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Fluctuating Asymmetry in the head shape and parotid gland secretion in introduced cane toads (*Rhinella marina*) from established populations in Caraga State University, Butuan City, Agusan del Norte, Philippines

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ABSTRACT

The study was supervised to determine and assess the ecological health of Caraga State University in Ampayon, Butuan City by looking into the level of fluctuating asymmetry in populations and affected landmarks of Rhinella marina. Various studies have shown that fluctuating asymmetry can be used to indirectly assess water quality as well as the overall health of the ecosystem. In this study, R. marina was utilized because of its abundance in Caraga State University. Hence, with the presence and use of the thin-plate spline (TPS) series, landmark analysis was obtained and subjected to Symmetry and Asymmetry in Geometric Data (SAGE) software. Wherein, the findings in Procrustes ANOVA showed that although individual symmetry delineate no significant difference, Sides (Directional Asymmetry) and Interaction (Fluctuating asymmetry) showed a highly significant difference (P = 0.0000***). Furthermore, the results of Principal Component Scores display a high percentage Fluctuating Asymmetry of pooled (79.964%). Thus, in the results, PC 1 (40.589%), PC2 (18.8634%), PC3 (12.7566%), and PC4 (7.755%), the interaction was explicitly found to have significant variations affecting the R. marina parotid gland, landmarks of the loreal crest, and supra-tympanic edge of the cane toad. A significant proportion of the variation in head shape can be explained by environmental variables, suggesting that conditions of the physical environment should also be considered as a source of morphological variation. To conclude, this study authenticated the use of fluctuating asymmetry in determining and assessing the status of the ecological health of the study area confirming that indeed results of high FA equates for the incidence of cane toads in the area.

INTRODUCTION

Fluctuating asymmetry has been proposed as an indicator of environmental stress and population health. However, a notable feature of research examining the relationship between fluctuating asymmetry and population fitness is that of inconsistency. In fluctuating asymmetry refers to these directionally random, subtle departures from perfect symmetry and is hypothesized to indicate the inability of an organism to maintain precise development (Bookstein FL, 1997). The fluctuating asymmetry is an essential morphometrics tool in terms to small, random deviation from the ideal morphology of organism due to its capability of giving absolute difference between the left- and right-hand side of a bilaterally symmetrical organism (Moller & Swaddle 1997, Palmer &Strobeck 2003). Also, to measure and evaluate the difference of the right- and left-hand side of bilaterally symmetrical organism using FA, there are three methods for measuring the dispersion: classic measurement linear measurement using variance, landmark methods, and continuous symmetry measure (Bookstein FL, 1996). The results in fluctuating asymmetry measurement will determined the condition of an individual organism.

The fluctuating asymmetry is very pivotal besides in assessing the water quality due to the massive establishments of pollutants in aquatic area. Furthermore, the estimation will be done by means of using bio-indicators. Additionally, these bio-indicators are the predominant inhabitants in the aquatic area. Within the aquatic areas, frogs are the second abounding organisms. Whenever the fluctuating asymmetry will be applied and accomplished, the high fluctuating asymmetry value means that the water is exceedingly polluted which will indicate the stressed state of the frogs. The upend condition is portrayed by low fluctuating asymmetry value. For this study, cane toad frog (*Rhinella marina*) was used to assess the water quality and ecological health in a certain area.

The cane toad frogs are different from native species; toad tadpoles are jet black and reach a maximum of about 30 mm long from head to tail. They have non-transparent abdomen skin, and their tails are nearly the same length as their body. The tail has a jet-black central muscle with totally transparent fins with no spots or pigment. The tadpoles of native frogs can be very dark (but not jet black) with lighter or transparent abdomen skin and longer tails. The existence of the cane toad frog in the aquatic area which serves as their habitat helps to distinctly assess the environment by means of regulating the condition of the cane toad frog (*R. marina*). This state was used to identify the status of the water quality or some of the water area here in Caraga State University, whether it is highly polluted or not.

