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## Seasonal Shift in the Age Structure of Calling Males Within a Spring Peeper (*Pseudacris crucifer*) Chorus

Many factors can influence male reproductive success including attractiveness of sexual displays, effectiveness of sensory and locomotory systems in scramble competition, physical condition in fight contests, or quality of resources offered to mates. In lek breeding as is found for many species of anurans, the ability to remain reproductively active within a breeding assemblage is one of the most important predictors of mating success (Halliday and Tejedo 1995; Andersson and Iwasa 1996; Friedl and Klump 2005; Castellano et al. 2009). Males of many anuran species congregate in wetlands and produce advertisement calls to attract females for reproduction. Males that spend more nights calling in a breeding chorus than average (hereafter chorus tenure) tend to have greater mating success (Greer and Wells 1980; Sherman 1980; Halliday and Tejedo 1995; Friedl and Klump 2005; Mangold et al. 2015; Botto and Castellano 2016). A positive relationship between chorus tenure and mating success is evident for most taxa where this has been evaluated (Table 1). Chorus tenure often surpasses all other factors in explaining male mating success variance, such as attributes of advertisement calls and body size that are often assumed to play a key role in sexual selection (Sullivan and Hinshaw 1992; Friedl and Klump 2005; Castellano et al. 2009; Ospina-L. et al. 2017). For example, chorus tenure explained about 50% of the variance in mating success in Natterjack Toads, *Epidalea calamita* (Arak 1983) and European Treefrogs, *Hyla arborea* (Jaquiéry et al. 2010). In Italian Treefrogs, *Hyla intermedia*, chorus tenure explained 19% of mating success variance, while call rate, one of the most important call attributes under direct female selection in the field, accounted for only 5% (Castellano et al. 2009).

Abbreviated chorus tenure, when males spend only a fraction of breeding season in a chorus, has been observed in many anuran species (Murphy 1994b): for example, over 50% of males called only 1–2 nights and the maximum chorus tenure was only 13 nights over a single 4-mo breeding season in Rosenberg's Treefrog, *Hypsiboas rosenbergi*, in Puntarenas Province, Costa Rica (Höbel 2000). The median chorus tenure was also only 2–3 nights in the Barking Treefrog, *Dryophytes gratosus*, in southwest Tallahassee (Florida, USA), whose breeding season lasted 49–96 nights from 1987 to 1990 (Murphy 1994a). Even for Great Plains Toads, *Anaxyrus cognatus*, whose breeding season was only 2–5 nights, over 90% males called for only a single night in southern Arizona and southwestern New Mexico, USA from 1980 to 1982 (Sullivan 1983). Non mutually-exclusive hypotheses for variance in male chorus tenure that have at least some empirical support include: 1) the energy limitation hypothesis proposing that some males cannot sustain costly calling behaviors for many nights (Green 1990; Murphy 1994b); 2) the predation risk hypothesis where males choose to leave choruses due to high predation risk (Green 1990); and 3) the mortality hypothesis where males are removed from choruses because of predation, parasitism, disease or desiccation (Murphy 1994b). To test the energy limitation hypothesis, feeding starvation experiments are commonly done to test whether feeding increases the number of nights males call, and male body size and body condition are examined to test whether they are correlated with chorus tenure (Green 1990; Murphy 1994b; Given 2002). In some species, smaller males lost weight more quickly than larger males and thus attended their respective chorus for shorter periods of time (e.g., Natterjack Toads; Tejedo 1992). In contrast, Morrison et al. (2001) found that smaller male Orange-thighed Frogs, *Litoria xanthomera*, of northern Queensland, Australia, attended choruses for longer due to slower energy consumption. In some species only body size predicts chorus tenure, but not body condition (e.g., European Treefrogs; Luquet et al. 2013), while in other species body size does not relate to chorus tenure at all (Arak 1988; Woodward 1982; Dyson et al. 1992; Given 2002; Grafe and Meuche 2005; Basto-Riascos et al. 2017). Thus, the effect of body size and body condition on chorus tenure appears to be species-specific. Empirical data for the predation risk hypothesis and the mortality hypothesis

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TABLE 1. List of anuran species that have been examined to test for a relationship between male chorus tenure (i.e., the number of nights producing calls) and mating success. This table is based on Halliday and Tejedo (1995; Table 3), augmented here with 15 studies.

