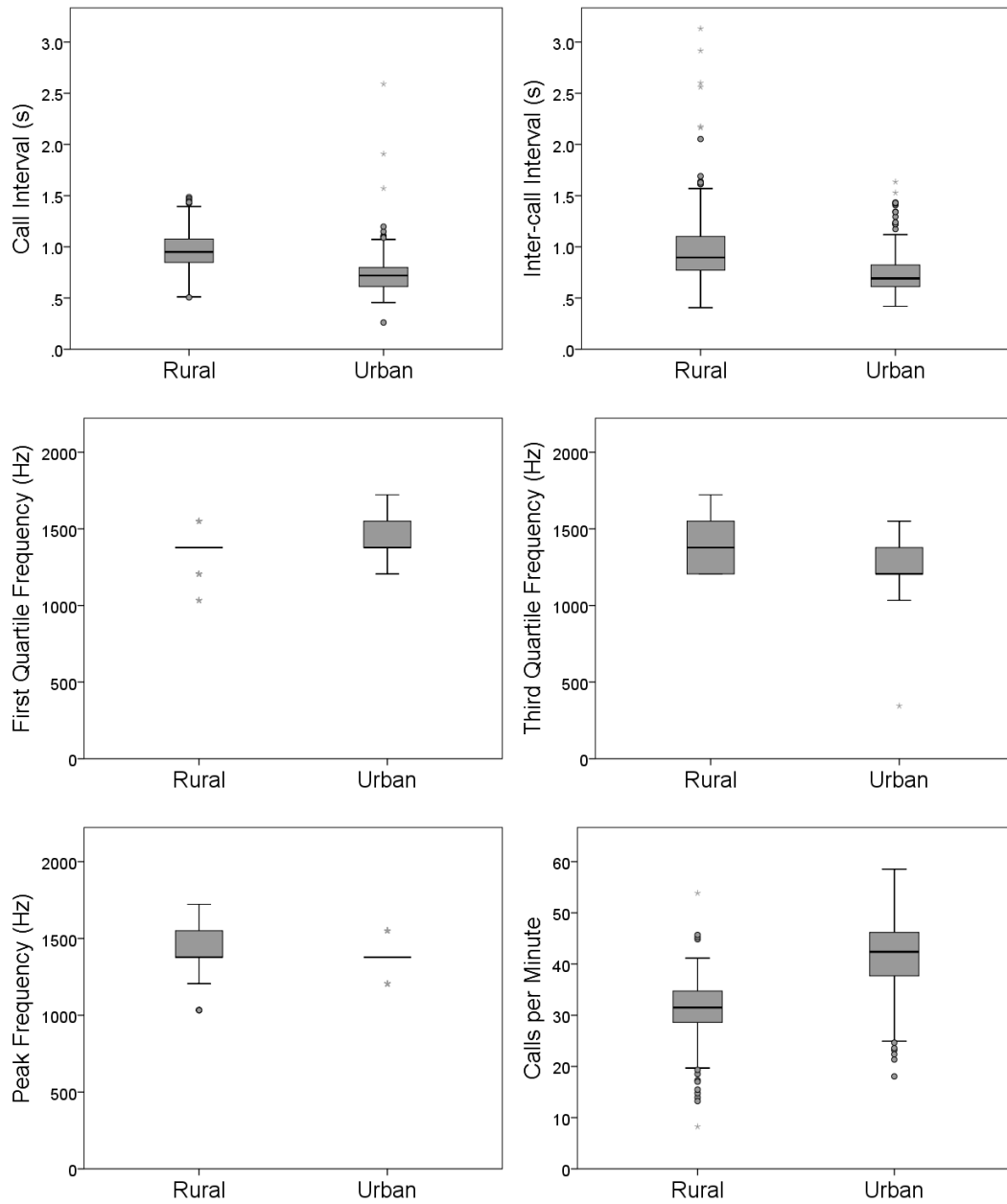


Fig. 11. Box and whisker plots of advertisement call variables measured from all recorded *Spea multiplicata*. Individuals were recorded on site at either urban or rural sites and were grouped based on classification of the site where they were located.

Table 3. Post hoc Tukey test significance values from ANCOVA of call variables of rural and urban *Spea multiplicata*, with light level as covariate (Sig. Light Level) and light level covariate removed (Sig. Population). Asterisk denotes significant values ($p < 0.05$).

	Sig. Light Level	Sig. Population
Call Interval	.000*	.004*
Inter-call Interval	.158	.000*
Q3 Frequency	.000*	.000*
Q1 Frequency	.457	.000*
Peak Frequency	.000*	.000*

