

## Article

# Does sexual dimorphism vary by population? Laryngeal and ear anatomy in cricket frogs

Blinda E. McCLELLAND<sup>a,\*</sup>, Michael J. RYAN<sup>a</sup>, and Walter WILCZYNSKI<sup>b</sup>

<sup>a</sup>Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA and

<sup>b</sup>Neuroscience Institute and Center for Behavioral Neuroscience, Georgia State University, Atlanta, GA 30303, USA

\*Address correspondence to Blinda E. McClelland. E-mail: mcclelland@austin.utexas.edu.

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## Abstract

Acoustic communication in many anuran species can show the effects of both natural and sexual selection. This is reflected in the sexually dimorphic anatomy of the larynx and ear structures, as well as the allometric relationship of these morphological traits to head or body size. In this study, we examined laryngeal and ear structures of cricket frogs *Acris crepitans* not only as sexually dimorphic characteristics, but also as they differ across populations in environmentally different habitats. We used 2-way ANOVA to determine whether the volumetric or linear measurements of these structures differed by sex and population. Females have significantly larger body, head, and ear sizes, but significantly smaller larynges than males. Furthermore, females as well as males show larger body and head sizes, ears, and larynges in a dryer open habitat. An ANCOVA analysis shows that males, but not females, differ in laryngeal size across populations beyond the allometric changes attributable to head size alone indicating that males have a greater degree of laryngeal population variation. In contrast, our covariate analysis found that in both sexes many of the ear differences are non-significant once head size is accounted for, suggesting that most of the population-level ear variation is due to allometric effects of body size. We conclude that although both sexes show size differences in the larynx related to selection for larger body size in dry, open habitats, selection on males for larger larynx size related to the production of lower frequency calls in those habitats does not result in correlated changes in the female larynx. The results suggest that in anurans, selection for changes in body and head size affects both sexes equally, male calls and the vocal structures responsible for them can further diversify without concordant changes in females.

**Key words:** acoustic communication, allometry, amphibians, population variation, sex differences.

The operation of natural and sexual selection can result in variation in behavioral, physiological, and morphological characteristics across populations and between sexes (Andersson 1994). Males and females may be subject to similar selection based on environmental characteristics but, at the same time, subject to very different sexual selection pressures that result in sexually dimorphic characteristics. This is particularly true when there is sexual selection on males driven by female mate choice. This is often seen when examining

aspects of animal communication that are essential for reproductive success (Bradbury and Vehrenkamp 2011). In that case, males and females might differ dramatically in structures for producing mate attraction signals in addition to any impact of natural selection that might influence these traits in both sexes. Anuran acoustic communication has been extensively studied as a model for understanding both the mechanisms of acoustic communication and the evolution of this type of social behavior (Fritzsche et al. 1988; Ryan 2001;

Gerhardt and Huber 2002). Anuran reproduction depends largely, if not exclusively, on the production and reception of vocal signals. Acoustic communication signals transmit both species- and sex-specific messages, are commonly produced by males, and females can use characteristics of the vocalization, such as dominant frequency, to make mate choice decisions (Ryan 1985; Gerhardt and Huber 2002; Wells and Schwartz 2007). In many species only the males produce loud advertisement calls as part of their reproductive social behavior, although there are many anuran species in which females vocalize. Female vocalizations can range from limited repertoires with simplified acoustic properties, to vocalizations that are as extensive or complex as in their male conspecifics (Tobias et al. 1988; Emerson and Boyd 1999; Bosch 2002; Preininger et al. 2016; Zhang et al. 2017; Zornik and Kelley 2017; Serrano and Penna 2018). This often results in gross sexual dimorphism in the size and structure of the larynx (McClelland and Wilczynski 1989; Wilczynski et al. 1993; McClelland et al. 1997; Zornik and Kelley 2017) which varies depending on the degree of female calling (Preininger et al. 2016). Female choice for particular call characteristics such as low frequencies can further impact male laryngeal evolution based on the sexual selection this causes (Ryan and Drewes 1990; Gridi-Papp et al. 2006). In addition, male vocalizations in anurans and many vertebrate groups evolve for optimal transmission in various habitats, which can result in further structural changes in the male larynx (McClelland et al. 1998). As a result, variation in male laryngeal structure should and can be seen among species (Wilczynski et al. 1993; McClelland et al. 1997; Ryan and Guerra 2014) and across populations of species in different habitats (McClelland et al. 1998), as well as between sexes (McClelland and Wilczynski 1989; McClelland et al. 1997; Zornik and Kelley 2017).

Larynges, however, are found in both males and females as these structures are necessary for the “pulse-pump” respiratory system characteristic of anurans (De Jongh and Gans 1969; West and Jones 1975; Kogo et al. 1997). Thus, both males and females share the correlated trait of having a larynx and using it for respiration even in those species where larynges are necessary for the production of acoustic signals only in males but not females. This presents an opportunity for investigating what happens to traits or structures that exist in both sexes, but are subject to sexual selection pressures in only one sex.

Although the production of vocal signals is heavily male-biased across anuran species, the reception of those signals guides behavior in both sexes. The reception of conspecific calls triggers a variety of behavioral responses in both sexes, reflecting the differing functions of vocalizations: Males variously use acoustic signals to establish call sites, produce calls in competition with other males, and/or to express aggression and possibly competition-related behaviors, while females are using the acoustic signals to find and choose mates (Wells 1977, 2007; Gerhardt and Huber 2002). Despite the reception of acoustic signals in both sexes, sexual dimorphisms in tuning have been documented in the responses of anuran auditory systems (reviewed in Wilczynski and Burmeister 2016). Some of these differences might be indirect results of sex and population size differences. In anurans, the diameter of the tympanic membrane and the volumes of the middle and inner ear can affect the frequencies of sound that reach the amphibian and basilar papillae, which are the functionally homologous structures to the cochlea in mammals (Wilczynski and Capranica 1984; Feng et al. 1990; Simmons et al. 2007), and therefore allometric changes in those structures could generate tuning differences (Keddy-Hector et al. 1992; Wilczynski et al. 1993; Hetherington 1994).