Family	Species	Reference
<b>Species with a positive relationship</b>		
Bufonidae	<i>Anaxyrus americanus</i>	Gatz (1981)
	<i>Anaxyrus canorus</i>	Sherman (1980)
	<i>Anaxyrus exsul</i>	Sherman (1980)
	<i>Anaxyrus fowleri</i>	Given (2002)
	<i>Anaxyrus woodhousii</i>	Woodward (1982); Sullivan (1987)*
	<i>Epidalea calamita</i>	Arak (1983, 1988); Tejedo (1992); Stevens et al. (2003)
	<i>Sclerophrys capensis</i>	Cherry (1993)
	<i>Sclerophrys gutturalis</i>	Telford and van Sickle (1989)
Allocentrolenidae	<i>Centrolene savagei</i>	Ospina-L. et al. (2017)
	<i>Espadarana prosoblepon</i>	Jacobson (1985); Basto-Riascos et al. (2017)
	<i>Hyalinobatrachium fleischmanni</i>	Greer and Wells (1980); Jacobson (1985)
	<i>Hyalinobatrachium valerioi</i>	Mangold et al. (2015)
Dendrobatidae	<i>Ameerega trivittata</i>	Roithmair (1994)
Eleutherodactylidae	<i>Eleutherodactylus coqui</i>	Townsend and Stewart (1994)
Hylidae	<i>Boana rosenbergi</i>	Kluge (1981); Höbel (2000)
	<i>Dryophytes chrysoscelis</i>	Godwin and Roble (1983); Morris (1989)**; Ritke and Semlitsch (1991)
	<i>Dryophytes gratiosus</i>	Murphy (1994a)
	<i>Dryophytes versicolor</i>	Sullivan and Hinshaw (1992); Bertram et al. (1996)
	<i>Hyla arborea</i>	Jaquiéry et al. (2010)
	<i>Hyla intermedia</i>	Castellano et al. (2009); Botto and Castellano (2016)
	<i>Litoria xanthomera</i>	Morrison et al. (2001)
	<i>Pithecopus rohdei</i>	Wogel et al. (2005)
Hyperoliidae	<i>Hyperolius marmoratus</i>	Dyson et al. (1992); Dyson et al. (1998)
Leptodactylidae	<i>Engystomops pustulosus</i>	Ryan (1983)
Ranidae	<i>Lithobates catesbeianus</i>	MacDonald (1997)
	<i>Lithobates virgatipes</i>	Given (1988)
Rhacophoridae	<i>Buergeria buergeri</i>	Fukuyama and Kusano (1989)
	<i>Chiromantis xerampelina</i>	Jennions et al. (1992)
<b>Species with a positive relationship between mating success and presence at the breeding site (not necessarily calling)</b>		
Hylidae	<i>Dryophytes cinereus</i>	Gerhardt et al. (1987)
<b>Species with a positive relationship between mating success and residency (the time between the first and last observation during which males might space out chorus)</b>		
Hylidae	<i>Boana faber</i>	Martins (1993)
<b>Species with no relationship between mating success and chorus tenure</b>		
Bufonidae	<i>Anaxyrus woodhousii</i>	Sullivan (1987*, 1989)
Hylidae	<i>Dryophytes versicolor</i>	Fellers (1979)***

\*Chorus tenure was significant only in simple regression with mating success but not in multiple regression analysis that incorporated mean call rate and body size.

\*\*Morris (1989) found positive relationships in all four years from 1984 to 1987 in *Dryophytes chrysoscelis* in central Indiana. This was mistakenly reported to be one out of four years, which was male body size, in Halliday and Tejedo (1995).

\*\*\*Significance level was  $0.05 < P < 0.10$ .

are scarce probably due to the difficulty in quantifying adult mortality rate and predation in the wild (but see Green 1990 and Murphy 1994b).

