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Fluctuating asymmetry as an Indicator of Ecological Stress in *Rhinocypha colorata* (Odonata: Chlorocyphidae) in Iligan City, Mindanao, Philippines

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ABSTRACT

Odonata species are known to be successful biological indicators because they are particularly sensitive to human disturbances due to their habitat selection which makes them vulnerable to changes. A useful trait to monitor developmental instability (DI) and ecological stress is fluctuating asymmetry (FA), which is a measure of the differences between the left and right side of bilateral symmetrical organisms. It refers to a slight number and non-directional deviations from strict bilateral symmetry of biological objects that occur as a result of stochastic microscopic processes. In this study, fore-wing variation of Rhinocypha colorata, a Philippine endemic species was investigated. It assessed developmental stability via fluctuating asymmetry in the fore- wings of R. colorata, in three populations from three areas: Buruun, Ditucalan, Dalipuga, Iligan City, Mindanao, Philippines. Analysis was based on Procrustes method that makes comparison of FA indices of homologous points. Using landmark method for shape asymmetry, anatomical landmarks were used and analyzed using Symmetry and Asymmetry in Geometric Data (SAGE) program. Twenty landmarks on the fore-wings were tested on samples for all populations. Results obtained showed variation and significantly high FA for all populations with relatively higher FA for Dalipuga. Principal component analysis (PCA) showed that barangay Dalipuga exhibited more variations (74.93%) than that of Ditucalan (72.19%) and Buru-un (67.97%). Possible reasons behind high FA values were anthropogenic activities in the area. FA has been considered as a good indicator of DI and thus acts as a biomarker for environmental stress. Hence, results may reflect inability of the organism to cope with stressing factors and any perturbations during development. Herewith, understanding the relationship between the species and its environment would help determine the health of a given ecosystem. Nonetheless, Odonata, as bioindicator species, can play an important role for biomonitoring purposes.

Keywords: Fluctuating Asymmetry, Biological indicator, Odonata, Procrustes ANOVA, SAGE

INTRODUCTION

Odonata species such as *Rhinocypha colorata*, are known to be effective biological indicator for environmental assessments wherein, it provide information about their habitats based on its sensitivity to a particular attribute, and then assessed to make inferences about that attribute [1]. The good thing about biological indicators is that they can provide reliable, quantitative characterizations of ecological conditions [2]. The Odonata fauna plays an important role in the balance of aquatic communities, and their general visibility, diversity and abundance around freshwater bodies makes them ideal for purposes of ecological indicators [3]. They are particularly sensitive to human disturbances, owing to the two habitats they occupy in their life cycle hence making them vulnerable to changes affecting either terrestrial or aquatic habitats [4]. They can also act as an umbrella species, for the purpose of protection of a habitat which is necessary for the survival of other species [5]. All of these characteristics make odonates a very useful group of insects for habitat assessment and biodiversity monitoring.

In this respect, studies had shown that the degree of developmental instability (DI), of individuals and populations is often estimated by the level of fluctuating asymmetry (FA) Whereby, it is perceived that perturbations resulting from stochastic cellular processes mostly act locally [6] thus effects will accumulate separately on the left and right sides of a developing individual. This consequently gives rise to left-right asymmetries in development. Here, FA refers to fine and random deviations from perfect symmetry of an organism's form and it may reflect both genetic and environmental stresses making it an important theory in evolutionary biology for decades [7]. Noteworthy, is that FA is one of the few morphological attributes for which the norm i.e. perfect symmetry is known, thus it is believed to be a more stress sensitive estimator compared to traditional use of fitness measures (e.g. survival) [8,9]. The use of FA as a conservation tool has been promoted in line with its potential to predict future stress-mediated changes in fitness [10,11].

Moreover, for biomonitoring purposes, there is growing evidence from various studies that FA, can act as a universal measure of developmental stability (DS) and predictor of stress-mediated changes in fitness [12]. DS is defined as the ability of individuals to buffer its development against any small and random perturbations due to cellular processes and thus, accurately develop an expected phenotype given their genotype and the environment [13, 14] and the converse of which is known as developmental instability (DI). Hypothesis assumes that corresponding body sides of an organism are under the same control of genetic pathways during development and presumably experience similar external effects thus, expected deviations from symmetry could be reflective of local perturbations that could break developmental homeostasis [15, 16]. Thus, it is in this context that the pattern of symmetry of bilateral structures (e.g. wings) has been widely used as a marker for DI [17].

