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Sexual dimorphism in external morphology of the American bullfrog *Rana (Aquarana) catesbeiana* and the possibility of sex determination based on tympanic membrane/eye size ratio

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ABSTRACT. The American bullfrog *Rana* (*Aquarana*) *catesbeiana* has been reported to show significant sexual dimorphism based on the size ratio between the tympanic membrane and the eye. In males the tympanic membrane is much larger than the eye, but not in females. The ratio has been used as a convenient criterion to discriminate sexes (sexing) in the American bullfrog, though its reliability is unknown. In this study, we examined 86 adult American bullfrogs to clarify whether the tympanic membrane long diameter/eye long diameter (D_{tm}/D_e) ratio is a reliable index to discriminate sexes in this species. In addition, we examined the growth of this sexually dimorphic trait. Results indicated that there is a significant difference but there is a small overlap in this ratio D_{tm}/D_e between sexes. The allometric comparisons showed the sexual dimorphism of the D_{tm}/D_e ratio was increased during growth and the dimorphism is attributable to the difference in the growth rate of the tympanic membrane (D_{tm}). Therefore, sex determination of American bullfrogs cannot be wholly reliably achieved by the D_{tm}/D_e ratio alone; other external morphological features are required in addition.

KEY WORDS: eye, Ranidae, sexual dimorphism, tympanum

The American bullfrog *Rana* (*Aquarana*) *catesbeiana* is native to North America but has introduced worldwide including Japan [8, 9]. Several authors have classified this species into the genus *Lithobates* because some members of New World frogs in the family Ranidae show a monophyletic grouping, according to recent phylogenetic analyses [2, 4]. However, Yuan *et al*. do not support the monophyly of American bullfrog and the true *Lithobates* group, recommending that the species should be classified as *Rana* (*Aquarana*) *catesbeiana* [18]. Therefore, we use *Rana* (*Aquarana*) *catesbeiana* as the name of the species in the present paper.

Female American bullfrogs have a greater average body size than males; however, the growth rate of wild American bullfrogs does not differ between sexes [5]. Thus, the difference in body size is a reflection of the difference in average age between the sexes [5]. In anurans, including genus *Rana*, there is a correlation between the differences in average body size and average age between sexes, therefore, the conclusion above is supported [11].

In one particular trait, American bullfrogs show sexual dimorphism in the size of the tympanic membrane [12, 13, 16, 17]. For American bullfrogs, previous authors have stated that the tympanic membrane diameter/eye diameter ratio is 1.3–1.7 in males and 0.9–1.2 in females [8, 9], whereas another author has stated ratios of 1.3–1.7 in males and 0.95–1.15 in females [6]. Accordingly, this ratio has been used as a convenient criterion to determine sexes (i.e., sexing) in the American bullfrog. However, its reliability remains unclear. Although Iwasawa stated that the sexes of individuals longer than 12 cm in the body length are easily distinguishable by this ratio, but his result showed overlapping of the index between sexes even in the larger individuals [6]. Therefore, it is important to clear the growth of this sexually dimorphic trait. In this study, we first compared the tympanic membrane diameter/eye diameter ratio between sexes to clarify whether the ratio is a reliable index for sex discrimination in the American bullfrog. Then, we compared the growth of this sexually dimorphic trait in the context of allometry.

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MATERIALS AND METHODS

We examined 86 adult (not tadpole) wild American bullfrogs (*R. catesbeiana*; 43 males and 43 females; no intersex individuals were observed) caught in Lake Kasumigaura, Ibaraki Prefecture, Japan, purchased from a dealer of experimental animals. The frogs were anesthetized, euthanized, and then they were used for an anatomy course at the university. The sex of each individual was determined by internal morphology (i.e., existence of testes or ovaries). Sexual maturation was not assessed. The snout-vent length (SVL) was measured by a scale ruler to the nearest 5 mm. Other external measurements were measured using a digital caliper (Mitutoyo CD-15CX, Kawasaki, Japan) after the anatomy course. The measured points were the long diameters of the tympanic membrane (D_{tm}) and eye (D_e: eyelid length). D_{tm} was called eye length (EL) and D_e was called tympanum diameter (TD) in the previous study [7].