We examined the relationship of sex and population differences in larynx and ear structure in cricket frogs *Acris crepitans*. Cricket frogs are distributed throughout much of the United States east of the Rockies. In the populations, we have studied males gather in choruses in extended breeding seasons that last from April to September in Central Texas, depending on rainfall. Males produce advertisement calls that consist of click-trains with characteristic inter-click intervals that vary systematically from beginning to end of the call, and across calls that are produced in strings of variable length that lengthen as the frogs engage in agonistic competition with other males around their call site in a “graded” communication system in which males gradually vary their advertisement calls to reflect their level of aggression (Wagner 1989a, 1989b; Perrill and Shepherd 1989; Ryan et al. 1992; Burmeister et al. 2002; Venator et al. 2017). Females respond to the male calls by approaching them and using them as a mate attractant (Ryan and Wilczynski 1988; Ryan et al. 1992; Witte et al. 2000). However, they produce no vocalizations of their own, and their lack of vocal production extends to the absence of distress and release calls, neither of which have been reported in the literature or observed by us working on multiple populations of this species.

We previously demonstrated population variation in call characteristics through an analysis of male cricket frogs across multiple populations in an east–west transect through Texas (Ryan and Wilczynski 1991; Ryan et al. 1992). This investigation documented call variation and body morphology in 17 populations distributed from the Texas–Louisiana border to West Texas and included habitats ranging from wet forested regions of East Texas, grasslands and isolated pine forest habitats of Central Texas, and dry rangeland areas of West Texas. Clinal variation was supplemented with a significant effect of habitat type, which influences acoustic transmission characteristics (Ryan and Wilczynski 1991). Subsequent analysis of 8 populations within this sample, representing different habitats, found that morphological structures in male cricket frog larynges correlate with acoustic characteristics of vocalizations both within and among populations (McClelland et al. 1996, 1998). We also found that gross anatomical features, such as head and body size, vary by habitat with larger male frogs found in drier habitats, presumably to reduce the probability of dehydration due to smaller surface-to-volume ratios (McClelland et al. 1998). Further, we have shown that male cricket frog laryngeal size and ear size varies by population in response to body size difference plus an additional factor related to population differences in the frequency and temporal characteristics of that population’s calls (McClelland et al. 1998).

The question then becomes: What happens to the anatomy of female larynges and ears in a species that shows male laryngeal size variation due to both habitat effects on body size and female mate choice on the call? In other words, can we tease apart and demonstrate effects that are the result of natural selection from those that result from sexual selection? We addressed this question by statistically analyzing the volumetric measurements of male and female laryngeal components and ear structures from cricket frog populations occupying 3 different habitats in which we previously examined male morphology. The 3 sites we sampled differ ecologically: Stengl Ranch is located in an East Texas pine forest habitat, Gill Ranch is an open grassland habitat, and Wimberley is a grassy habitat interspersed with deciduous vegetation. We had previously shown that male advertisement vocalizations and body size differ significantly among these populations with Gill Ranch males larger and having lower frequency calls (Ryan and Wilczynski 1988, 1991; McClelland et al. 1998). Furthermore, there was sufficient

population diversity in male laryngeal and ear structures of males (McClelland et al. 1996, 1998) against which to compare these structures to those in females. We used this analysis to test between 2 competing hypotheses. The first is that female laryngeal size shows a strictly allometric relationship with body size and does not reflect the additional population-level variation seen in males. That is, although males and females both have a larynx, any selection leading to population-level trait variation in males is independent of any change in females. The alternative hypothesis is that variation in female laryngeal size across populations reflects the same pattern seen in males, variation due to habitat-related body size differences plus variation related to sexual selection on the male call. That is, the female larynx is essentially evolving in response to indirect selection on the male larynx as it changes across populations, similar to the “between sex genetic correlation” hypothesis that has been proposed to explain the evolution of multiple mating in females (Halliday and Arnold 1987; Forstmeier et al. 2011) and the evolution of vocal behavior in some frogs (Emerson and Boyd 1999).

## Materials and Methods

### Morphological analysis

We examined 24 male and 39 female cricket frogs collected from 3 sites located, from east to west, at Stengl Ranch (97.1 longitude; 10M, 14F), Gill Ranch (98.08 longitude, 13M, 13F), and near Wimberley (98.1 longitude, 10M, 12F) close to a transect at  $30.5 \pm 0.05^\circ$  latitude across central Texas, USA. Males had been collected as part of a larger survey of population variation in cricket frog morphology and call characteristics (Ryan and Wilczynski 1991). Male individuals from the populations analyzed here are collected between May and September in 1988 and 1989; females from these same populations were collected in July 1991. In both cases, animals were stored in 10% formalin for variable time periods ranging from 3 to 6 months, then dissected, decalcified, sectioned, and stained as described below. Measurements for this study were done on the resultant slides. Larynx and ear data from the males were reported in McClelland et al. (1998) as part of a description of clinal variation in calls, larynx, and ear morphology, and used in McClelland et al. (1996) to assess correlations between call characteristics and laryngeal structure.

Upon capture, the animals were sacrificed via overdose in an aqueous solution of 2.5% tricaine methanesulfonate (MS-222). We measured snout–vent length (SVL) and head width with digital calipers. We then removed the head and throat of each animal, removing as much skin as possible and placed the heads in a decalcifying agent (Cal-Ex, Fisher Diagnostics) for 24–36 h, after which we dehydrated the specimens in a series of ethyl alcohols and xylenes before embedding them in paraffin. We sectioned the heads on a rotary microtome at  $25 \mu\text{m}$  through the ear and laryngeal region and mounted serial sections on gelatin-subbed slides. We stained the sections using Pollak’s trichrome (Humason 1972) and Cresyl Violet to differentiate muscles and cartilage. We processed the tissues in batches from randomly selected individuals to avoid the potentially confounding problem of inter-batch variation in the effects of dehydration and embedding procedures on the morphological measurements.