Hence, this study utilizes FA as a potential indicator of DI and stress in populations of *R. colorata*, which is an endemic species of the Philippines [18]. This study will generate knowledge and provide information on the variation and nature of species in view of the assumption that FA has costs and reflects the quality of individuals. Information obtained may also aide in the development of tailor-fit conservation programs.

Fig. 1. Geographical map showing the three sampling sites: Buru-un, Ditucalan and Dalipuga, Iligan City, Philippines

MATERIALS AND METHODS

Collection, Preservation and Mounting

Sampling was conducted in Iligan City, Philippines. Three sites were randomly selected according to presence of large freshwater bodies. *R. colorata* males were collected in Buru-un (8°10'3.77"N 124°10'39.16"E), Ditucalan (8°9'46.81"N 124°11'31.53"E) and Dalipuga (8°17'59.35"N 124°16'15.44"E) (Figure 1). For this study only males

(N=30 per site) were utilized because in comparison to female *R. colorata*, the male species are more effective as biological indicator since they stay more often alongside freshwater systems and females only tend to visit freshwater areas during mating season [19]. The samples were collected using sweep nets and were placed in paper triangles. Technical grade acetone was used to preserve the samples. Fore- wings (left and right) of the samples were separated carefully and mounted on a 7x6 inches transparent glass. Only the fore-wings were utilized because they are said to be more prone to adapt to environmental changes and geographic variation especially for wide-ranging species [20]. It reflects adaptations of a population to local environments and biotic factors [21, 22]. Adult specimens were identified as *R. colorata*, using existing illustrated keys and guides.

Digitization and Acquisition of Landmark Data

Digital images were obtained using HP Scanjet G2410 flatbed scanner at 1200 resolution (dpi) for the mounted wings. Geometric morphometric data consist of 2D or 3D Cartesian landmark coordinates (relative to some arbitrarily chosen origin and axes). Landmarks are points of correspondence on each specimen that match between and within populations or, equivalently, biologically homologous anatomical loci recognizable on all specimens in the study [23, 24]. Building TPS files and landmark assignment were done using tpsUtil and tpsDig2 software. Sample images were processed in triplicates to minimize error. Descriptions of landmark locations were shown in Table 1 and Figure 2. There are a total of 20 landmarks in the fore- wing.

Table 1: Description of assigned landmarks on both left and right fore- wing of *R. colorata* male

Landmark #	Position
1	1st junction of costa
2	junction of analis and anal crossing
3	Proximal end of the 1st Anal vein (A1)
4	Proximal end of the Subcosta (Sc)
5	nodus
6	subnodus
7	nodal junction of costa
8	basal end of the anal vein
9	split of 2nd and 3rd branch of radius
10	beginning of 2nd intercalary radial vein
11	postero-lateral and proximal end of the pterostigma
12	proximal, exterior corner of pterostigma
13	distal, exterior corner of pterostigma
14	postero-lateral and distal end of the pterostigma
15	termination of 2nd radial vein
16	termination of 3rd radial vein
17	distal end of radial branch
18	termination of cubitus
19	junction of cubitus and 5th post-nodal cross vein
20	junction of costa and 5th post-nodal cross vein

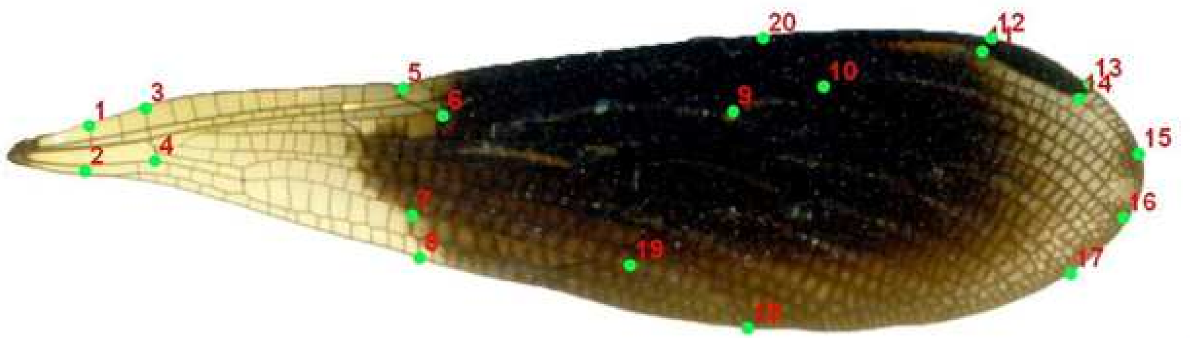

Fig. 2. Locations of the twenty (20) landmarks in the fore- wing of *R. colorata* male

