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INFLUENCE OF NATURAL AND ANTHROPOGENIC LIGHT LEVELS ON FEMALE PREFERENCES IN EASTERN GRAY TREEFROGS (*HYLA VERSICOLOR*)

by

Victoria Underhill

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ABSTRACT

INFLUENCE OF NATURAL AND ANTHROPOGENIC LIGHT LEVELS ON FEMALE PREFERENCES IN EASTERN GRAY TREEFROGS (*HYLA VERSICOLOR*)

by

Victoria Underhill

The University of Wisconsin-Milwaukee, 2018
Under the Supervision of Professor Gerlinde Höbel

Chapter 1: While the influence of environmental variables, particularly temperature and rainfall, on the breeding behavior of amphibians is widely recognized, relatively few studies have addressed how the moon affects amphibian behavior. Yet, the lunar cycle provides several rhythmic temporal cues that animals could use to time important group events such as spawning, and the substantial changes in light levels associated with the different moon phases may also affect the behavior of nocturnal frogs. Using seven years of field observation data, we tested for lunar effects on the reproductive activity of male and female Eastern gray treefrogs (Hyla versicolor). We found that chorusing and breeding activity was statistically more likely to occur around the first quarter of the moon and during intermediately bright nights, but that reproductive activity also occurred during various other times during the lunar cycle. We discuss these findings in relation to the two main hypotheses of lunar effects on animals: predator avoidance and temporal synchronization of breeding.

Chapter 2: Nocturnal light levels vary throughout the course of the lunar cycle, being darkest during the new moon and brightest during the full moon. Many nocturnal animals change their

behavior in response to this natural variation in moonlight intensity. Frequently, these behavioral changes can be attributed to the way in which moonlight affects the ability of predators to spot potential prey. Mate sampling females may expose themselves to predators, making mate choice a behavior likely influenced by moonlight. Because mate choice is an important cause of sexual selection, understanding the causes of variation in mate choice decisions can yield a better understanding of the strength and direction of sexual selection under natural conditions. We predicted that female Eastern gray treefrogs (Hyla versicolor) would prefer longer calls (i.e., more attractive males) and/or be choosier, under darker conditions, because cover of darkness may aid in predator evasion. However, light treatment did not affect how females responded to variation in call duration, nor did it affect female choosiness or aspects of their approach behavior. This suggests that in gray treefrogs, variation in light levels associated with the changing phases of the moon does not alter the sexual selection regime on male call traits.

Chapter 3: Human activities are drastically changing the amount of artificial light entering natural habitats. Because light pollution alters the sensory environment, it may interfere with behaviors ranging from prey detection and vigilance to mate choice. Here we test the hypothesis that anthropogenic light pollution affects the mate choice behavior of female Eastern gray treefrogs (*Hyla versicolor*). We tested this hypothesis under two experimental light treatments that simulate the light pollution created by streetlights (i.e., expansion of lit areas and increased light intensity), and the light pollution created by headlights of passing vehicles (i.e., rapid fluctuations between bright and dark conditions). The hypothesis predicts that females tested under conditions simulating light pollution will show behavioral changes geared towards mitigating detection by predators, such as relaxed preferences, decreased choosiness for the normally preferred call, and

differences in approach behavior (either more directional, faster, or stealthier movements, or no approach at all). Contrary to our prediction, we found that light pollution did not affect mate choice behavior in gray treefrogs, and should therefore neither interfere with population persistence nor affect the sexual selection regimes on male call traits of this species. However, we caution that this result does not imply that anthropogenic light pollution is of no concern for amphibian conservation, because behavioral responses to variation in nocturnal light levels (both in the natural as well as anthropogenically enhanced range) seem to be highly species-specific in anurans. We encourage additional studies to help gage the vulnerability of anurans to anthropogenic light pollution.

To

my family,

my fiancé,

and especially my mother

for always encouraging me

to pursue my passion

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ACKNOWLEDGEMENTS

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I would also like to thank my family, especially my mother for supporting me and always encouraging me to pursue my love for animals and biology, and my fiancé for supporting and encouraging me through this process.

Chapter 1: Moonlighting? - Consequences of lunar cues on anuran reproductive activity

1. Introduction

The environmental variation in nighttime light levels associated with waning and waxing phases of the moon influences the behavior of many nocturnal animals. For example, moonlight intensity affects the activity patterns of Mexican wolves (*Canis lupus baileyi*), monkey owls (*Aotus spp*), cottonmouth snakes (*Agkistrodon piscivorus conanti*), and Italian treefrogs (*Hyla intermedia*) (Fernández-Duque et al., 2010; Lillywhite and Brischoux, 2012; Ferrer et al., 2016; Onorati and Vignoli, 2017), and the vocalization patterns of coyotes (*Canis latrans*), eagle owls (*Bubo bubo*) and several species of frogs and toads (Bender et al., 1996; Johnson and Batie, 2001; Penteriani et al., 2010). The most likely impact that variation in nocturnal light levels has on animal behavior is via its effect on the animal's visibility to predators, causing prey species to either limit their activity to darker times during the lunar cycle to avoid visually-orienting predators, or to brighter times to be able to spot the approaching predator sooner (Tuttle et al., 1982; Clarke, 1983). Not surprisingly, most studies investigating how moonlight affects animal behavior do so in the framework of foraging, predation risk, or risk assessment (see Kronfeld-Schor et al., 2013 and references therein).

In addition to the more immediate effects of lunar light, the lunar cycle also provides several rhythmic temporal cues that animals could use to time important group events such as spawning and migration. Indeed, many animals synchronize their reproduction in lunar or semilunar spawning cycles, most notably marine fish and invertebrates (i.e., Robertson et al., 1990; Naylor, 1999). Sometimes lunar spawning periodicity is related to tides (Skov et al., 2005), but some species show lunar synchronization that is not tidally mediated (i.e., Hines et al., 1985; Rahman et al., 2000), with some reef fish responding specifically to lunar light (Takemura et al., 2004). Various amphibians, birds and mammals also show lunar periodicity of reproduction (Church, 1960; Cowgill et al., 1962; Erkert, 1974; Archibald, 1976; Dixon et al., 2006). The particular aspect of the reproductive phenology that is influenced by the moon can differ between species. In anurans, for example, the moon likely influences the timing of ovulation in Javanese toads (*Bufo melanostictus*) (Church, 1960), the timing of breeding migrations in the European toad (*Bufo bufo*) (Sinsch, 1988) and the level of breeding activity in the Quacking frog (*Crinia georgiana*) (Byrne, 2002).

Most anuran amphibians (frogs and toads) are nocturnal, have breeding seasons that are temporally restricted, and are preyed upon by a variety of nocturnal predators (Wells, 2007). This makes them likely candidates to be influenced by lunar cues. In fact, a recent review found that there are significantly more examples of amphibian behavior being affected, rather than unaffected, by lunar cues (Grant et al., 2012). Nevertheless, there are almost no studies that explicitly set out to investigate the influence of lunar cues on amphibians (but see Onorati and Vignoli, 2017). Lack of information pertains in particular to the effect of moonlight; frequently full moon is categorized as bright and new moon as dark, and differences in behavior between moon phases are then attributed to assumed differences in moon-phase associated light levels (i.e., Johnson et al., 2013). Consequently, it still remains relatively unclear whether the lunar cycle itself or associated variation in moonlight intensity accounts for the observed lunar effects on amphibians, and whether predator avoidance or reproductive synchronization are the aspects most strongly affected by the moon (Grant et al., 2012).

Here we ask whether the moon affects the reproductive behavior of Eastern gray treefrogs (*Hyla versicolor*). In particular we were interested in testing predictions for the two main hypotheses of lunar effects: predator avoidance and reproductive synchronization. The predator avoidance hypothesis makes the prediction that variation in nocturnal light levels should drive variation in behavior. The reproductive synchronization hypothesis makes two predictions: (i) frogs should show increased reproductive activity during certain times in the lunar cycle, and (ii) males and females should respond in similar ways to the same lunar cues. The latter prediction is rarely tested, because it requires concurrent sampling of both males and females.

2. Methods

2.1. Study species and study site

We examined lunar effects on the reproductive activity of Eastern gray treefrogs, *Hyla versicolor*, at a pond adjacent to the University of Wisconsin Milwaukee Field Station (Saukville, WI, USA, 43.39°N, 88.03°W). It is located in a wooded area with no artificial light visible from any location within the pond. The pond is surrounded by deciduous forest (mostly Basswood, *Tilia americana*, White Ash, *Fraxinus americana*, and Sugar Maple, *Acer saccharum*), and has several patches of sedges throughout.

Experimental procedures were in accordance with guidelines of the Guide for the Care and Use of Laboratory Animals, and were approved by University of Wisconsin-Milwaukee's IACUC protocols number 06–07 #37, 09–10#25, and 13–14 #38. The sampling window for which lunar effects were tested encompassed the entire breeding season (defined as the time from the first to last evidence of calling males or amplexed females), extended by seven nights before and after. Although our data set spanned several years, it was still drawn from a single pond, and we acknowledge that this may somewhat limit the generality of our interpretation.

2.2. Collection of frog behavior data

We sampled frog breeding and calling activity during seven breeding seasons (2009–2015). Although the duration of the breeding season varied between years, on average (± SD) 1.80 ± 0.34 lunar cycles (range 1.36–2.37) occurred during each season. Note that we did not set out to test what lunar cues initiate the start of the gray treefrog breeding season (our 7-year data set would be too short to accomplish this). Rather, we wanted to examine whether lunar cues affected their calling and breeding activity throughout the breeding season.

We sampled female breeding activity over seven seasons (May–July, 2009–2015). Female breeding activity was determined by teams of 2–4 people using headlamps to search for pairs in amplexus. Searching occurred between 2100 and 0100 h on nights in which weather conditions were amenable for frog reproductive activity (i.e., temperature and rainfall intensity allowing for choruses to form). Sampling was not randomized; rather, we attempted to find as many pairs as possible.

We sampled male chorusing activity over three seasons (May-July 2011-2013). Data on chorus formation and chorus size were obtained from a long-term recorder (SongMeter SM2+, Wildlife Acoustics, Inc., Massachusetts, USA) stationed at the pond. Gain settings and location of the recorder was identical across years. The recorder was set to record for one minute every hour on the hour. Detailed analysis of hourly calling activity throughout three breeding seasons showed that (i) at our study site peak calling activity generally starts at 2100 h and ex- tends to 0100 h (on rare occasions frogs start as early as 1900 h and chorus as late as 0600 h), and (ii) irrespective of the chorus start/end times, calling will peak around 2200 h (unpubl. data). Consequently, we used the 2200 hrs-recordings to estimate the occurrence and intensity of male calling activity for any given sampling date. We scored whether a chorus formed, and for the nights with chorus activity, we estimated chorus size by average call amplitude (in rel. dB) in the frequency range of 500 Hz–2200 Hz (using Raven Pro 1.5; Cornell Lab of Ornithology). We chose this frequency range because it is only occupied by male *H. versicolor* calls, and excludes ranid calls (*Rana castesbeiana*, *Rana clamitans*; lower frequencies) and *Pseudacris crucifer* calls (higher frequencies) that may otherwise confound the relative amplitude measures. We are aware that this amplitude method does not provide a measure of the absolute number of males present in the chorus. Nevertheless, it allowed for a more fine-scale estimate of chorus size than the standard three-point NAAMP index (Weir and Mossman, 2005). For our data set, chorus size estimates from call amplitude measurements correlated well with the NAAMP index ($r = 0.85$, $P < 0.0001$; data not shown).

As mentioned above, our focal species (*Hyla versicolor*) shares the breeding pond with several other anurans, one of which (*P. crucifer*), due to its local abundance and loud vocalizations, may cause acoustic interference (the hypothesis of acoustic interference predicts a negative correlation between the calling activity of *P. crucifer* and *H. versicolor*). To verify that avoidance of acoustic interference did not skew our results of lunar effects, we obtained *P. crucifer* call intensity data from the same nights during which *H. versicolor* were sampled. This analysis revealed a positive relationship between the calling intensity of both species, i.e., when more *P. crucifer* were calling, so were more *H. versicolor* (Least square means regression: $F_{1,52.97} = 7.98$, $p = 0.007$). This suggests that our analysis of lunar effects on *H. versicolor* is not affected by acoustic interference from *P. crucifer*.

We examined female and male reproductive activity at two levels: occurrence and intensity. Occurrence refers to whether breeding took place or whether choruses formed, respectively. Occurrence data were scored as either the presence (score of 1) or absence (score of 0) of females in amplexus or male chorus formation. Intensity refers to how much breeding took place or how large the chorus was, respectively. Intensity data were taken as the number of females found in amplexus each night, or male chorus size estimated as average call amplitude (see above). The number of females in amplexus, as well as chorus size, varied between years. To be able to pool the data between years, we normalized the reproductive intensity measures and expressed them as a percentage of the total observed population in each year (see Grant et al., 2009). To do this we set the highest number of females in am- plexus each year to 100, and expressed all other female captures relative to this; likewise, for male chorus activity we set the highest call amplitude to 100, and expressed all other measures relative to this.

2.3. Collection of environmental data (lunar light and lunar phase)

We examined how female and male reproductive activity was affected by two types of lunar effects: nocturnal light levels and lunar cycle. We estimated nocturnal light levels from the fraction of the lunar disk that is illuminated (i.e., Brooke et al., 2000; Granda et al., 2008), adjusted for moonrise times. During the full moon, moonlight is available throughout the night, and during new moon, there is no moonlight at all. Half (50%) of the lunar disk is illuminated during first and third quarter. However, during the first quarter, the moon sets in the middle of the night (around 0100 h), while at the third quarter, the moon rises in the middle of the night (around 0100 h). Consequently, during the activity period of our frogs (2000-0100 h), differences in moonrise time generate radically different light environments: the sky is moonlit during the first quarter but not the third quarter (Fig. S1). We looked up moonrise times and gave all nights in which the moon rose after 0100hrs an illumination value of starlight (0% lunar disk illuminated). We obtained lunar disk illumination and moonrise data for the study area (Saukville, WI) from the website http://staging.timeanddate. com. In addition, we obtained cloud cover data from the NOAA's

Center for Weather and Climate website (https://www.ncdc.noaa.gov/data- access/quick-links#dsi-3505; West Bend Municipal Airport, the closest location that records cloud cover data; 10 km distance from the study pond). The NOAA's Center for Weather and Climate website reports cloud cover on a 4-point scale (clear, scattered, broken, and overcast). Increasing cloud cover may limit the amount of lunar light reaching the ground (Onorati and Vignoli, 2017). Some studies try to correct for cloud cover by multiplying the lunar disk illumination data by a certain correction factor; for example, overcast nights are generally multiplied by a factor of 0 (i.e., Granda et al., 2008). This correction factor of 0 seemed overly strict to us, but we also did not want to pick any other (equally arbitrary) correction factor. We therefore explored our data set to see whether cloud correction would even be required. We did so in two steps: First we tested whether cloud cover per se affected frog behavior (mixed model, the four cloud stages coded as ordinal terms). It did not (Occurrence of breeding: x^2 3 = 1.29, P = 0.73, Intensity of breeding: $F_{1,3}$ = 0.68, P = 0.57, Occurrence of chorusing x^2 3 = 2.50, P = 0.48, intensity of chorusing $F_{1,3}$ = 2.34, P = 0.08). Then we ran our analysis of lunar effects on frog reproductive behavior twice, once using the full data set, and once only including the nights with clear skies/scattered clouds (i.e., nights for which cloud corrections would not have been warranted anyway; 83% of our observation nights fall into those categories). The results were very similar (see Tables S1, S2 and S3 in supplementary material, which show the results from the analysis including only clear nights). We therefore decided to refrain from performing a cloud-correction on our moonlight estimates.

To express lunar cycle, we obtained moon phase data from the website http://staging.timeanddate.com. Then we assigned each observation night a numerical value, calculated as the days since full moon divided by 29.5 (where 0 represents the full moon, and the number of days in the lunar cycle is 29.5). Lastly, we multiplied the results obtained by 360° (0°

= 360° = full moon; 180° = new moon) (see Grant et al., 2009). We performed the transformation of lunar cycle to a 360° scale in order to analyze amphibian phenology data by means of circular statistics.

2.4. Statistical analysis

To examine the effect of nocturnal light levels and lunar phase on frog behavior, we used logistic regressions (for occurrence data) and standard least squares regressions (for intensity data) in JMP 12 software (SAS Institute Inc., Cary, NC). We entered a linear term for lunar phase, as well as linear and quadratic terms for nocturnal light. The rationale for including both linear and quadratic terms for nocturnal light was that light levels might influence activity in a linear way (i.e., largest choruses form during darkest nights) or in a curvilinear way (i.e., largest choruses form during nights with intermediate illumination, while very bright or very dark nights only see smaller choruses). We visualize the behavior of the frogs in response to variation in nocturnal light levels by fitting non-parametric cubic splines to the data, using the program PFunc (Kilmer et al., 2017).

We examined the effect of lunar phase on frog activity in more detail by computing circular statistics in the program Oriana (Kovach Computing Services, Anglesey, Wales). We used Rao's spacing test to examine whether calling and breeding took place uniformly across the lunar cycle, or whether it was clustered during certain lunar phases (Batschelet, 1981). Rao's spacing test is more robust than other circular goodness-of-fit-tests, such as the Rayleigh test, and able to analyze bi- modal and multimodal distributions (Bergin, 1991). To test whether occurrence and intensity of calling or breeding, respectively, both peaked during the same period in the lunar cycle, we computed Wat- son's U^2 test. Watson's U^2 tests for difference between distributions (Batschelet, 1981). We provide the angle of the mean vector (μ), and the length of the mean vector (r) for each test. The length of the mean vector is a measure of angular dispersion (similar to standard deviation); its value can range from 0 to 1, where r=0 indicated uniform dispersion, and r = 1 indicates complete concentration in one direction. In terms of our study, a high r-value denotes that re- productive activity is limited to a particular lunar phase, while a low r- value indicates that reproduction is distributed across the lunar cycle.

We also examined whether the sexes responded differently to lunar cues. For this comparison, we limited the data set to the three years for which data for both males and female was available (2011–2013). To test for sex differences in response to nocturnal light levels and lunar phase, we used logistic regressions (for occurrence data) and standard least squares regressions (for intensity data) in JMP 12 software (SAS Institute Inc., Cary, NC). We entered a term for sex, a linear term for lunar phase, linear and quadratic terms for nocturnal light, and sex × lunar phase or sex × light interaction terms in the model. Here, the effect of the interactions terms are of main interest: a significant sex × lunar phase interaction term indicates that males and females are reproductively active during different times in the lunar cycle, and a significant sex × light interaction term indicates that males and females are reproductively active under different nocturnal light levels. In addition, we examined sex differences in response to the lunar cycle with circular statistics, using Watson's U^2 test (Batschelet, 1981) calculated in Oriana.