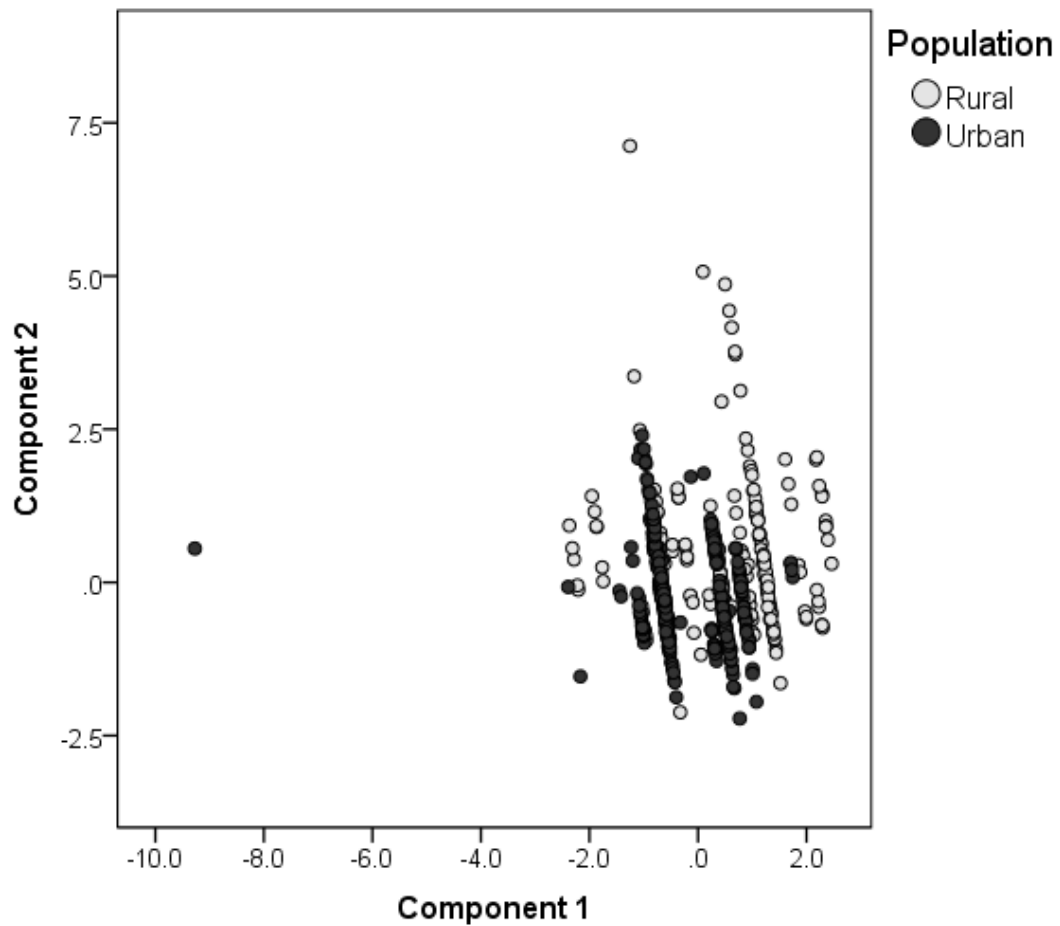


Fig. 12. Scatterplot of PCA with log transformed call variables from rural and urban populations of *Spea multiplicata*. Component one and two are depicted.

Table 4. Rescaled loading values of components one through three from PCA of log transformed call variables from rural and urban *Spea multiplicata* populations. Shadowed loadings are the most significant loading value for each variable measured.

Rescaled Components			
	1	2	3
Log Call Interval	-.101	.174	.979
Log Inter-call Interval	.156	.986	.049
Log Q3 Frequency	.936	.186	.067
Log Q1 Frequency	.544	-.159	-.326
Log Peak Frequency	.781	.160	-.045
Log Calls per Minute	-.054	-.827	-.554

Table 5. Initial eigenvalues representing the percentage variance of rescaled components one through three, with cumulative eigenvalues for each additional component of log transformed call data from rural and urban populations of *Spea multiplicata*.

Initial Eigenvalues		
Rescaled Components	% Variance	Cumulative %
1	65.171	65.171
2	24.877	90.048
3	6.789	96.837

Hybrid call analyses

An ANOVA, using log-transformed call variables, found that each species had significantly different call structures and were calling at significantly different rates in terms of pulses per second (Table 6, 7; Figs. 13, 14). ANOVA comparing means of call variables indicated significant differences between species ($p < 0.05$). A Tukey post hoc test found differences in call interval, third quartile frequency, and calls per minute to be significant between all three species. Significant differences between *Spea bombifrons* and *S. multiplicata*, and between *S. bombifrons* and hybrids were found when comparing peak frequency. Additionally, significant differences between *S. bombifrons* and *S. multiplicata*, and between *S.*

multiplicata and hybrids were found when comparing inter-call interval and first quartile frequency (Table 6; Fig. 13). A PCA compared log-transformed call variables with varimax rotation was run using the feature which allowed variables to be identified by species in the component scatter plot (Fig. 15). This allows for the comparisons of the component loadings by species. Three factors were extracted and account for 98.435% of the variance (Table 8). The rescaled component matrix showed component one loadings for log first quartile frequency and log peak frequency in the excellent range and log third quartile frequency to be considered good. Component two showed log call interval and log calls per minute loadings as excellent. Component three loading of log inter-call interval was in the excellent range (Table 9).

The advertisement call of hybrid *Spea bombifrons* x *S. multiplicata* is described from five individuals recorded from Berino Tank. Hybrid calls are distinct from the parental calls in both temporal and spectral characteristics. To the human ear, a hybrid call sounds like a quick repetition of robust high pitch snores, with a distinct build followed immediately by a shorter tail. Call interval varies from 0.209–0.653 sec ($\mu = 0.482 \pm 0.05$ SD; $n = 5$). The following description is based on a high quality recording (length of 18.597 sec), recorded at Berino Tank on 15 August, 2012 (03:42 h; 26.3° C) (Fig. 16). Call rate [(total number of calls – 1) / (time from beginning of first call to beginning of last call)] for 17 sec of continuous calling is 0.85 calls per second and inter-call interval ranges from 0.521–0.867. Call interval varies from 0.471–0.545. Hybrids first quartile frequencies ranged between 1033.6–1378.1, third quartile frequencies ranged from 1378.1–1550.4, and peak frequencies ranged from 1205.9–1550.4.

Table 6. Significance values from post hoc Tukey test from ANOVA of measured log-transformed call variables from *Spea bombifrons*, *S. multiplicata*, and hybrids. Asterisk denotes significant values ($p < 0.05$).