To measure the volumes of laryngeal and ear structures, we projected an image from every 10th section through the area of interest onto a PC-interfaced Summagraphic 2201 digitizing pad and captured traced outlines of each structure using Sigma-scan (version 3.0, Jandel Scientific) graphics software to yield traced area

measurements at intervals of  $250 \mu\text{m}$ . We measured volumes of the following larynx structures: laryngeal constrictor muscles, laryngeal dilator muscles, and arytenoid cartilages. Sizes of male vocal cords and the basal cartilage of the arytenoid are reported in McClelland et al. (1998). We did not include them here as females lack both. We also measured the following ear structures: middle and inner ear cavities, the extracolumella cartilages, and the tympanic membranes. The columella was nearly always fragmented or otherwise compromised in our sections so we did not include it in our measurements. Whenever possible structures were measured on both sides of the head and compared. A previous analysis (Ryan et al. 1995) of males of this species, including those from the populations analyzed here, found that neither larynx nor ear structures demonstrated fixed asymmetries, although fluctuating asymmetries ranging from 2% to 18% were seen across measured structures. The asymmetries did not correlate with call parameters. The values in the specimens measured here were consistently within 10% of each other. We therefore averaged the resulting values for each side to yield 1 value for each structure per animal. We calculated the volumes ( $V_{\text{total}}$ ) of each anatomical structure from the area measurements of the serial sections using the formula:

$$V_{\text{total}} = \sum V_{\text{slice}} = \sum_{i=0}^{n+1} T \frac{\sqrt{(A_i)(A_{i+1}) + A_i + A_{i+1}}}{3},$$

where  $i$  is a running index of the order of the measured slices,  $i = 1$  is the first section with measureable area,  $n$  is the last section with measureable area,  $A_i$  is the area of a structure (in  $\text{mm}^2$ ) in 1 section,  $A_{i+1}$  is the area of the structure in the next measured section, and  $T$  is the distance between the sections (i.e.,  $250 \mu\text{m}$ ). This formula for the volume of a frustum ( $V_{\text{slice}}$ ) does not rely on the assumption that the area measurements for each slice are equal in order to calculate the volume accurately. We also measured the diameter of the tympanic membrane using a linear setting of the Sigma-scan program. As all morphological measurements except for SVL and Head Width were made on decalcified, dehydrated, paraffin-embedded specimens, we expect considerable shrinkage. We did not correct for this, however, we assume that the amount of shrinkage would be equal in all specimens for any morphological character. We nevertheless note that the values obtained and reported here represent the measured values obtained from the paraffin sections and not the values in living animals, which we assume to be much greater. All procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

### Statistical analyses

We used GraphPad Prism (version 7.03) to obtain descriptive statistics and correlations among all measured variables. We calculated Spearman correlation coefficients for all pair-wise comparisons among all variables. Correlations were calculated separately for males and females.

We used SPSS for all other statistical analyses. We performed our statistical analyses on population and sex differences in stages. First, we tested for site and sex differences in SVL and head width using separate 2-way analysis of variance (ANOVA) to determine if sex differences in measures of head and body size were present and consistent across populations. Second, we tested whether there were population and sex differences in the volumes of laryngeal structures (constrictor muscle, dilator muscle, and arytenoid cartilage) using separate 2-way ANOVAs for each structure. Third, we performed separate 2-way ANOVAs for ear structures to test whether there

were population and sex differences in the volumes of the middle and inner ear cavities and extra-columella and in the diameter of the tympanic membrane. Because the 2-way ANOVAs indicated significant interactions likely due to differences in the degree of population differences in males and females, we performed separate follow-up 1-way ANOVAs for each sex for each laryngeal and ear structure to test whether the significant effects of site found in the 2-way ANOVAs were present when data from only 1 sex were analyzed. Finally, to test whether population differences in male and female larynx and ear structures were due to population differences in the sizes of males and females, we performed separate ANCOVAs with head width as the covariate for each larynx and ear structure. We elected to use head width for the covariate analysis under the assumption that population variation in head size would be a more meaningful driver of structures in or near the head such as the ear and larynx. ANCOVAs were performed separately in males and females to determine if (1) population differences in male larynx or ear remained once head width was removed from the analysis and (2) population differences in female larynx or ear remained once head width was removed from the analysis.

## Results

Size data for body/head, larynx structures, and ear structures for each site and sex are presented in Table 1. Spearman  $r$ -values for pairwise comparisons across all variables for males and females are presented in Table 2. The correlation analysis shows that all morphological variables are significantly inter-correlated in both sexes.

A 2-way ANOVA indicated that there were significant sex differences and population differences in both measures of body size, snout-vent length (SVL), and head width. For SVL, there was a significant difference among populations ( $F = 17.73$ ,  $P < 0.0001$ ) and between sexes ( $F = 55.64$ ,  $P < 0.001$ ), with no significant sex by population interaction ( $F = 1.80$ ,  $P = 0.173$ ). Similarly, head width differed significantly among populations ( $F = 10.82$ ,  $P < 0.0001$ ) and between sexes ( $F = 34.05$ ,  $P < 0.0001$ ) with no sex by population interaction ( $F = 0.39$ ,  $P = 0.678$ ). In all populations, females were larger than males. As seen in Figure 1, changes across populations were largely parallel in males and females, and more consistent for head width. Tukey HSD *post hoc* tests indicated that the population size differences were driven by Gill Ranch individuals being larger than both the Stengl (head width: mean difference = 0.059, SE 0.021,  $P = 0.020$ ) and Wimberly (head width: mean

difference = 0.093, SE 0.022,  $P < 0.0001$ ) individuals, which were not significantly different from each other (mean difference = 0.35, SE 0.022, head width:  $P = 0.27$ ). The male size difference in these 3 populations were reported previously (Ryan and Wilczynski 1991; McClelland et al. 1996, 1998). Our new data show that the pattern is identical for females, with Gill Ranch females being larger in head width and body length than females from the other populations.