3. Results

3.1. Lunar effects on male behavior

Over three seasons (from 2011 to 2013), we observed a total of 59 nights with chorusing activity (Mean: 20, Range: 15–28; on 20 additional nights individual males were calling, but no choruses formed) (nota bene: this refers only to the nights when frogs actually called; it does not represent the duration of the breeding season, which was considerably longer). Choruses were observed over a wide range of nocturnal light levels (Fig. 1 A), and through most of the lunar cycle (Fig. 1B and C). Nevertheless, choruses were statistically more likely to form during nights with intermediate nocturnal light levels (Table 1; Fig. 1A, gray line), and chorus size was also larger during nights with intermediate light levels (Table 1; Fig. 1A, black line). In these comparisons (i.e., when effects of lunar light and lunar phase are examined in the same statistical model), chorusing was never affected by lunar phase (Table 1).

Results from the data analysis using circular statistics are in line with the above results, in as much as frog activity patterns also reflect a weak preference for nights that are expected to have intermediate nocturnal light levels. Chorus formation was not uniform over the course of the lunar cycle (Rao's spacing test: $U = 183.1$, $N = 59$, $p < 0.01$), but not highly clustered around a certain lunar phase either. Choruses were statistically more likely to form before the first quarter of the moon ($\mu = 254°$, $r = 0.14$, although inspection of the circular histograms also shows a secondary peak before the third quarter of the moon; Fig. 1 B). Chorus size showed a similar pattern (Rao's spacing test: $U = 341.9$, $N = 576$; $p < 0.01$), with choruses being larger around the first quarter ($\mu = 247°$, $r = 0.14$; Fig. 1 C), but with secondary peaks around the new moon and the third quarter. The occurrence and the intensity of chorus formation throughout the lunar cycle was similar (Watson's U^2 test: $U^2 = 0.002$, $N_{chorus} = 59$, $N_{chorus\ size} = 576$, $p > 0.5$).

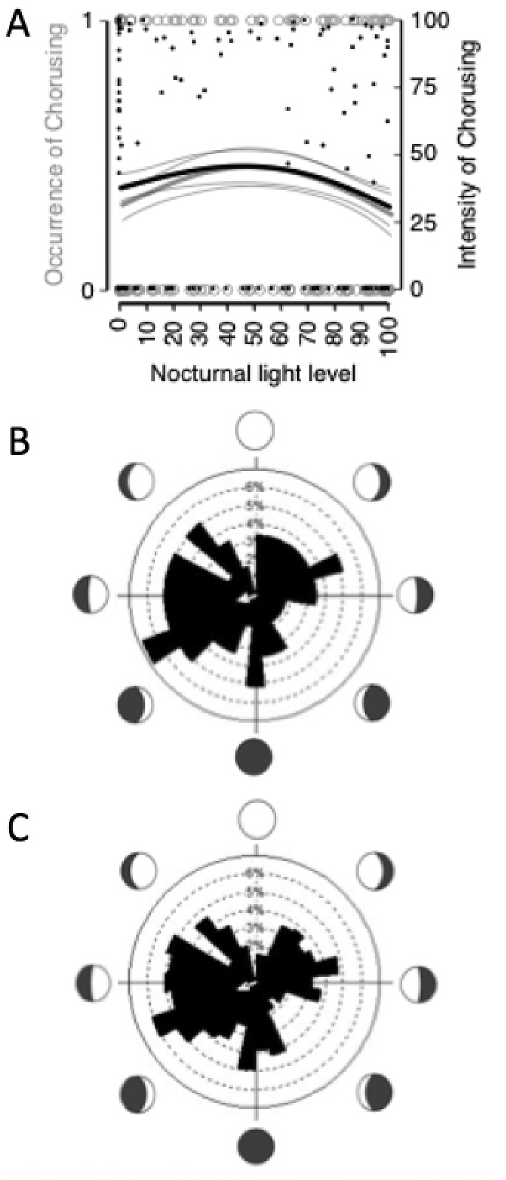
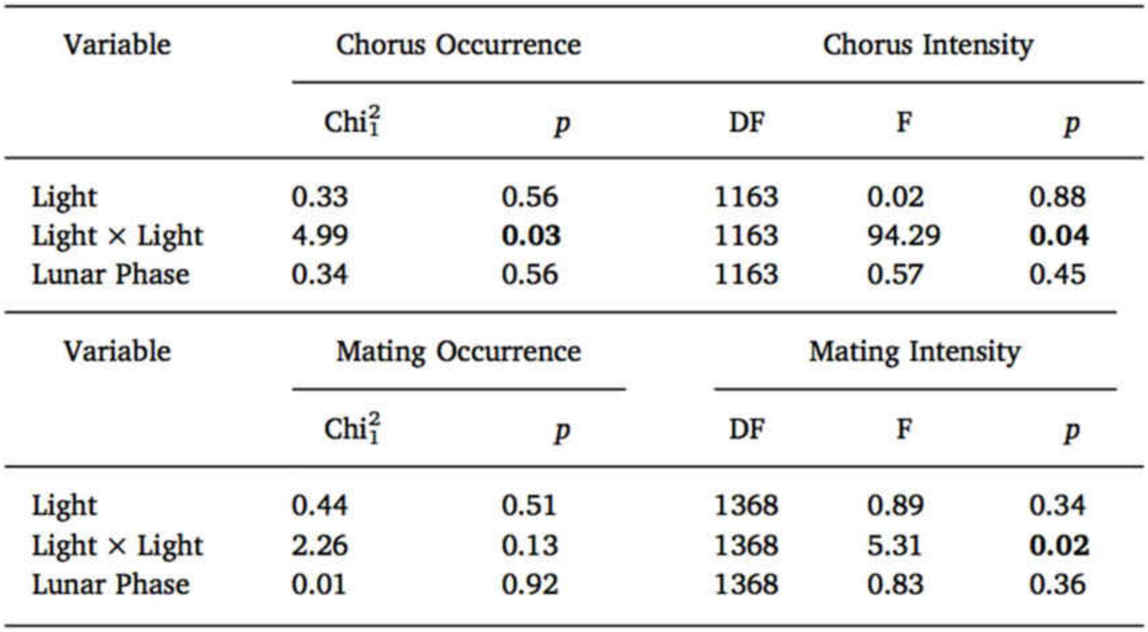


Figure 1: Lunar effects on chorus formation and chorus size in male Eastern gray treefrogs (*Hyla versicolor*). (A) Both the likelihood that chorus formed (occurrence; gray symbols and line), as well as the size of the chorus (intensity; black symbols and line) were larger at intermediate light levels. Light levels are expressed as moonrise-adjusted % lunar disk illumination values. Curves depict mean ± 1 SE. (B) Choruses were statistically more likely to form before the first quarter of the moon (μ=254°, r=0.14). (C) Larger choruses tended to form during the first quarter of the moon (μ=247°, r=0.14). Circular histograms showing the distribution (black bars) of male reproductive activity; mean vector (μ) and vector length (r) are indicated by the white arrow.



Variable	Chorus Occurrence		Chorus Intensity		
	Chi$^2_1$	*p*	DF	F	*p*
Light	0.33	0.56	1163	0.02	0.88
Light × Light	4.99	**0.03**	1163	94.29	**0.04**
Lunar Phase	0.34	0.56	1163	0.57	0.45
Variable	Mating Occurrence		Mating Intensity		
	Chi$^2_1$	*p*	DF	F	*p*
Light	0.44	0.51	1368	0.89	0.34
Light × Light	2.26	0.13	1368	5.31	**0.02**
Lunar Phase	0.01	0.92	1368	0.83	0.36

Table 1: Results of logistic regression (left) and ANOVA (right) testing whether nocturnal light levels or lunar phase affects the occurrence or intensity of reproductive activity in male (top) and female (bottom) Eastern gray treefrogs (*Hyla versicolor*). Significant effects are set in bold.

3.2. Lunar effects on female behavior

Over seven seasons (from 2009 to 2015), we observed a total of 674 pairs on a total of 67 "breeding nights" on which at least one pair was observed (Mean: 10, Median: 7, Range: 1–38). The occurrence of breeding was not significantly affected by nocturnal light levels (Table 1; Fig. 2A, gray line) but the intensity (Table 1; Fig. 2A, black line) of breeding was higher during moderately bright nights. In these comparisons (i.e., when effects of lunar light and lunar phase are examined in the same statistical model), breeding was never affected by lunar phase (Table 1).

Results from the data analysis using circular statistics are in line with the above results. Breeding was not uniform over the course of the lunar cycle (Rao's spacing test: $U = 220.3$, $N = 67$, $p < 0.01$), but not highly clustered around a certain moon phase either. Breeding was statistically more likely to occur around the first quarter of the moon ($\mu = 241°$, $r = 0.03$; Fig. 2B), but inspection of the circular histograms also shows that many breeding events occurred during the second half of the lunar cycle; this is further indicated by the very poor vector length (r) value only 0.03. Breeding intensity showed a similar pattern of being not uniform (Rao's spacing test: U = 346.7, N = 706, p < 0.01; Fig. 2C), but not highly clumped either. Many females breed before the third quarter (μ = 106°, r = 0.085), but secondary peaks can be observed at several other times. The occurrence and the intensity of breeding throughout the lunar cycle were similar (Watson's U^2 test: $U^2 = 0.059$, $N_{occurrence} = 67$, $N_{intensity} = 706$, $p > 0.5$).

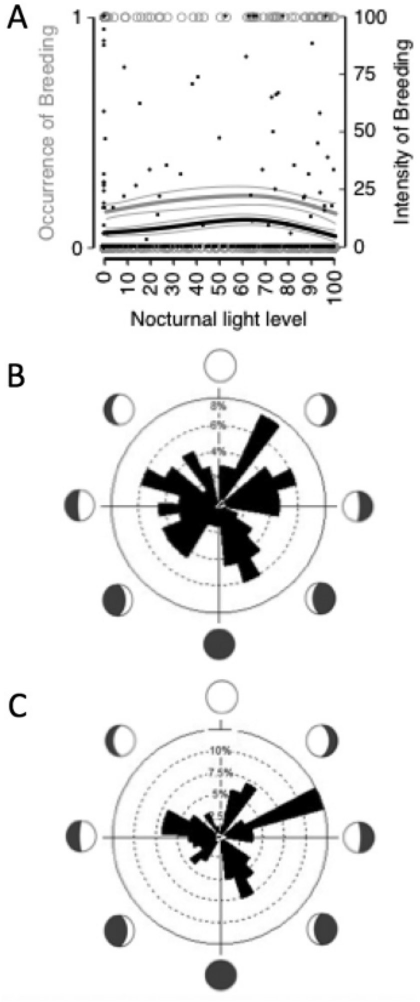
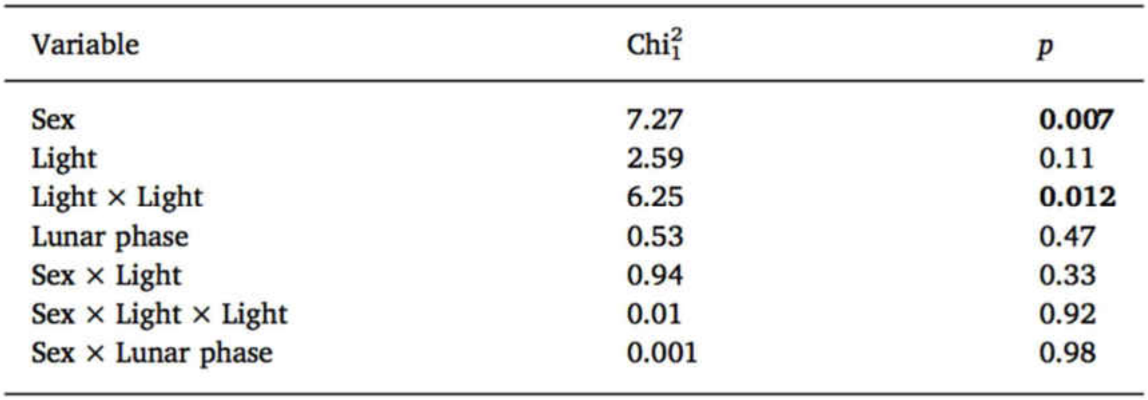


Figure 2: Lunar effects on the occurrence and intensity of breeding activity in female Eastern gray treefrogs (*Hyla versicolor*). (A) Whether females came to breed was not affected by nocturnal light levels (occurrence; gray symbols and line), but the number of breeding females (intensity; black symbols and line) was higher during moderately

bright nights. Light levels are expressed as moonrise-adjusted % lunar disk illumination values. Curves depict mean ± 1 SE. Shown is the 7-year data set. (B) Female breeding activity was statistically more likely to occur around the first quarter of the moon ($\mu = 241°$, $r = 0.03$). (C) Breeding intensity peaked around the third quarter of the moon ($\mu = 106°$, $r = 0.085$). Circular histograms showing the distribution (black bars) of female reproductive activity; mean vector (μ) and vector length (r) are indicated by the white arrow.



Variable	Chi_1^2	*p*
Sex	7.27	**0.007**
Light	2.59	0.11
Light × Light	6.25	**0.012**
Lunar phase	0.53	0.47
Sex × Light	0.94	0.33
Sex × Light × Light	0.01	0.92
Sex × Lunar phase	0.001	0.98

Table 2: Results of logistic regression testing whether lunar phase or nocturnal light levels differentially affects the occurrence of reproductive activity in male and female Eastern gray treefrogs (*Hyla versicolor*). Significant effects are set in bold.

3.3. Sex differences in response to lunar cues

In the three years for which data for both males and females was available (2011–2013), the male calling season and the female breeding season commenced on the same night. While the onset of the male calling season was always characterized by intense calling activity (i.e., a full chorus), the intensity of the onset of the female breeding season was less uniform. In 2011, almost 30% of that year's breeding females arrived on the first night, while in the other two years only 6–7% of each year's females were captured during the first night.

On average, the occurrence of reproductive activity was higher in males than in females (significant sex term in Table 2), and re- productive activity occurred more often during moderately bright nights (significant light × light term in Table 2). Although visual inspection of the graph (Fig. 3 A) suggests that occurrence of male re- productive activity peaks at lower light levels than that of females, this difference was not statistically significant (non-significant sex × lunar cue interaction terms in Table 2; Fig. 3 A). Likewise, on average the intensity of reproductive activity was higher in males than in females (significant sex term in Table 3) and was strongest during moderately bright nights (significant light × light term in Table 3). Again, although visual inspection of the graph (Fig. 3 D) suggests that the intensity of male and female reproductive activity peaks at somewhat different light levels, this difference was not statistically different (non-significant sex × lunar cue interaction terms in Table 3). Consequently, the sexes differ in their overall level of activity (higher in males), but neither occurrence nor intensity of reproductive activity in male and female Eastern gray treefrogs was affected differentially by variation in nocturnal light levels (non-significant sex × light interaction terms in Tables 2 and 3). In these comparisons (i.e., when effects of lunar light and lunar phase are examined in the same statistical model), lunar phase did not differentially affect breeding activity in male and female gray treefrog (see Tables 2 and 3).

Results from the data analysis using circular statistics only partially corroborates the above results. The occurrence of reproductive activity in males and females (i.e., when choruses formed and when re- productively active females were observed) did not differ significantly in relation to the lunar cycle (Watson's U^2 test: $U^2 = 0.039$, $N_{chorus} = 59$, $N_{mating} = 20$; $p > 0.5$; Fig. 3B and C). However, the intensity of reproductive activity (i.e., when during the lunar cycle the size of the male chorus and the number of breeding females on site was largest) was statistically significantly different (Watson's U^2 test: $U^2 = 1.16$, $N_{chorus\ size} = 300$, $N_{breeding\ females} = 576$, $p < 0.001$). Inspection of the circular histograms suggests that this result is due to male calling activity being somewhat higher around the first quarter of the lunar cycle, while more females were breeding before the third quarter of the lunar cycle (Fig. 3E and F).

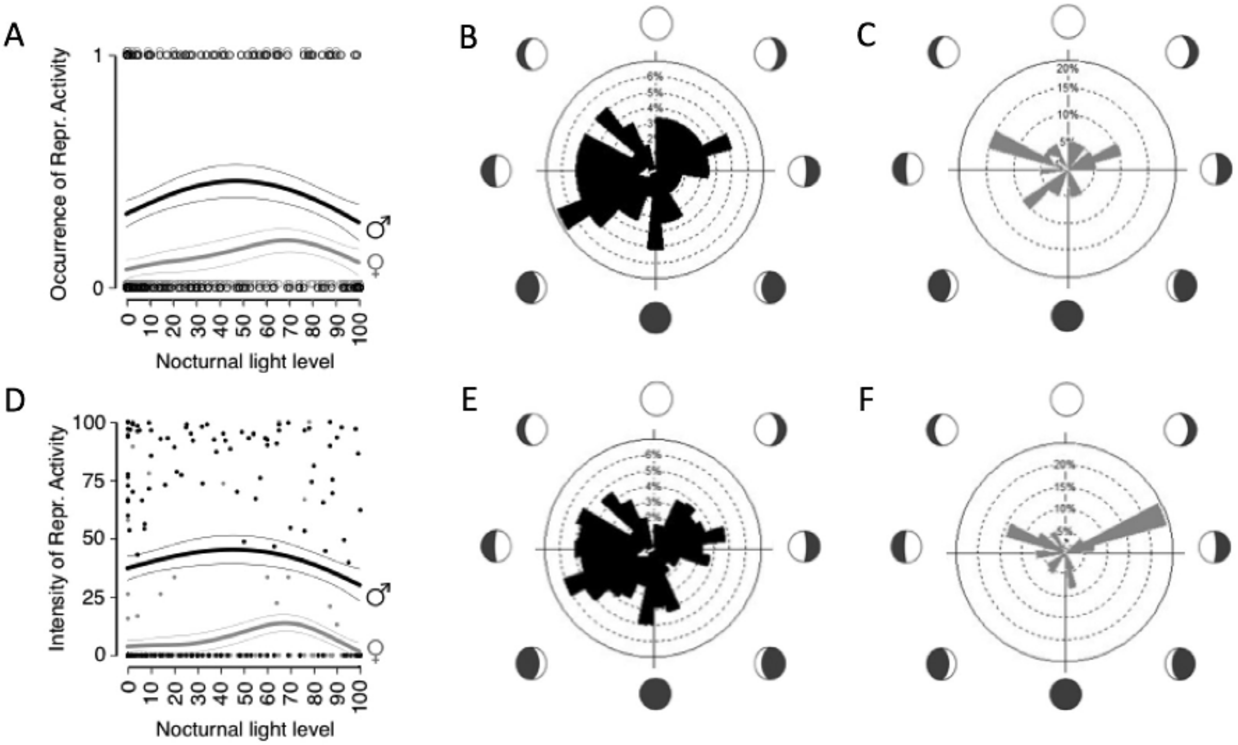
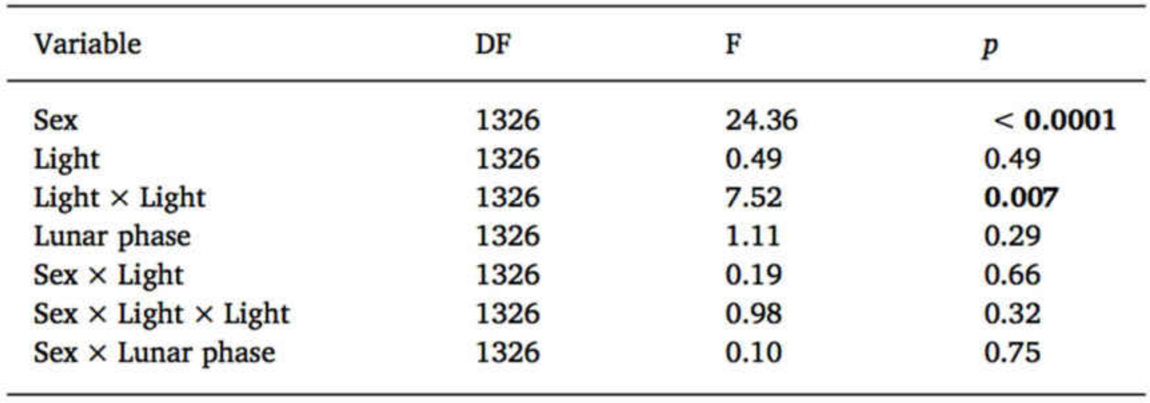


Figure 3: Sex differences in response to lunar cues. (A) The occurrence of reproductive activity in male and female Eastern gray treefrogs (*Hyla versicolor*) was not affected differentially by variation in nocturnal light levels. Both sexes tended to be reproductively active during intermediately bright nights. (B) Choruses formed throughout most of the lunar cycle, but tended to occur more frequently during the first quarter. (C) Female breeding activity tended to occur more often during the first quarter of the lunar cycle. (D) The intensity of reproductive activity in male and female Eastern gray treefrogs (*Hyla versicolor*) was not affected differentially by variation in nocturnal light levels. Both sexes tended to be reproductively active during intermediately bright nights. (E) Large choruses can form during most of the lunar cycle, but chorus size tended to be larger during the first quarter. (F) More females bred during the third quarter of the lunar cycle. Light levels are expressed as moonrise-adjusted % lunar disk illumination values. Curves depict mean ± 1 SE. Circular histograms show the distribution (bars) of reproductive activity, the mean vector (μ) and the vector length (r) are indicated by white arrow. Shown is data from 2011 to 2013, the years when data from both males and females was available.