Sig. Call Interval	<i>Bombifrons</i> -Hybrid	.000*
	<i>Bombifrons</i> - <i>Multiplicata</i>	.000*
	Hybrid- <i>Multiplicata</i>	.000*
Sig Inter-call Interval	<i>Bombifrons</i> -Hybrid	0.814
	<i>Bombifrons</i> - <i>Multiplicata</i>	.000*
	Hybrid- <i>Multiplicata</i>	.001*
Sig. Third Quartile Frequency	<i>Bombifrons</i> -Hybrid	.000*
	<i>Bombifrons</i> - <i>Multiplicata</i>	.001*
	Hybrid- <i>Multiplicata</i>	.000*
Sig. First Quartile Frequency	<i>Bombifrons</i> -Hybrid	0.927
	<i>Bombifrons</i> - <i>Multiplicata</i>	.000*
	Hybrid- <i>Multiplicata</i>	.033*
Sig. Peak Frequency	<i>Bombifrons</i> -Hybrid	.000*
	<i>Bombifrons</i> - <i>Multiplicata</i>	.000*
	Hybrid- <i>Multiplicata</i>	0.381
Sig. Calls per Minute	<i>Bombifrons</i> -Hybrid	.000*
	<i>Bombifrons</i> - <i>Multiplicata</i>	.000*
	Hybrid- <i>Multiplicata</i>	.000*

Table 7. Significance values from post hoc Tukey test from ANOVA of pulses per second from rural *Spea bombifrons*, *S. multiplicata*, and hybrids. Asterisk denotes significant values ($p < 0.05$).

Sig. Pulses per Second	<i>Spea bombifrons</i>	.000*
	<i>Spea multiplicata</i>	.000*
	Hybrid	.000*

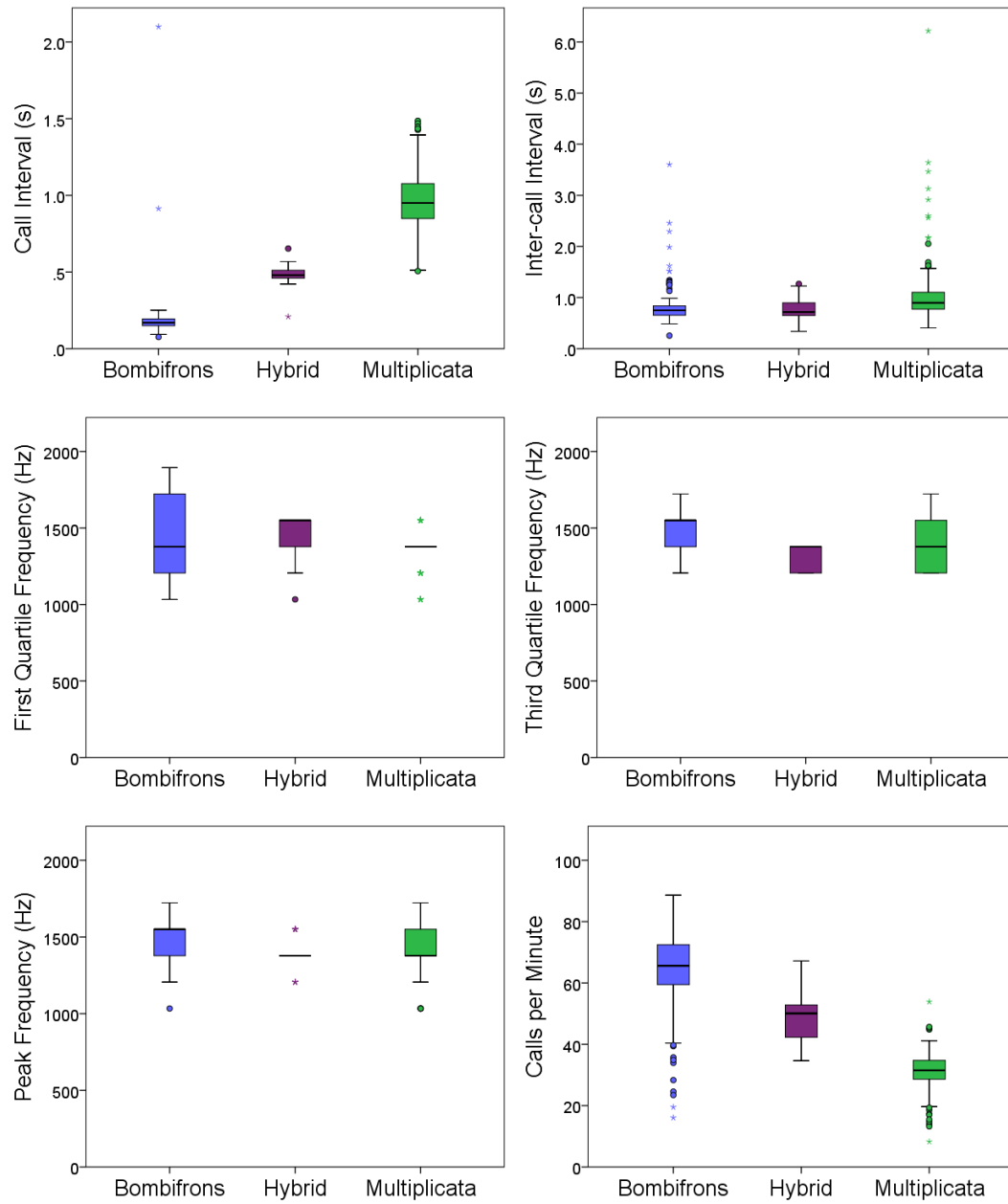


Fig. 13. Box and whisker plots of advertisement call variables measured from *Spea bombifrons*, *S. multiplicata*, and hybrids.