The cane toads in the Philippines are said to be one of the most successful species, but it is restricted to some isolated aquatic areas. Nonetheless, cane toad frogs are easily

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identified due of its rough skin and rocky configuration. They are found in some of aquatic environment and estuaries. In this study, Caraga State University was the primary concern of the study. The ecological health reflects the essential link between the groups of living organisms and the health of their environments. It basically relates to the biological diversity, ecosystem productivity and its flexibility on defying negative impacts under a variety of pressure. Proper functioning of a natural system is one of the foundations to a community's economic prosperity as we rely on the environment on the wide range of services. However, due to the continued threats from pollution, erosion and the introduction of invasive species, coupled with the lack of local concern, education and sustainable protection, pose a level of ambiguity on the survival of this cane toad (Bagarinao 2001; Guerrero III 2002). In addition, cane toad frog is exposed to water pollution especially when the practice of overstocking and overfeeding the frogs from the several frogs' cages are present in the area aside from municipal effluents causing degradation of the carabao center in Caraga State University. The proper management programs have to be adopted to impose several conservation procedures. This way, the pollution will be properly monitored and early precautions will be imposed. This study aimed to determine percentage fluctuating asymmetry of cane toad frog found in Caraga State University, Ampayon, Butuan City and compared the intraspecific asymmetrical variations, and individual symmetry. Hence, the results of the fluctuating asymmetry of cane toad frog can determine if the species has undergone environmental and genetic perturbations in Caraga State University.

MATERIALS AND METHODS

Study Area. Caraga State University is located at Ampayon, Butuan City which geographically lies between 06°13'N and 124°42'E. Sampling was carried out from May 15-17, 2019 at agricultural area within the institution.

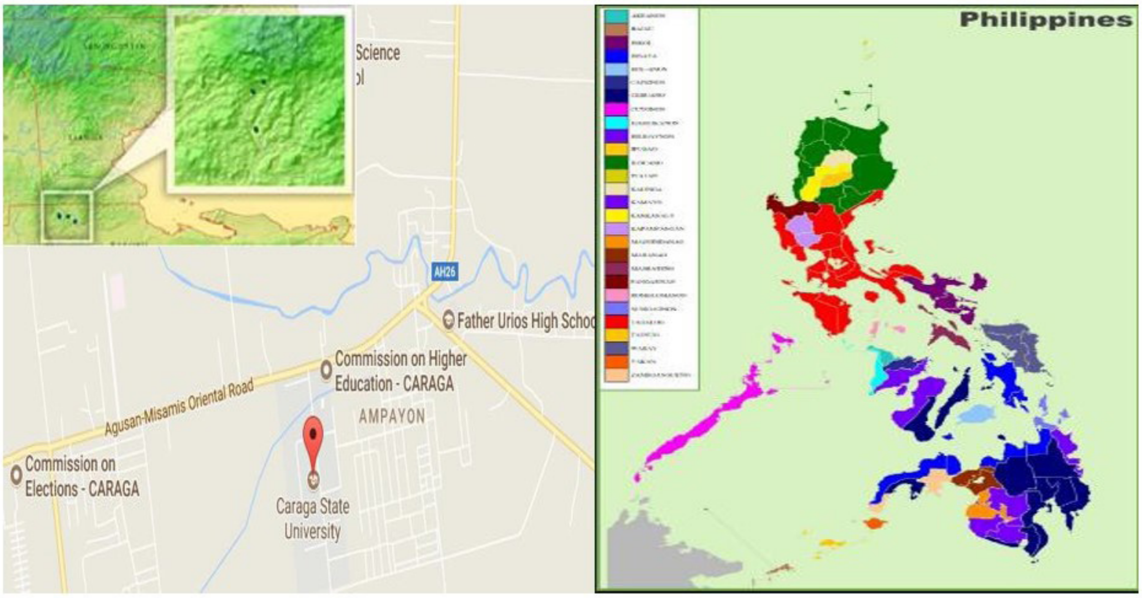


Figure 1. Map of the sampling station at Caraga State University, Ampayon, Butuan City

Fluctuating Asymmetry of cane toad frogs in CSU (Carabao Center)

Sample Processing. Sixty samples of R. marina pooled were sampled and collected. Cane toad frog individuals were preserved using chloroform and were placed in a square plate Styrofoam. Digital imaging was done using Olympus digital cam (SP-800uz, 14 megapixels). The captured images were digitized using tpsDig2 program (version 2.0, Rohlf, 2004) and were saved as a TPS file. After having photographed the cane toad frog's samples, pooled was determined and identified based on www.frogbase.com. Pooled were identified based on its external morphology and later confirmed by direct examination of the head shape and parotid gland.