Variable	DF	F	*p*
Sex	1326	24.36	**< 0.0001**
Light	1326	0.49	0.49
Light × Light	1326	7.52	**0.007**
Lunar phase	1326	1.11	0.29
Sex × Light	1326	0.19	0.66
Sex × Light × Light	1326	0.98	0.32
Sex × Lunar phase	1326	0.10	0.75

Table 3: Results of ANOVA testing whether lunar phase or nocturnal light levels differentially affects the intensity of reproductive activity in male and female Eastern gray treefrogs (*Hyla versicolor*). Significant effects are set in bold.

4. Discussion

Reproductive activity of Eastern gray treefrogs (*H. versicolor*) was weakly affected by lunar cues, and the effect was somewhat stronger on males than females. Males formed choruses more often under supposed intermediate nocturnal light levels, and choruses were also larger during these times. Whether females came to breed or not was unaffected by nocturnal light, but, as in males, the number of females breeding was higher during nights with supposed intermediate light levels. Furthermore, chorusing and breeding was not clumped around a particular lunar phase. Below we discuss these findings in relation to the two main hypotheses of lunar effects on animals: predator avoidance and temporal synchronization of breeding.

4.1. Effect of moonlight intensity

It is intuitive to assume that nocturnal animals such as frogs would be affected by the brightness of moonlight, yet quantitative data is surprisingly scarce. A recent review summarized data on male calling activity (43 species), and female breeding activity (8 species), but most studies were either qualitative, or equated full moon with bright conditions and new moon with dark conditions

(Grant et al., 2012 and references therein). Few studies quantitatively assess lunar light conditions, either by measuring moonlight on each sampling night (Onorati and Vignoli, 2017), by categorizing nocturnal light conditions while sampling, and then measuring light intensity for representative conditions (Tuttle et al., 1982), or by using correlates of lunar light intensity such as the fraction of the moon's visible disk illuminated (i.e., Brooke et al., 2000; Granda et al., 2008). Focusing on the nine species for which the effect of moonlight on male calling activity has been assessed quantitatively, results were mixed: as moonlight increased, calling de- creased in five species (56%) (Granda et al., 2008; Onorati and Vignoli, 2017), was unaffected in one species (13%) (Höbel, 2017), and increased in three species (37%) (Tuttle et al., 1982; Brooke et al., 2000; Granda et al., 2008). This subset of species mirrors the pattern found in the larger data set from Grant et al. (2012), and indicates that (i) although some species are neutral with respect to lunar light, in most species lunar light affects behavior, and (ii) there are substantial between-species differences in response to lunar light (spanning the gamut from positive to negative responses).

Our study found that male gray treefrogs are more reproductively active under intermediate nocturnal light levels, which is an activity pattern that has rarely been described for frogs (but see Onorati and Vignoli, 2017). We do not think, however, that this is an unusual pat- tern of lunar-light related activity. Rather, we suspect that many of the frog species listed as neutral with respect to lunar light in the Grant et al. (2012) review might actually show intermediate-level preferences that were obscured by using qualitative or overly coarse quantitative estimates of lunar light, or by only asking whether there is a linear relationship between frog activity and lunar light intensity.

To our knowledge, whether lunar light specifically affects female breeding activity (either via sampling amplexed pairs or newly-oviposited clutches) has been examined in only four species, and they differ in their behavior. In neotropical Gladiator frogs (*Hypsiboas rosenbergi*), breeding intensity is not affected by nocturnal light levels (Höbel, 2017). The gray treefrogs investigated here increase breeding intensity in nights with intermediate nocturnal light levels (breeding occurrence, however, is not affected by moonlight). In the Agile Frog (*Rana dalmatina*) (Vignoli and Luiselli, 2013) and the Italian Treefrog, *Hyla intermedia* (Vignoli and Luiselli, 2013; Onorati and Vignoli, 2017), reproductive activity intensifies during times of lower moonlight in- tensity (new moon or cloudy nights). Italian Treefrogs also showed an interesting light-mediated difference in microhabitat use – during brighter nights, females selected more concealed microhabitats for egg-laying (Vignoli and Luiselli, 2013; Onorati and Vignoli, 2017).

The last observation makes another important point: even if moonlight does not affect whether breeding aggregations form, or how large they are, nocturnal light may still influence behaviors that alter social interactions within these breeding aggregations or that change male attractiveness (female frogs generally prefer calls that are longer and produced more frequently, Gerhardt and Huber, 2002). For example, male *Smilisca sila* treefrogs call for longer, and from more open sites, on moonlit nights (Da Silva Nunes, 1988), and they increase both the rate and complexity of calls when moonlight is simulated (Tuttle and Ryan, 1982). In male túngara frogs (*Engystomops [Physalaemus] pustulosus*), the arrival of predatory bats suppresses calling during moonlit nights, while there is little change in chorusing behavior on moonless nights (Tuttle et al., 1982; Ryan, 1985). Male Green frogs (*Rana clamitans*) are more likely to call, but less likely to move, when exposed to artificial light (Baker and Richardson, 2006), and male *Hyla squirella* call less when nearby stadium lights illuminate their calling sites (Buchanan, pers com, reported in Longcore and Rich, 2004). And finally, Italian treefrogs (*H. intermedia*) exhibit shifts in microhabitat use: during bright nights, frogs are found more often on emerging vegetation in open aquatic sites, while they prefer closed vegetation during dark nights (Vignoli and Luiselli, 2013).

Changes in female mate searching and spawning behavior in response to variation in nocturnal light levels are equally diverse. For example, under brighter conditions, female túngara frogs (*E. pustulosus*) choose mates faster (Bonachea and Ryan, 2011a, b), they are less likely to travel longer distances for otherwise more attractive males (Rand et al., 1997), and they are also more likely to commit to initial mate choices, even with lower quality males (Baugh and Ryan, 2010). Likewise, during brighter conditions female reed frogs (*Hyla marmoratus*) prefer to approach a broadcast call via elevated perches, yet in darkness perch availability has no effect on phonotaxis movements (Backwell and Passmore, 1990). At the other extreme, Giant bullfrogs (*Pyxicephalus adspersus*), that normally spawn during the day, prolong the duration of spawning on bright moonlit nights (Yetman and Ferguson, 2011). And then there are species, like gray treefrogs (*Hyla versicolor*), where variation in nocturnal light levels does not affect female call preferences, nor subtle aspects of approach behavior such as whether females crawl or hop towards the speaker broadcasting a male call (Underhill and Höbel, 2017).

4.2. Lunar cycle response

In their review, Grant and colleagues found that 71% of studies (including 64 species of anurans) report some type of response to the lunar cycle (Grant et al., 2012). Again, there are substantial species differences, and no significant difference between the numbers of species that increase and those that decrease activity or reproductive behavior (including migration, breeding, and calling) during a full moon (see Grant et al., 2012 and references therein). Despite the accumulating number of studies documenting lunar effects, it is still unclear what underlies the variation in response to the lunar cycle. Grant et al. (2012) suggested that taxonomic affiliation does not account for the observed variation in lunar cycle responses, as the same genus (such as *Bufo* or *Hyla*) can contain species with both positive and negative responses to the full moon. They suggested that lunar responses may re- late more to a species' ecology. In the Eastern gray treefrogs investigated here, both occurrence and intensity of reproductive activity increased slightly around periods of half moon. This is somewhat different to the lunar cycle response of Cope's gray treefrogs (*Hyla chrysoscelis*), where breeding is more likely to occur around the new moon (Johnson et al., 2013). Both species are closely related: Cope's gray treefrog (*H. chrysoscelis*) is the diploid, and the Eastern gray treefrog (*H. versicolor*) is the tetraploid member of a cryptic diploid-tetraploid species complex (Ptacek et al., 1994; Holloway et al., 2006). The two species overlap in parts of their geographic range and have similar ecology and habitat requirements (Conant and Collins, 1998). Our data thus does not support the hypothesis that similarities in ecology underlie similar lunar responses.

4.3. Predator avoidance

Both increasing activity under brighter conditions (i.e., Tuttle and Ryan, 1982; Grant et al., 2009), as well as increasing activity as under darker conditions (i.e., Backwell and Passmore, 1990; Rand et al., 1997; Baugh and Ryan, 2010; Bonachea and Ryan, 2011a, b; Johnson et al., 2013) have been interpreted as anti-predator strategies in frogs. Bright conditions may help frogs spot approaching predators, while dark conditions may allow them to remain undetected by their predators. As pointed out by Grant et al. (2012), the large variety of predators that prey on frogs (fish, turtles, snakes, raccoons, bats, birds, aquatic insects, etc.), and their diverse hunting strategies (visual, olfactory, vibrational, echolocation, etc.) makes it difficult to speculate about the general utility of light-mediated changes in activity patterns and calling/phonotaxis behavior as a predator avoidance strategy. At first glance, the pattern found here in gray treefrogs, where both males and females increase reproductive activity under intermediate moonlight levels, may suggest a compromise between the conflicting demands of needing light to spot potential predators, and having a greater chance of remaining undetected from predators in the dark. However, male and female frogs are engaged in vastly different behaviors during the mating season. Because males loudly advertise for females, with signals that have acoustic, visual and vibrational components (Höbel and Kolodziej, 2013; Reichert and Höbel, 2015), the very activity they engage in while breeding should render them more conspicuous to predators. Males, thus, cannot use cover of darkness as an anti-predator strategy. This leaves early predator detection as the only viable strategy, which is predicted to be most effective during brighter nights. Yet, gray treefrog males preferred to chorus during nights that were only intermediately bright, and chorusing activity actually dropped during the brightest nights. Predator avoidance thus does not seem to be a very important driver of nocturnal light preferences in male Eastern gray treefrogs. Mate-searching females, on the other hand, move about stealthily. For females, both cover of darkness as well as early predator spotting during brighter nights are potentially beneficial, and being more reproductively active under intermediately bright conditions might represent a compromise between both strategies.

4.4. Reproductive synchronization

Reproductive synchronization may increase reproductive success by maximizing the number of breeding adults in an aggregation while minimizing predation risk via the dilution effect (Lima and Dill, 1990). Currently, support for lunar-driven reproductive synchronization in anurans is mixed. For example, in several European ranids and bufonids large arrival and spawning events, as well as the number of animals in amplexus, show lunar periodicity (Grant et al., 2009). Likewise, in the Australian quacking frog (*Crinia georgiana*), the number of males as well as the number of females was significantly correlated with lunar phase (Byrne and Roberts, 2004). A real test of the reproductive synchronization hypothesis would have required a direct comparison of the sexes, but results are nevertheless suggestive of reproductive synchronization via lunar effects. Then again, in the Eastern gray treefrogs investigated here, the dates in the lunar cycle during which females bred and males formed choruses were statistically similar, although the dates during which each behavior peaked were not. One might thus argue that there is lunar-driven reproductive synchronization in the occurrence (although not intensity) of reproductive activity in Eastern gray treefrogs. However, the rather uniform distribution of reproductive activity across the lunar cycle, and the fact that we did not identify a moon phase at which male and female activity peaked together, all reject a strong role of the moon in reproductive synchronization in gray treefrogs.

Three aspects of the breeding ecology of Eastern gray treefrogs may account for the apparently low degree of reproductive synchronization. First, reproductive synchronization, in general, as well as via lunar cues, may be more prevalent in explosive breeders whose breeding season only lasts a couple of nights. Indeed, the species for which Grant et al. (2009) reports lunar driven reproductive synchronization are mostly explosive breeders. Prolonged breeders like the gray treefrogs, whose season extends for several weeks, may not require such a high degree of reproductive synchronization. Second, because of the heavily male-biased operational sex ratio typical for breeding aggregations of prolonged breeding anurans (Wells, 2007), even small (i.e., low-intensity) choruses will have a surplus of males that allows for successful reproduction even in the absence of tight reproductive synchronization. Finally, a significant limitation of lunar cues driving reproductive synchronization in anurans, especially in temperate species, may be that their breeding activity is often also affected by climatic factors like temperature and rainfall (Blankenhorn, 1972; Oseen and Wassersug, 2002). At our study site in Wisconsin, for example, warm conditions after prolonged cold spells often generate large breeding events (Höbel, pers. obs.). It would be interesting to see whether lunar effects are more easily detected in tropical species, where climatic factors are more uniformly amenable for reproduction, compared to temperate ones, for which temperature may play a larger role in determining reproductive activity.

5. Conclusion

Although we found evidence for lunar effects on the reproductive activity of Eastern gray treefrogs, the influence was generally quite weak. In addition, the pattern in which moonlight or lunar phase influenced breeding and chorusing activity, while not rejecting either the predator avoidance or the reproductive synchronization hypothesis, also did not lend strong support in favor of either hypothesis. We suggest that lunar cues may have some effect on anuran breeding behavior, but that other factors, such as temperature, rainfall, or even social cues (Höbel, 2017), likely modify its effect.

In this study, we only focused on whether the general occurrence and intensity of anuran reproductive activity is affected by lunar cues. However, the social interactions within these breeding aggregations, play a crucial role in reproduction (Gerhardt and Huber, 2002). There is a small but growing body of evidence documenting that in some species, variation in light levels affects microhabitat choice, female phonotaxis behavior, and male calling behavior (i.e., Rand et al., 1997; Baker and Richardson, 2006; Onorati and Vignoli, 2017). This suggests that the role moonlight plays in anuran reproductive behavior may not be primarily via its effect on large-scale phenomena like the occurrence and intensity of frog reproductive activity, but by subtly modifying behaviors that determine mating success and thus impact the evolution of mating displays.

Chapter 2: Variation in nocturnal light levels does not alter mate choice behavior in female Eastern gray treefrogs (*Hyla versicolor*)

1. Introduction

Mate choice is an important cause of sexual selection (Darwin, 1871; West-Eberhard, 1983; Andersson, 1994), and understanding the causes of variation in mate choice decisions can yield a better understanding of the strength and direction of sexual selection (Jennions and Petrie, 1997; Coleman et al., 2004). Variation in mate choice decisions can arise from a range of sources spanning the gamut from internal factors, such as age, size, or body condition (Hunt, 2005; Uetz and Norton, 2007) to context-dependent factors such as social experience with conspecifics or with predators (e.g., Johnson and Basolo, 2003; Fowler–Finn and Rodríguez, 2012).

Context-dependent variation in mate choice decisions is quite common, particularly changes in female mate choice behavior associated with predator exposure. Interestingly, such effects can occur both when directly confronted with predators (e.g., Forsgren, 1992; Godin and Briggs, 1996; Gong and Gibson, 1996; Johnson and Basolo, 2003) as well as when increased predator exposure is merely inferred, such as when being placed in a situation that increases perceived predation risk (i.e., Backwell and Passmore, 1990; Hedrick and Dill, 1993; Rand et al., 1997; Rundus et al., 2010; Bonachea and Ryan, 2011a, b). For example, female crickets are less likely to choose a previously preferred call when it is associated with little or no cover (Hedrick and Dill 1993), and female wolf spiders (*Schizocosa floridana)* appear to forego male assessment and mate more quickly under daylight compared to darkness (Rundus et al., 2010).

The changing phases of the moon can drastically alter the visual environment, and bright moonlight is widely believed to increase predation risk for nocturnal animals by increasing the ability of predators to detect prey (Prugh and Golden, 2014). A number of studies have examined moonlight-mediated changes in foraging behavior, often finding that predators increase activity, while prey species decrease activity, during brighter phases of the lunar cycle. For example, cottonmouth snakes increase foraging activity during brighter nights (Lillywhite and Brischoux, 2012), while many rodents decrease foraging intensity during brighter nights, particularly when foraging in open habitats (Longland and Price, 1991; Kotler et al., 2010).

Mate searching involves a number of behaviors that should render the individual more conspicuous to predators, such as traveling long distances and remaining in proximity to conspicuously displaying mates. Consequently, the comparative dearth of studies examining the effect of lunar illumination on mate choice behavior is surprising. To our knowledge, how lunar light affects mate choice behavior has been investigated in only two species (both anuran amphibians) (Backwell and Passmore, 1990; Rand et al., 1997; Baugh and Ryan, 2010; Bonachea and Ryan, 2011a, b, c). The way in which the behavior of female frogs differed between dark and bright nights is consistent with the interpretation that selection has acted to adjust their behavior to less perilous dark conditions. For example, in the dark, female túngara frogs (*Physalaemus pustulosus*) were more likely to engage in phonotaxis, they took more time moving towards the sound source, and they were overall more selective (Rand et al., 1997; Bonachea and Ryan, 2011a, b). And female reed frogs (*Hyperolius marmoratus*) preferred to approach a broadcast call via elevated perches during bright moonlit nights, but perches had no effect on phonotaxis in darkness (Backwell and Passmore, 1990).