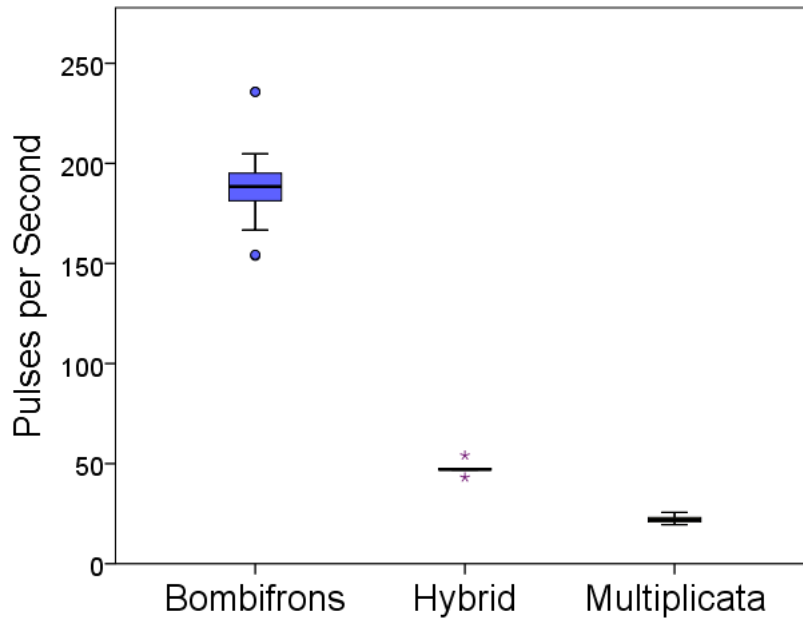


Fig. 14. Box and whisker plot depicting average number of pulses per second recorded from *Spea bombifrons*, *S. multiplicata*, and hybrids.

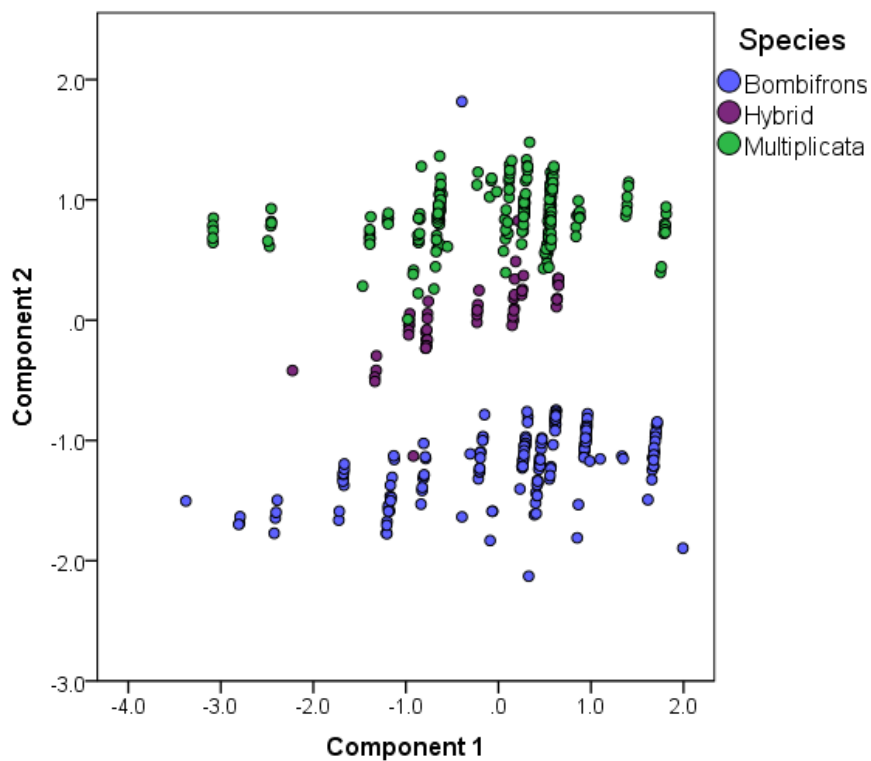


Fig. 15. Scatterplot of PCA with log-transformed call variables of *Spea bombifrons*, *S. multiplicata*, and hybrids. Component one and two are depicted.

Table 8. Initial eigenvalues representing the percentage variance of rescaled components one through three, with cumulative eigenvalues for each additional component of call variables from *Spea bombifrons*, *S. multiplicata*, and hybrids.

Initial Eigenvalues		
Rescaled Components	% Variance	Cumulative %
1	82.449	82.449
2	13.976	96.425
3	2.009	98.435

Table 9. Rescaled loading values of components one through three of a principal components analysis consisting of log transformed call variables from *Spea bombifrons*, *S. multiplicata*, and hybrids. Shadowed loadings are the most significant loading value for each variable measured.

Rescaled Components			
	1	2	3
Log Call Interval	-.219	.971	.100
Log Inter-call Interval	.066	.158	.982
Log Q3 Frequency	.618	-.117	.062
Log Q1 Frequency	.830	-.083	-.089
Log Peak Frequency	.919	-.144	.042
Log Calls per Minute	.121	-.756	-.635