The fourth non-exclusive hypothesis, the residual reproductive value hypothesis, looks at sexual displays from a life history perspective: an individual has a finite lifespan and limited expendable resources; allocating resources towards sexual advertisement diminishes an individual's capacity to invest in current survival or growth as well as future reproduction because of the life-history trade-off; when the residual reproductive value, i.e., the opportunity for future reproduction, decreases with age, current reproductive investment should increase (Fisher 1930; Williams 1966; Fischer et al. 2008). The empirical evidence for this hypothesis includes greater persistence and aggressiveness in aerial courtship despite lower body dry mass and lower fat content in older male butterflies (Squinting Bush Brown, *Bicyclus anynana*; Fischer et al. 2008), and greater willingness to assume risk of injury and death to fight for vertebrate carcasses for laying eggs in older female burying beetles compared to similar sized younger females (*Nicrophorus orbicollis*; Trumbo 2012). For anurans, few studies have examined the impact of age on calling efforts and chorus tenure from a life history vantage. Although older males seem to not produce greater call effort (i.e., the product of call length and call rate) in Gray Treefrogs *Dryophytes versicolor* (Kuczynski et al. 2015), older males had greater chorus tenure in one of two years in European Treefrog in southern Germany, although this relationship may have been confounded by the positive relation between age and body size (Friedl and Klump 2005). According to the residual reproductive value hypothesis, an older male anuran reaching the end of its lifespan with little or no residual reproductive value should invest in current reproductive opportunities and attend a chorus regardless of mortality risks or energy reserves. However, an old male might also be larger given indeterminate growth in amphibians (Halliday and Verrell 1988; Friedl and Klump 2005) and thus show greater stamina in chorus tenure.

To test whether chorus tenure varies among age classes, instead of tracking chorus tenure of individuals from different age class through mark-recapture, we investigate nightly age structure of a chorus across a breeding season, and approach that is more feasible for populations with a large number of calling males. We hypothesize that older males will have longer chorus tenure, predicting that age structure within a chorus will shift towards a greater proportion of older males over the duration of a breeding season. Our focal species is the Spring Peeper, *Pseudacris crucifer*, a broadly-distributed, temperate North American hylid frog that has a maximum 4-year life span (Lykens and Forester 1987). It is a cold season breeder and males assemble in choruses of varying sizes, sometimes up to hundreds of individuals (Forester and Lykens 1986; Lance and Wells 1993). The mating system is lek-like in that males aggregate in wetlands and produce advertisement calls to attract females. Chorus tenure in the Spring Peeper is known to vary, with some males participating in a chorus for short periods while others were present for almost the entire season in a population in Michigan (Delzell 1958). The Spring Peeper does not exhibit endosteal resorption (Lykens and Forester 1987), which makes it an excellent candidate for using skeletochronology to estimate age.

## MATERIALS AND METHODS

We sampled calling male Spring Peepers in 2018 from an upland marsh near the Queen's University Biological Station, Ontario, Canada (44.5786°N, 76.3297°W). Spring Peeper calling was delayed in 2018 due to an ice storm in early-mid April. We started sampling near the beginning of male chorusing (21 April) and finished when only a few Spring Peeper males remained calling (12 May), coincident with the onset of Gray Treefrog calling. Our sampling was not continuous, comprising 4 consecutive nights of sampling, one night with no sampling, 2 nights of sampling, 2 nights with no sampling, 3 nights of sampling, 2 nights with no sampling, 3 nights of sampling, one night with no sampling, and finally 4 nights of sampling. The total sampling included 16 nights. The males were sampled randomly across the marshland to avoid bias towards specific calling site. Males were located using their call and hand captured. We then clipped 2 toes with both toe pad and second phalange and stored them in 100% ethanol for skeletochronology. Toe clipping does not affect adult frog survival and recapture probability (Guimarães et al. 2014). Individuals were released near their point of capture.

