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Moonlighting? - Consequences of lunar cues on anuran reproductive activity

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ABSTRACT

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.

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

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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 extends 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 catesbeiana*, *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 amplexus 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 bimodal 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 Watson'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 reproductive 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: $U^2 = 0.002$, $N_{chorus} = 59$, $N_{chorus\ size} = 576$, $p > 0.5$).

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

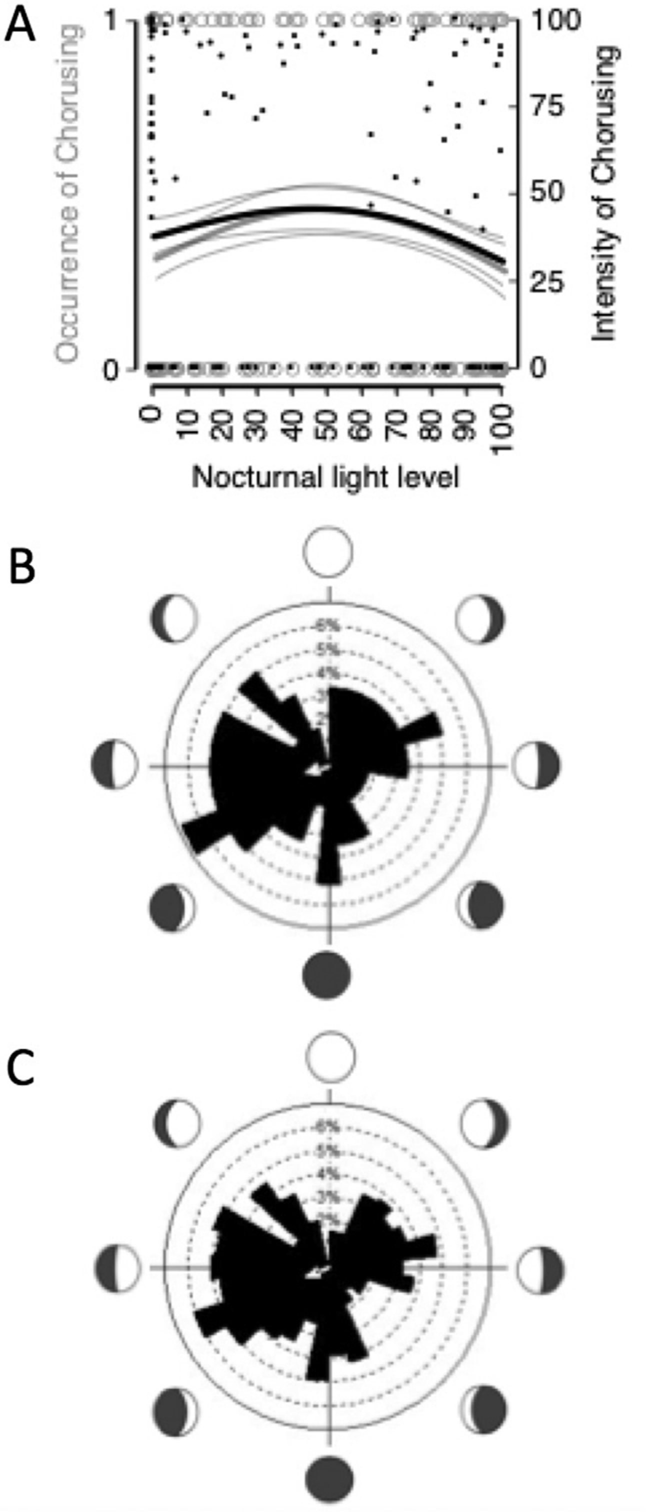

Fig. 1. Lunar effects on chorus formation and chorus size in male Eastern Gray Treefrogs (*Hyla versicolor*). (A) Both the likelihood that chorus formed (occurrence; gray symbols and line), as well as the size of the chorus (intensity; black symbols and line) were larger at intermediate light levels. 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.

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.

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

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 (μ = 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: $U^2 = 0.059$, $N_{\mathrm{occurrence}} = 67$, $N_{\mathrm{intensity}} = 706$, $p > 0.5$).

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 reproductive 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 reproductive 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