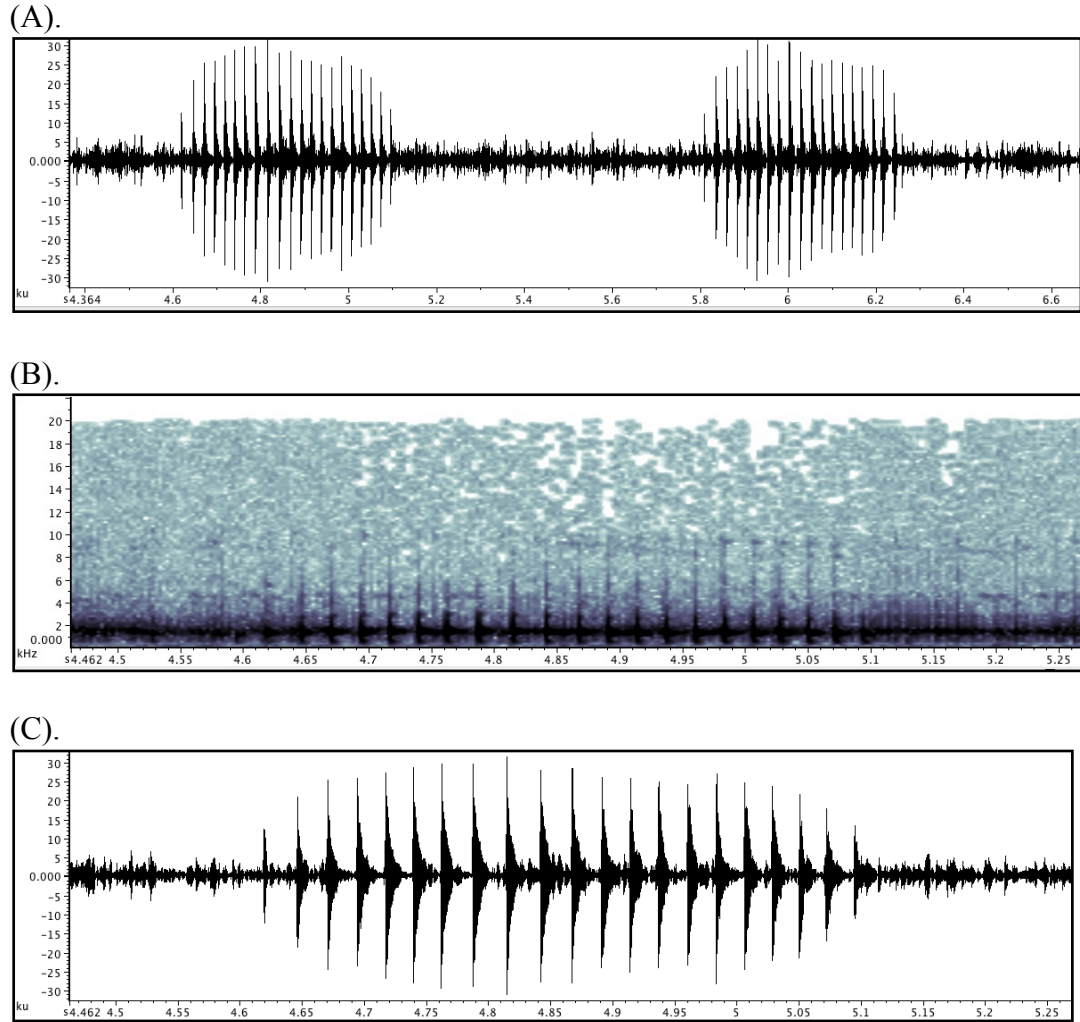


Fig. 16. The advertisement call of *Spea bombifrons* x *Spea multiplicata* hybrid, recorded at water temperature 26.3°C on 15, August 2012. (A) = waveform oscillogram, measured in relative amplitude vs. time in seconds, of two individual calls over 2.2 sec., (B) = audio spectrogram measuring 0.788 sec (frequency in kHz vs. time in sec) of the first call in A, (C) = expanded waveform oscillogram below, of the first call depicted in A.

Table 10. Significance values from post hoc Tukey test from ANOVA's of body condition, mass (g), and SVL (mm) from rural *Spea bombifrons*, *S. multiplicata*, and hybrids. Asterisk denotes significant values ($p < 0.05$).

Sig. Values Mass (g)	<i>Bombifrons</i> -Hybrids	.971
	<i>Bombifrons</i> - <i>Multiplicata</i>	.001*
	Hybrid- <i>Multiplicata</i>	.040*
Sig. Values SVL (mm)	<i>Bombifrons</i> -Hybrids	.783
	<i>Bombifrons</i> - <i>Multiplicata</i>	.021*
	Hybrid- <i>Multiplicata</i>	.074
Sig. Values Body Condition	<i>Bombifrons</i> -Hybrids	.995
	<i>Bombifrons</i> - <i>Multiplicata</i>	.000*
	Hybrid- <i>Multiplicata</i>	.040*

Hybrid body analyses

Assessments of body size (body condition, mass, and SVL) were compared between species using ANOVA (Table 10; Fig. 17). Body condition varied significantly between *S. bombifrons* and *S. multiplicata*, and between *S. multiplicata* and hybrids ($p < 0.05$), but was not significant between hybrids and *S. bombifrons*. Hybrids and *S. bombifrons* have a higher mean body condition ($\mu = .691$ and $\mu = .687$, respectively) than *S. multiplicata* ($\mu = .599$) (Fig. 17). Mass was significantly different between *S. bombifrons* and *S. multiplicata*, and between *S. multiplicata* and hybrids ($p < 0.05$). Hybrids had the highest average body mass ($\mu = 14.99$), followed by *S. bombifrons* ($\mu = 14.56$), and *S. multiplicata* ($\mu = 10.31$) (Fig. 17). SVL was significantly different only between *S. bombifrons* ($\mu = 48.5$) and *S. multiplicata* ($\mu = 44.29$) ($p < 0.05$); hybrid mean SVL was 50.22 mm (Fig. 17).