Inspection of laryngeal sections revealed that female larynges in all populations were qualitatively different than male larynges. Female larynges were much smaller and lacked both vocal folds and basal arytenoid cartilages. We therefore restricted our statistical comparisons to constrictor and dilator muscles and the arytenoid cartilages.

Two-way ANOVA showed that there were significant site differences and sex differences in constrictor muscle volume (site:  $F = 16.28$ ; sex:  $F = 219.71$ , both  $P < 0.0001$ ), dilator muscle volume (site:  $F = 10.61$ ; sex:  $F = 131.30$ , both  $P < 0.001$ ), and arytenoid cartilage volume (site:  $F = 16.39$ ; sex:  $F = 483.11$ , both  $P < 0.001$ ), with significant sex by site interactions (constrictor muscles:  $F = 11.79$ ,  $P < 0.0001$ ; dilator muscles:  $F = 5.82$ ;  $P = 0.005$ ; arytenoids:  $F = 12.24$ ,  $P < 0.0001$ ). The sex differences reflect the substantially larger size of all laryngeal components in males (Table 1 and Figure 2). *Post hoc* analysis indicated that the site difference was driven by the large laryngeal size in the Gill Ranch population compared with the Stengl Ranch and Wimberly populations, which were not significantly different from each other. The significant interaction reflects the larger population differences seen in males than in females (Figure 2). Because the size variation across sites appeared different in males and females, separate 1-way ANOVAs for each sex were performed for each larynx structure. As suggested by inspection of the 2-way ANOVA results, all 3 tested larynx structures were significantly different across populations in males (constrictors:  $F = 12.63$ ,  $P < 0.0001$ ; dilators:  $F = 7.29$ ,  $P = 0.003$ ; arytenoids:  $F = 12.69$ ,  $P < 0.0001$ ), with *post hoc* analysis indicating that the difference was due to the large size of the Gill Ranch male larynx compared with the Stengl Ranch and Wimberly males. For females, dilator muscle ( $F = 5.30$ ,  $P = 0.01$ ) and arytenoid cartilage ( $F = 3.89$ ,  $P = 0.03$ ) volumes were significantly different across sites, whereas constrictor muscle volumes did not reach significance ( $F = 2.82$ ,  $P = 0.073$ ).

Male and female ears did not appear qualitatively different, but quantitative statistical analysis of the ear data via 2-way ANOVA revealed a complicated pattern of group differences. As for the

**Table 1.** Mean (SD) for all morphological measures in males and females from 3 populations (Gill Ranch, Stengl Ranch, and Wimberly)

	Gill Ranch		Stengl Ranch		Wimberly	
	Male	Female	Male	Female	Male	Female
SVL	2.295 (0.095)	2.853 (0.133)	2.108 (0.160)	2.473 (0.216)	2.031 (0.213)	2.350 (0.429)
HEAD	0.758 (0.039)	0.850 (0.049)	0.671 (0.077)	0.798 (0.092)	0.659 (0.071)	0.753 (0.102)
CONSTMUS	0.499 (0.115)	0.079 (0.016)	0.329 (0.132)	0.065 (0.022)	0.253 (0.117)	0.059 (0.025)
DILAMUS	0.176 (0.049)	0.038 (0.009)	0.124 (0.060)	0.028 (0.008)	0.092 (0.052)	0.026 (0.013)
ARYTCART	0.619 (0.087)	0.060 (0.014)	0.478 (0.148)	0.045 (0.018)	0.362 (0.135)	0.042 (0.020)
MIDEAR	0.264 (0.064)	0.488 (0.054)	0.202 (0.057)	0.301 (0.18)	0.170 (0.060)	0.309 (0.177)
INNEAR	1.09 (0.197)	1.409 (0.122)	1.040 (0.227)	1.21 (0.18)	0.937 (0.213)	1.160 (0.014)
EXTRACOL	0.019 (0.008)	0.035 (0.001)	0.016 (0.004)	0.017 (0.006)	0.009 (0.007)	0.0201 (0.014)
TYMPDIAM	1.102 (0.083)	1.406 (0.093)	1.041 (0.101)	1.25 (0.11)	0.900 (0.117)	1.093 (0.265)

Notes: SVL, snout-vent length (cm); HEAD, head width (cm); CONSTMUS, constrictor muscle volume ( $\text{mm}^3$ ); DILAMUS, dilator muscle volume ( $\text{mm}^3$ ); arytenoid cartilage volume ( $\text{mm}^3$ ); MIDEAR, middle ear volume ( $\text{mm}^3$ ); INNEAR, inner ear volume ( $\text{mm}^3$ ); EXTRACOL, extracolumella volume ( $\text{mm}^3$ ); TYMPDIAM, tympanic membrane diameter (mm).