To clarify the relationship between D_{tm} and D_e, the measurement data were log-transformed, and an allometric comparison of D_{tm} and D_e, D_{tm} and SVL, and D_e and SVL between the sexes was performed. Differences in the regression lines between the sexes were tested by analysis of covariance (ANCOVA). Regression analysis was performed using the ordinary least-squares method. The D_{tm}/D_e ratio and the other parameters were also compared between the sexes (Mann–Whitney *U*-test). Statistical analysis was performed using Minitab 18 (Minitab Inc., State College, PA, USA) and R (R Core Team, Vienna, Austria, 2016).

Prior permission was obtained from the Chubu Regional Environment Office, Ministry of the Environmental Government of Japan (No. 1505081), for the temporary maintenance of American bullfrogs in captivity. The drug treatment of the American bullfrogs and the anatomy course were performed according to the guidelines on animal experimentation by Aichi Gakuin University and the guidelines on animal experimentation by Division of Liberal Arts and Sciences, Aichi Gakuin University.

RESULTS

In male American bullfrogs, the average D_{tm} was larger than the average D_e, whereas, in females, the average D_{tm} was smaller than the average D_e (Table 1). The D_{tm}/D_e ratio in males was significantly larger than that in females ($P<0.001$: Mann–Whitney *U*-test; Table 1, Fig. 1). In the allometric comparisons, the slopes of regression lines were significantly greater than 0 in both sexes, indicating a correlation between D_{tm} and D_e, D_{tm} and SVL, and D_e and SVL, respectively ($P<0.001$: ANCOVA; Fig. 2A–C; Table 2). The slopes were significantly different between sexes in the comparison of Log D_e vs. Log D_{tm} and Log SVL vs. Log D_{tm}, but were not significantly different in that of Log SVL vs. Log D_e (ANCOVA; Fig. 2A–C; Table2). The intercepts were not significantly different between sexes in the comparison of Log SVL vs. Log D_e (ANCOVA; Fig. 2A–C; Table 2). Because there was some overlap of individual points between sexes, the sexes were not clearly separated by D_{tm}/D_e ratio alone (Fig. 1A and 1B). D_{tm}/D_e ratio was significantly correlated to SVL in both sexes ($P<0.001$; Fig. 2D). Overlapping of males and females was observed when the D_{tm}/D_e ratio was between 0.90 and 1.08. 51.1% of the total individuals were included in this range but 80.5% of the total male individuals were separated from female individuals by the ratio (Fig. 1B).

DISCUSSION

The result supported previous studies [6, 8, 9, 12, 17] that have stated that there is sexual dimorphism in the tympanic membrane diameter/eye diameter ratio of the American bullfrog; the tympanic membrane is larger than the eye in males, but there is no prominent difference in females (Table 1; Fig. 1).

The individual-level allometric analysis showed difference between sexes in the slopes of regression lines in the plot of Log D_e vs. Log D_{tm} and Log SVL vs. Log D_{tm} (Fig. 2A and 2B). However, the result did not show difference between sexes in regression

Table 1. Long diameter of the tympanic membrane (D_{tm}) and eye (D_e), body size (SVL: snout-vent length) (mm) in the American bullfrog *Rana* (*Aquarana*) *catesbeiana* from Ibaraki prefecture, Japan