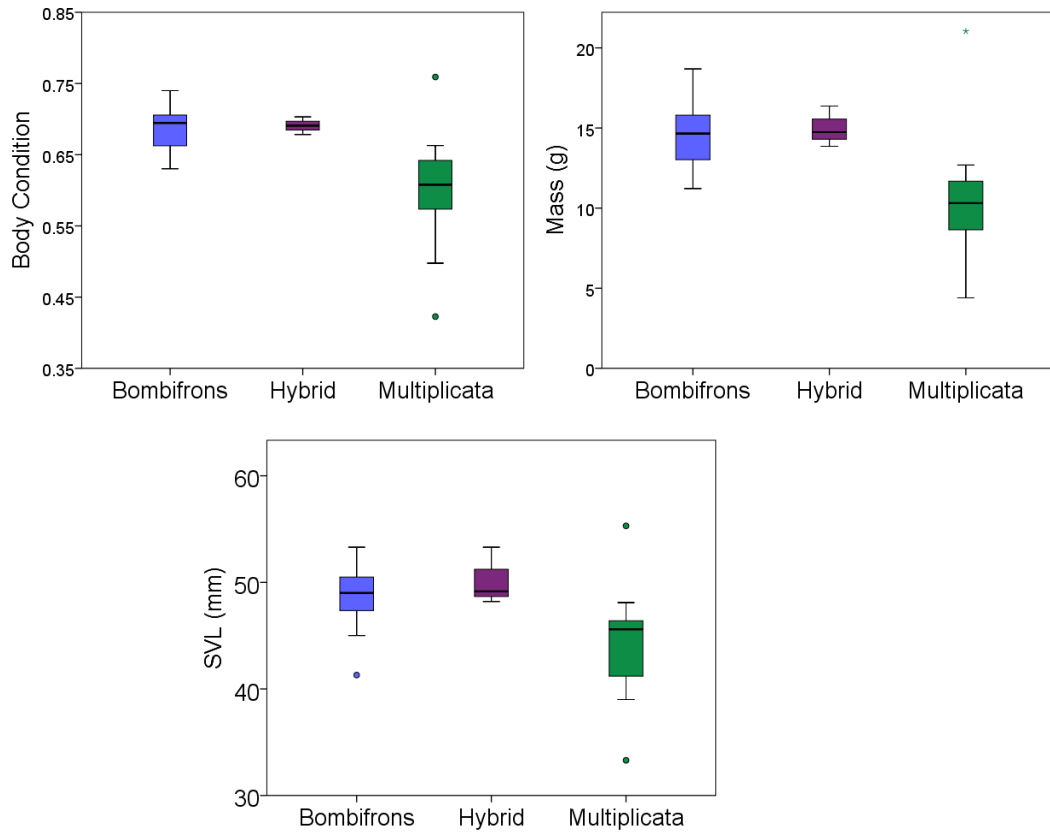


Fig. 17. Box and whisker plot depicting body conditions (measured as $\log \text{SVL} / \log \text{mass}$), mass (g), and SVL (mm) of *Spea bombifrons*, *S. multiplicata*, and hybrids.

Hybrid verification by DNA

Newly sequenced material used in this study consisted of three *S. bombifrons* (UTEP 20768–70), three *S. multiplicata* (UTEP 20764–66) and one hybrid (UTEP 20767). Hybrid origin was confirmed by sequencing 754 base pairs (bp) of mitochondrial gene *cyt b* and 800 base pairs of the nuclear gene *RAG1*. Examination of the *RAG1* gene recovered twenty variable sites between *S. bombifrons* and *S. multiplicata* that were taxonomically informative. The putative hybrid was polymorphic at 55% (Fig. 18), consistent with *S. multiplicata* at 20%, and consistent with *S. bombifrons* at 25% of informative sites. Cytochrome *b* sequences were identical between the hybrid and *S. bombifrons* sampled, confirming the maternal lineage.

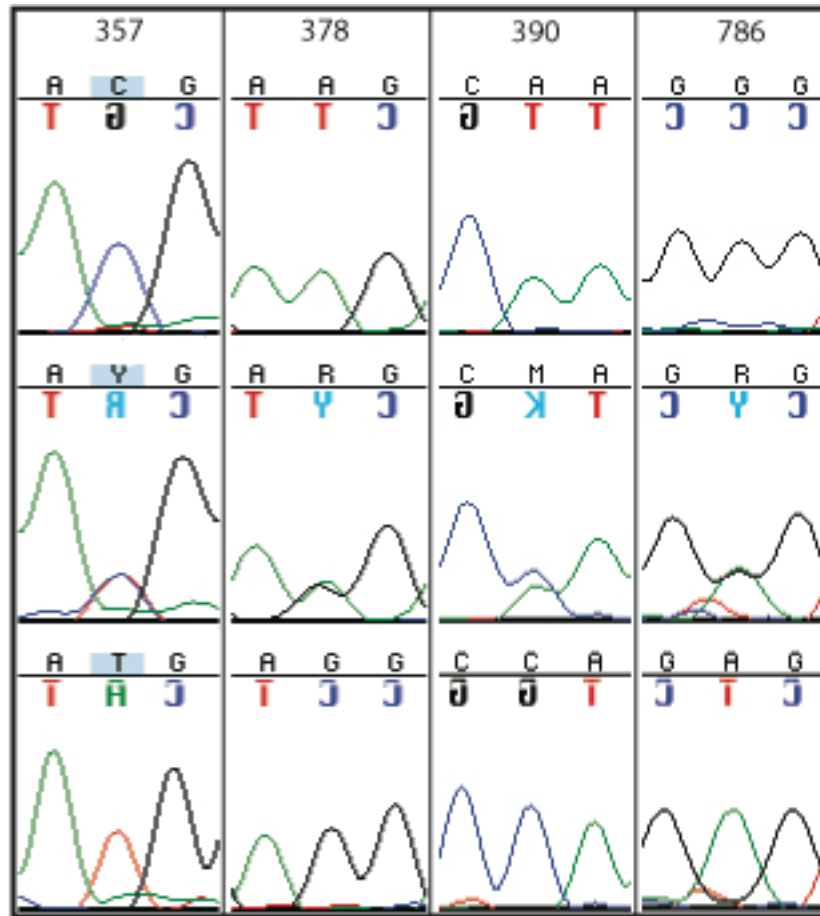


Fig. 18. Example of polymorphisms at variable sites. *Spea bombifrons* represents the top sequence, *S. bombifrons* x *S. multiplicata* hybrid the middle sequence, and *S. multiplicata* represents the bottom sequence.

DISCUSSION

*Mating calls of rural and urban *Spea multiplicata**

The evolution of male anuran advertisement calls is principally driven by sexual selection and the individual characteristics of each species' call are similarly molded by female choice (Gerhardt and Huber, 2002). One of the call characteristics analyzed in this study was acoustic energy interval, measured in first and third quartile frequencies, and peak frequency. There is a direct relationship between the metabolic energy needed to produce a call and the acoustic energy measured from the sound of the call. Metabolic energy can be measured in terms of calling rate, call duration, and oxygen consumption (Taigen and Wells, 1985). Generally, females prefer calls containing more acoustic energy. Higher acoustic energy requires more metabolic energy to produce and females interpret this as a more fit male (Ryan and Keddy-Hector, 1993; Hoback and Wagner Jr., 1997). Acoustic frequency has been reported to be linked to the size of the organism producing it (Wells, 2007), although the differences observed in this study were not likely due to difference in age classes, as means of SVL, mass, and body conditions were not significant between populations of *S. multiplicata* and fall within the published range of SVL (Stebbins, 2003) (Table 1; Fig. 8).