**Table 2.** Spearman *r*-values for pairwise correlations for body measurements, larynx, and ear structures

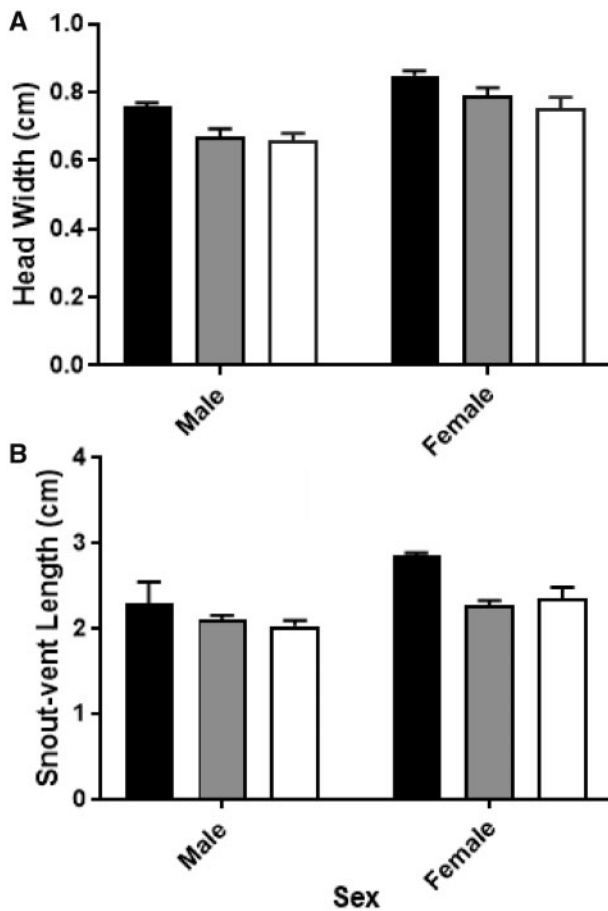
	SVL	HEAD	ARYTCART	CONSTMUS	DILAMUS	MIDEAR	INNEAR	EXTRACOL	TYMPDIAM
Males									
SVL		0.728769	0.803079	0.75376544	0.7818596	0.776886	0.54325	0.56996124	0.67935535
HEAD	0.728769		0.5102905	0.49332964	0.5050438	0.49851	0.337566	0.36063758	0.53674746
ARYTCART	0.803079	0.51029		0.84890524	0.8080234	0.731783	0.605242	0.71987011	0.5819135
CONSTMUS	0.753765	0.49333	0.8489052		0.9504305	0.75943	0.674733	0.68709869	0.63456691
DILAMUS	0.78186	0.505044	0.8080234	0.9504305		0.746495	0.649995	0.67705176	0.63614362
MIDEAR	0.776886	0.49851	0.7317833	0.75943029	0.7464946		0.55379	0.52398388	0.69351265
INNEAR	0.54325	0.337566	0.6052419	0.67473282	0.649995	0.55379		0.57027088	0.38966314
EXTRACOL	0.569961	0.360638	0.7198701	0.68709869	0.6770518	0.523984	0.570271		0.45881115
TYMPDIAM	0.679355	0.536747	0.5819135	0.63456691	0.6361436	0.693513	0.389663	0.45881115	
Females									
SVL		0.69084	0.719566	0.67247846	0.7454482	0.85867	0.797387	0.78148603	0.75134273
HEAD	0.69084		0.7178526	0.67356598	0.5885279	0.64796	0.67728	0.57120374	0.54984337
ARYTCART	0.719566	0.717853		0.85328269	0.7584213	0.67181	0.669387	0.56046989	0.67768769
CONSTMUS	0.672478	0.673566	0.8532827		0.7992293	0.615983	0.646772	0.47245468	0.61104857
DILAMUS	0.745448	0.588528	0.7584213	0.79922934		0.741496	0.651398	0.55980999	0.723041
MIDEAR	0.85867	0.64796	0.6718105	0.6159826	0.7414958		0.750752	0.86370592	0.78727283
INNEAR	0.797387	0.67728	0.6693874	0.64677233	0.6513979	0.750752		0.66339545	0.61252191
EXTRACOL	0.781486	0.571204	0.5604699	0.47245468	0.55981	0.863706	0.663395		0.70011356
TYMPDIAM	0.751343	0.549843	0.6776877	0.61104857	0.723041	0.787273	0.612522	0.70011356	

Notes: All values are significant at  $P < 0.05$ . SVL, snout-vent length (cm); HEAD, head width (cm); CONSTMUS, constrictor muscle volume ( $\text{mm}^3$ ); DILAMUS, dilator muscle volume ( $\text{mm}^3$ ); arytoid cartilage volume ( $\text{mm}^3$ ); MIDEAR, middle ear volume ( $\text{mm}^3$ ); INNEAR, inner ear volume ( $\text{mm}^3$ ); EXTRACOL, extracolumella volume ( $\text{mm}^3$ ); TYMPDIAM, tympanic membrane diameter (mm).

larynx structures, a sex difference was apparent (Table 1 and Figure 3), but with all ear structures being significantly larger in females than in males (middle ear:  $F = 43.16$ ; inner ear:  $F = 22.27$ ; extracolumella:  $F = 21.95$ ; tympanic membrane:  $F = 47.88$ ; all  $P < 0.0001$ ). For middle ear ( $F = 3.32$ ,  $P = 0.042$ ) and extracolumella ( $F = 3.98$ ,  $P = 0.23$ ) there was a significant site by sex interaction. In addition, all structures were significantly different across sites (middle ear:  $F = 16.95$ ,  $P < 0.0001$ ; inner ear:  $F = 5.414$ ,  $P < 0.007$ ; extracolumella:  $F = 12.95$ ,  $P < 0.0001$ ; tympanic membrane:  $F = 19.26$ ,  $P < 0.001$ ). Gill Ranch individuals had the largest ear structures, but unlike the laryngeal structures Gill Ranch means were not always significantly different from both Stengl Ranch and Wimberly means, and Stengl and Wimberly means were significantly different for some structures. For middle ear volume ( $P < 0.0001$  for both site comparisons) and extracolumella volume (Gill vs. Stengl:  $P = 0.001$ ; Gill vs. Wimberly:  $P < 0.0001$ ), Gill Ranch animals were significantly larger than Stengl Ranch and Wimberly animals, and Stengl Ranch and Wimberly means were not significantly different (middle ear:  $P = 0.99$ ; extracolumella:  $P = 0.88$ ). For inner ear volume, however, the Gill Ranch population was significantly different from the Wimberly population ( $P = 0.14$ ), whereas the Stengl Ranch population was intermediate in size and not significantly different from either (vs. Gill:  $P = 0.15$ ; vs. Wimberly:  $P = 0.52$ ). Tympanic membrane diameter was significantly different between all populations with Gill Ranch animals having the largest and Wimberly animals having the smallest diameter (Gill vs. Stengl:  $P = 0.022$ , Gill vs. Wimberly:  $P < 0.0001$ ; Stengl vs. Wimberly:  $P = 0.006$ ). There was not a significant site by sex interaction for inner ear volume or tympanic membrane diameter. However, for middle ear ( $F = 3.32$ ,  $P = 0.042$ ) and extracolumella ( $F = 3.98$ ,  $P = 0.023$ ), there was a significant site by sex interaction.