We fixed each toe sample in 10% neutrally buffered formalin for 1 h and then rinsed it in distilled water for 30 min. We decalcified the toes in 3% nitric acid for 90 min and rinsed them in distilled water overnight. We then dehydrated the toes sequentially in 35% ethanol, 50% ethanol and 70% ethanol baths, each step lasting 1 hour, followed by dehydration, clearing, and infiltration in a General Data Healthcare™ Automatic Tissue Processor (i.e., rinsing toes 2 times in 70% ethanol, 1 time in 80% ethanol, 1 time in 95% ethanol, 3 times in 100% ethanol, 2 times in xylene, and 3 times in paraffin, with each rinsing step lasting 1 h for a total of 12 hours). We then embedded each toe in paraffin and sectioned it to 4- $\mu$ m thickness using a Leica RM2125 RTS microtome (Leica Biosystems, Buffalo Grove, USA). We tried to section all toes at the middle of the phalange where the periosteal bone is at maximum thickness and all lines of arrested growth (LAGs) should be present (Rozenbult and Ogielska 2005). The cross-sections were then washed twice in toluene and twice in 100% ethanol, each wash lasting 3 min, followed by staining in 0.1% cresylviolet for 20 min. We photographed all cross-sections with a Motic Pro Microscope Camera at 200 $\times$  magnification. YC and RBC examined the images independently and the results were compared. For the individuals with discrepancies (mostly not-well sectioned or not-well stained), we re-examined the images together, compared them with good cross-sections, and discussed the results until a consensus was reached on the age of each individual. For the unsatisfactory cross-sections of 18 individuals, either with a thin periosteal bone or with lines of arrested growth not well-stained, we re-sectioned and stained the toe sections. We compared new and original results to gain some estimate of repeatability. For all subsequent analyses, we used the age obtained in the first round of skeletochronology preparations.

The statistical analyses and visualizations were conducted in R 3.5.2 (R Core Team 2018). For our analysis, we coded the 16 sampling nights as days into the breeding season to account for the nonconsecutive sampling. The first sampling night (21 April) was coded as day 1 of the breeding season, and the sixteenth sampling night (12 May) was coded as day 22. We calculated percentage of males through dividing number of males at each age by total number of frogs sampled that night to account for