Landmark Selection and Digitization. Using thin-plate spline (TPS) series, landmark analyses were obtained in order to incorporate curving features within the images. Both evolutionary and functional significance were

Figure 2. The unbridged image of the cane toad frog *Rhinella marina*.

obtained through the standard forms of the digitized landmarks used in frog morphometric. The landmarks were selected to provide homogenous outline of the head shape and parotid gland. A total of 24 landmarks (equivalent to 60 pooled) were identified in order to best represent the external configuration of the head and parotid gland. Landmark description was shown in table

Table 1: Description of the landmark points according to Bandeira et.al (2012) with modifications.

Landmark Number	Description
1	Top of rostrum
2 and 24	Beginning of the loreal crest
3 and 23	Midpoint of the loreal crest
4 and 22	Landmark positioned in the mandible at 90 degrees
5 and 21	Junction between the loreal crest and the anterior edges of the eye
6 and 20	Junction point between the supra-tympanic edges and posterior edge of the eye
7 and 19	Midpoint between landmarks 5 and 6
8 and 18	Beginning of the cephalic crest
9 and 17	End of cephalic crest
10 and 16	Beginning of the supra-tympanic edge
11 and 15	Top of the parotid gland
12 and 14	Bottom of the parotid gland
13	Midpoint of the shortest distance between parotid glands

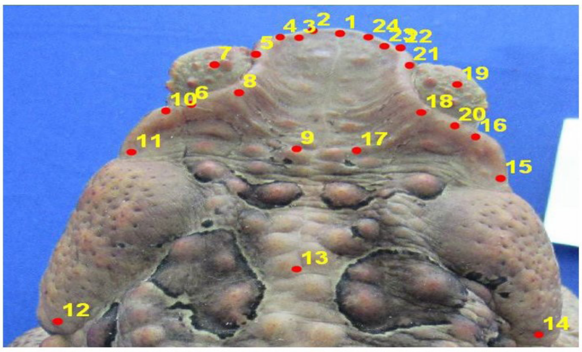


Figure 3. Landmark point of the cane toad frog (head shape and parotid gland) of *Rhinella marina.*

1. Pooled coordinates of landmarks on images were then obtained for further analysis. To lessen the inconsistencies and or errors in plotting the landmark points, digitization were copied in triplicates for each frog samples.

Head Shape and Parotid Gland Analysis

Generated pooled coordinates had served as baseline data in analyzing fluctuating asymmetry of cane toad frog. Pooled form landmark coordinates of the TPS version were subjected to Symmetry and Asymmetry in Geometric Data (SAGE) software (version 1.04, Marquez, 2007) to identify geometric data of object with emphasis on its asymmetry. Sage generates symmetrized data sets, residuals from symmetric components, in addition to head shape configuration and parotid gland of each component of variation (Symmetric, Asymmetric, and error) as well as expected covariance matrices. Procrustes ANOVA were employed with triplicates and with 99 permutations to calculate and quantify the residual asymmetry. This indicates the variation between sides and is the measure of directional asymmetry. Percentage (%) FA were obtained and compared between the pooled.

Intraspecific Variation

By using Paleontological Statistics (PAST) software (Hammer et al., 2001), comparisons between pooled and individual symmetry were analyzed by generating relevant statistical representations such as histogram, box plot and scattered plot.

RESULTS AND DISCUSSION

Individual head shape and parotid gland configuration fluctuations through Procrustes ANOVA were shown in table 2. Individual symmetry of L-R size and shape were depicted to have significance. However, variations of the pooled were depicted to have high significance (0.0000**) on the side scores indicating the occurrence of fluctuating asymmetry (FA) in the head shape and parotid gland configuration of the cane toad frog.