Given the different degree of sex dimorphism in some ear traits indicated by the 2 significant interactions, we performed follow-up 1-way ANOVAs for each sex separately for each ear measure. Males showed significant population differences for middle ear volume ( $F = 6.96$ ,  $P = 0.003$ ), extracolumella volume ( $F = 5.64$ ,  $P = 0.008$ ), and tympanic membrane diameter ( $F = 11.99$ ,  $P < 0.001$ ), but not inner ear volume ( $F = 1.47$ ,  $P = 0.245$ ). Females differed significantly in inner ear volume ( $F = 11.10$ ,  $P < 0.0001$ ), extracolumella volume ( $F = 4.70$ ,  $P = 0.015$ ), and tympanic membrane diameter ( $F = 10.11$ ,  $P < 0.0001$ ), but not middle ear volume ( $F = 1.94$ ,  $P = 0.158$ ).

Both head width and SVL differed across populations and between sexes, and the 2 measures of animal size were significantly correlated in both males (Spearman  $r = 0.729$ ) and females (Spearman  $r = 0.691$ ). Furthermore, both measures correlated significantly with all larynx and ear structures in both sexes (Table 2). We elected to use head width for the covariate as we felt that its value could be more accurately and consistently measured in the formalin fixed, preserved specimens, and because head size and shape might reflect population-based, overall body size changes (as the head-width vs. body size correlations show to be true) as well as any other differential selection related to the habitats such as feeding strategies, or availability of narrow refuges. We are not aware of such environmental differences, but cannot reject them. Using head width as the covariate, we performed ANCOVA on all larynx and ear structures separately for males and females to determine if the significant site differences remained for either sex once size was accounted for. Male population differences in larynx component volumes remained significantly different for constrictor muscle volume ( $F = 6.10$ ,  $P = 0.006$ ) and arytoid cartilage volume ( $F = 6.25$ ,  $P = 0.006$ ), but dilator muscle volume differences did not reach significance ( $F = 2.63$ ,  $P = 0.089$ ). None of the female laryngeal



**Figure 1.** Head and body size means ( $\pm$ SEM) for males and females in 3 cricket frog populations. Black bars, Gill Ranch; gray bars, Stengl Ranch; and white bars, Wimberley. (A) Headwidth; (B) snout-vent length. Site and sex were significantly different (2-way ANOVA) with no significant interaction in both cases. See text for details of *post hoc* tests.

measures remained significantly different across populations after using head size as a covariate (constrictors:  $F=0.19$ ,  $P=0.826$ ; dilators:  $F=1.92$ ,  $P=0.16$ ; arytenoids:  $F=1.08$ ,  $P=0.35$ ). Population differences in male ear measures were no longer significantly different when using head width as the covariate with the exception of tympanic membrane diameter which remained significantly different across populations ( $F=5.73$ ,  $P=0.008$ ). In females, population differences in inner ear volume ( $F=8.92$ ,  $P=0.001$ ) and tympanic membrane diameter ( $F=7.06$ ,  $P=0.003$ ) remained significant after using head width as a covariate, but middle ear volume and extracolumella volume did not.

## Discussion

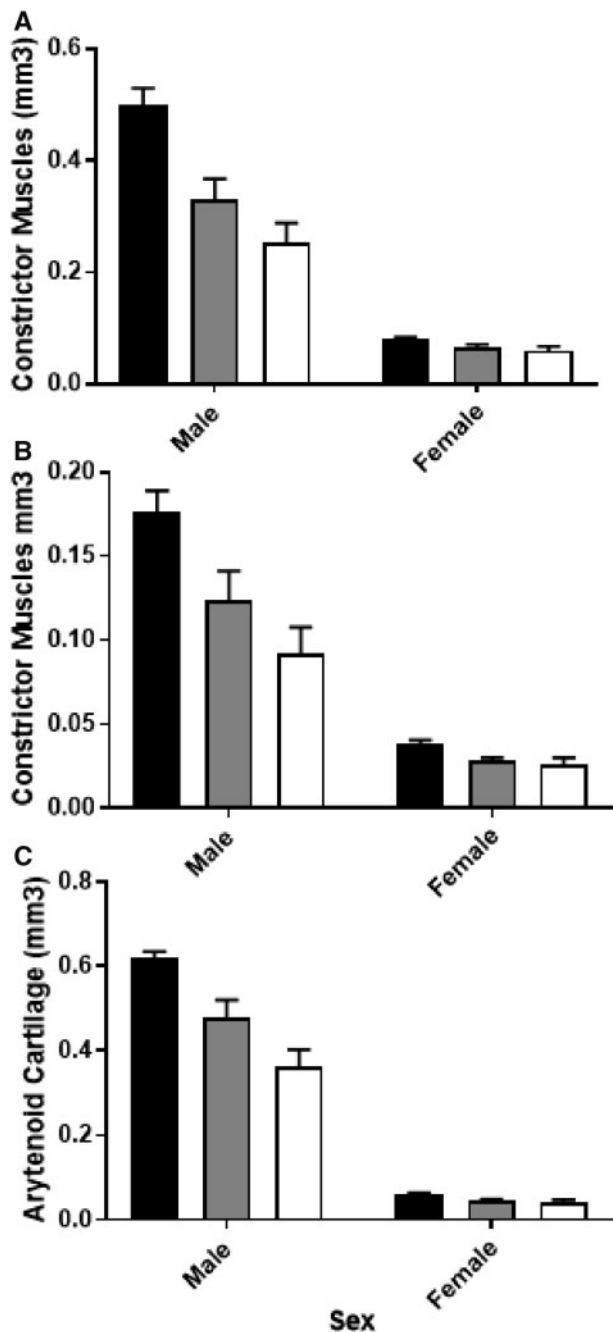
Our study of population and sex differences in cricket frog laryngeal structures allowed us to distinguish between 2 competing hypotheses about the evolution of correlated traits underlying communication in this species. One is that selection on the male larynx leading to different calls in different habitats moves the female larynx to diversify as well even though females do not use it to vocalize; the other is that selection on the males results only in male larynx changes such that the female larynx reflects only the shared allometric changes related to overall body size. Our results show that the

second hypothesis is true: although both males and females change body, head, larynx, and ear sizes across populations in a similar fashion, only the male larynx components reflect additional changes related to differences in calls among populations.