Measurement of Fluctuating Asymmetry (FA)

FA levels of *R. colorata,* were obtained using the SAGE (Symmetry and Asymmetry in Geometric Data) program. This software analyzed the x- and y-coordinates, using a configuration protocol that corresponds to both sides of the male fore-wings. Matching symmetry protocol was applied in this case for both the left and right fore-wings.

Procrustes methods were used to analyze shape by superimposing configurations of landmarks into two or more specimens to achieve an overall best fit. Procrustes superimposition analysis was performed with the original and mirrored configurations simultaneously [25].

Herewith, the squared average of Procrustes distances for all specimens is the individual contribution to the FA component of variation within a sample. In order detect the components of variances and deviations, a Procrustes ANOVA was used. The ANOVA used mostly for FA is a two-way, mixed model ANOVA with three replicates [13, 26, 27]. The effect called "*sides*" is the variation between the two sides of the individual; it is a measure of directional asymmetry (DA). The effect called "*individuals*" is the variation among individual genotypes (size and shape variation). The individual's mean square is a measure of total phenotypic variation and it is random. The "*individual by sides*" interaction is the failure of the effect of individuals to be the same from side to side; it is a measure of fluctuating asymmetry and antisymmetry; variations could be dependent to both environmental and genetic conditions [28].

Moreover, PCAs of the covariance matrix associated with the component of FA variation were performed, to carry out an interpolation based on a thin-plate spline analysis to visualize shape changes as landmark displacement in deformation grids simultaneously [25].

RESULTS AND DISCUSSION

This study utilizes FA as a potential indicator of DI and stress in populations of *R. colorata,* which is an endemic species of the Philippines. The use of FA as a conservation tool has been promoted in line with its potential to predict future stress-mediated changes in fitness [10, 11]. An underlying assumption of FA analysis is that the development of the two sides of bilateral symmetrical organisms are often influenced by identical genes and thus, non-directional differences between the sides must be environmental in origin and may reflect accidents occurring during development [14, 29, 30]. FA of the right and left fore-wings of *R. colorata* were assessed through Procrustes method using SAGE software and wing variations between sampling sites were also determined.

The FA indices were determined using the landmark coordinates including the product of the coordinates of left and right homologous points which provided the final result of the Procrustes ANOVA (Table 2). Noteworthy, is the *individual by sides* interaction which is the failure of the effect of individuals to be the same from side to side. It is a measure of fluctuating asymmetry and antisymmetry thus, a mixed effect. The error term is the measurement, and is a random effect. Only '*individual by sides*' interaction denotes FA [13, 31]. Hereby, the interaction of '*individual by sides*' showed a high value of mean square and a low value of mean square measurement error. Thus, the F value suggested highly significant FA for all samples of *R. colorata* from three sampling sites in Iligan City with *P<0.001 where, Dalipuga exhibited a higher FA value. The results of the Procrustes ANOVA indicated a random variation (FA) between the left and the right sides of the landmark parts of the fore-wings from the three sampling sites rather than non-random differences among sides.

Table 2: Procrustes ANOVA results for the fore-wings of *R. colorata* from the three sampling sites

EFFECTS	SS	DF	MS	F	Significance
Dalipuga					
Sides	0.3296	32	1.61E-04	0.99065	
Individual x Sides	0.07272	448	0.00016232	4.8207	High
Measurement error	0.064649	1920	3.37E-05	--	
Buruun					
Sides	0.0035793	32	1.11E-04	1.0558	
Individual x Sides	0.047461	448	0.00010594	2.8441	High
Measurement error	0.071517	1920	3.72E-05	--	
Ditucalan					
Sides	0.010481	32	0.00032754	2.3246	
Individual x Sides	0.063124	448	0.0001409	4.1492	High
Measurement error	0.065201	1920	3.40E-05	--	

*Note: side= directional asymmetry; individual x sides interaction =fluctuating asymmetry; * P < 0.001, ns – statistically insignificant (P> 0.05); significance was tested with 99 permutations.*

The fore-wings were used in this study because fore-wings are more prone to adapt to environmental changes and geographic variation in wide-ranging species [20]. This often reflects adaptations of a population to local environments and biotic factors. Such adaptations are expected to end up in (sub) speciation, but it is not always easy to determine when this process has taken place. A proper comprehensive analysis of wing shape may thus provide an insight in phenotypic variation related to flight performance, a character that should be under selection [21, 22].