This study presents that the differences in the head shape and parotid glands of the pooled can be attributed to perturbations in the environment resulting from poor water quality. Stressed environment according to Barrett (2005) translates to the inability of the species to develop phenotypically to a desired path as these stressors acts upon the individual species during their development (Bonada and Williams, 2002). In the study of Van Valene (1962), these asymmetries developed errors in the developmental processes because of the species' inability to thrive and buffer environmental disturbances. Developmental instability of the R. marina to these fluctuating asymmetries.

Table 2. Procrustes ANOVA for configuration of R. marina in pool

FACTORS	SS	DF	MS	F	P-VALUE
			Pooled		
Individuals	4.0687	1298	0.0031	7.2643	0.0001**
Sides	0.0982	22	0.0045	10.3477	0.0001**
Individual x Sides	0.5601	1298	0.0004	6.4501	0.0001**
Measurement Error	0.3532	5280	0.0001	---	---

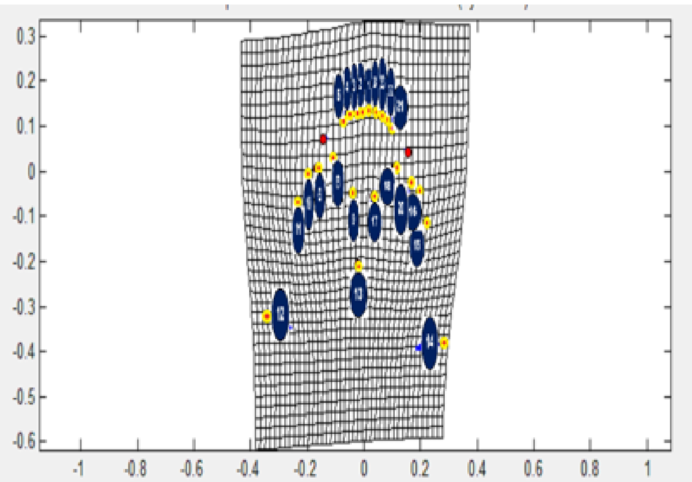
*** Highly significant (P<0.005), ns not significant

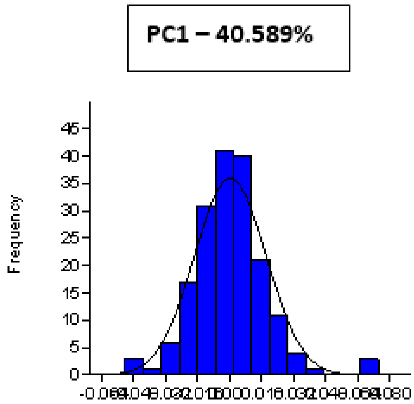
A total of 79.964% of fluctuating asymmetry interaction from upper 5% effective principal components (PC1-PC4) of R. marina in pooled is measured. In table 3 shows the Principal Components for the variation for pooled samples. According to the results in PC 1, asymmetry can found greatest in the area covered by landmark: 1 (top of rostrum), 2 and 24 (beginning of the loreal crest), 3 and 23 (midpoint of the loreal crest), 4 and 22 (landmark positioned in the mandible at 90 degrees) 5 and 21 (junction between the loreal crest and the anterior edge of the eye), 6 and 20 (junction point between the supra-tympanic edge and posterior edge of the eye), 8 and 18 (beginning of the cephalic crest) 9 and 17 (end of the cephalic crest) 10 and 16 (beginning of the supra-tympanic edge) 11 and 15 (top of the parotid gland) 12 and 14 (bottom of the parotid gland) and 13 (midpoint of the shortest distance between parotid gland); PC2 on the landmarks 1, 2, 3, 6, 8, 9, 10, 11,12,