Many frog species, including *S. multiplicata*, show an inverse relationship between body size and dominant call frequency (Sullivan and Sullivan, 1985; Gingras et al., 2013), which was true in this study as well (Fig. 10). Females are likely choosing the fittest males based on a variety of signals both auditory and visual, including assessing male size (Pfennig, 2008).

Call rate is dependent on body temperature, which is directly influenced by the air or water temperature from which the frog is calling (Navas, 1996). This premise is supported by the results of this study; water temperature does affect the call rate of spadefoot toads. Urban spadefoots are calling faster, but also are calling in warmer water than rural populations. Interestingly, when the effect of water temperature is removed, calls per minute remain statistically significant between populations.

Previous studies on a variety of taxa have found the effects of ecological light pollution to adversely disturb several types of behaviors (e.g., Longcore and Rich, 2004; Navara and Nelson, 2007; Smith, 2008). However, few studies addressing ecological light pollution have focused on anurans. Baker and Richardson (2006) found Green Frogs (*Lithobates clamitans*) exposed to the beam of a flashlight stop producing complex calls that are preferred by females and switch to a simpler, less complex call. Buchanan (1993) observed that captive Gray Tree Frogs (*Hyla chrysoscelis*) exposed to night light treatments were less successful at foraging. The results of the present study suggest that ecological light pollution is influencing several call characteristics of *Spea multiplicata* in urban populations: including call interval, third quartile frequencies, peak frequencies, and calls per minute (Fig. 11). Furthermore, all call variables are significantly different between urban and rural populations when the effect of light is removed (Table 3). This suggests additional, unidentified variables are influencing the call characteristics of urban populations of *S. multiplicata*.

For the purposes of this study, it is assumed that light does influence call variables, but likely others, although unknown at this point, are effecting vocalizations as well. Frogs in the rural populations are producing calls with a wider range of acoustic energy intervals measured

from the third quartile and first quartile frequencies. The rural population also produced longer, slower calls, with longer call and inter-call intervals, than the urban population (Fig. 11).

Female spadefoots from rural populations may also prefer a variety of frequencies, and are likely gathering multiple genetic and phenotypic messages from calls (Pfennig, 1998, 2000). Parris et al. (2009) demonstrated that Southern Brown Tree Frogs (*Litoria ewingii*) call at higher pitches when competing with busy highway noise and suggested that because advertisement calls are instinctual, the change in frequency is an evolutionary adaptation to compensate for human induced noise pollution. However, because traffic noise in the present study was not measured and that the urban location was in a quiet residential neighborhood with a low traffic volume, their results are not directly comparable; it is likely that variables other than traffic noise are affecting call energy. From the findings of this study, it can be inferred there are numerous undefined variables affecting the call characteristics of the urban and rural populations of *S. multiplicata*. Field-based ecological investigations are notoriously difficult to control for external variables, as they are interdependent and in a constant state of flux (Smith, 1966).

Hybridization of Spea in the southwestern U.S.

Although *Spea bombifrons* and *S. multiplicata* are currently sympatric, Rice and Pfennig (2008) suggested that *S. bombifrons* had an ancestral range in the southern Great Plains of North America and underwent a major, starburst-shaped expansion during the Pleistocene. They suggested that *S. bombifrons* moved northward from their ancestral range following glacial retreat, and expanded into southwestern deserts after anthropogenic changes modified the landscape. Historically, water-retaining bodies in the desert southwest were temporary (Gehlbach, 1981), and only rapidly developing anuran larvae could persist in those conditions

(Rice and Pfennig, 2008). Of the three species of spadefoots native to the Chihuahuan Desert, *S. bombifrons* requires the most time to complete metamorphosis (15-28 days) (Voss, 1961; Justus et al., 1977; Pfennig and Simovich, 2002). This vulnerable larval interval takes considerably longer than *Scaphiopus couchii* (6-8 days) to complete metamorphosis, which is arguably the most well adapted desert anuran (Justus et al., 1977; Degenhardt, et al., 1996).

The expansion of cattle ranching around the end of the 19th century necessitated developing additional water sources, so many earthen cattle tanks were constructed by ranchers trying to make a living in desert environments (Gehlbach, 1981). The addition of those man-made, long-lasting water sources was a key factor for expansion of *S. bombifrons* into the southwest (Rice and Pfennig, 2008). It is thought that this recent range expansion lead to *S. bombifrons* and *S. multiplicata* becoming syntopic and hybridizing when breeding in the same pools (Bragg 1965; Simovich 1994; Rice and Pfennig, 2008). The calls of the two parent species differ significantly, with little to no overlap in every variable analyzed (Table 6; Fig. 13). Where those species occur together, calls of *S. bombifrons* contrast more from *S. multiplicata* than where *S. bombifrons* occurs alone (Pierce 1976), corroborating the idea that individuals of closely related, syntopic species have stronger reinforcement mechanisms than individuals of similar species not occupying the same breeding site (Noor, 1999). Female spadefoots in ponds containing closely related species are likely deciphering many call variables to find conspecific mates and avoid producing potentially costly hybrid offspring (Pfennig, 2000).

Forester (1973), after demonstrating female spadefoots could discriminate between conspecifics and closely related species, postulated that advertisement calls are important pre-mating mechanism. In the present study, each species called at different rates (Fig. 14), with *S. bombifrons* calling the fastest, *S. multiplicata* calling the slowest, and hybrids calling at

intermediate rates, which confirmed the situation reported by Pfennig (2000). Furthermore, female *S. multiplicata* select against high quality conspecific males physically resembling *S. bombifrons* when syntopic with *S. bombifrons*, but not in allopatry suggesting an evolution of these mating behaviors, thus reducing detrimental hybridization (Pfennig, 2000). This begs the question, why do these two species continue to hybridize?