Results suggested that such variations or asymmetry were possibly due to various stressors present in the areas. Sadeghi *et al*., 2009 demonstrated that environmental stress affects the species phenotypically as they strive to adapt to changes [32]. Human interventions were present in the three sampling sites. Noteworthy, is that freshwater systems in Barangay Dalipuga were commercialized as recreational sites and some parts were used as public washing area by the populace. Meanwhile, barangay Ditucalan freshwater systems were also considered as public washing area and Barangay Buru-un was also commercialized as tourist destination. High level of environmental stress may give rise to low developmental stability in organisms, which is measured as a high level of fluctuating asymmetry [33]. Herewith, individual and population levels of bilateral asymmetry have been shown to relate positively to a wide range of abiotic, biotic, and genetic stresses, although the strength of the association varies considerably between taxa, traits, and or types of stress [34-39].

Accordingly, stress in this field can be clearly manifested as high levels of asymmetry. A possibility behind high levels of FA arises from the differences in genetic composition of the populations resulting in different tolerance to stress. Populations in their respective locations might have experienced developmental perturbations/noise early in life which resulted to the observed deviations from bilateral symmetry based on the trait examined. The possible sources of developmental noise include exogenous and endogenous stresses such as low habitat quality to low genetic heterozygosity among others [40]. In addition, according to Mpho et al., 2000, the possible causes of developmental instability were well studied and include a wide range of environmental factors (e.g. deviant climatic conditions, food deficiency, parasitism, pesticides) and genetic factors (e.g. inbreeding, hybridization, novel mutants). Such factors may also increase stress to populations. Hence, FA can be utilized as an indicator of individual quality and adaptation thereby, also demonstrating the potential for FA as a bioindicator of stress and developmental instability of populations [41].

Moreover, another way to determine and examine variability in the wings for each population was through Principal Component Analysis (PCA) derived from Procrustes analysis. This analysis illustrates shape variation in accordance to the landmark points (Table 3 and Figure 3). First principal component depicts vectors at landmarks that show the magnitude and direction in which that landmark is displaced relative to the others. The second depicts the difference via the thin plate splines, an interpolation function that models change between landmarks from the data of changes in coordinates of landmarks. Here, the red dots represent the morphological landmarks used in the study. The percentage values of PCA represent the level of variability in the data [25, 42].

Based on the percentage of overall variation exhibited by PC1 and PC2 the population from Dalipuga exhibited more variation (74.93%) than Barangay Ditucalan (72.19%) and Buru-un (67.97%). Thus, higher FA value, were also observed for Dalipuga samples.

Analyses of the results from the species population in three different sites provided consistent evidence that the wings may undergo adaptive development, and the fore-wings were much affected with ecological stress also shown as of high value based on the overall principal components. Data on PCA suggested that the morphological features are said to be adaptive to changing ecological conditions. Significantly high levels of FA in the population may indicate that individuals are having more difficulty maintaining particular development and homeostasis, resulting in negative effects on the population over time [43].

Table 3: Variance explained by the first two principal components of *R. colorata* fore-wings from three barangays in Iligan City, Philippines

Site	PC1	PC2	Total
Buru-un	41.15%	26.81%	67.97%
Ditucalan	59.67%	12.53%	72.19%
Dalipuga	62.56%	12.36%	74.93%