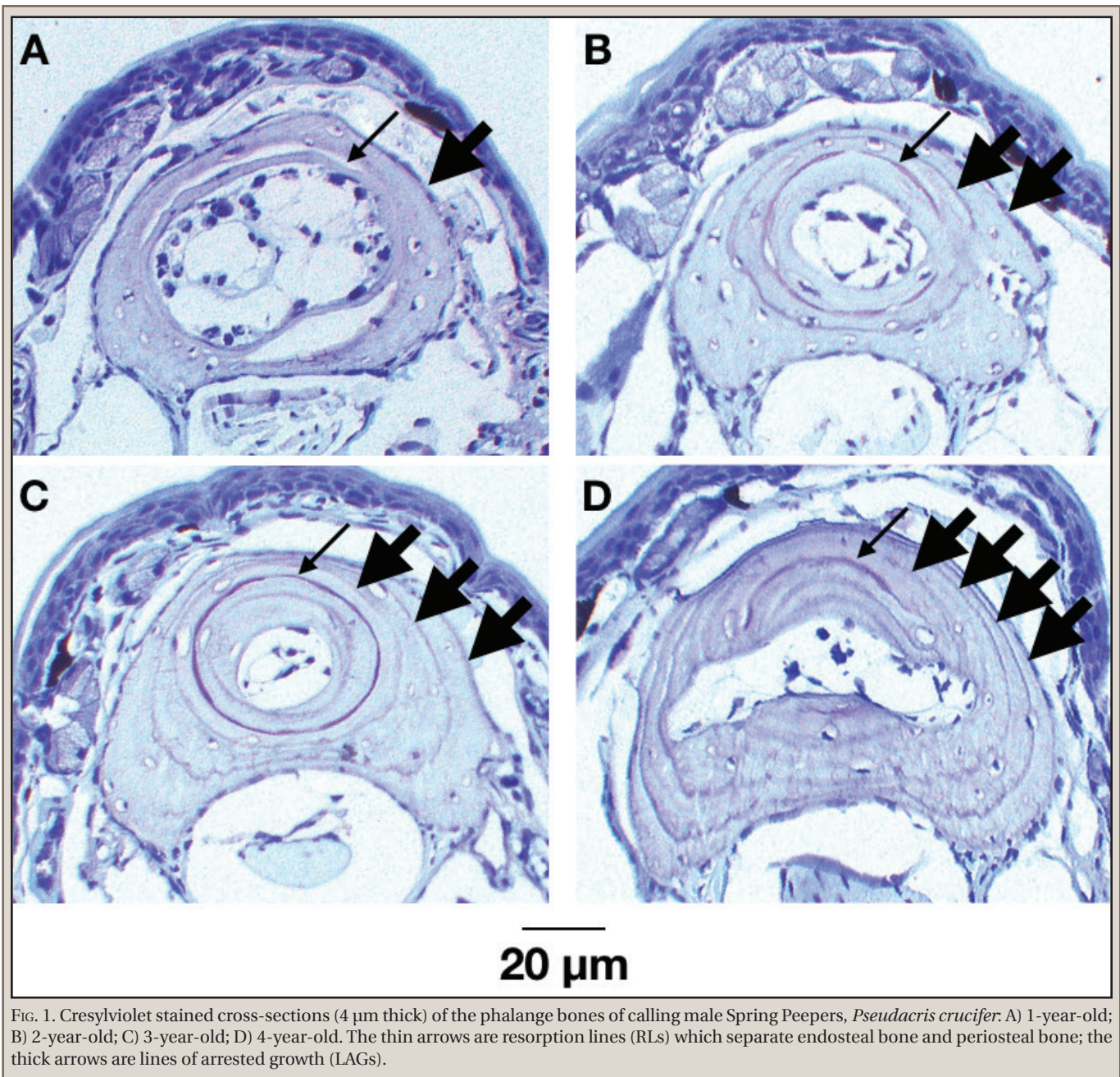


FIG. 1. Cresylviolet stained cross-sections (4  $\mu$ m thick) of the phalange bones of calling male Spring Peepers, *Pseudacris crucifer*. A) 1-year-old; B) 2-year-old; C) 3-year-old; D) 4-year-old. The thin arrows are resorption lines (RLs) which separate endosteal bone and periosteal bone; the thick arrows are lines of arrested growth (LAGs).

uneven sampling size across nights. We tested the relation of percentage of male frogs to age class (coded as a categorical factor), days into the season, and the interaction between days into the season and age using a quasi-binomial generalized linear model (glm command, basic R installation). We also tested whether there is temporal autocorrelation in the model (acf command, basic R installation).

#### RESULTS

We collected data from 126 calling male Spring Peepers. During sampling we encountered only 4 recaptures suggesting a large census population size at our study site and that males attended the chorus for an abbreviated period. The stained phalange sections gave clear LAGs that we could use to infer age (thick arrows in Fig. 1). Resorption lines (RLs) are evident from darker staining than LAGs, separating the endosteal and

periosteal bone (thin arrows in Fig. 1). Tear artifacts occurred at RLs in some sections, and these were used to differentiate between endosteal and periosteal bone (Fig. 1A; Rozenbult and Ogielska 2005). We did not find evidence of endosteal bone resorption in the phalange samples, consistent with what has been observed in femur sections of Spring Peepers (Lykens and Forester 1987). We observed double lines in a few individuals, which were counted as a single LAG. The repeatability for our skeletochronology estimates was 83% with 3 of 18 individuals showing inconsistency between repeated sections. Of the three inconsistent individuals, two were older than the estimate from the first-round sections and the other was younger. Paterson and Blouin-Demers (2018) found 84% repeatability in a mark-recapture study of the Tree Lizard (*Urosaurus ornatus*) where they used skeletochronology to estimate age, very close to the value we found.