	Sex	Average	Range	SD	Coefficient of variation	Sexual dimorphism (*P* value)
Tympanic membrane (D_{tm})	Male	17.34	11.4–22.83	3.04	0.175	<0.001
	Female	13.52	9.46–16.46	1.49	0.111	
Eye (D_e)	Male	13.92	11.89–16.58	1.01	0.072	0.168
	Female	14.23	11.69–16.55	1.19	0.084	
Tympanic membrane/Eye (D_{tm}/D_e)	Male	1.238	0.90–1.46	0.15	0.124	<0.001
	Female	0.949	0.81–1.08	0.05	0.054	
Body size: Snout-vent length (SVL)	Male	141.28	105–175	14.72	0.104	0.032
	Female	148.72	110–190	17.19	0.116	
Tympanic membrane/Body size (D_{tm}/SVL)	Male	0.122	0.091–0.165	0.02	0.124	<0.001
	Female	0.091	0.074–0.110	0.01	0.069	
Eye/ Body size (D_{tm}/SVL)	Male	0.099	0.086–0.113	0.01	0.067	0.056
	Female	0.096	0.080–0.112	0.01	0.072	

D_{tm}, tympanic membrane long diameter; D_e, eye long diameter; SVL, snout-vent length.

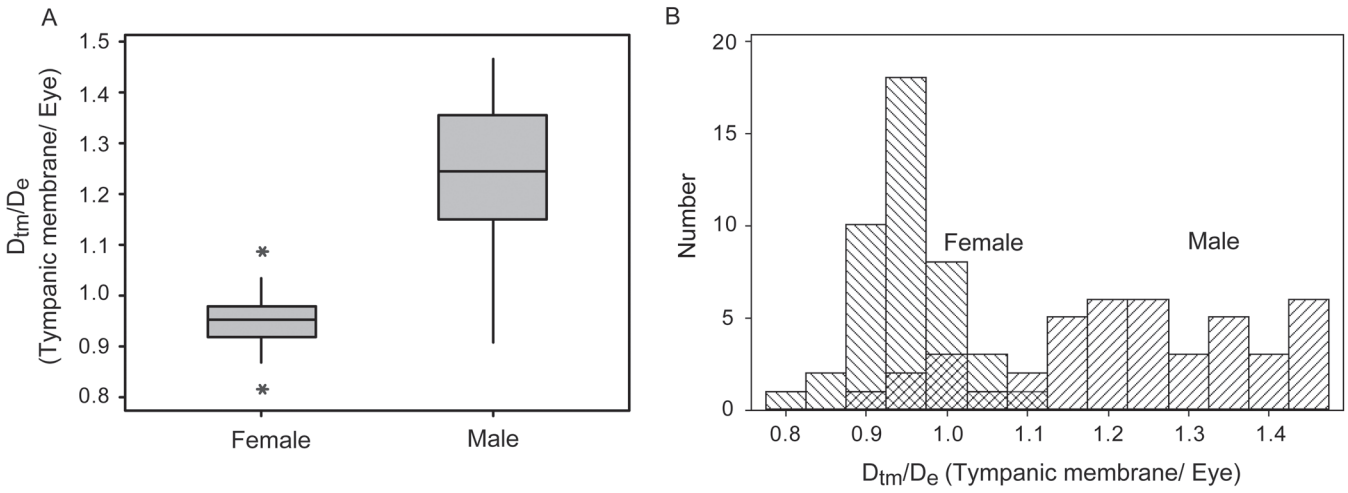

Fig. 1. Sexual dimorphism of size ratio between the tympanic membrane (D_{tm}) and eye (D_e) in the American bullfrog *Rana* (*Aquarana*) *catesbeiana* from Lake Kasumigaura, Ibaraki Prefecture, Japan. A. Box plots of the ratio of long diameter of tympanic membrane/long diameter of eye (D_{tm}/D_e). Boxes indicate quartiles, central–lateral bars indicate means, vertical bars indicate ranges, and the asterisk indicates an outlier. B. Histogram of the ratio of long diameter of tympanic membrane/ long diameter of eye (D_{tm}/D_e), showing an overlap of the ratios between males and females.