Although the above studies provide an interesting glimpse into the potential importance of moonlight-mediated variation in mate choice behavior, they tend to focus on behaviors associated with female's willingness to approach an attractive call in the face of danger. Mate choice decisions, however, are the product of a complex interaction between mate preference, i.e., the order in which females rank prospective mates, and choosiness, i.e., the effort females are willing to invest to reach their preferred mate (Jennions and Petrie, 1997). Both preference and choosiness may vary with nocturnal light levels, and they could do so either independently or in a correlated fashion. For example, a female's mate preference may remain constant across environmental conditions, but she may be less choosy under increased perceived predation risk. In addition, other aspects of the mate searching process, such as how directly a female approaches a chosen mate, or how careful and stealthy her locomotion movements are, may be influenced by the environmental context in which mate choice takes place.

We were interested in whether mate choice behavior of female Eastern gray treefrogs (*Hyla versicolor*) is affected by variation in moonlight intensity. *Hyla versicolor* is a common North American treefrog species, and has been the focus of intense research on neurophysiological (Diekamp and Gerhardt, 1995), behavioral (Runkle et al., 1994; Schwartz et al., 2001), and evolutionary (Sullivan and Hinshaw, 1992; Gerhardt et al., 2000; Gerhardt, 2005; Reichert and Höbel, 2015) aspects of mate choice and sexual selection. Studies examining female mate choice behavior in frogs are traditionally conducted in darkness (under infrared light that frogs cannot see). If moonlight-dependent variation in mate choice behavior is indeed widespread in frogs (or nocturnal animals in general), this would suggest that sexual selection regimes inferred from those experiments are only acting during a fraction of the lunar cycle (i.e., new moon), and much about the strength or direction of sexual selection acting in nature is in fact unknown even for well-studied species such as Eastern gray treefrogs.

Here we test the hypothesis that changes in nocturnal light levels associated with the changing phases of the moon affect female gray treefrog mate choice behavior. Based on previous studies of light effects on frog mate choice behavior (i.e., Backwell and Passmore, 1990; Rand et al., 1997; Baugh and Ryan, 2010; Bonachea and Ryan, 2011a, b, c), we predicted that females would behave differently under simulated dim and bright moonlight. In particular, we predicted that under bright moonlight females would show behaviors that mitigate the increased danger of being detected by predators, such as relaxed preferences, decreased choosiness, and more directional, faster or stealthier phonotactic approach movements.

2. Methods

2.1. Study site and species

The study was carried out from May-June 2016, during the natural breeding season of *H. versicolor*. We captured females in a pond adjacent to the University of Wisconsin Milwaukee Field Station (Saukville, WI). Pairs were then brought to the laboratory on the night of capture and placed on melting ice to prevent oviposition prior to testing.

2.2. Light treatments

We assessed variation in female mate choice behavior (preference functions, choosiness, approach behavior) under two light levels almost spanning the natural range of nocturnal light (0.2 lux and 2.0 lux). Light levels vary throughout the course of the lunar cycle from 0.05 lux (new moon) to 2.10 lux (full moon) (Campbell et al., 2008). We set light conditions in the testing chamber using a 25 W incandescent bulb and a dimmer (Leviton TBL03), and measured light levels using an EXTECH EasyView EA31 Digital light meter.

Nota Bene: The vast majority of studies examining the effect of nocturnal light on frog mate choice behavior have been conducted with only one species (túngara frogs), and although light source or light intensity may not be crucial for túngara frogs (see below), we acknowledge that it could be important for other species. Nevertheless, when choosing the light source for our experiments, we used the túngara literature as our guide. Based on those studies, it appears that neither variation in the type of light source used, nor variation in light intensity may be crucial for designing experiments investigating light effects in frogs. A range of different light sources, each with different spectral characteristics, have been used to experimentally simulate nocturnal light conditions, including nightlights, LED lights, and incandescent bulbs (Rand et al., 1997; Baugh and Ryan, 2010; Bonachea and Ryan, 2011a, b, c). Use of different light sources yielded similar results (Rand et al., 1997; Baugh and Ryan, 2010; Bonachea and Ryan, 2011a, b, c). Light intensity effects show a similar pattern, although comparison of light intensities used in the different studies is not straightforward, because they are reported in different units that are not easily converted (conversion factors vary depending on the wavelength, and thus, the type of light source involved). Our best attempt at conversion yields light intensities ranging from roughly 0.004 lux (irradiance of 5.8×10^{-10} W/cm^2; Baugh and Ryan, 2010) to roughly 3 lux (0.04-0.05 microeinsteins; Rand et al., 1997). Again, studies obtained similar results although they used light intensities differing by orders of magnitude (0.004 – 3 lux). Moreover, although the visual acuity of most anurans remains poorly understood, the range of light intensities used in all studies should have been above the visual sensitivity of nocturnal frogs. Cummings et al. (2008) measured visual sensitivity of túngara frogs using the optomotor response and found that the frogs exhibited visual sensitivities corresponding to light intensities below moonless conditions. Thus, while the minimum visual sensitivity of gray treefrogs are unknown, if they are similar to that of túngara frogs, then all light treatments should have provided females with more light than their sensory system requires for effective orientation and vigilance.

2.3. Stimulus generation

Male *H. versicolor* have pulsed advertisement calls consisting of a series of short pulses. At 20°C, the average temperature at of chorus formation of *H. versicolor*, as well as our testing temperature, pulses are approximately 25 msec in duration, and are repeated after a pause of 25 msec (thus, the duration of the call can be expressed in number of pulses or in milliseconds). Two frequency peaks are emphasized in the call (1100Hz and 2200Hz). Females are selective for multiple aspects of male calls, including pulse rate, call frequency, and call rate. Call duration, however, is the trait for which females have the strongest preferences (Gerhardt et al. 2000; Reichert and Höbel 2015), and accordingly we focused on call duration preferences in this experiment.

We generated acoustic stimuli in R (Version 3.1.0) software (R Development Core Team 2015), using the seewave package (Sueur et al., 2008). For all stimuli, we set call frequency and call period to the average values of our study population (i.e., first frequency peak of 1071Hz, second (dominant) frequency peak of 2142 Hz (2^{nd} peak 10dB louder); Call period of 7750 msec (see Reichert and Höbel, 2015). Call duration ranged from 6 to 30 pulses per call, increasing in increments of 3 pulses (i.e. 6, 9, 12, 15, 18, 21, 24, 27, and 30).

2.4. General testing procedure

Females were tested in a circular playback arena (2m diameter), set up inside a semi-anechoic chamber. The arena wall was constructed from hardware cloth covered in black fabric (visually opaque but acoustically transparent). We placed speakers (JBL Control 1Xtreme) just outside the arena wall and angled them towards the center of the arena. Call stimuli were played from a laptop computer, and amplified by a Behringer Reference Amplifier (Behringer, A500 Model). Call amplitude, measured at the release point of the female, was adjusted using a 407764 Sound Level

Meter (EXTECH instruments, RS232/Data logger; C-weighting, fast RMS). Unless otherwise specified, amplitude was set at 85dB SPL. Because phonotaxis trials require exact knowledge of the presented call alternatives it was not possible to record data blind.

Prior to testing, females were warmed to a testing temperature of 20°C. For testing, females were placed in an acoustically transparent release cage in the center of the arena. An acoustic stimulus was broadcast to the female while confined in the release cage; the experimental choice time began when the female was released from the cage by pulling on a rope attached to the lid. A choice was defined as entering a choice zone, marked by tape on the arena floor, within 10 cm of a speaker. Trials where a female failed to reach the choice zone or did not make a distinct choice within 5 minutes, but instead crossed the choice zone while wandering around the arena, were considered non-responses. Females that did not respond were rested for 5 minutes and rerun on that stimulus. Females that had 3 consecutive non-responses were not tested further and their data set was discarded. Only 6 females out of the initial 61 tested stopped responding, so our sample size was 55 responsive females (Preference Function trials: n=20; Choosiness 12 vs. 24 trials: n=20; Choosiness 6 vs. 18 trials: n=15). Non-responses were not included in the analysis. Female movements were observed and recorded from outside the chamber using a video camera (EQ150, EverFocus USA, Duarte, CA, U.S.A.) mounted above the arena. Following testing, pairs were returned to the pond.

It took 1-2 hrs for a given females to complete her set of trials, and individual females had 5 to10 min rest between consecutive trials. To ensure that the females' eyes were adapted to the treatment light level, we placed them into the testing chamber (in small individual transparent boxes) at least 5 min. prior to starting the experiment. Between tests, we kept females inside the testing chamber, but placed them in individual transparent boxes inside a larger lidless, thick-walled cooler. The cooler was placed in the quietest location inside the test chamber (i.e., outside the actual testing arena and at 90-degree angle from the speaker(s)). Although this did substantially attenuate the calls broadcast to the female currently being tested, it did not completely prevent females in the cooler from hearing the playbacks. Rapid shifts in light intensity (which are unavoidable when transporting frogs to and from the test chamber to a holding area outside the chamber) affect frog behavior (Buchanan, 1989, 1993). Keeping frogs in the test chamber was the only logistically feasible way to maintain females' eyes continuously adapted to experimental light levels for the duration of the experiment.

2.5. Testing variation in call duration preferences

We assayed female call duration preferences using a single-speaker design, where call stimuli varying in duration are presented sequentially, and the latency of a female's approach towards the speaker are noted as a measure of preference. To obtain data on approach latency, we used a stop watch to time from the moment the release cage was lifted and the females was free to move around the arena until she reached the choice zone at the speaker.

We examine variation in call duration preferences using preference functions, which are curves that describe female responses as a function of variation in call traits (i.e., Ritchie, 1992; Meyer and Kirkpatrick, 2005; Rodríguez et al., 2006; Reichert and Höbel, 2015). In this function-valued approach, the entire preference function is considered as the trait of interest, which allows us to extract further preference function traits to describe female preferences (Fowler-Finn and Rodríguez, 2012). We visualize preference functions by fitting non-parametric cubic splines to the response data, using the program PFunc (Kilmer et al., 2017). After generating individual preference functions (two functions per female, one for each light treatment), the program also extracts several traits from those preference functions: (1) Peak describes the female's most preferred call duration value (i.e., call duration eliciting the fastest response); (2) Strength describes the extent to which a female's response is reduced to signals that deviate from the peak preferred signal; (3) Tolerance describes the range of call duration values that still elicits a high level of response (within 1/3 of the function peak); and (4) Responsiveness quantifies the mean response across the range of stimuli. In this experiment, a strong preference for a signal is expressed by a fast approach towards the broadcast call (i.e., a short response latency). However, because interpretation of results is more intuitive if a female's most preferred value is shown as the highest point in a curve, not the lowest, we converted raw latency (in seconds) to 1/latency before generating preference functions.

A total of 20 females contributed to the data set, each female responded to the full complement of 9 call duration stimuli (6-30 pulses; presented in random order) under each of two light treatments (randomly assigned to start with the dim or bright treatment). Thus, each female provided two preference functions (one for each light treatment).

Latency is a commonly used measure of preference in studies of anuran mate choice (see Gerhardt, 1992; Bosch et al., 2000; Lynch et al., 2005; Reichert and Höbel, 2015), and we consequently focused our detailed description of call duration preferences on this measure. However, since we were interested in behaviors that might affect "risk", we also examined other aspects of female phonotaxis behavior. The rationale for this is that a female taking a short, direct path with frequent stops, and another female taking a long, winding path walking continuously does show very different approach behaviors, yet may end up spending the same amount of time in phonotaxis (i.e., same latency value). To obtain data for approach path length and directionality, we used the program Avidemux (http://fixounet.free.fr/avidemux/) to break videos into individual frame JPGs, and then used the Extended Depth of Field plugin for ImageJ (Schneider et al. 2012) to stack the single-frame JPGs into a composite image that showed the approach path taken by the female. We then used ImageJ to measure the approach path length (by tracing the path the female took from the release cage to the choice zone), and the leave angle (the angle, relative to the playback speaker, at which the female left the release cage).

Statistical Analysis- To test for differences in preference function shape between light treatments, we entered the phonotaxis measures (latency, path length, and leave angle, respectively) as response variables in a mixed model (standard least squares) implementing REML. As predictor variables, we entered linear and quadratic terms for call duration, a term for light treatment, and a call duration × treatment interaction term. We entered both a linear and a quadratic term for call duration to capture the effect of preference function shape: a significant linear term would indicate that females prefer longer calls, while a significant quadratic term indicates that females prefer an intermediate call duration value. We also entered female identity as a random term, to account for each female having provided data for two full preference functions (in dim and bright conditions).

We analyzed each preference function trait (derived from the latency-based preference function) using a mixed model (implementing REML) with the preference function trait (i.e., peak, strength, tolerance, or responsiveness) as the response variable, and the treatment (dim or bright condition) as the independent variable. Because each female contributed two data points per preference function trait (one from the preference function under dim and one under bright light), we also included female identity as a random term in the model. All statistical tests were implemented in JMP 11 (SAS Institute Inc., Cary, NC).

2.6. Testing variation in choosiness

We assayed choosiness using a two-speaker design, that is based on the common observation that female frogs trade off call attractiveness with distance to source (i.e., females may approach a less attractive stimulus if it is perceived as being closer, or played at relatively higher amplitude; Gerhardt, 1987). Here, an attractive and an unattractive call are played antiphonally (from speakers separated by 180 degrees, each facing the center of the arena). The amplitude of the unattractive call remains constant at 85dB SPL, while the amplitude of the attractive call is attenuated in successive trials (in 3 dB steps), until the female no longer approaches the attractive call.

Females frequently discriminate more strongly against very unattractive calls, while showing only slight preferences between average and attractive calls (Gerhardt et al., 2000; Reichert and Höbel, 2015). Thus, females seem to evaluate absolute as well as relative attractiveness of available alternatives. We took this behavior into account by testing choosiness in trials that pitted (i) a very unattractive call against an average call (6 vs. 18 pulses/call), and (ii) a call that was shorter than average against one that was longer than average (12 vs. 24 pulses/call). We expected the 6 vs. 18 pulses/call trials to present a stronger trade-off for the tested females.

A total of 15 females in the 6 vs. 18 and 20 females in the 12 vs. 24 trials contributed to the data set. Females were randomly assigned to start with the dim or bright light treatment. Each female's choosiness was determined within a given light treatment; depending on the female's responses. This required 2-4 trials in which attenuation levels were adjusted until the female no longer approached the attractive call. Then the process was repeated in the other light treatment. Thus, each female provided two choosiness measures (one for each light treatment) to the final data set. Following testing, pairs were returned to the pond.

Statistical Analysis- We performed a population-based analysis in which we expressed choosiness data as the percentage of females still approaching the attractive stimulus at each attenuation level. For each experiment (6 vs. 18, and 12 vs. 24, respectively) we used a mixed model implemented in JMP 11 (SAS Institute Inc., Cary, NC). We entered the percentage of females approaching the attractive call as the dependent variable, and terms for light treatment, attenuation and the treatment × attenuation interaction as test variables.

2.7. Phonotaxis behavior

Using the phonotaxis videos and the corresponding stacked pictures of the 30-pulse trials from the preference function experiment, we mapped the cumulative distance moved by the females and the cumulative time it took them to reach the speaker (see Murphy and Gerhardt, 2002). We distinguished three types of movement: hops, crawls and repositions. Hops are quick movement that results in a displacement of >1 body length; crawls are slow movement that results in a displacement of >1 body length; and repositions are movements that do not result in displacement, such as moving the head to look in a different direction, or adjusting arm/leg position. For each phonotaxis approach, we noted (1) the number of hops, (2) the number of crawls, and (3) the number of repositions. In addition, we noted (4) the time after lifting the lid of the release box until the females started to move towards the speaker, (5) the average time between displacement movements (hops and crawls), and (6) the average distance covered by displacement movements (hops and crawls).

Statistical Analysis- We used JMP 11 (SAS Institute Inc., Cary, NC) to calculate a series of ANOVAs testing whether phonotaxis movement behaviors, or their timing, were affected by light treatments.

2.8. Effect sizes

We calculated effect sizes for the differences in preference function traits, choosiness and approach behavior, respectively, between dim and bright moonlight treatments. We first calculated Cohen's d by using mean values and a pooled standard deviation between light treatment types and then calculated the correlation coefficient r from Cohen's d (Cohen, 1988). Values of r range from 0 to 1 and have similar interpretations as r^2 in a simple linear regression. Correlation values that range from 0 - 0.3 indicate small effect sizes, 0.3 - 0.5 indicate intermediate effect sizes, and values greater than 0.5 indicate large effect sizes.

To put the above effect sizes into context, we also computed some comparisons that focused on the effect of call traits, not light treatment. These values serve to illustrate the differences that we are able to detect with our experiments (at similar or even lower sample size), if they involve variables that are biologically relevant to the frogs. We calculated the correlation coefficient r (from Cohen's d) for preference function traits obtained in Reichert and Höbel (2015). Those are based on call duration preference functions obtained using the same call stimuli as used here, but the treatment variable was presence/absence of a co-occurring visual stimulus instead of the dim/bright moonlight treatments used in the present study. We also calculated effect sizes for a comparison of choosiness during our 6 vs. 18 compared to the 12 vs. 24 pulse trials, focusing on the effect of the auditory stimuli the frogs were presented with.

3. Results

3.1. Preference Functions

Female Eastern gray treefrogs (*Hyla versicolor*) prefer longer duration calls (Fig. 4A), irrespective of the behavior (latency, path length or angle) that is scored to assess their preferences (significant linear and quadratic stimulus terms in Table 4). However, light treatment did not affect how females responded to variation in call duration (non-significant treatment and stimulus x treatment interactions terms in Table 4; Fig 5 A-C). As may be expected from the similar shapes of the preference functions, there was also no significant difference in the preference function traits of peak preference ($F_{1,19}$=0.0071, p=0.94; Fig. 4B), responsiveness ($F_{1,19}$=0.028, p=0.87; Fig. 4C), tolerance ($F_{1,19}$=0.61, p=0.45; Fig. 4D), and strength ($F_{1,19}$=0.53, p=0.48; Fig. 4E). Effect sizes were small in each case: peak preference (r =0.04), responsiveness (r =0.02), tolerance (r = 0.14), strength (r = 0.12). For comparison, effect sizes from Reichert and Höbel (2015) preference function traits were generally of intermediate size: peak preference (r =0.18), responsiveness (r =0.40), tolerance (r = 0.40), strength (r = 0.37).