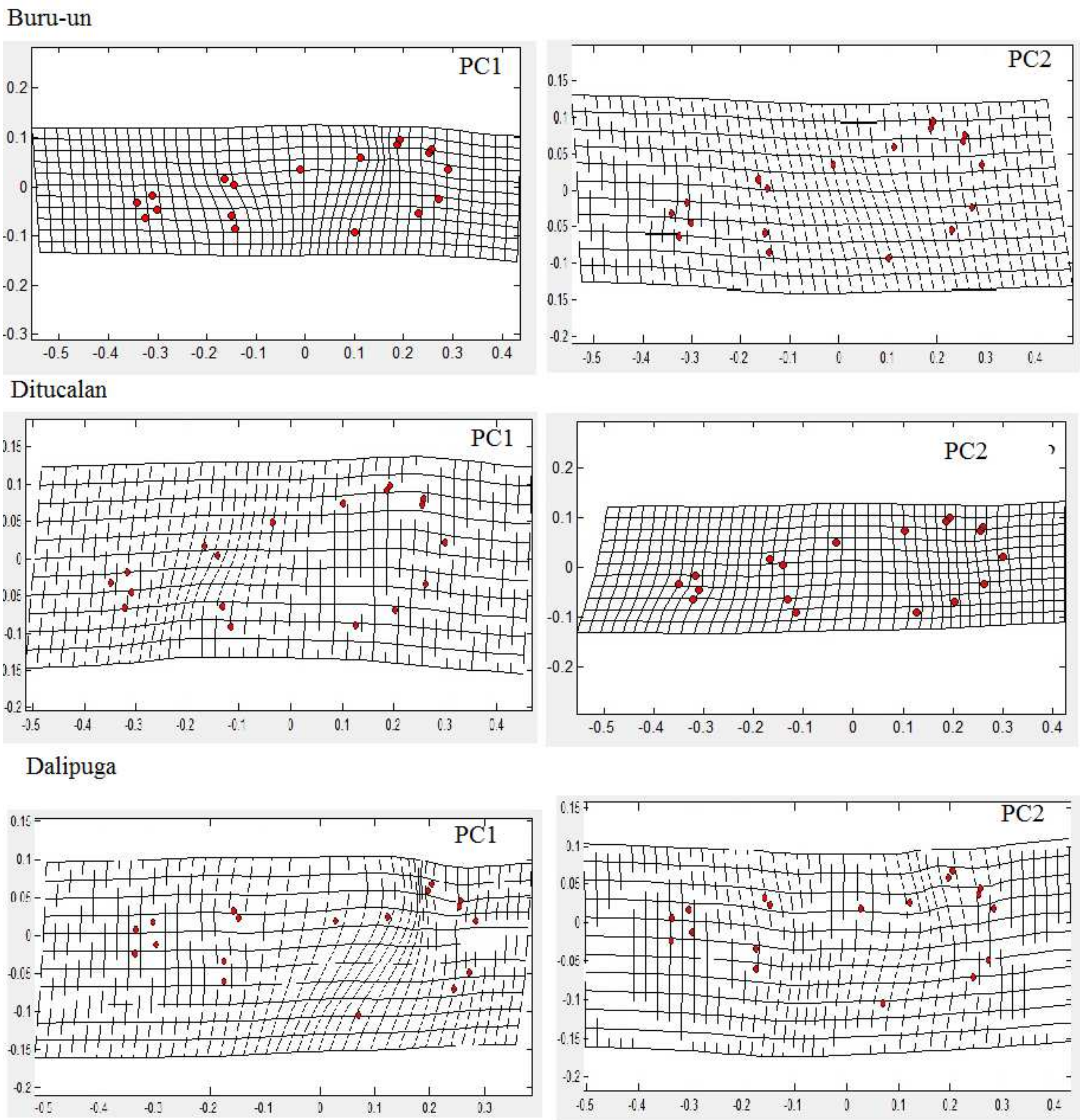

Fig. 3. PCA implied on for *individual by side* interaction of fluctuating asymmetry of the three different populations

CONCLUSION

Results have demonstrated the potential of FA as a universal measure of developmental stability (DS) and predictor of stress-mediated changes in fitness in *R colorata* fore-wings. Results yield significant FA for all populations with relatively high FA for Dalipuga. Principal component analysis (PCA) showed that barangay Dalipuga exhibited more variations (74.93%) than that of Ditucalan (72.19%) and Buru-un (67.97%). FA has been considered as a good indicator of DI and thus acts as a biomarker for environmental stress. Herewith, stress present could be attributed to pollution in general, and/or declination of habitat quality for all sampling sites or a result of the interplay of developmental "noise" and stabilizing processes, brought about by anthropogenic activities in the areas resulting to high levels of pollutants or disturbances. Moreover, results may reflect inability of the organism to cope with stressing factors and any perturbations during development. Thus, understanding the relationship between the species and its environment would help determine the health of a given ecosystem. Nonetheless, Odonata species, as a bioindicator, can play an important role for biomonitoring purposes.

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