Table 2. Results of the regression analysis

	Sex	Slope	95% CI	Sexual dimorphism (*P* value)	Intercept	95% CI	Sexual dimorphism (*P* value)
D_e vs. D_{tm}	Male	2.163	1.734–2.591	<0.001	−1.239	−1.723– −0.750	NA
	Female	1.197	1.005–1.389		−0.251	−0.470– −0.030	
SVL vs. D_{tm}	Male	1.332	0.984–1.679	<0.001	−1.628	−2.373– −0.882	NA
	Female	0.801	0.629–0.972		−0.610	−0.983– −0.237	
SVL vs. D_e	Male	0.529	0.397–0.662	0.513	0.005	−0.280–0.290	0.668
	Female	0.568	0.428–0.708		−0.083	−0.386–0.221	

D_{tm}, tympanic membrane long diameter; D_e, eye long diameter; SVL, snout-vent length; NA, not applicable

lines in the plots of Log SVL and Log D_e (Fig. 2C). The result indicates that sexual dimorphism in D_{tm}/D_e ratio is attributable to the different growth rates of the tympanic membrane between sexes. A previous author has stated that the regressions of tympanic membrane area on SVL exhibited significantly different intercepts in male and female frogs over 100 mm SVL; however, the slopes were not significantly different for frogs of this size-range [10]. In another allometric analysis using three frog species, including the American bullfrog, the traits for which significant sexual dimorphism was observed did not necessarily show greater slopes of allometric lines than other traits; therefore, the report concluded that sexual dimorphism could not be based on positive allometry [15]. However, Boatright-Horowitz and Simmons [1] concluded that sexual dimorphism in the tympanic membrane of American bullfrogs showed more prominent positive allometry on SVL in males than in females. Iwasawa [6] investigated the growth of the tympanic membrane using American bullfrog specimens from broader ranges of growth stages, including younger individuals, than those used in the abovementioned studies. His results indicated that male American bullfrogs showed more prominent positive allometry of the tympanic membrane on head-body length than the females at a particular growth stage (when the head-body length is approximately 120 mm). However, the slopes of the tympanic membrane growth lines in both sexes were identical after that stage. He concluded that the difference in the slopes when the head-body length was approximately 120 mm generated sexual dimorphism of the tympanic membrane in American bullfrogs. In the present study, we used American bullfrog specimens whose head-body length was from 105 to 175 mm, a growth stage including the stage when sexual dimorphism of the tympanic membrane increases [6]. Our allometry results, with different slopes of regression line between both sexes, may be considered to be a consequence of our specimens being wider growth stages. The differentiation of the tympanic membrane may be caused by its functional demand for increased sensitivity to intraspecific calls or vocalizations for mating [e.g. 3, 14].

The present study also supported the existence of sexual dimorphism in the D_{tm}/D_e ratio. However, males and females could not be entirely separated by this ratio (Fig. 1A and 1B). Therefore, sex determination (sexing) could not be performed using only this ratio. It should be mentioned that the measurement method might be slightly different between the present study and the previous study. However, the sexual dimorphism increases as the body size increases (Fig. 2A and 2D). Overlap in the ratio between the sexes was observed in relatively small individuals (Fig. 2D). This result is consistent with the previous study [6]. In conclusion, sex discrimination (sexing) of American bullfrogs can be partially achieved by the D_{tm}/D_e ratio; however, because the separation is not