We find that there are significant sex differences in cricket frogs in each of 3 categories of measurements made in this study, with females having larger head sizes and body lengths while having larger ears but smaller larynges than males. Our previous paper (McClelland et al. 1998) described significant variation in body length, head width, and the sizes of multiple larynx and ear morphological characteristics in male cricket frogs across multiple populations, including the 3 reported in this paper, spanning an east-west transect across Texas and encompassing a range of habitats. Our new data find that females also manifest population differences in all 3 domains in the same way seen in males, with larger body and head sizes, larger ears, and larger larynges in the dryer open habitat typical of the Gill Ranch site, compared with the wetter or more densely vegetated habitats in Wimberley and Stengl Ranch. In both sexes, population variation in head and body size correlates with population changes in ear and larynx size: larger animals, whether male or female, have larger laryngeal and ear structures, but males show changes in larynx size beyond the allometric changes attributable to head size alone. Females do not show this same pattern of allometric changes, which results in a greater degree of laryngeal population variation in males than in females. We (Ryan and Wilczynski 1991; McClelland et al. 1998) previously suggested that male cricket frog calls vary across habitats beyond the simple effects of body size due to selection for call characteristics to match habitat acoustics, and that this is reflected in laryngeal changes independently of those predicted by body size differences alone. Females, which do not call, are not subjected to this same selection pressure. Our new data find that as a result female larynges vary only with the size of the animals. This results in laryngeal muscle and cartilage size differences across populations in both sexes due to body size variation, but female larynges have not responded pleiotropically in the same way as males that respond to the additional mate selection pressures demonstrated by a larynx size increase above that expected from body and head size changes alone.

It is routinely found in anurans that females are, on average, larger than males (Duellman and Trueb 1994). We find that this sexual size difference is maintained in all 3 populations investigated. Furthermore, both male and female cricket frogs from the dryer, more open habitat of Gill Ranch are larger than those from the wetter, more densely vegetated Stengl Ranch and Wimberley habitats (reported for males in McClelland et al. 1998). This habitat-based male variation in body length and head size is a pattern previously seen in frogs (Nevo 1973; Nevo and Capranica 1985). Here, we report that the habitat-based body size differences are apparent for females as well. As for the males, the Gill Ranch females are larger than the females from the Stengl Ranch and Wimberley populations. The lack of a statistically significant site-by-sex interaction indicates that the trend in body and head size is the same in both sexes.

Despite having a smaller body size, males have substantially larger larynges than females. This laryngeal sex difference has been reported in many other anuran species, as has the absence of vocal folds and other qualitative dimorphic differences (McClelland and Wilczynski 1989; Ryan and Drewes 1990; Wilczynski et al. 1993; McClelland et al. 1997; Ryan and Guerra 2014; Zornik and Kelley 2017). This structural dimorphism reflects the great behavioral sex difference in most anurans. Although there are many anuran species in which females vocalize (Preininger et al. 2016), in most only the



**Figure 2.** Mean ( $\pm$ SEM) larynx structure volumes for males and females in 3 cricket frog populations. Black bars, Gill Ranch; light gray bars, Stengl Ranch; and white bars, Wimberley. (A) Constrictor muscle volume; (B) dilator muscle volume; (C) arytenoid cartilage volume. Site and sex were significantly different (2-way ANOVA) in each case. See text for details of interactions and *post hoc* tests.

males produce loud advertisement calls as part of their reproductive social behavior. Females have greatly reduced vocal behavior to the point of being mute in many species, including cricket frogs.

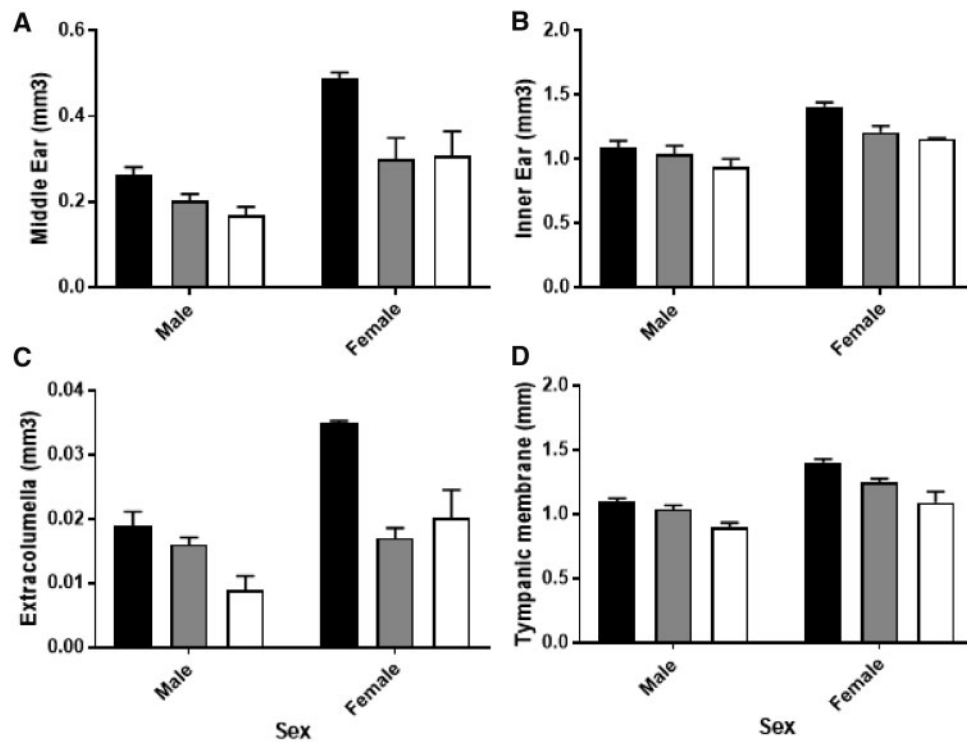
Both males and females show significant population differences in the size of laryngeal structures. Populations with larger mean head and body sizes have larger laryngeal structures in both sexes, with only female constrictor muscle volume differences not reaching significance across populations. Because in both sexes body and head