The age of 126 sampled calling male Spring Peepers ranged from 1–4 years old. Only two individuals were estimated to be 1

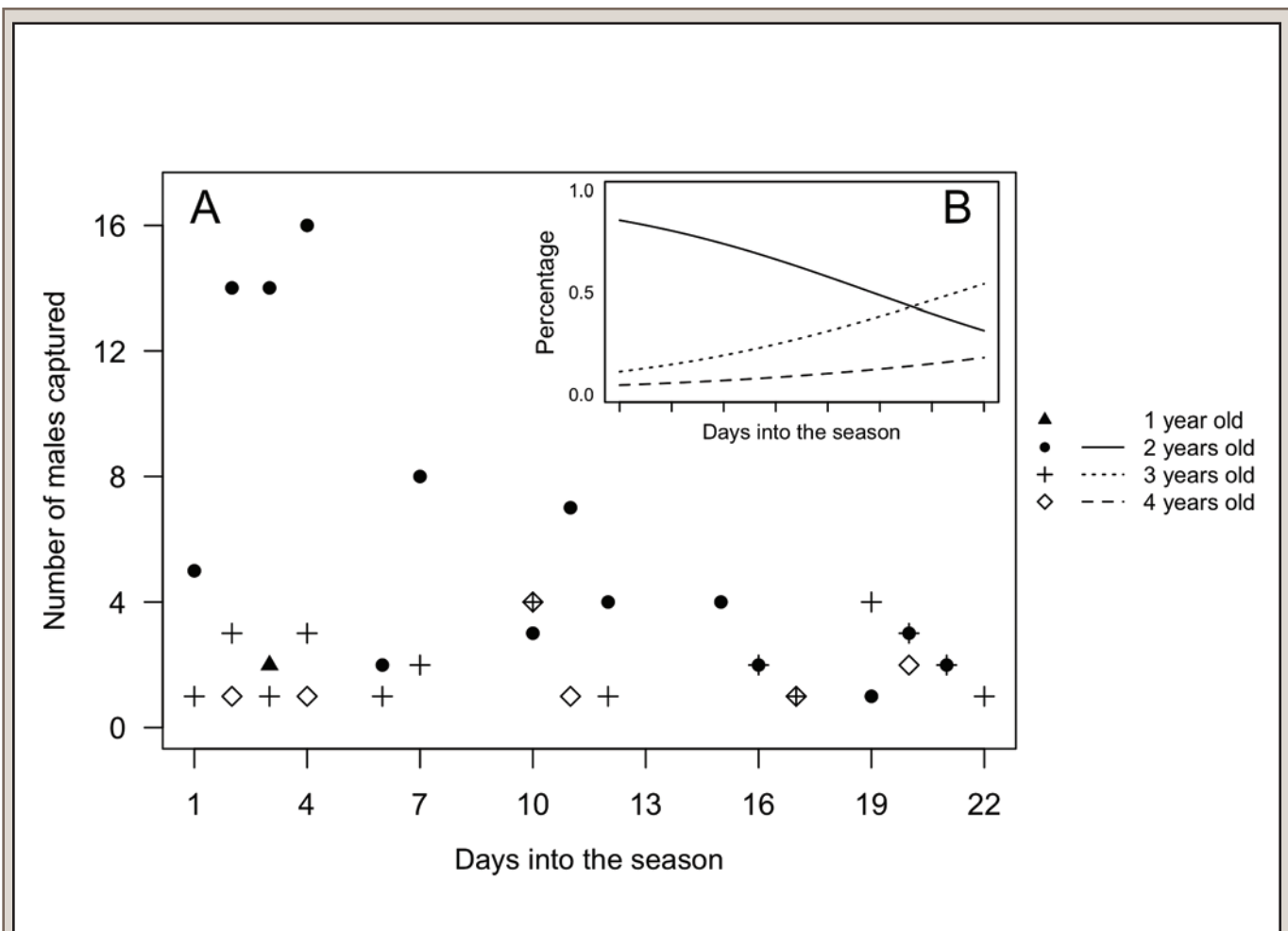


FIG. 2. A) Number of calling male Spring Peepers sampled for each age class with days into the mating season. The first sampling night (21 April) was coded as 1 day into the mating season, and the sixteenth sampling night (12 May) as 22 days into the mating season; B) predicted generalized linear model curves of percentage of calling male Spring Peepers with days into the season for three age classes in the calling assemblage.

year old (Fig. 2A). Over half (67%;  $N = 85$ ) of sampled males were 2 years old, with 29 individuals aged at 3 years, and 10 at 4 years old (Fig. 2A). The two 1-year-old males were excluded from the generalized linear model analyses as they were from the same sampling day and thus, we could not investigate the temporal pattern of this age class. The interaction of age and days into the season was significant in explaining the percentage of male peepers captured across the season ( $\chi^2(2) = 28.98$ ,  $P < 0.001$ ). The percentage of 2-year-old males in the chorus decreased across the season while the percentage of 3- and 4-year-old males increased (Fig. 2B). There was no temporal autocorrelation in the model.