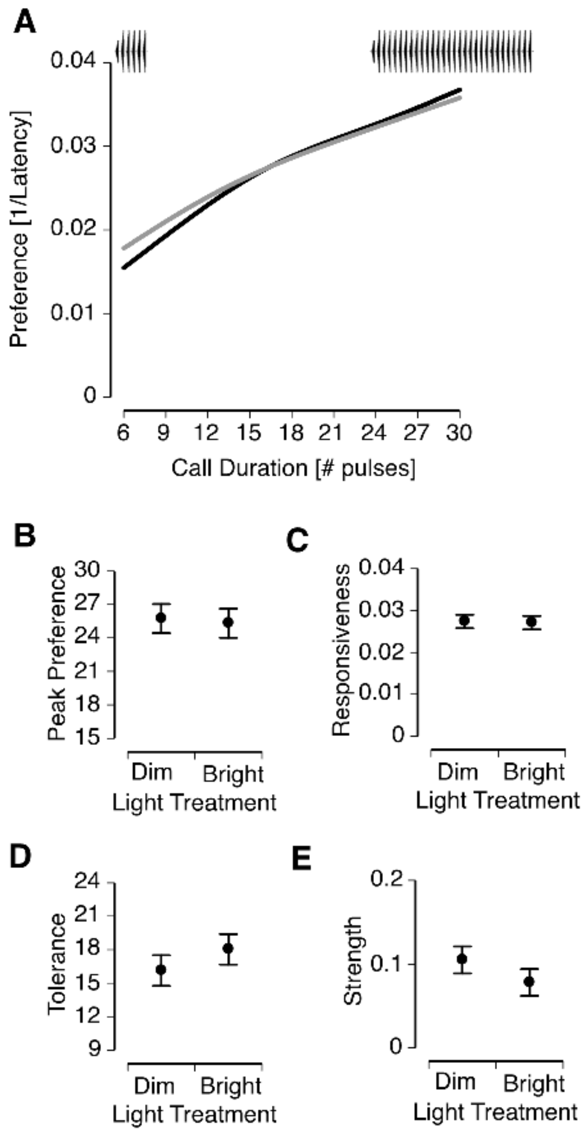
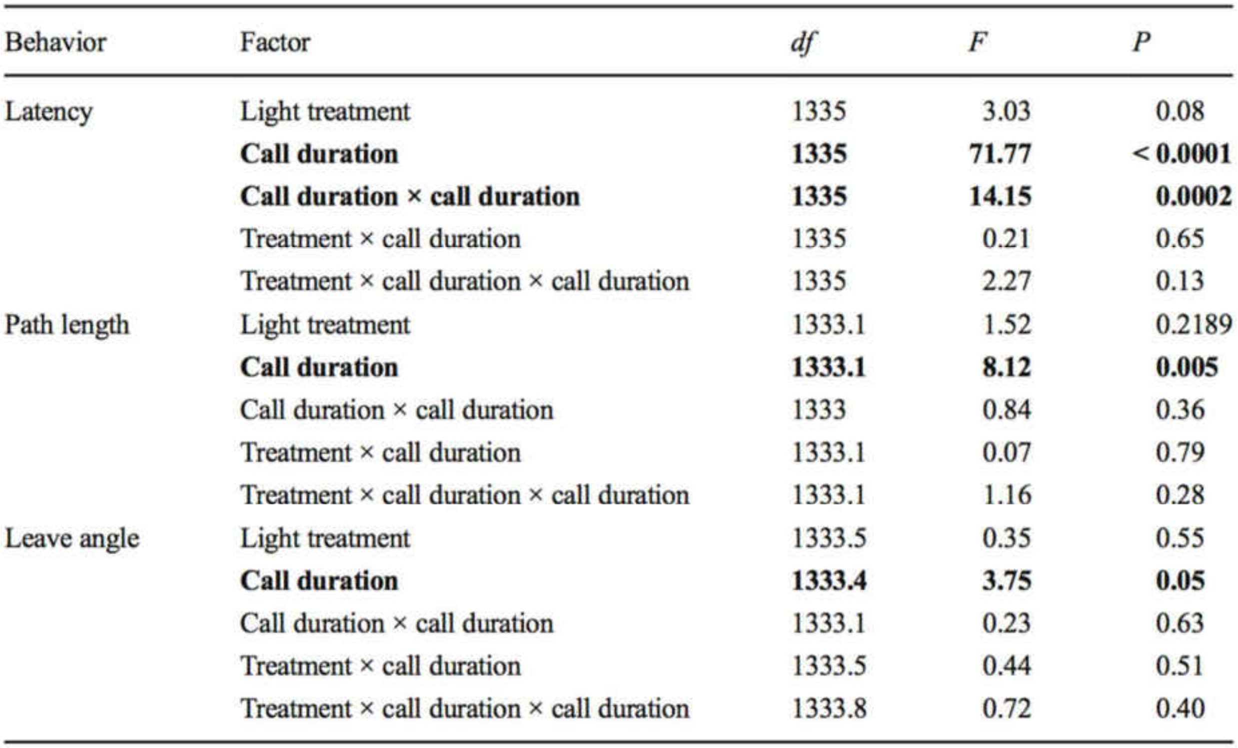


Figure 4: Call duration preferences of female Eastern gray treefrogs (*Hyla versicolor)* under simulated dim and bright moonlight. Shown are preference functions (a) and traits derived from those preference functions (b–e) based on the latency it took females to reach the speaker broadcasting the test calls. Dim light treatment is indicated by a black line, the bright treatment by a gray line. Preference functions were almost identical under the two light conditions (a), and preference function traits did not differ either (b–e). The waveforms in a illustrate the range of call stimuli tested, from a short 6-pulse call to an along 30- pulse call. Shown are means ± 95% CI (imperceptibly small in a)



Behavior	Factor	*df*	*F*	*P*
Latency	Light treatment	1335	3.03	0.08
	Call duration	**1335**	**71.77**	**< 0.0001**
	Call duration × call duration	**1335**	**14.15**	**0.0002**
	Treatment × call duration	1335	0.21	0.65
	Treatment × call duration × call duration	1335	2.27	0.13
Path length	Light treatment	1333.1	1.52	0.2189
	Call duration	**1333.1**	**8.12**	**0.005**
	Call duration × call duration	1333	0.84	0.36
	Treatment × call duration	1333.1	0.07	0.79
	Treatment × call duration × call duration	1333.1	1.16	0.28
Leave angle	Light treatment	1333.5	0.35	0.55
	Call duration	**1333.4**	**3.75**	**0.05**
	Call duration × call duration	1333.1	0.23	0.63
	Treatment × call duration	1333.5	0.44	0.51
	Treatment × call duration × call duration	1333.8	0.72	0.40

Table 4: Effect of variation in nocturnal light levels on preference for call duration in female Eastern gray treefrogs (*Hyla versicolor*). The assays used to describe preferences are (i) the latency until females reached the sound source); (ii) the length of the path traveled between the release box and the sound source; and (iii) the angle at which the female left the release box (relative to the speaker). Females were faster, walked a shorter path, and were more directional when approaching longer calls, but light treatment did not affect call duration preferences. Significant terms are set in bold.

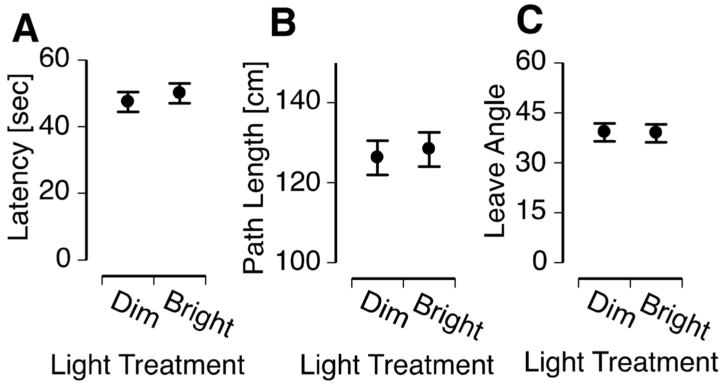


Figure 5: Light treatments (simulated dim or bright moonlight) did not affect how fast or how directly female Eastern gray treefrogs (*Hyla versicolor*) approached the speaker broadcasting the call stimulus. Neither response latency (a; the time it took females to reach the speaker broadcasting the test calls), nor path length (b; the distance females walked until they reached the speaker), nor the angle (relative to the sound source) at which they left the release box to start their approach to the speaker (c) differed between light treatments. Shown are least square means ± SE

3.2. Choosiness

As amplitude differences between an attractive and an unattractive call increased, fewer females continued to approach the attractive call (Fig. 6A, C; Table 5: significant effect of Attenuation). Light levels did not affect choosiness (Table 5: non-significant effect of Treatment): either in the 6 vs. 18 (Fig. 6 A, B; Table 5, left columns) nor in the 12 vs. 24 pulse trials (Fig. 6 C, D; Table 5, right columns). Effect sizes were small in each case: 6 vs. 18 (r = 0.03); 12 vs. 24 pulse trials (r = 0.06);

By contrast, the range of tested call duration values did affect choosiness: choosiness was higher in the 6 vs. 18- pulse duration trials compared to the 12 vs. 24- pulse trials; for example, at an amplitude difference of 9dB, 90% of females still approached the attractive call in the 6 vs. 18 pulse trials, while only 50% still did so in the 12 vs. 24 pulse trials (see Fig. 3 A,C), and average choosiness was also different (Fig. 6 B,D). Here, effect size was always large: 6 vs. 18 compared to 12 vs. 24 pulse trials in dim light (r = 0.59); 6 vs. 18 compared to 12 vs. 24 pulse trials in bright light (r = 0.70).

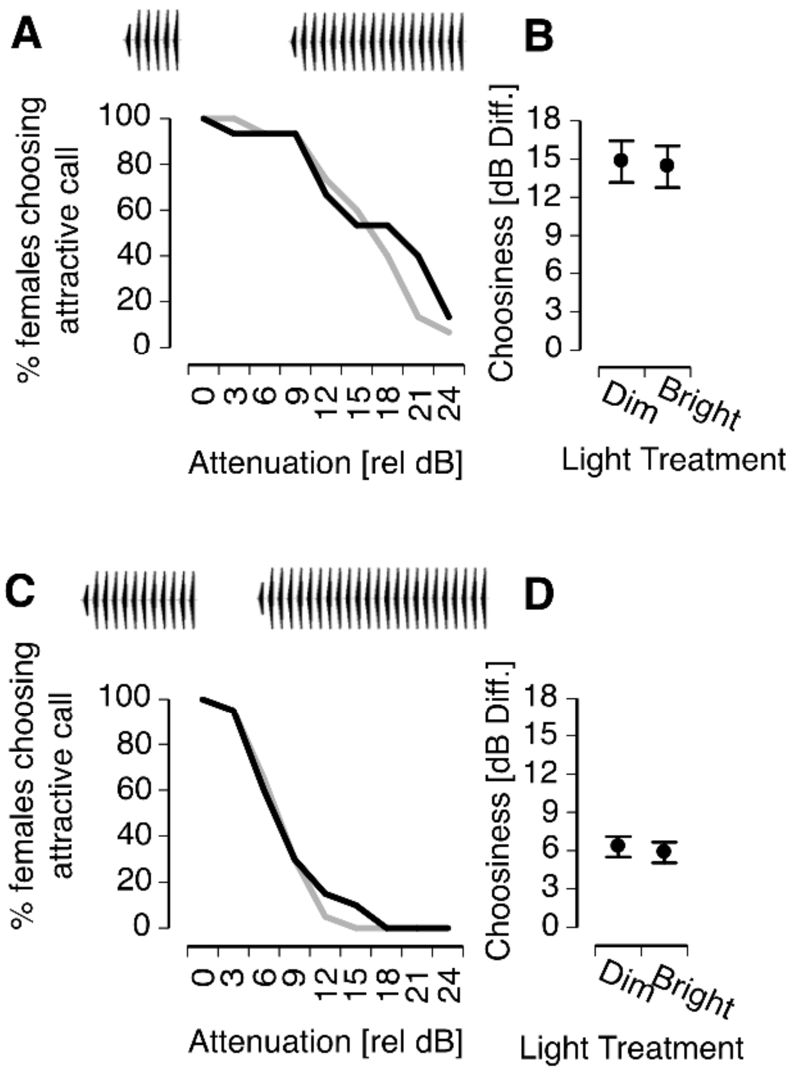


Figure 6: Choosiness of female Eastern gray treefrogs (*Hyla versicolor*), expressed as the percentage of females still approaching the more attractive stimulus as it is gradually attenuated, did not differ under simulated dim and bright moonlight. Waveforms show the call stimuli females were offered to choose from. The call alternatives tested in each trial differed by 12 pulses, but in one (a, b) they represent an unattractive (6p) and an attractive (18p) call, while in the other (c, d) they represent two attractive (12p and 24p) calls. In a, c, dim light treatment is indicated by a black line, the bright treatment by a gray line; b, d show least square means ± SE.

	6 vs. 18 pulses			12 vs. 24 pulses		
	df	*F*	*P*	*df*	*F*	*P*
Light treatment	1.14	0.37	0.55	1.14	0.04	0.85
Attenuation	**1.14**	**154.25**	**< 0.0001**	**1.14**	**73.39**	**< 0.0001**
Treatment × attenuation	1.14	1.69	0.21	1.14	0.01	0.92

Table 5: Effect of light treatment on choosiness. As amplitude differences between an attractive and an unattractive alternative increased, fewer females continued to approach the attractive call. Light treatment, however, had no effect on choosiness. Shown are results from trials that pitted a very unattractive call against an average call (6 vs. 18 pulses; left columns), and a call that was shorter than average against one that was longer than average (12 vs. 24 pulses; right columns). Significant terms are set in bold. See also Fig. 6.

3.3. Phonotaxis behavior

Females hopped/crawled on average (± SD) 6.2 ± 3.2 (range: 3-17) times before reaching the speaker located 1m away from them. Average (± SD) distance moved per movements was 22 ± 9 cm (range 8-40cm), and the average (± SD) time between successive movements was 10 ± 16 sec (range 2-23 sec). Females also adjusted head position during phonotaxis (without forward movement); there were an average (± SD) of 2.8 ± 1.7 (range: 0-7) head movements during each phonotaxis trial. These head movements either resulted in the female facing the speaker (58%), or away from the speaker, such that one ear was turned towards the speaker (42%), suggesting that these movements are involved in sound source localization.

Although there was substantial individual variation in approach behavior (see Fig. 7 A), none of the sampled approach behaviors were affected by light treatments (Fig. 7 B-D; Table 6). Effect sizes were small in each case (see Table 6, right column).

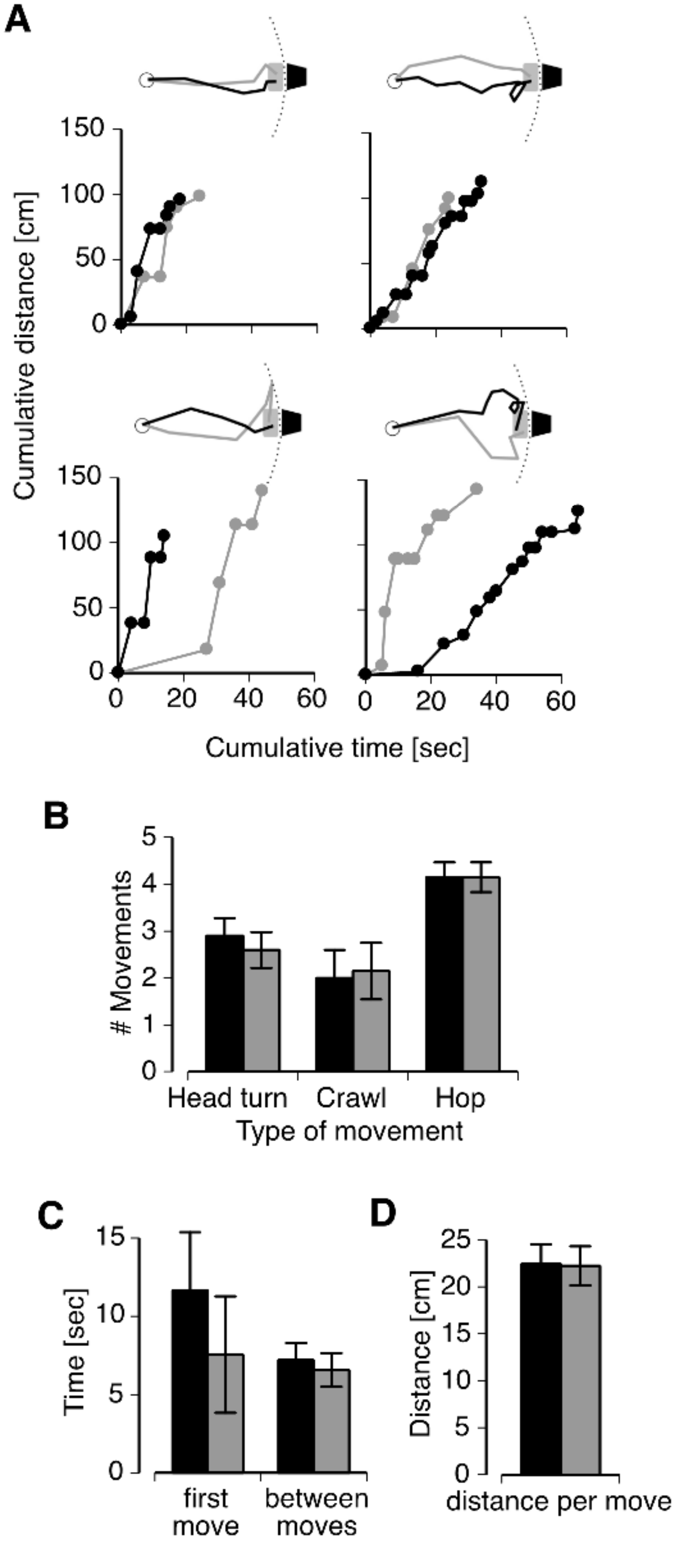
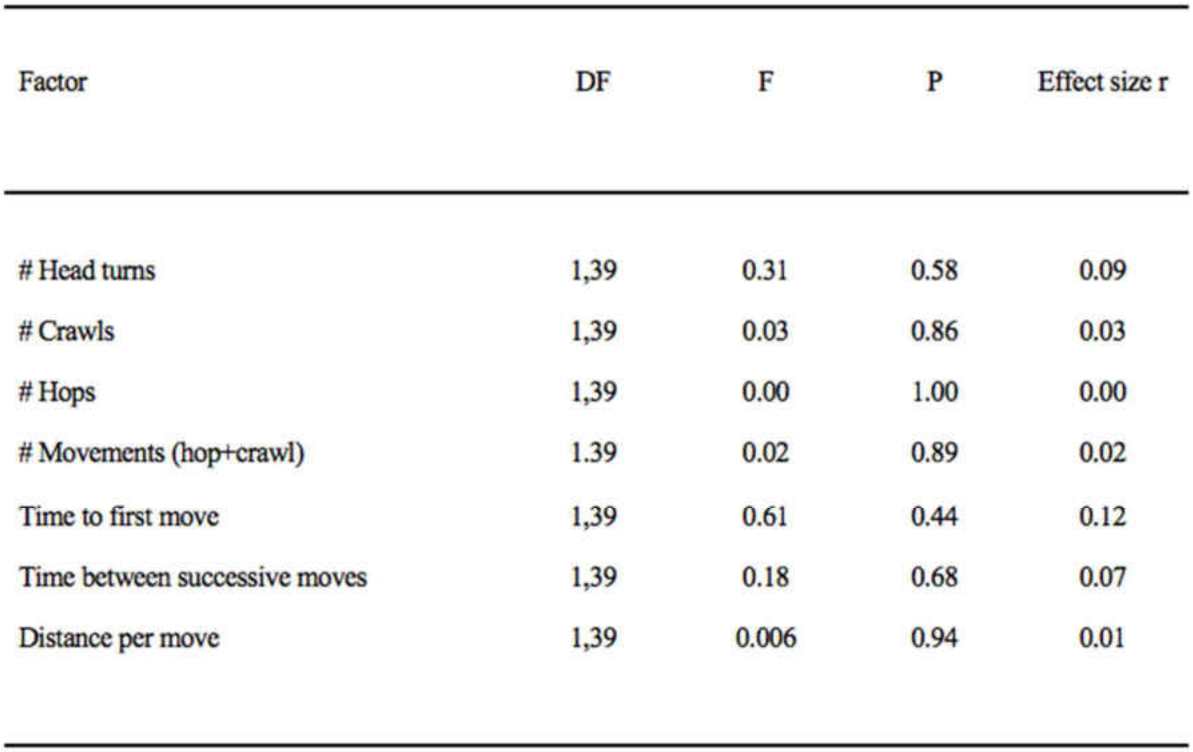


Figure 7: Movement patterns of female Eastern gray treefrogs (*Hyla versicolor*) during phonotaxis under simulated dim and bright moonlight. a Paths taken by four females approaching the playback speaker under dim (black line) and bright (gray line) simulated moonlight. The dashed outer lines represent the edge of the playback arena; the

inner circle depicts the position of the release box, and the gray square indicates the target zone in front of the speaker. Graphs below path diagrams depict the cumulative distance moved by females plotted against cumulative time since leaving the release box. b Average numbers of stationary movements (repositions) or locomotion movements (crawls, hops) did not differ between light treatments. c Neither the time until the females left the release box nor the average times between forward locomotion movements differed between light treatments. d The average distance covered by a forward locomotion movement did not differ between light treatments. Shown are least square means ± SE



Factor	DF	F	P	Effect size r
# Head turns	1,39	0.31	0.58	0.09
# Crawls	1,39	0.03	0.86	0.03
# Hops	1,39	0.00	1.00	0.00
# Movements (hop+crawl)	1.39	0.02	0.89	0.02
Time to first move	1,39	0.61	0.44	0.12
Time between successive moves	1,39	0.18	0.68	0.07
Distance per move	1,39	0.006	0.94	0.01

Table 6: Results of ANOVAs testing whether phonotaxis movements differed between light treatments (simulated dim and bright moonlight). See also Fig. 7.