sizes correlate with the volumes of all larynx components, which in turn are all significantly inter-correlated, we conclude that overall animal size allometrically affects larynx size in both sexes. Our data here, however, show that males manifest larger larynx variation across the populations than females despite both sexes showing similar population variation in head width and body length. This is apparent in the graphed data (Figure 2) and confirmed by the significant site-by-sex statistical interactions. The ANCOVA using head width as the covariate sheds light on the sexually differentiated patterns of habitat variation. Male population variation in larynx component sizes is greater than that due to head size alone; for females, this is not the case. After correcting for population variation in head size, female larynx size differences among the populations are rendered non-significant.

Male larynx size correlates with the dominant frequency of the male advertisement calls, and both call dominant frequency and larynx size correlate with overall body size (McClelland et al. 1996). McClelland et al. (1998) reported, however, that habitat-based call and larynx differences in males exceeded differences due to body size alone based on a comparison across multiple populations including the 3 analyzed in this study. They suggested that this resulted from selection on males for lower-frequency calls in open habitats. Female larynges are not used in vocal production in cricket frogs, and are not subject to the same additional call-based selection impacting males. As for other terrestrial anurans (De Jongh and Gans 1969), we assume that cricket frog females (and males) use the larynx as a valve for controlling lung inflation and deflation. Female larynx size only reflects allometric changes related to head size differences. What is interesting is that female larynges are not pulled along as the male larynx increases due to the additional male-only call-based sexual selection pressure.

We find that the size of the basic morphological components of the ear are also sexually dimorphic, but unlike the larynx, ears are larger in females, consistent with their larger body size. Auditory tuning (the frequency to which the ear is most sensitive) is inversely correlated with body size in cricket frogs (Keddy-Hector et al. 1992). Moreover, the female cricket frog auditory system is tuned to lower frequencies compared with males (Wilczynski et al. 1992). Sexually differentiated tuning of the ear in other anuran species generally follows the same pattern: females are larger and have ears tuned to lower frequencies compared with the smaller males (Narins and Capranica 1976; Wilczynski et al. 1992; McClelland et al. 1997; Arch and Narins 2009; Shen et al. 2011; Liu et al. 2014). Sex differences in the size of the measured ear structures could be in part responsible for the sex difference by biasing the resonant frequencies of the transducing structures (Hetherington 1994; Mason et al. 2003). Sex differences in tuning might also reflect differences in inner ear sensory organs or their constituent hair cells, which we could not assess.

McClelland et al. (1998) reported size differences in male cricket frog ears in these 3 populations along with those in other populations correlating with changes in body/head size and larynx size. As for the larynx results, here we find that population-level ear variation in male cricket frogs is matched in females. Unlike the case for larynx structures, our covariate analysis found that in both sexes many of the ear differences are rendered non-significant after head size is accounted for, suggesting that most of the population-level ear variation in both sexes is due to allometric effects as the average sizes of the individuals change. The tympanic membrane is the only ear structure that remains significantly different across populations in both males and females after the effect of head size is removed by



**Figure 3.** Mean ( $\pm$ SEM) ear structure sizes for males and females in 3 cricket frog populations. Black bars, Gill Ranch; gray bars, Stengl Ranch; and white bars, Wimberley. (A) Middle ear volume; (B) inner ear volume; (C) extracolumella volume; and (D) tympanic membrane diameter. See text for statistical results.

the ANCOVA. Inner ear volume from the 3 populations remained significantly different in females after head width is controlled, but not in males. At present there is no clear explanation why these particular ear structures differ across populations beyond that predicted by the overall size of males or females.

The analysis of correlated male and female traits used in reproductive communication has most often been focused on relationships between male signals and female preference (Ryan et al. 1990; Kirkpatrick and Barton 1997; Wilczynski et al. 2001; Betancourth-Cundar et al. 2016). How shared traits—such as the larynx structures measured here—might change in 1 sex when selection yields changes in the other sex was outlined in Lande's classic theoretical paper (Lande 1980). Lande noted that selection on 1 sex should yield coincident changes in the other sex when traits are correlated, but that selection on the opposite sex could mitigate that correlated change until some optimum is reached. Several studies have tested this idea using behavioral traits and the results have been mixed. Halliday and Arnold (1987) outlined how selection for promiscuity in males could result in changes in female mating behavior via correlated evolution across the sexes. Forstmeier et al. (2011) tested this and found that positive selection for promiscuity in male zebra finch has led to increased promiscuity in females. In contrast, Harano and Miyatake (2007) reported that artificial selection on female beetles *Callosobruchus chinensis* for multiple matings did not result in correlated behavioral changes in male mating frequency. Correlated changes in signaling structures, which is 1 function of anuran larynges, have often been assumed to occur, resulting, for example, in female ornamentation (Lande 1980; Amundsen 2000). Amundsen's review of female bird ornaments finds, however, that selection can act independently on females to shape their signaling structures. A general conclusion arising from this is that shared male and female traits can be affected independently. That appears to be the case for

*Acris* larynges. Both sexes show allometric changes in the larynx related to selection for larger animal size in dry, open habitats; but selection on males for additional size increases related to the production of lower frequency calls does not result in correlated changes in the female larynx. As a result, the traditional sex difference found in anuran laryngeal size and shape remains in all populations and, in all cases, habitat selection on body size influences laryngeal structure equally in males and females. As additional selection targets the male larynx, however, the degree of sex dimorphism changes at the level of within species populations.

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