#### DISCUSSION

We sampled 126 calling males in a single wetland in Southeastern Ontario over the duration of the male calling season in the Spring Peeper. Across all nights collectively, most males in the chorus were estimated to be 2 years old; however, the dominant age class shifted from more 2-year-old males in early season to more 3- and 4-year-old males in later season. We found more than 65% of calling males were 2-year-old males which is concordant with the age structure of both calling and

non-calling males of one population sampled in Harrison, Maryland, USA (Lykens and Forester 1987). We found two 1-year-old male Spring Peepers in the chorus, although most males seem to start breeding at 2 years old (Lykens and Forester 1987). While it is possible that there is variation in the age of first calling or breeding among male Spring Peepers, this observation may also be attributable to technical artifacts in skeletochronology. The snout-urostyle lengths of the two “1-year-old” individuals were both greater than 22 mm (YC, unpubl. data), a body size at which male Spring Peepers attain sexually maturity (Dodd 2013). Nevertheless, sub-adult male Spring Peepers entering their second growing season tend to remain terrestrial while mature males are calling (Delzell 1958), suggesting that participation in choruses does not start until males are in their third growing season (i.e., 2 years old). Future studies should further assess variation in the age of first-time calling in Spring Peepers and other short-lived treefrogs.

We found that the age structure in the calling male Spring Peeper assemblage shifted from young 2-year-old male dominance to 3- and 4-year-old male dominance. This suggests that the timing of males joining the chorus might differ among age class and/or that chorus tenure of young male Spring Peepers is shorter than older males and more older males persist

calling at the end of the breeding season (Lucas et al. 1996). Although data on the timing variance in males joining chorus is scarce, Friedl and Klump (2005) observed a positive relationship between chorus tenure and age in *H. arborea*; 2-year-old males attended the chorus less often than older males in 1991 but not 1990 in southern Germany.

One possible reason for short chorus tenure of young males is that young male peepers remained at our focal wetland but adopted satellite behavior, as 10–20% of males have been estimated to do so in other populations of this species (Fellers 1975; Forester and Lykens 1986); this would not be reflected in our dataset as we sampled only calling males. However, Forester and Lykens (1986) found the number of satellite males strongly correlates with chorus size, presumably peaked in the mid-season, but never exceeds 14%. Lance and Wells (1993) found that satellite males were not present late in the breeding season. That is to say that the temporal shift of age structure that we found is probably not attributable to satellite behavior.

Energetic cost of calling is high in the Spring Peeper and thus energy constraints might be a cause of the observed shifting age structure and chorus tenure variation among age classes. About 90% of the energy for calling comes from lipids stored in trunk muscles and only 10% from glycogen (Wells and Bevier 1997). By the end of breeding season, as much as 75% of lipid reserves can be expended, accompanied by decreasing body mass (Wells and Bevier 1997). Most males in the early season have an empty stomach as breeding starts in early spring immediately after emergence when the temperature is still low and invertebrate food is relatively scarce (Lance and Wells 1993); thus, foraging that could supplement glycogen for calling has limited contribution to the energy pool compared to lipid stores (Wells and Bevier 1997). That means that the energy needed to produce calls in Spring Peepers mostly derives from energy stores from the previous year (also see a study on the Túngara Frog, *Engystomops pustulosus*, Green 1990). Further, energy expenditure increases with faster call rate under warmer temperatures, which means that there are higher hourly costs to calling towards the end of breeding season compared to the beginning (Taigen et al. 1996). With such high energetic expenses later in the season, one would expect that some younger and smaller peepers with lower initial lipid stores and faster energy depletion would be unable or more reluctant to produce calls later in the season (e.g., *Lithobates catesbeianus*: Judge and Brooks 2001; *Hyla arborea*: Brepson et al. 2013). To our knowledge, no data are available on inter-male variation in initial lipid restoration among age classes before breeding and rates of energy depletion in Spring Peepers.