4. Discussion

Although not widely appreciated, mate choice decisions in frogs are surprisingly flexible. Female frogs generally show strong preferences for particular call traits, often favoring calls of lower frequency (associated with larger male size) or calls that are longer or more frequently repeated (associated with higher energy expenditure) (Gerhardt and Huber, 2002; Wells, 2010). Yet, both the preference as well as the choosiness aspects of these call trait preferences can be modified. For example, female treefrogs generally become more tolerant towards accepting less attractive calls if those are paired with a visual signal component (Gomez et al., 2009; Taylor et al., 2011; Reichert and Höbel, 2015, Reichert et al., 2016). And female green treefrogs (*Hyla cinerea*) adjust choosiness as a function of variation in the social environment they experience when approaching the breeding chorus to mate: presence of attractive males increases choosiness, while absence of attractive males reduces choosiness (Neelon and Höbel, 2017). Evidently, female frogs are perfectly capable of adjusting their mate choice behavior in contexts in which selection has favored such behavioral plasticity.

Here we tested the hypothesis that female Eastern gray treefrogs show context-dependent variation in mate choice behavior, in particular whether their behavior is affected by variation in nocturnal light levels. Contrary to our prediction that females would prefer more attractive males and/or be choosier under dimmer moonlight, we actually found that none of the mate choice behaviors scored during our experiments were affected by the treatment light intensity. Females approached a simulated male call in the same way in dim and bright conditions, showing similar speed, directionality and even similar number and timing of phonotaxis movements (i.e., hops, crawls and head scans). Females also had similar call duration preferences, including details of preference function shape such as peak preference and tolerance for deviation from the peak, etc. Finally, females also showed similar effort in obtaining their preferred mate, i.e., choosiness did not vary with light treatment.

The choosiness trials are particularly instructive in terms of the importance female gray treefrogs seem to give to ambient light. We tested choosiness in two experiments in which the overall difference between tested alternatives was the same (call duration differed by 12 pulses), but in one experiment females were offered an unattractive (6 pulse) and an attractive (18 pulse) call, while in the other experiment they had to choose between two attractive calls (12 and 24 pulse). Choosiness was much higher in the experiment that involved the unattractive call, indicating that females put a premium on call traits, but disregard ambient light conditions, at least within the range of light intensities tested here. This is also corroborated by effect size calculations: while effect sizes comparing light treatment effects were small, the effect sizes comparing call quality were of large magnitude.

As is tradition in anuran mate choice experiments, our experiments were conducted in a comparatively small playback arena (i.e., Rand et al., 1997; Reichert and Höbel, 2015). Observations thus only encompass the last few meters of phonotactic approach, which is only a fraction of the distance a mate searching female will cover in the night she approaches the breeding pond. Consequently, not documenting behavioral differences in phonotaxis-related behaviors during the very last portion of approach still leaves many aspects of female mate choice behavior unexamined. For example, the approach path through the surrounding forest towards the pond may still differ between dark and moonlit nights. In addition, we know that the night of migration towards our pond may be affected by lunar light: although breeding occurs during any point in the lunar cycle (and thus, under the full range of natural nocturnal light levels), it is statistically more likely to occur during intermediately bright nights (incidentally, male calling activity mirrors this pattern; Underhill and Höbel, 2018). Potentially, selection for when to migrate to the breeding pond, and how to get there from a distance, is stronger than how to move during the last meter before reaching the male.

At first glance, the lack of nocturnal light effects documented for gray treefrogs differs markedly from previous studies examining the effect of nocturnal light on anuran mate choice behavior. However, studies are difficult to compare because of differences in experimental design.

For example, we tested choosiness in a relatively benign scenario (i.e., asking females to choose between two conspecific calls). The experimental design used by Bonachea and Ryan (2011a, b) to test risk assessment during mate choice in female túngara frogs included light treatments, but additionally placed females in more challenging choice scenarios, such as making females choose between con- and heterospecific calls, or adding predator sounds to the calls females were asked to approach. Potentially, the effects of nocturnal light may manifest in gray treefrogs under more challenging conditions as well. On the other hand, there are comparable trial conditions where túngara frogs nevertheless behaved differently from gray treefrogs. For example, Rand et al. (1997) showed that the willingness of female túngara frogs to move and perform phonotaxis in dark and illuminated trials was quite different: almost half the tested túngara frog females did not respond in the illuminated trials, while the vast majority of gray treefrogs tested in our trials approached the playback speaker irrespective of light conditions.

Despite the difficulty of comparing studies, there seems to be genuine differences between female gray treefrogs and the two other species studied before, suggesting that mate choice behavior in relation to lunar light may be highly species-specific. Given what is known about lunar responses in amphibians in general, such between-species variation is not unexpected. In their review paper, Grant et al. (2012) found 79 examples across diverse amphibian taxa where behavior and ecology in relation to the moon had been studied - note that *any* response (ovulation, breeding migrations, phonotaxis behavior, etc.) to *any* lunar cue (moon phase, lunar light) was included in the study. The majority of species were affected by lunar cues, but there was no significant difference between the numbers of species that increased, and those that decreased activity during a full moon. There was also no clear trend as to which behavioral responses (i.e., breeding migrations, spawning events, calling, etc.) were particularly frequent during specific moon phases.

And there was no taxonomic signature either, as the same family or even genus can contain species that increased or decreased activity under full moon (Grant et al., 2012).

The three frog species whose mate choice behavior in relation to nocturnal light has been studied (see above) are members of different anuran families (*Hylidae, Hyperolidae, Leptodactylidae*), they hail from different geographic areas (North America, Central America, Africa), and inhabit tropical and temperate regions. There is thus a multitude of reasons that may explain the observed between-species differences in how mate choice behavior is affected by nocturnal light.

Different predator regimes may select for species differences in moonlight-related behavior. Cover of darkness may provide respite from visually orienting predators, but should not protect from acoustically or chemically orienting ones. In the case of the Central American túngara frog, it seems that visually-orienting frog-eating bats are a major threat (Ryan, 1985), and there are also frog eating bat species in Africa that eat reed frogs (Channing, 2001). By contrast, this predator guild is absent in North America, and consequently gray treefrogs may not have been under selection to avoid predators during bright nights. Gray treefrogs are preyed upon by a diverse array of nocturnal predators [raccoons, snakes, larger frogs (bullfrogs, green frogs), and even giant water bugs], encompassing a range of prey detection mechanisms (acoustic, chemical, visual). Maybe the lack of a predominant predator did not result in selection for increased vigilance under brighter conditions, at least not in females. In this regard it is interesting to note that male gray treefrogs from our pond seem to use visual cues to detect potential predators and adjust their calling behavior accordingly: while the majority of males in the predator-absent trials did call, only a fraction of the ones in the predator-present trials did (Höbel and Barta, 2014). Then again, broadcast of bullfrog vocalizations (another known predator on gray treefrogs), failed to influence female phonotaxis as well as male calling behavior in gray treefrogs from another population (Schwartz et al., 2000). These observations suggest that acoustic predator cues are less salient for gray treefrogs than visual cues, and also that there may be a sex difference in response to visual predator cues. However, more research is needed to tackle these hypotheses.

The distribution range of Eastern gray treefrogs is enormous, from the Gulf of Mexico in the south to some parts of Canada in the north, and it is possible that the geographic location of our study population affected our results. First, frogs from our more northern population may experience overall reduced predation pressure, particularly from snakes and mammals, and may thus not experience strong selection for increased predator vigilance during brighter nights. Second, although gray treefrogs are categorized as prolonged breeders, in our Wisconsin population breeding takes place only during a portion of the 4-6 week long breeding season. This is mostly because warm nights amenable to reproduction are separated by cold spells during which frogs are inactive (GH, pers. obs.). Gray treefrogs at our site thus have a shorter period of breeding activity, and may actually be under selection to disregard variation in nocturnal light in order to take advantage of best climatic conditions. In addition, females from our population have a comparatively low life expectancy (1-3 years) and likely only reproduce once in their lifetime (GH et al., unpubl. data), which may put a premium on obtaining the best possible sire for their offspring. Overall few chances to reproduce in conjunction with potentially low predation pressure may constitute a combination of environmental factors that did not select for differential mate choice behavior under dark and moonlit conditions at our study site. The behavior of our northern frogs may thus not reflect the behavior across the species' range. Comparative studies in sites with higher predation pressure, and / or longer duration breeding seasons could yield interesting results on the importance of additional environmental factors on the evolution of female mate choice behavior.

5. Conclusion

In conclusion, we have shown that neither call duration preferences, nor choosiness for longer over shorter calls, nor the minutia of phonotaxis behavior change when female gray treefrogs search for mates under different nocturnal light levels. A key take-home message from our study is thus that in gray treefrogs, variation in light levels associated with the changing phases of the moon, does not affect the sexual selection regime on male call traits. In a previous study we have shown that female gray treefrogs breed under a wide range of nocturnal light levels, but that there is a slight increase in the numbers of females arriving to breed during intermediately bright nights (Underhill and Höbel, 2018). However, this should not lead to lunar-cycle driven variation in the strength or direction of sexual selection, because female gray treefrogs have similarly strong preferences for longer calls irrespective of the moonlight available during the night they come to breed.

Chapter 3: Mate choice behavior of female Eastern gray treefrogs (*Hyla versicolor*) is robust to anthropogenic light pollution

1. Introduction

Urbanization is rapidly altering natural environments. One of its consequences is ecological light pollution, the alteration of natural light levels due to the introduction of artificial light at night (Longcore and Rich, 2004). In recent years, increases in human population growth, industrialization, and urban and suburban sprawl have drastically changed the amount of artificial light entering natural habitats (Cinzano et al., 2001). The extent of ecological light pollution is global (Elvidge et al., 1997), nearly 20% of land on earth is considered polluted by light (Cinzano et al., 2001), and this pollution is increasing every year (Hölker et al., 2010).

Natural light has strong biological relevance for daily and annual biological rhythms, and artificial lighting may interfere with daily activity, sleep patterns, and the timing of reproduction (i.e., Dominoni et al., 2013; Kempenaers et al., 2010; Nordt et al., 2013; Raap et al., 2015; Russ et al., 2015). Because it alters the sensory environment, artificial light may also interfere with predator-prey dynamics. Predators may take advantage of improved prey detection – bats, for example, are more successful at capturing their insect prey when artificial light is present at night (Minnaar et al., 2014; Rydell, 1992). In response, prey may adjust their anti-predator strategies. For example, some male frogs change their calling behavior when exposed to artificial nocturnal light (Bonachea and Richardson, 2006; Tuttle and Ryan 1982).

The effect of light pollution on mate choice behavior has received little attention so far. This is surprising because mate choice is often thought to drive the evolution of extravagant sexual ornaments and elaborate mating displays (Andersson, 1994; West-Eberhard, 1983), which can foster divergence and reproductive isolation (Seddon et al., 2013; West-Eberhard, 2014;). Ultimately, this may lead to differences between populations in polluted and unpolluted sites.

Moreover, the act of sampling potential mates can be a dangerous undertaking because it exposes mate-searching females to predators, both via spending additional time in the open during the sampling process, and via lingering in the vicinity of conspicuously displaying males.

Most anurans (frogs and toads) are partly or completely nocturnal, and thus likely affected by artificially high or fluctuating illumination from light pollution (Perry et al., 2008). Despite its potential impact on population maintenance and divergence, the effect of anthropogenic light pollution on anuran mate choice behavior has to our knowledge never been examined. Here we conduct such a test with female Eastern gray treefrogs (*Hyla versicolor*). We focus on three aspects of female mate choice behavior: (i) mate preference functions, which describe the order in which a female ranks prospective mates, (ii) choosiness, which indicates how much effort a female is willing to invest in acquiring her preferred mate; and (iii) approach behavior, which focuses on the type and pattern of female mate searching movements. Our general prediction is that when faced with light pollution, females will show behaviors that mitigate the increased danger of being detected by predators, such as relaxed preferences, decreased choosiness, and more directional, faster, or stealthier movements towards potential mates.

Artificial light is created by a multitude of sources that likely have different biological effects, and we took this into account when assessing differences in mate choice behavior. Streetlights, lighted buildings, and security lights result in expansion of lit areas and increased light intensity. These sources thus generate a static increase in light levels, and likely affect a number of behaviors that are normally mediated by the diurnal/seasonal pattern of natural light, or with detection of prey or predators. By contrast, the headlight of cars and other vehicles lead to rapid fluctuations between bright and dark conditions. Changes of illumination over brief periods of time can interfere with the visual capacities of dark adapted eyes and temporarily blind animals (Buchanan, 2006), and its effects thus likely center on vigilance and predator detection.

We tested female mate choice behavior in two experiments that mimic the different temporal patterns of light pollution. In Experiment 1 we examined the effects of static light pollution, such as would be generated by street lamps. This experiment involved three light-intensity treatments, spanning the gamut from light levels comparable to natural moonlight to standing under a streetlight (0.2-15 lux). Consequently, in the static light treatment experiment we examined whether *presence* and *intensity* of artificial light affects mate choice behavior, and we predict that higher light levels result in relaxed preferences, decreased choosiness, and stealthier phonotactic approach movements. In Experiment 2 we examined whether *temporal fluctuation* in light pollution affects frog behavior. This experiment involved two light treatments with identical light intensity (15 lux), but different temporal pattern (continuous, as under a street light, or intermittent, as from cars driving by). Here we predict that the occurrence of temporal fluctuations in light levels would result in relaxed preferences, decreased choosiness, and stealthier phonotactic approach movements.

2. Methods

2.1. Study site and species

This study was carried out from May-June 2016 and 2017, during the natural breeding season of *H. versicolor*. During the same time, we also conducted a study examining the mate choice behavior of females under natural nocturnal light conditions (Underhill and Höbel, 2017). The data reported there derives from a separate set of females. All females were captured in amplexus in a pond adjacent to the University of Wisconsin Milwaukee Field Station (Saukville, WI). Pairs were held in containers placed on melting ice to prevent oviposition prior to testing. Following testing, pairs were returned to the pond. All applicable national and institutional guidelines for the care and use of animals were followed. We received ethical clearance from the Institutional Animal Care and Use Committee of the University of Wisconsin – Milwaukee (protocol number 15-16 #43).

2.2. Stimulus generation

Male *H. versicolor* have advertisement calls consisting of a series of short pulses. At a testing temperature of 20 °C, pulses are approximately 25 msec in duration, and are repeated after a pause of 25 msec (thus, the duration of the call can be expressed in number of pulses or in milliseconds). Two frequency peaks are emphasized in the call (1100Hz and 2200Hz). Females are selective for multiple aspects of male calls, including pulse rate, call frequency, and call rate. Call duration, however, is the trait for which females have the strongest preferences (Gerhardt et al., 2000; Reichert and Höbel, 2015), and accordingly we focused on this call trait.

Acoustic stimuli were generated in R (Version 3.1.0) software (R Development Core Team., 2015), using the seewave package (Sueur et al., 2008). For all stimuli, we set call frequency and call period to the average values of our study population (i.e., first frequency peak of 1071Hz, second (dominant) frequency peak of 2142 Hz (2^{nd} peak 10dB louder); Call period of 7750 ± 3027 msec (see Reichert and Höbel, 2015). Call duration ranged from 6 to 30 pulses per call, increasing in increments of 3 pulses (i.e. 6, 9, 12, 15, 18, 21, 24, 27, and 30).

2.3. General testing procedure

Females were tested in a circular playback arena (2m diameter), set up inside a semi-anechoic chamber. The arena wall was constructed from hardware cloth covered in black fabric (visually opaque but acoustically transparent). We placed speakers (JBL Control 1Xtreme) just outside the arena wall and angled them towards the center of the arena. Call stimuli were played from a laptop computer, and amplified by a Behringer Reference Amplifier (Behringer, A500 Model). Call amplitude, measured at the release point of the female, was adjusted using a 407764 Sound Level Meter (EXTECH instruments, RS232/Data logger). Unless otherwise specified, amplitude was set at 85dB SPL.