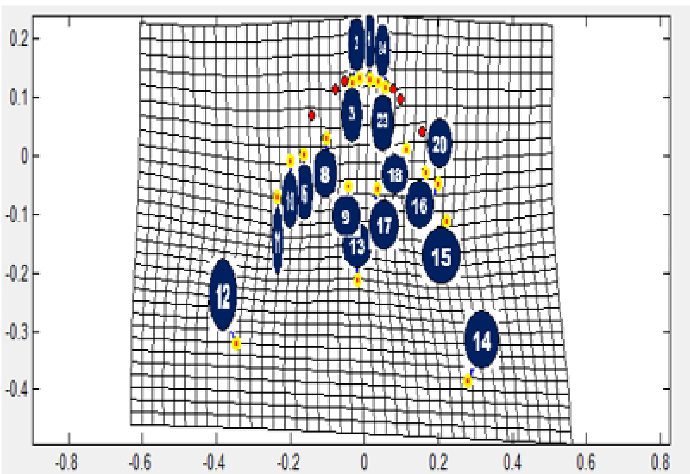
Table 3. The principal component scores showing the values of symmetry and asymmetry scores with the summary of the affected landmarks.

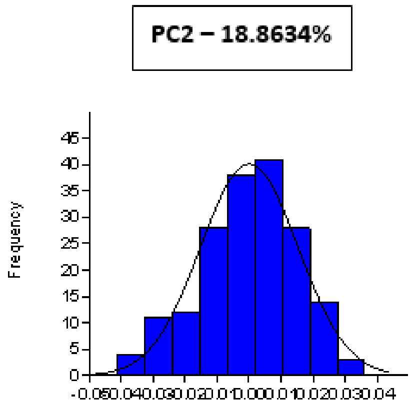
PCA	Individual (Symmetry)	Sides (Directional	Interaction (Fluctuating Asymmetry)	Affected Landmarks
		Pooled		
PC1	48.7376%	100%	40.589%	1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23, 24
PC2	19.4203%		18.8634%	1, 2, 3, 6, 8, 9, 10, 11,12, 13, 14, 15, 16,17, 18, 20, 23, 24
PC3	13.0545%		12.7566%	6, 9, 10, 11, 12, 14, 15, 16, 17, 20
PC4	6.8666%		7.755%	7, 8, 11, 12, 13, 15, 17, 18, 19
	88.079%		79.964%	

13,14,15,16,17,18,20,23,24 are the affected landmarks in the profile; PC3 on the landmarks got a result of affected landmarks 6,9,10,11,12,14,15,16,17,20 this will indicates the low portion of affected landmarks in the head shape and parotid gland configuration; in PC4 has a minimal affected landmarks 7,8,11,12,13,15,17,18,19. And the most common affected landmarks out of the (PC1 to PC4) are the 11, 12,13,14,15 that is found in the parotid gland because parotid gland is secretion defense of mechanism of the cane toad frog. Fluctuating asymmetry interaction that shows movement of landmarks on the different regions depicted on the following landmarks:









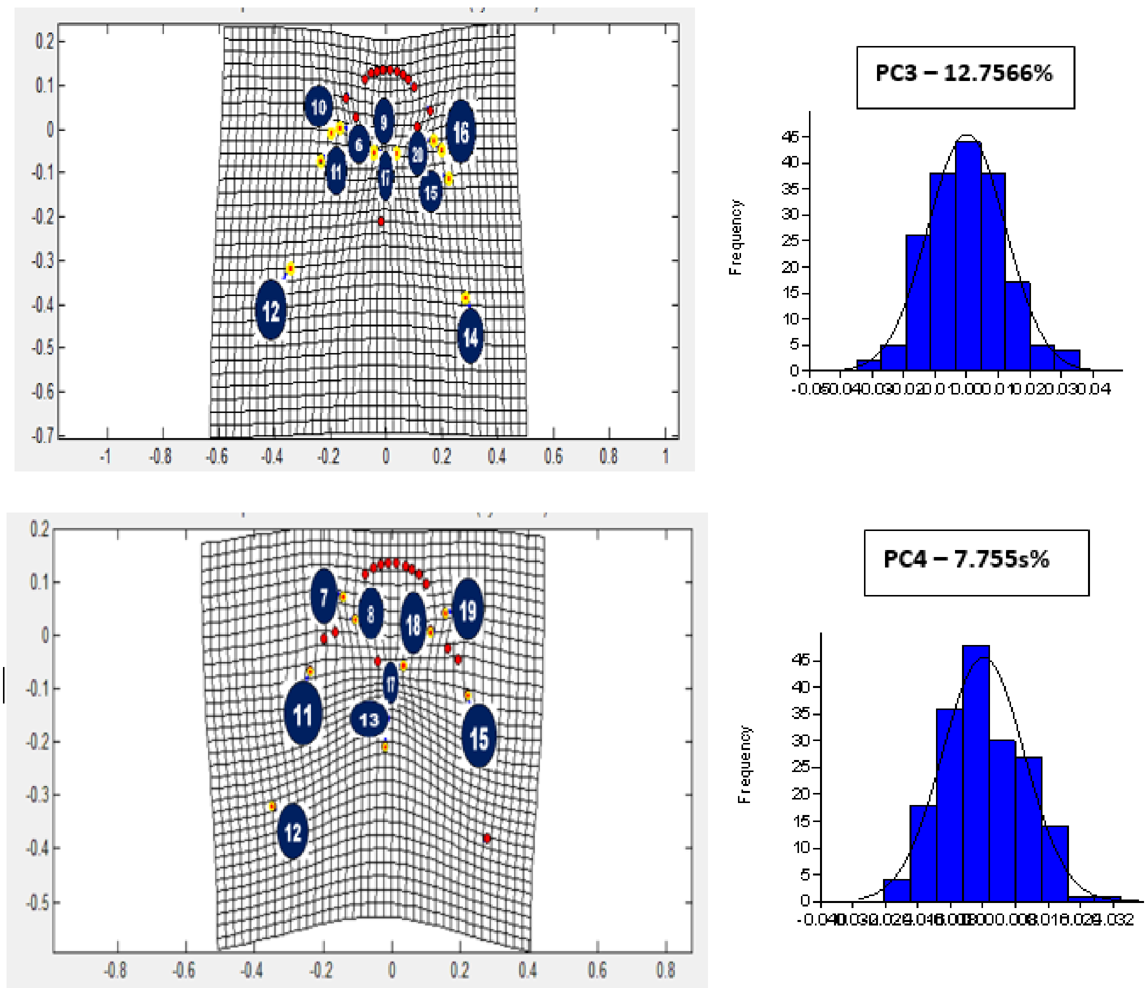


Figure 4. The principal components (PC) implied deformation grid and histogram of individual (symmetric) in *R. marina* pooled species showing distribution of asymmetry. The percentages indicate the proportions of variation for which the respective principal components account.

PC1 to PC4. These landmarks can indicate the number of affected landmarks in the head shape and parotid gland of the R. marina. To visualize the actual affected landmarks, Figure 4 shows the actual digitized image of the pooled cane toad frogs with the PCA deformation grid.

In this the assistive mechanism of the frog in order to compensate with the stressful environment probably result to significant levels of fluctuating asymmetry in its morphology. Frog mobility is considered an advantage for determining direct impact of stressors of the cane toad R. marina specifically in the parotid gland region and in some portion of the head shape configuration.

The figure 4 shows the distribution of asymmetrical configuration of R. marina frogs in pooled. The number of symmetric individuals is shown as the bar graph emanating from point 0. The variations are represented by the blue marks signifying the movement of the affected landmarks. Also, adjacent is the histogram to visualize the central location, spread, and shape of the individual symmetry. The PC1, PC2, PC3, and PC4 in pooled have an individual (symmetry) score that were skewed which signify effect on its asymmetry.

CONCLUSION

The statistical results showed high variations ($P < 0.005$) on the pooled fluctuating asymmetry with a percentage of 79.964%. The results also showed higher asymmetry in pooled *Rhinella marina*. Additionally, the most affected landmarks out of (PC1 to PC4) are 11,12,13,14, and 15 that are found in the parotid gland. Moreover, because of the poor water quality in the area and air pollution that would affect the ecological health of Rhinella marina thus, there's affected landmarks of the cane toad frogs. This study validates the use of Fluctuating Asymmetry in determining the status of the ecological health in Caraga State University and focused on the affected landmarks in the head shape and parotid gland. In addition, confirming that indeed results of high fluctuating asymmetry equates for the incidence of cane toad frog *R. marina* kills in the area.

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