Predation risk can also affect male chorus tenure (Green 1990). Compared to early in the season when nighttime air temperatures are typically lower than 10°C, predation risk in Spring Peeper populations at our latitude is probably higher later in the season when temperatures exceed 15°C and more predators are active, including Eastern Ribbonsnakes (*Thamnophis saurita*), Common Gartersnakes (*Thamnophis sirtalis*) and Northern Watersnakes (*Nerodia sipedon*; YC and SCL, pers. obs.). Predation during calling can be substantial. For example, about 20% of calling male Barking Treefrogs, *Dryophytes gratiosus*, were estimated to have been predated in one breeding season (Murphy 1994b). Green (1990) suggested that male Túngara Frogs (*Engystomops pustulosus*) chose nights to call based on environmental variables to reduce predation risk. Thus, in Spring Peepers, high predation risk might affect calling behavior especially in the later season. We think the

observed shifting age structure of male peepers in the season reflects distinct mating strategies adopted under different residual reproductive opportunities. For 2-year-old males with potentially one or even two more breeding seasons, adopting a conservative tactic by entering a chorus early and/or dropping out of a chorus later to enhance survival and future reproductive opportunities makes sense. In contrast, 3 and 4-year-old males with shorter residual life spans would benefit more from investing in present reproductive opportunities. Lucas et al. (1996) modelled male anuran mating tactics under the interacting influences of energetic states, female arrival dates, weather and predation conditions. The models reveal that, when weather conditions are continually favorable over a prolonged breeding season, first-year males are more likely to stop calling when energy consumption and predation risk are high, while second-year males tend to remain calling across the season regardless energy and predation due to lower residual reproductive value (Lucas et al. 1996). The resulting temporal age structure pattern predicted from those models is that the highest proportion of first year calling males will be at the beginning of the season with the proportion of second-year calling males increasing over time (Lucas et al. 1996). This predicted pattern is supported by our observations; moreover, the breeding ecology of the Spring Peeper fits their model assumptions well, including a prolonged breeding season (Harding 1997) and high energetic cost of calling (Wells and Bevier 1997). Hence, our data are consistent with the residual reproductive value hypothesis, although we cannot rule out the energetic constraint and predation risk hypotheses.

Higher mating success attributable to longer chorus tenure is probable in the Spring Peeper. Females arrive in sporadic waves throughout the breeding season (Lykens and Forester 1987). Sullivan and Hinshaw (1990) found that actual mating in Spring Peepers did not start until 7 days and 21 days after males began calling in two different years in Maine, USA. There is no apparent size difference between amplexic and non-amplectic males in the Spring Peeper (Forester and Czarowsky 1985; Sullivan and Hinshaw 1990) suggesting no sexual selection on male body size among males. Rather we posit that ability to stay in the chorus is the focus of selection. For example, endurance rivalry through chorus tenure plays a more important role in sexual selection than female choice towards advertisement calls in Italian Treefrogs (Castellano et al. 2009). With uncertain dates of arrival of females, increasing the number of calling nights would lead to higher probability of mating. We thus assume such a relationship would exist in the population that we studied, and further hypothesize that older males have a higher reproductive success because of longer durations of chorus tenure. If this is true, the important evolutionary insight is that selection would act on male survivorship because females would have increased probability of mating with males who have longer chorus tenure and thus who are more likely to be older and/or have traits that enhance survivorship (discussed in Friedl and Klump 2005).

In conclusion, 2-year-old young males numerically dominated the Spring Peeper chorus that we sampled, but young males dropped out of the chorus as the breeding season progressed and the age structure of calling males shifted towards more 3- and 4-year-old in later season. The possible causes for this pattern include energetic constraints limiting ability to call over many nights, predation risks affecting propensity to call, and different mating strategies reflecting life-history trade-offs between current and future mating opportunities. Future studies should incorporate mating success data such

as observations comparing chorus tenure between amplexic males and non-amplectic males (assuming amplexus leads to successful fertilization) to test the hypothesis that mating success correlates with chorus tenure in the Spring Peeper and that older males have greater mating success.

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