Prior to testing, we warmed females to a testing temperature of 20°C. For testing, females were placed in an acoustically transparent release cage in the center of the arena. An acoustic stimulus was broadcast to the female while confined in the release cage; the experimental choice time began when the female was released from the cage by pulling on a rope attached to the top. A choice was defined as entering a 10-cm choice zone (marked by tape on the arena floor), after having shown directed phonotaxis movements (i.e., trials where females crossed the choice zone while aimlessly wandering around the arena were not counted). Female movements were observed and recorded outside the chamber using an infrared video camera (EQ150, EverFocus USA, Duarte, CA, U.S.A.) mounted above the arena. Females that did not respond were rested for 5 minutes and rerun on that stimulus. Females that had 3 consecutive non-responses were not tested further and their data set was discarded (6 out of 72 tested females did not complete the trials and were removed). Sample size for the four experiments was (# females tested / # responsive females included in study): Exp. 1 - Preferences (21/20), Exp. 1 - Choosiness (13/11), Exp. 2 - Preferences (21/20), Exp. 2 - Choosiness (17/15).

2.4. Light treatments

We used a 100W incandescent bulb mounted in the center of the testing arena to simulate light pollution. We measured light levels using an EasyView Digital light meter (EXTECH instruments, Model EA31) and used a dimmer (Leviton TBL03) to adjust light levels to the desired brightness.

We assessed variation in female mate choice behavior in two experiments. In Experiment 1 (*Static light pollution*), we tested females under three light levels: one in the natural nocturnal range (new moon/starlight at 0.2 lux) and two increased light levels (5 lux and 15 lux, comparable to standing about 3.5 m next to a streetlight, and directly under a streetlight, respectively). While testing, the light was on continuously.

In Experiment 2 (*Dynamic light pollution*) we assessed variation in female mate choice behavior under two light conditions that were identical in the intensity of the light pollution used (15 lux), but differed in their temporal presentation: (i) static light pollution was simulated by a light source set at a constant brightness of 15 lux; (ii) dynamic light pollution was simulated by alternating between darkness and 15 lux. We used a dimmer to steadily increase light to 15 lux over 3 seconds, then quickly switching the light off and maintaining darkness for 7 seconds (resulting in a 3s on/ 7s off pattern that repeated every 10 seconds). To verify the consistency of our manual dimmer manipulation, we reviewed the phonotaxis videos of the dynamic trials and scored the proportion of frames that appeared bright and dark, respectively. Mean ± SD of bright frames was 27.1 ± 3.73 %, of dark frames it was 72.9 ± 3.73 %.

Rapid shifts in light intensity (which are unavoidable when transporting frogs to and from the test chamber to a holding area outside the chamber) may affect frog behavior (Buchanan,1993; 1998). To ensure that the females' eyes maintained adapted to the treatment light level, we placed them into the testing chamber at least 5 min. prior to starting the experiment, and kept them (in individual transparent boxes inside a large, thick-walled cooler with the top open) inside the testing chamber for the duration of the experiment. The cooler was placed in the quietest location inside the test chamber (i.e., outside the actual testing arena and at 90-degree angle from the speaker(s)). Although this did substantially attenuate the calls broadcast to the female currently being tested, it did not completely prevent females in the cooler from hearing the playbacks. However, keeping frogs in the test chamber was the only logistically feasible way to maintain females' eyes continuously adapted to treatment light conditions.

2.5. Testing variation in female mate choice behavior

2.5.1. Testing variation in call duration preferences

We used a single-speaker design to assay female call duration preferences. Here, call stimuli varying in duration are presented sequentially, and the timing and directionality of a female's approach towards the speaker are noted as a measure of preference. We used a stop watch to measure choice time (time from the moment the release cage was lifted and the females was free to move around the arena until she reached the choice zone at the speaker).

Using the program PFunc (Kilmer et al., 2017), we examined variation in call duration preferences with preference functions. This program generates individual preference function curves, and also extracts several traits from those preference functions: (1) Peak describes the female's most preferred call duration value (i.e., duration eliciting the fastest response); (2) Strength describes the extent to which a female's response is reduced to signals that deviate from the peak preferred signal; (3) Tolerance describes the range of call duration values that still elicits a high level of response (within 1/3 of the function peak); and (4) Responsiveness quantifies the mean response across the range of stimuli. In this experiment, a strong preference for a signal is expressed by a fast approach towards the broadcast call (i.e., a short choice time). However, because interpretation of results is more intuitive if a female's most preferred value is shown as the highest point in a curve, not the lowest, we converted raw choice time (in seconds) to 1/choice time before generating preference functions.

Each female responded to the full complement of 9 call duration stimuli (6-30 pulses; presented in random order) under each light treatment. Since Experiment 1 had three light treatments (0.2, 5 and 15 lux), each female tested in this experiment provided three preference functions (one for each light treatment) to the final data set. Experiment 2 had two light conditions (static on or dynamic blinking), and accordingly each female provided two preference functions to the final data set.

To test for differences in preference function shape between light treatments, we entered choice time as response variables in a mixed model (standard least squares) implementing REML. As predictor variables, we entered linear and quadratic terms for call duration, a term for light treatment, and a call duration × treatment interaction terms. We entered both a linear and a quadratic term for call duration to capture the effect of preference function shape: a significant linear term would indicate that females prefer longer calls, while a significant quadratic term indicates that females prefer an intermediate call duration value. We also entered female identity as a random term, to account for each female having provided data for several preference functions (three in Experiment 1 and two in Experiment 2)

We analyzed each preference function trait using a mixed model (implementing REML) with the preference function trait (i.e., peak, strength, tolerance, or responsiveness) as the response variable, and the light treatment (0.2, 5, and 15 lux in Experiment 1, and static and dynamic in

Experiment 2, respectively) as the independent variable, and female identity as a random term. All statistical tests were implemented in JMP 11 (SAS Institute Inc., Cary, NC).

2.5.2. Testing variation in Choosiness

We assayed choosiness using a two-speaker design, that is based on the common observation that female frogs trade off call attractiveness with distance to source (i.e., females may approach a less attractive stimulus if it is perceived as being closer, or played at relatively higher amplitude (Gerhardt, 1987). Here, an attractive (18 pulse duration) and an unattractive call (6 pulse duration) are played antiphonally (from speakers set at 180-degree to each other, each facing the center of the arena). The amplitude of the unattractive call remains constant at 85dB SPL, while the amplitude of the attractive call is attenuated in successive trials (in 3 dB steps), until the female no longer approaches the attractive call.

Each female's choosiness was determined within a given light treatment; depending on the females' responses, this required 2-4 trials in which attenuation levels were adjusted until the female no longer approached the attractive call. Then the process was repeated in another light treatment. Since Experiment 1 had three light treatments (0.2, 5 and 15 lux), each female provided three data points to the final data set. Experiment 2 had two light conditions (static on or dynamic blinking), and each female provided two data points to the final data set.

We performed a population-based analysis in which we expressed choosiness data as the percentage of females still approaching the attractive stimulus at each attenuation level. We used a mixed model implemented in JMP 11 (SAS Institute Inc., Cary, NC). We entered the percentage of females approaching the attractive call as the dependent variable, and terms for light treatment, attenuation and the treatment × attenuation interaction as test variables.

2.5.3. Phonotaxis behavior

Reviewing the phonotaxis videos of the 6-pulse (unattractive) and 30-pulse (very attractive) trials from the light pollution preference function data sets (see 2.5.1.), we distinguished two types of movements: hops, and crawls. Hops are quick movements that result in a displacement of >1 body length; crawls are slow movements that result in a displacement of <1 body length. For each phonotaxis approach, we noted (1) the number of hops, and (2) the number of crawls, (3) the leave time, i.e., the time after lifting the lid of the release box until the females started to move towards the speaker, and (4) the choice time, i.e., the time until the female reached the speaker.

We used JMP 11 (SAS Institute Inc., Cary, NC) to calculate a series of mixed models, testing whether phonotaxis movement behaviors, or their timing, were affected by light treatments, call attractiveness, or an interaction of the two.

Finally, we conducted an even more detailed analysis focusing only on the dynamic trials of Experiment 2, since those contained periods of both bright illumination and darkness. We re-analyzed the phonotaxis videos of the 6- and 30-pulse trials, now noting for the bright and dark portions of the trials whether (1) females moved or not, and (2) how many locomotion movements (hops and crawls) they made in each condition. Because of the 30/70 bright/dark pattern of those trials (see 2.4.), we calculated the observed % of movements in the dark (# movements in dark / total # movements), and used JMP 11 (SAS Institute Inc., Cary, NC) to test whether this differed from the expected % of movements in the dark (using the % of darkness in the bright/dark cycle extracted from each trial video).

3. Results

Across all experiments, response rate (i.e., the proportion of females that successfully completed a set of trials) was 91 ± 5 % (range 85-95%). This is comparable to the response rate typical for our population (Höbel, pers. obs), and suggests that females were not scared by the light treatments to the point that they refused to move or tried to flee the arena.

3.1. Static Light Pollution

In the trials testing the effect of static light pollution on female call duration preferences, the intensity of the light stimuli between treatments differed by almost two orders of magnitude (0.2 – 15 lux). Nevertheless, while there was almost always a strong effect of call attractiveness on female behavior, light treatments had little if any effect.

The trials testing preference functions showed that female Eastern gray treefrogs (*Hyla versicolor*) prefer longer duration calls (Fig. 8A, Table 7), irrespective of light treatment (Fig 8A). As may be expected from the similar shapes of the preference functions, there was also no significant difference in the preference function traits of peak preference ($F_{2,38}$=1.58, p=0.22; Fig. 8B); strength ($F_{2,38}$=2.29, p=0.12; Fig. 8C), tolerance ($F_{2,38}$=0.46, p=0.63; Fig. 8D); and responsiveness ($F_{2,38}$=0.69, p=0.51; Fig. 8E).

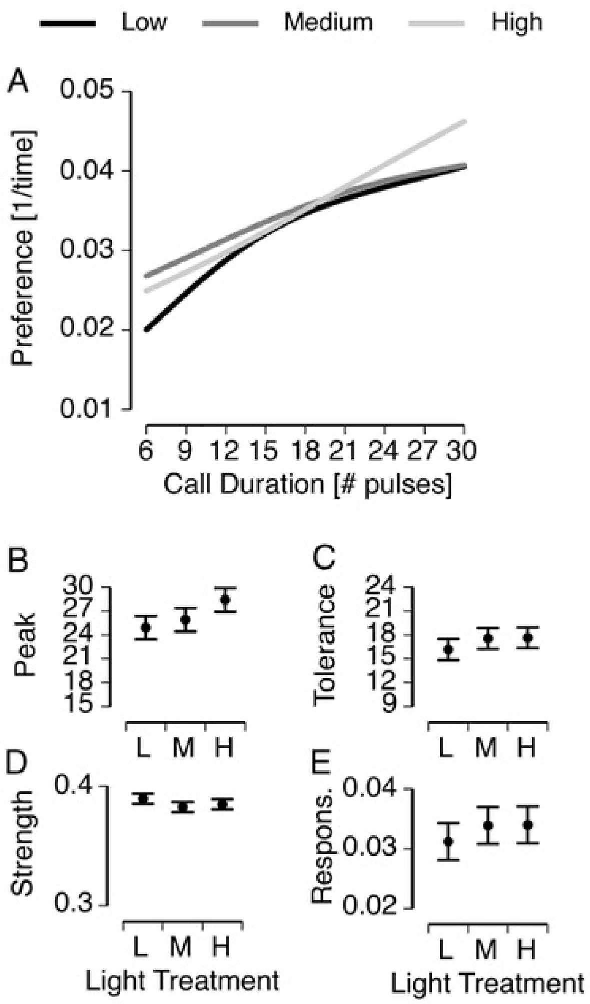


Figure 8: Call duration preferences of female Eastern gray treefrogs (*Hyla versicolor*) under static 0.2 lux (low), 5 lux (medium), and 15 lux (high) light levels. Shown are preference functions (A) and traits derived from those preference functions (B-E) based on the choice tie it took females to reach the speaker broadcasting the test calls. Preferences functions were almost identical under the three light conditions (A), and preference function traits did not differ either (B-E).

Factor	DF	F	P
Light Treatment	2,326.3	0.94	0.39
Call Duration	**1,326.2**	**60.24**	**<.0001**
Call Duration × Call Duration	**1,326.2**	**10.53**	**0.0013**
Treatment ×Call Duration	2,326.2	1.64	0.20
Treatment ×Call Duration × Call Duration	2,326.2	2.66	0.07

Table 7: Effect of temporal variation in light pollution on preference for call duration in female Eastern gray treefrogs (*Hyla versicolor*). Females preferred longer duration calls, but light treatment did not affect call duration preferences. Significant terms are set in bold. See also Fig 8.

The trials testing choosiness showed that as amplitude differences between an attractive and an unattractive call increased, the proportion of females continuing to approach the attractive call decreased (Figure 9A; Table 8: significant effect of Attenuation). However, light levels did not affect choosiness (Table 8: non-significant effect of Treatment).

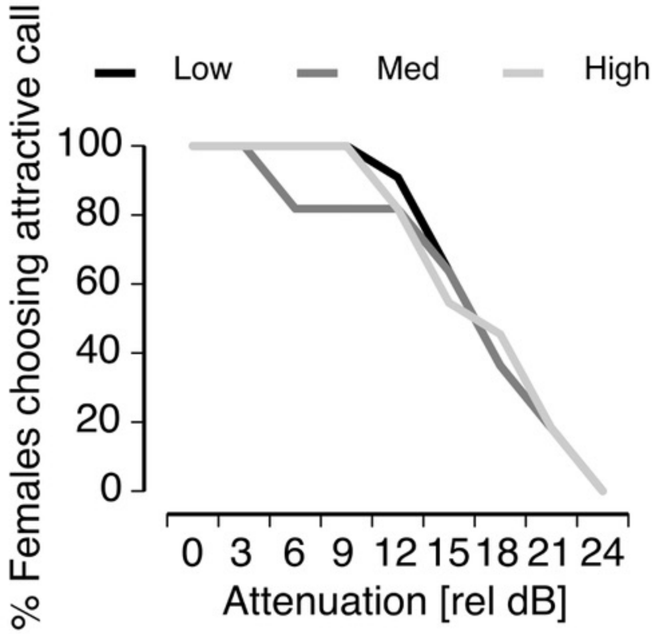


Figure 9: Choosiness of female Eastern gray treefrogs (*Hyla versicolor*), expressed as the percentage of females still choosing the more attractive stimulus as it is gradually attenuated, did not differ under 0.2 lux (low), 5 lux (medium), and 15 lux (high) light levels. Panel B shows the least square means ± SE.

Factor	**DF**	**F**	**P**
Light Treatment	2,21	0.32	0.73
Attenuation	**1,21**	**154.0**	**<0.0001**
Treatment x Attenuation	2,21	0.07	0.93

Table 8: Effect of attenuation and light pollution treatments on choosiness: As attenuation difference increases, fewer females choose the attractive but softer call. Light treatment had no effect on choosiness. See also Fig 9.

The attractiveness of the call stimuli frequently affected approach behaviors (i.e., females left the release box faster when presented with a more attractive call, they approached the attractive call faster, and they used fewer hops to reach the speaker). By contrast, treatment light levels only affected choice time (Table 9). Overall, choice time increased significantly with more intense light pollution. However, this statistical effect was likely driven by the very long choice time in response to unattractive calls at low light levels (Fig. 10).

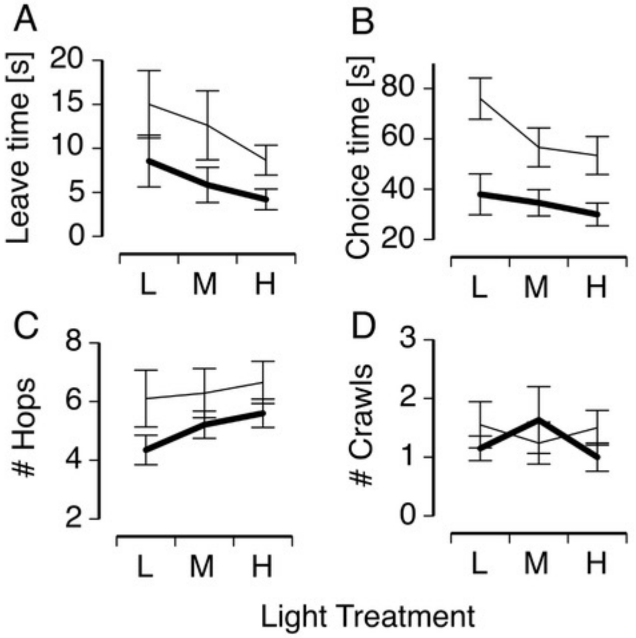


Figure 10: Light treatments (0.2, 5, and 15 lux) had little effect on how female Eastern gray treefrogs (*Hyla versicolor*) moved towards the playback speaker. Leave time (A), the number of hops (C), and the number of crawls (D) did not differ between light treatments. Only choice time (B) was faster under higher light pollution. Shown are least square means ± SE. Thick lines indicate responses to the attractive 30 pulse call, thin lines responses to the unattractive 6 pulse call.

Behavior	**Factor**	**DF**	**F**	**P**
Leave time	Light Treatment	2,95.01	2.01	0.14
	Call Duration	**1,95.61**	**7.37**	**0.007**
	Light Treatment × Call Duration	2,95.6	0.12	0.89
Choice time	**Light Treatment**	**2,95.01**	**4.37**	**0.015**
	Call Duration	**1,95.2**	**39.70**	**<0.0001**
	Light Treatment × Call Duration	2,95.2	1.30	0.28
# hops	Light Treatment	2,95.01	1.17	0.31
	Call Duration	**1,95.31**	**7.15**	**0.009**
	Light Treatment × Call Duration	2.95.3	0.24	0.79
# crawls	Light Treatment	2,95.02	0.12	0.88
	Call Duration	1,95.92	0.34	0.56
	Light Treatment × Call Duration	2,95.91	0.93	0.40

Table 9: Results of mixed models testing whether phonotaxis movements of female Eastern gray treefrogs (*Hyla versicolor*) differed as a function of light treatments and call duration (unattractive 6 pulse call and attractive 30 pulse call). Note that there was generally a significant effect of call duration (i.e., females approached the attractive call faster and with fewer movements), while light treatment only affected choice time . See also Fig. 10.

3.2. Dynamic Light Pollution

In the trials testing the effect of dynamic vs static light pollution on female call duration preferences, the intensity of the light stimuli was set to 15 lux in all trials, but in the static trials the light remained constantly switched on (i.e., 100% of each trial was illuminated), while in the dynamic trials light was switched on about 30% of the time, but the frogs spent 70% of each trial in darkness. Nevertheless, while there was almost always a strong effect of call attractiveness on female behavior, light treatments had few effects.

The trials testing preference functions showed that female Eastern gray treefrogs (*Hyla versicolor*) prefer longer duration calls (Fig. 11A, Table 10), and the temporal pattern of the light pollution treatment did not affect how females responded to variation in call duration (Fig 11A). As may be expected from the similar shapes of the preference functions, there was also no significant difference in the preference function traits of peak preference ($F_{1,19}$=0.07, p=0.80; Fig. 11B); strength ($F_{1,19}$=1.6, p=0.19; Fig. 11C), tolerance ($F_{1,19}$=0.79, p=0.39; Fig. 11D); and responsiveness ($F_{1,19}$=1.00, p=0.33; Fig. 11E).