Because hybrid offspring between *S. bombifrons* and *S. multiplicata* are hypothetically less fit than offspring of pure-species, pre-mating mechanisms (reinforcement) should prevent hybridization and result in reproductive isolation between the species (Liou and Price, 1994; Higgie et al., 2000). In spite of this, male spadefoots will indiscriminately amplex heterospecific females (often by interception) that can lead to viable hybrid offspring (Bragg 1965; Simovich 1994). Consequently, the effectiveness of calls as an isolation mechanism may be reduced (Wasserman, 1957; Wells 1977). Mismatches during amplexus can occur in 10–30% of pairings (Simovich, 1985), and frequency of F₁ hybrid tadpoles can range from 0.3–40% (Pfennig and Simovich, 2002). Despite reinforcement mechanisms in place, these species continue to hybridize. In a controlled mate choice study conducted by Pfennig (2000) the hybrid offspring between female *S. multiplicata* and male *S. bombifrons* were 11% larger than pure *S. multiplicata* offspring, but had a lower survival rate and took longer to develop. If *S. multiplicata* x *bombifrons* hybrids did survive metamorphosis, males were sterile, and females were found to be less fecund. Female *S. multiplicata* were found to be less likely to hybridize with male *S. bombifrons*, regardless of environmental conditions. Despite a lower survival rate, hybrids may be better adapted to surviving rapidly drying ponds. This suggests there may be selection for female *S. bombifrons* to mate with male *S. multiplicata* when breeding pools are likely to evaporate quickly after eggs are laid. The hybrids of female *S. bombifrons* and male *S.*

multiplicata may indeed be better adapted for survival at the larval stage than pure *S. bombifrons* tadpoles. Because spadefoot toads regularly breed in fast-drying ephemeral pools, many tadpoles succumb to the drying pool before they can escape (Pfennig, 2003); therefore earlier metamorphosis may be advantageous even if the tradeoff is a smaller body size. Although no tadpoles were surveyed in the present study, adult hybrids were consistently larger overall, being heavier, longer, and had a higher body condition than either parent species (Fig. 15), possibly due to hybrid vigor as F₁ generation hybrids can have higher fitness levels than either parents (Arnold and Hodges, 1995). The hybrid mitochondrial gene analyzed in this study matched with *S. bombifrons*, and at the 20 variable sites of the nuclear gene, matched at five sites (25%) with *S. bombifrons* and four sites (20%) with *S. multiplicata* (Fig. 17). Parental lineage of this hybrid is congruent with the findings from Pfennig and Simovich (2002), that hybrids are more likely to have *S. bombifrons* as the maternal lineage. The pedigree of the hybrid makes sense, as female *S. bombifrons* have an advantage interbreeding with *S. multiplicata* in a quickly drying pool. Previous studies have found the levels of hybridization between *Spea bombifrons* and *S. multiplicata* are relatively low, averaging 4.9% of 39 surveyed pools containing spadefoot assemblages, with hybrids accounting for 9.7% of the toads recorded (Pfennig and Simovich, 2002).

Conclusions and future directions

The present study is a single moment of an intricate and dynamic interaction between these two species, and the exceedingly complex and ever changing urban and rural environments in which desert spadefoots now live. Urban environments are particularly dynamic and urgently need additional investigations to help determine which anurans can adapt and flourish in that

setting; specifically how other variables are affecting their ecological dynamics, such as where they forage, critical estivation sites, and dangers from urban predators. Furthermore, additional research is needed on the extent of hybridization between desert spadefoots and reasons for the breakdown of reproductive isolating mechanisms in rural environments. The southwest is one of the most rapidly developing areas in the United States, and major urban areas including Albuquerque, El Paso, Las Vegas, Lubbock, Phoenix, and Tucson have one or more species of native spadefoots. Although these species are currently listed as least concern by (IUCN, 2013), there is no knowing how these frogs will contend with increasing urban sprawl. Additional concerns include: are spadefoots susceptible to chytrid (*Batrachochytrium dendrobatidis*) infections; which populations are at most environmental risk; and are hybrids at a higher or lower risk than parent species, and why? With the development of large cities in the southwest, more standing water is becoming available, along with the introduction of invasive species (e. g., *Lithobates catesbeianus*, *Xenopus laevis*), which could act as predators and as vectors of chytrid fungus. An ever-increasing threat to life on earth is the progression of global climate change, as the earth's deserts are predicted to become drier and rainfall events more intense and sporadic, with water sources in North American deserts likely drying up at a faster rate (Schlesinger et al., 1990, Peterson et al., 2002). That scenario may not be conducive to survival of slower developing *S. bombifrons* tadpoles. These climatic changes may also lead to increases in hybridization events between *S. bombifrons* and *S. multiplicata*. Therefore continued research on hybrids and reasons for hybridization can provide insight into one facet of how climate change will affect future Chihuahuan Desert communities.

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VITA

Katie Anderson began her college career in 2003, completing a Bachelors degree in biology at the University of Wisconsin-Stevens Point (December 2007). During her time at UWSP, she worked closely with the UWSP Herpetology Society. In the fall of 2005, Katie took an active role in the UWSP Herpetology Society where she held the offices of advertising coordinator, vivarium manager, treasurer and president prior to graduation. As an undergraduate, Katie participated in a summer internship at the Henry Vilas Zoo, where she contributed to an ongoing captive breeding project of endangered Costa Rican anurans. After receiving her B.S. she worked at the El Paso zoo as a full-time zookeeper. Sumatran Tigers, Cotton-top Tamarins, and Inca Terns were among her favorite species to work with. After working at the zoo, Katie began her pursuit of a Master of Science degree in biology under the guidance of Dr. Jerry Johnson at the University of Texas at El Paso in August of 2011. As a Masters student, Katie has served as a teaching assistant for Introductory Biology (Biol. 1107) and Organismal Biology (Biol. 1108). She has presented preliminary results from her research at the Southwestern Association of Naturalists Meeting (April 2013) and the Joint Meeting of Ichthyologists and Herpetologists (July 2013). She will be seeking a research-based career in a zoo setting following graduation.

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