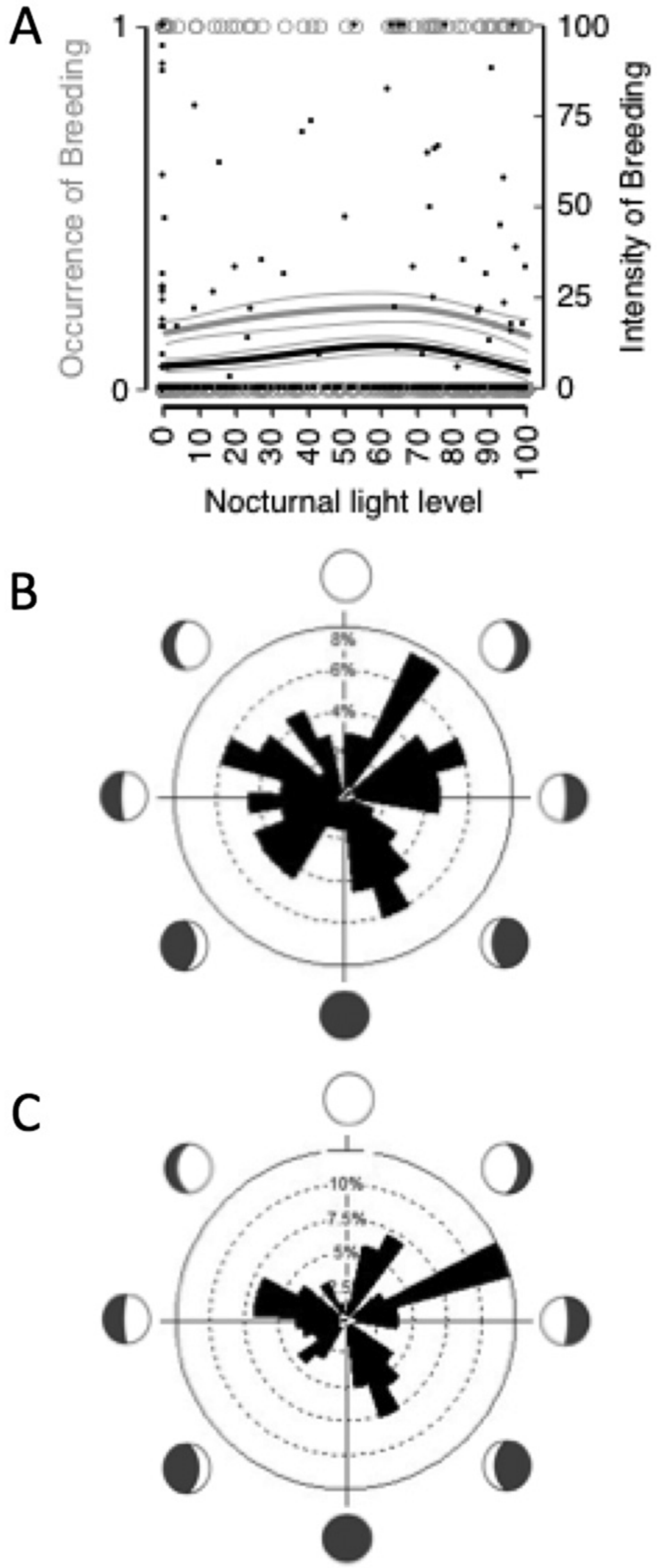

Fig. 2. Lunar effects on the occurrence and intensity of breeding activity in female Eastern Gray Treefrogs (*Hyla versicolor*). (A) Whether females came to breed was not affected by nocturnal light levels (occurrence; gray symbols and line), but the number of breeding females (intensity; black symbols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

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.

Variable	Chi$^2_1$	*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

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 reproductively active females were observed) did not differ significantly in relation to the lunar cycle (Watson's U^2 test: $U^2 = 0.039$, $N_{\text{chorus}} = 59$, $N_{\text{mating}} = 20$; $p > 0.5$; 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: $U^2 = 1.16$, $N_{\text{chorus size}} = 300$, $N_{\text{breeding females}} = 576$, $p < 0.001$). 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).

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 decreased in five species (56%) (Granda et al., 2008; Onorati and Vignoli,

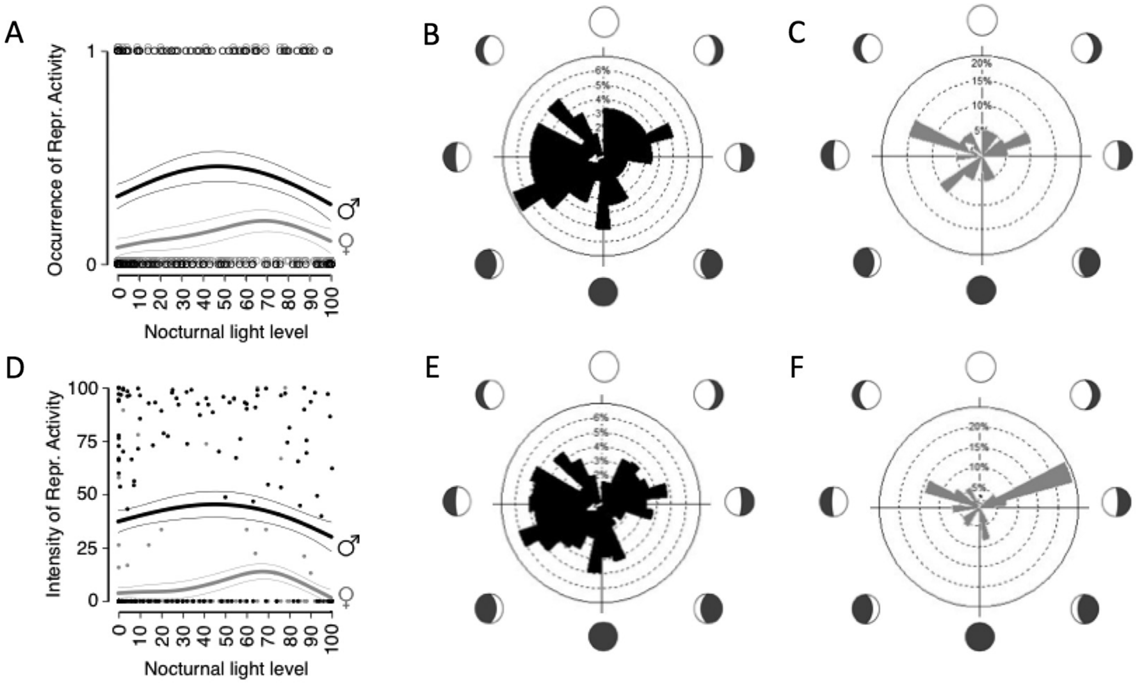

Fig. 3. Sex differences in response to lunar cues. (A) The occurrence of reproductive activity in male and female Eastern Gray Treefrogs (*Hyla versicolor*) was not affected differentially by variation in nocturnal light levels. Both sexes tended to be reproductively active during intermediately bright 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.

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.

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

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 pattern 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 intensity (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 relate 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.

Author contribution

GH designed the study with contribution from VAU; VAU analyzed the data; GH performed statistical analysis; GH and VAU wrote the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.actao.2018.02.001.

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