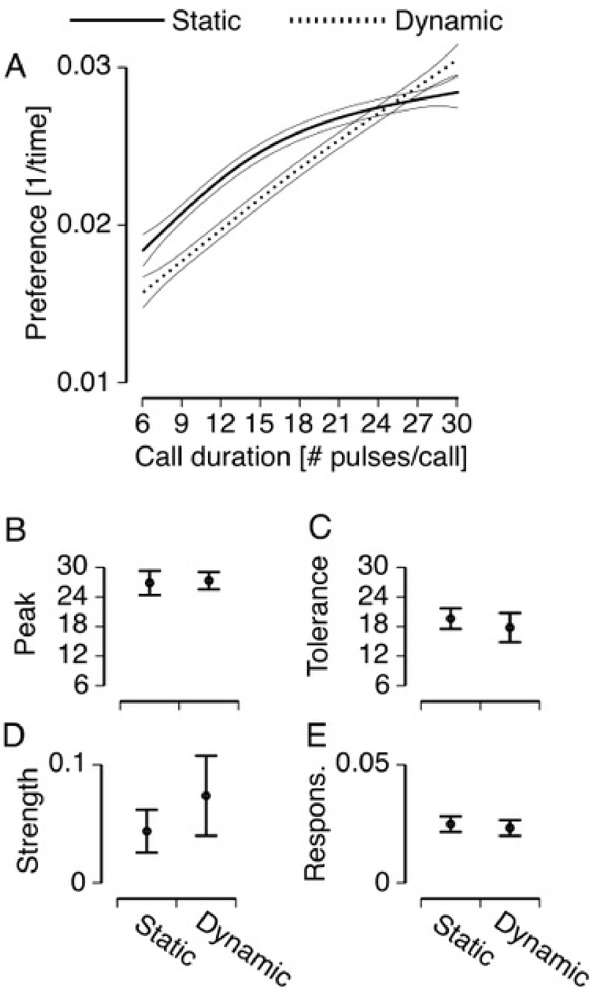


Figure 11: Call duration preferences of female Eastern gray treefrogs (*Hyla versicolor*) under static and dynamic light treatments. Preferences functions were almost identical under the three light conditions (A), and preference function traits did not differ (B-E).

Factor	**DF**	**F**	**P**
Light Treatment	1,335	1.95	0.16
Call Duration	1,335	**37.77**	**<0.0001**
Call Duration × Call Duration	1,335	1.63	0.20
Treatment ×Call Duration	1,335	1.57	0.21
Treatment ×Call Duration × Call Duration	1,335	0.78	0.38

Table 10: Effect of temporal variation in light pollution on preference for call duration in female Eastern gray treefrogs (*Hyla versicolor*). Females preferred longer duration calls, but call duration preferences did not differ whether light pollution was static or dynamic. Significant terms are set in bold. See also Fig. 11.

The trials testing choosiness showed that as amplitude differences between an attractive and an unattractive call increased, the proportion of females continuing to approach the attractive call decreased (Figure 12; Table 11: significant effect of Attenuation). However, the temporal pattern of the light pollution treatment did not affect choosiness (Table 11: non-significant effect of Treatment).

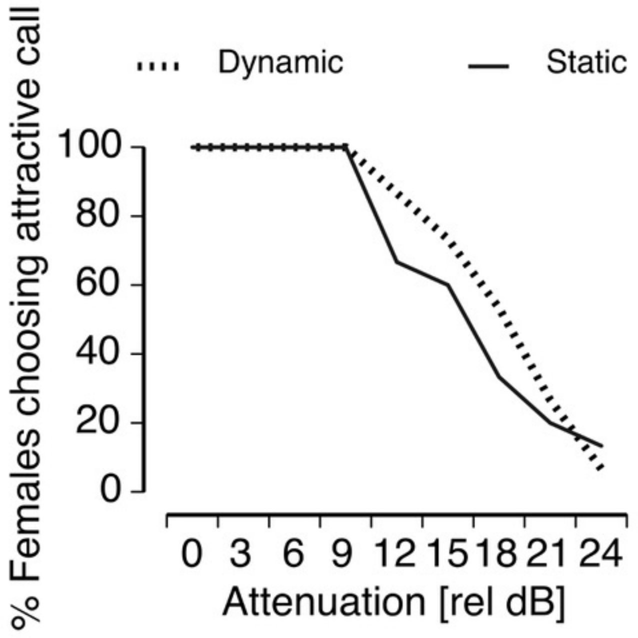


Figure 12: Choosiness of female Eastern gray treefrogs (*Hyla versicolor*), expressed as the percentage of females still choosing the more attractive stimulus as it is gradually attenuated, did not differ under static and dynamic light treatments.

Factor	**DF**	**F**	**P**
Light Treatment	1,14	0.87	0.37
Attenuation	1,14	**99.92**	**<0.0001**
Treatment x Attenuation	1,14	0.10	0.76

Table 11: Effect of attenuation and light pollution treatments on choosiness: As attenuation difference increase, fewer females choose the attractive but softer call. Light treatment had no effect on females choosiness. See also Fig. 12.

The attractiveness of the test stimuli frequently affected approach behaviors (i.e., females approached the attractive call faster and with fewer hops (Table 12). Light treatment, on the other hand, only affected the number of hops during phonotaxis. Females moved less during the dynamic light pollution treatment, although this effect was likely driven by the low variance of female movements towards attractive calls in the dynamic trials (Fig. 13).

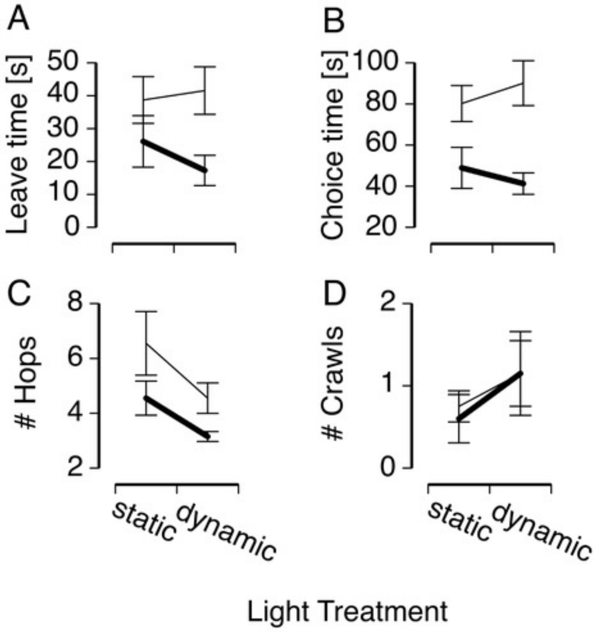


Figure 13: Light treatment (static vs. dynamic) had little effect on how female Eastern gray treefrogs (*Hyla versicolor*) moved towards the playback speaker. Leave time (A), choice time (B), and the number of crawls (D) did not differ between light treatments. Only the number of hops (C) was higher in the static light treatment. Shown are least square means ± SE. Thick lines indicate responses to the attractive 30 pulse call, thin lines responses to the unattractive 6 pulse call.

Behavior	**Factor**	**DF**	**F**	**P**
Leave time	Light Treatment	1,57	0.26	0.61
	Call Duration	1,57	**10.09**	**0.002**
	Light Treatment × Call Duration	1,57	1.01	0.32
Choice time	Light Treatment	1,56.68	0.01	0.91
	Call Duration	**1,56.69**	**21.01**	**<0.0001**
	Light Treatment × Call Duration	1,56.69	1.01	0.32
# hops	**Light Treatment**	**1,57**	**6.01**	**0.017**
	Call Duration	**1,57**	**6.01**	**0.017**
	Light Treatment × Call Duration	1,57	0.19	0.67
# crawls	Light Treatment	1,57	1.94	0.17
	Call Duration	1,57	0.05	0.83
	Light Treatment × Call Duration	1,57	0.05	0.83

Table 12: Results of mixed models testing whether phonotaxis movements of female Eastern gray treefrogs (*Hyla versicolor*) differed as a function of light treatments (static vs dynamic light pollution at 15 lux) and call duration (unattractive 6 pulse call and attractive 30 pulse call). Note that there was generally a significant effect of call duration (i.e., females approached the attractive call in faster times and with fewer movements), while light treatment only affected the number of hops (more hops during static light treatment). See also Fig. 13.

Focusing on the behaviors females exhibited during the light and dark portions of the same phonotaxis approach (i.e., only the dynamic trials), gave somewhat conflicting results. Qualitatively, there seemed to be a preference for moving in the dark: while all 20 females performed at least one locomotion movement during the dark portion of the trials, only about half of them also moved during the bright periods, and none of the females moved only during bright periods (Fig. 14A). Quantitatively, however, movement frequency did not appear to be influenced by light. Once taking into account that there was more darkness during the trials than bright periods (roughly 7:3 dark:bright), the expected and observed proportion of movements performed in darkness did not differ significantly (6P: $F_{1,39} = 1.26$, $P = 0.27$; 30P: $F_{1,39} = 1.45$, $P = 0.24$; Fig. 14B).

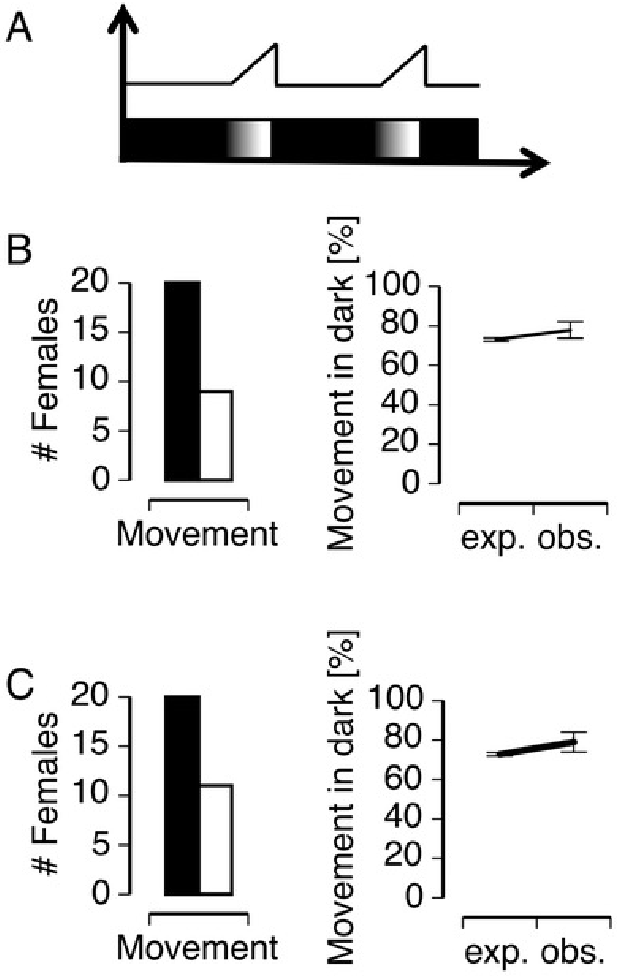


Figure 14: Temporal pattern of dark and bright periods during the dynamic light pollution trials (A). Movement pattern during the 6 Pulse (B) and 30 Pulse (C) stimuli of the dynamic trials. More females moved during the dark periods than the light ones (left panels): All females moved at least once during the dark portion of the trial (black bar), while only about half the females also moved during the bright portion of the trial (white bar). Observed frequency of movement in the dark (right panels; means ± SE), however, did not differ from expected (given that 70% of trials time was in the dark).

4. Discussion

We tested the hypothesis that mate choice behavior of female Eastern gray treefrogs (*Hyla versicolor*) is affected by anthropogenic light pollution. Contrary to our prediction that females would show relaxed preferences, decreased choosiness, and stealthier phonotactic approach movements under brighter and / or fluctuating light conditions, we actually found that almost none of the mate choice behaviors scored during our experiments were affected by light treatment.

4.1. Experiment 1: Static light pollution

In this experiment, we examined whether *presence* and *intensity* of artificial light affects mate choice behavior in *Hyla versicolor.* In our focal species, persistently increased nocturnal light levels spanning the gamut from light levels comparable to natural moonlight to standing under a streetlight (0.2-15 lux) did not change acoustic call preferences or modify choosiness for longer-duration calls. This is in line with the species' response to variation in natural light levels, where female mate choice behavior as similar under conditions equivalent to starlight and to full moon (Underhill and Höbel, 2017).

To our knowledge only one other study has examined female mate choice behavior in responses to artificially high light levels (roughly 3 lux, Rand et al., 1997). There, female túngara frogs changed their behavior under different light conditions in a way that suggested that they felt safer under darker conditions (Rand et al., 1997). Interestingly, female túngara frogs are also more cautious when tested under conditions simulating variation in natural light levels (i.e., equivalent to full moon; Baugh and Ryan, 2010; Bonachea and Ryan, 2011a, b, c), suggesting that there are genuine differences between female gray treefrogs and túngara frogs, and that the effect of light on mate choice behavior may be highly species-specific.

Some frogs use visual cues/signals during mate choice (Gomez et al., 2009; Taylor et al., 2007) and during male–male competition (Hödl and Amézquita, 2001). However, there is substantial between-species variation in the use of visual cues, as well as the relative reliance on visual vs. acoustic signal components. In *H. versicolor*, visual mate choice is weak (Reichert et al., 2014, Reichert and Höbel, 2015), and we therefore expect that females would not attend more to visual cues under increased light levels. Nevertheless, there are species that do attend to visual cues (Gomez et al., 2009; Taylor et al., 2007), and for them increased nocturnal light should facilitate the use of inter- or intrasexual displays. A possible consequence of light pollution on visually signaling species may therefore take the form of altered selection regimes, potentially resulting in divergence between natural and polluted sites. Increased light may facilitate the use of visual over acoustic signal components, thus bringing visual components under stronger selection and/or lowering the strength of selection on acoustic traits. We would predict that a relaxation of acoustic mate preferences is the more likely outcome, because studies examining multimodal communication in frogs frequently report that co-localization of a visual cue makes a call more attractive (i.e., Gomez et al., 2009; Taylor et al., 2007; Reichert and Höbel, 2015).

Another aspect of static light pollution we did not test in our experiments is phototaxis, i.e., movement in response to light. In a series of studies, Jaeger and Hailman (1973, 1976) tested more than 120 species of frogs and toads and found that the vast majority were photopositive, i.e., they approached the light source (there were also species that avoided light and those that preferred intermediate light levels). Thus, while some frog species may avoid artificial lights, many others may be attracted to them. This could lead to denser aggregations around light sources, with unexplored or unknown consequences (see also Buchanan, 2006). For example, denser aggregations of displaying males may attract more predators, thus leading to higher predation pressure on both the males and the females attracted by them. Denser choruses may also lead to increased male-male aggression, and the resulting increase in males producing aggressive instead of advertisement signals may hinder the ability of females to engage in acoustic mate choice. Then again, higher density choruses may facilitate mate choice or decrease the cost of mate assessment, because potential mates are spatially concentrated.

It is also important to remember that female mate preferences are only half of the equation of successful mate choice and population maintenance. Chorus wide effects of light pollution that merit more attention are male calling behavior, including male spacing and male calling perch choice, as all can affect the ability of females to localize and assess potential mates. Detailed observations and experimental tests on changes of male calling behavior are largely missing (but see Baker and Richardson, 2006; Tuttle and Ryan, 1982). However, a number of observations suggest that changes in ambient light levels may affect how frequently males call, how often they move between calling perches, and how exposed or hidden in vegetation they choose to call (Baker and Richardson, 2006, Buchanan, 1993, 2006; da Silva Nunes, 1988; Onorati and Vignoli, 2017; Tuttle and Ryan, 1982).

4.2. Experiment 2: Dynamic light pollution

In this experiment we examined whether *temporal fluctuation* in light pollution affects frog behavior. This experiment involved two light treatments with identical light intensity (15 lux), but different temporal pattern (continuous, as under a street light, or intermittent, as from cars driving by). In our focal species, rapid fluctuations in light levels did not change acoustic call preferences or modify choosiness for longer-duration calls.

Frogs conduct their reproductive behavior at night, and their eyes are expected to be adjusted to ambient condition, i.e., to be dark- adapted. Rapid changes in light level require dark or light adaptation, respectively, or frogs will suffer reduced visual capabilities (Fain et al., 2001; Fite, 1976). When dark-adapted eyes are suddenly exposed to light, the dark-adapted, dilated pupil allows more light to enter the eye, and excessive amount of light can causes photopigment bleaching, which can take hours to return eyes to a dark-adapted state (Cornell and Hailman, 1984; Donner and Reuter, 1962). This suggests that quick changes in light intensity should affect visual acuity of nocturnal frogs. A study examining the effect of rapidly increased ambient light levels on prey capture found that frogs behaved consistent with the interpretation that they were temporarily blinded by the light and required a substantial recovery period: frogs took significantly longer to detect or to attempt to capture prey after rapid increases in illumination (Buchanan, 1998). This was the case when frogs were exposed to 12 lux, which is less bright than our experiments, suggesting that if females during phonotaxis suffer similar consequences than during foraging, we should have observed slower or more hesitant approach movements. This suggests that mate choice, at least in the gray treefrogs tested here, is mediated predominantly by acoustic cues. This is in line with previous studies on multimodal communication in this species, that showed that although females are not indifferent to visual cues co-localized with an acoustic signal (Reichert and Höbel, 2015), there is no evidence for visual mate choice (Reichert et al., 2014).

5. Conclusion

We have shown that in gray Treefrogs, light pollution does not affect mate choice behavior, and should therefore neither interfere with population persistence nor affect the sexual selection regimes on male call traits. However, there are several reasons why we consider it premature to assume that anthropogenic light pollution is of no concern for amphibian conservation. First, as is tradition in anuran mate choice experiments, (i.e., Gerhardt, 1987; Rand et al., 1997; Reichert and Höbel, 2015), our trials were conducted in a comparatively small playback arena (2m diameter). Observations thus only encompass a small fraction of the distance a mate-searching female will cover in the night she approaches the breeding pond. Consequently, not documenting behavioral differences in phonotaxis-related behaviors during the very last portion of approach still leaves many aspects of female mate choice behavior unexamined. For example, the approach path from the surrounding forest towards the breeding pond may still differ between natural and polluted sites, or females may simply choose not to approach brightly illuminated sites. Second, behavioral responses to variation in nocturnal light levels (both in the natural as well as anthropogenically enhanced range) seem to be highly species-specific, with some species showing behavioral changes (i.e., Backwell and Passmore, 1990; Rand et al., 1997), and other not (this study). Interestingly, the species that did change its behavior in response to variation in the natural range (starlight to moonlight; Baugh and Ryan, 2010; Bonachea and Ryan 2011a, b, c) also changed behavior (in similar ways) when confronted with artificially high light levels (Rand et al., 1997), while the one that did not change behavior in response to light variation in the natural range (Underhill and Höbel, 2017) was also unaffected by artificially increase light levels. Potentially, knowledge of a species' response to variation in the natural range might help gage its vulnerability to anthropogenic light pollution.

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