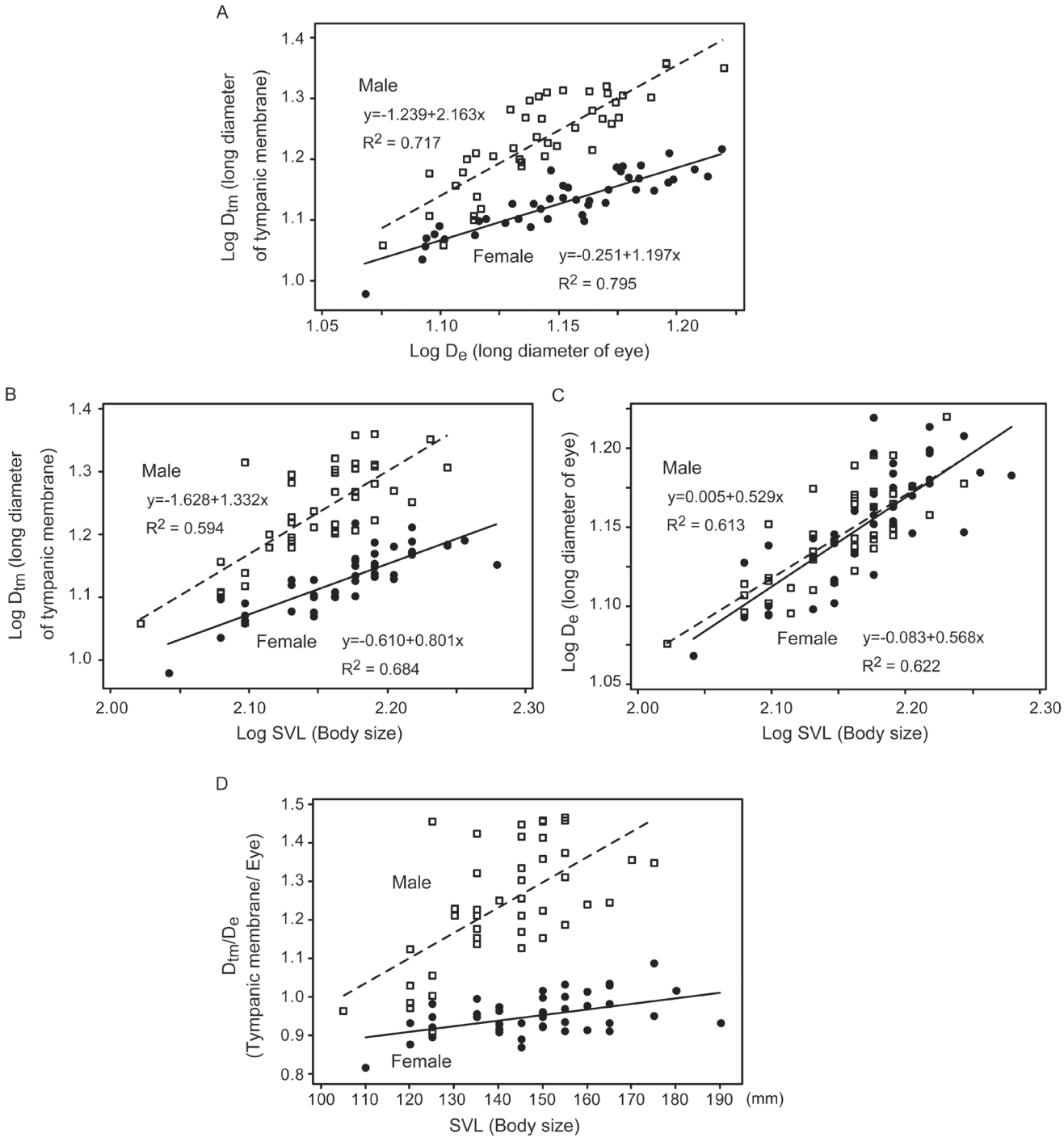

Fig. 2. Allometric comparison between sizes of the tympanic membrane and eye. Bivariate plot of Log long diameter of tympanic membrane (D_{tm}) and Log long diameter of eye (D_e) (A), Log D_{tm} and Log snout-vent length (SVL) (B), Log D_e and Log SVL (C), and D_{tm}/D_e and SVL (D). The result showing the sexual dimorphism of the ratio D_{tm}/D_e is increased during the growth and is attributable to the growth rate of the tympanic membrane.

entirely reliable, other external morphological features, such as the body size (SVL) or the development of the prepollex and throat color, are required for certainty.

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