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*<sub>tm</sub>/*D*<sub>e</sub>) 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*<sub>tm</sub>/*D*<sub>e</sub> between sexes. The allometric comparisons showed the sexual dimorphism of the *D*<sub>tm</sub>/*D*<sub>e</sub> ratio was increased during growth and the dimorphism is attributable to the difference in the growth rate of the tympanic membrane (*D*<sub>tm</sub>). Therefore, sex determination of American bullfrogs cannot be wholly reliably achieved by the *D*<sub>tm</sub>/*D*<sub>e</sub> 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.
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 (Dtm) and eye (De; eyelid length). Dtm was called eye length (EL) and De was called tympanum diameter (TD) in the previous study [7].

To clarify the relationship between Dtm and De, the measurement data were log-transformed, and an allometric comparison of Dtm and De, Dtm and SVL, and De 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 Dtm/De 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 Dtm was larger than the average De, whereas, in females, the average Dtm was smaller than the average De (Table 1). The Dtm/De ratio in males was significantly larger than that in females (*P*<0.001: Mann–Whitney U-test; Table 1, Fig. 1). The slopes were significantly different between sexes in the comparison of Dtm vs. SVL and Dtm vs. De, indicating a correlation between Dtm and De, Dtm and body size, and De and body size, respectively (*P*<0.001: ANCOVA; Fig. 2A–C; Table 2). The slopes were significantly different between sexes in the comparison of Log Dtm vs. Log De, Log Dtm vs. Log SVL, but were not significantly different in that of Log SVL vs. Log De (ANCOVA; Fig. 2A–C; Table 2). The intercepts were not significantly different between sexes in the comparison of Log SVL vs. Log De (ANCOVA; Fig. 2A–C; Table 2). Because there was some overlap of individual points between sexes, the sexes were not clearly separated by Dtm/De ratio alone (Fig. 1A and 1B). Dtm/De ratio was significantly correlated to SVL in both sexes (*P*<0.001; Fig. 2D). Overlapping of males and females was observed when the Dtm/De 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 De vs. Log Dtm and Log SVL vs. Log Dtm (Fig. 2A and 2B). However, the result did not show difference between sexes in regression

| Table 1. Long diameter of the tympanic membrane (Dtm) and eye (De), body size (SVL: snout-vent length) (mm) in the American bullfrog *Rana (Aquarana) catesbeiana* from Ibaraki prefecture, Japan |
|-----------------|--------|----------------|--------|-----------|------------------|
|                 | Sex    | Average         | Range  | SD        | Coefficient of variance | Sexual dimorphism |
|-----------------|--------|-----------------|--------|-----------|-------------------------|--------------------|
| **Tympanic membrane (Dtm)** |        |                 |        |           |                         |                    |
| 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 (De)**    |        |                 |        |           |                         |                    |
| 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 (Dtm/De)** |        |                 |        |           |                         |                    |
| 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 (Dtm/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 (De/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     |                         |                    |

* Dtm, tympanic membrane long diameter; De, eye long diameter; SVL, snout-vent length.
The results of the regression analysis are shown in Table 2. The slopes of the regression lines for the ratio of long diameter of tympanic membrane (Dtm) to eye (De) are calculated for both sexes as follows: Male (sphygmo = 2.163, 95% CI: 1.734–2.591, P < 0.001; intercept = −1.239, 95% CI: −1.723–−0.750, P < 0.001) and Female (sphygmo = 1.197, 95% CI: 1.005–1.389, P < 0.001; intercept = −0.251, 95% CI: −0.470–−0.030, P < 0.001). The slopes of the regression lines for the ratio of snout-vent length (SVL) to Dtm are calculated for both sexes as follows: Male (sphygmo = 1.332, 95% CI: 0.984–1.679, P < 0.001; intercept = −1.628, 95% CI: −2.373–−0.882, P < 0.001) and Female (sphygmo = 0.801, 95% CI: 0.629–0.972, P < 0.001; intercept = −0.610, 95% CI: −0.983–−0.237, P < 0.001). The slopes of the regression lines for the ratio of SVL to De are calculated for both sexes as follows: Male (sphygmo = 0.529, 95% CI: 0.397–0.662, P = 0.513; intercept = 0.005, 95% CI: −0.280–0.290, P = 0.668) and Female (sphygmo = 0.568, 95% CI: 0.428–0.708, P = 0.083; intercept = −0.386–0.221, P = 0.668). Dtm, tympanic membrane long diameter; De, eye long diameter; SVL, snout-vent length; NA, not applicable.
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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Fig. 2. Allometric comparison between sizes of the tympanic membrane and eye. Bivariate plot of Log long diameter of tympanic membrane (Dtm) and Log long diameter of eye (De) (A), Log Dtm and Log snout-vent length (SVL) (B), Log Dm and Log SVL (C), and Dtm/De and SVL (D). The result showing the sexual dimorphism of the ratio Dtm/De is increased during the growth and is attributable to the growth rate of the tympanic membrane.
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