Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

J E Z S Journal of Entomology and Zoology Studies

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2015; 3(6): 34-39

Received: 16-09-2015
Accepted: 17-10-2015

Divine Grace Quirog
Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology.

Sharon Rose Tabugo
Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology.

Correspondence
Sharon Rose Tabugo
Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology.

Population analysis via fluctuating asymmetry in the wings of *Aedes albopictus* from selected barangays in Iligan City, Philippines

Divine Grace Quirog, Sharon Rose Tabugo

Abstract
Fluctuating asymmetry (FA) measures deviations from the ideal state of symmetry, a common tool that measure environmental and genetic stresses experienced by individuals or population that affect their morphology during development. It had been used to show how developmentally stable an individual is. This study was made to evaluate developmental stability by measuring fluctuating asymmetry in the wings of *Ae. albopictus* from three different breeding sites (Tipanoy, Del Carmen, Tambacan). This is to understand the nature and variation of *Ae. albopictus* found in Iligan City. Only female adults (N=60) per site were utilized in this study. FA levels of samples from each site were measured for comparison. In this study, anatomical landmarks were subjected to Procrustes superimposition and Principal component analysis (PCA) using "Symmetry and Asymmetry in Geometric Data" (SAGE) program. Results yield highly significant FA in the wings of *Ae. albopictus* collected in the three barangays. Results implied that species populations present in the three areas can be considered as developmentally unstable.

Keywords: Fluctuating asymmetry, Procrustes ANOVA, Aedes albopictus, SAGE.

1. Introduction
Perturbations refer to local changes communicated from the environment or from the gene [1] while stability, as one of the most general characteristics of individual's development [2], acts in a system to correct minor fluctuations done by any perturbations [3]. However, when perturbation forms as a severe stress, it may impair the developmental stability of an organism. Developmental stability is defined as the ability of an organism to buffer against its genetic or environmental perturbations and produce the genetically determined phenotype [4]. Stressed individuals not shunting energy away from growth may have lower lifetime fitness [5, 6]. Stress works by changing the patterns in gene-expression [7] or by changing the flux through metabolic pathways [8] which dissipates energy away from feedback loops, thus it lessens the developmental stability of a species. In addition [9], define stress as "any environmental factor that causes a reduction in the efficient use of energy, causing a reduction in developmental homeostasis, and finally reducing long-term, total inclusive fitness." Thus, developmental stability can be affected both with genetic stresses and environmental stresses. Dobzhansky (1950) in his study, shows the basis of fitness that is associated with heterozygosity and coadaptation [10]. Heterozygous individuals are believed to buffer against environmental variation and thus to be phenotypically plastic i.e. maintain the optimal phenotype in the face of environmental fluctuation. Genomic coadaptation refers to the coadapted, harmonious interactions among gene loci. Selection at one locus influences selection at other loci, in a system of mutual interaction. Inbreeding and hybridization disturb these interactions, and both have been associated with increased developmental instability [11, 12]. The degree of fitness or stresses experience by an individual is believed to be measured by their FA or their fluctuating asymmetry value.
Fluctuating Asymmetry (FA) is defined as small random deviations from perfect symmetry in bilaterally paired structures due to subtle variations in the developmental environment [13]. FA which is known as biomarkers [14] has become a popular method for measuring phenotypic response to environmental stress [12]. It has been used as a measure of individual quality and indicator of genetic stress [15]. Asymmetry of an individual is measured as the right minus the left value of the bilaterally paired trait [13]. By studying the distribution of these asymmetries at the population level, we can distinguish between three types of biological asymmetry: fluctuating asymmetry, directional asymmetry, and antisymmetry.

These small random deviations result in a normal or leptokurtic distribution of asymmetry around a mean of zero. Directional asymmetry is characterized by a symmetry distribution that is not centered around zero but is biased significantly, towards larger traits either on the left or the right side. Antisymmetry is characterized by being centered around a mean of zero. Higher FA value indicate the species undergone some stresses while lower value shows species was under good developmental homeostasis condition [16]. Stresses may include temperature, insecticide and etc. The researcher used population estimates for fluctuating asymmetry because the used of population are usually far more useful and reliable than individual estimates. Studies of fluctuating asymmetry have been done with arthropods (insects, millipedes, arachnids), molluscs, and vertebrates (fish, reptiles, birds, and mammals) [17]. In insects, wing measurements have been used in the majority of geometric morphometric studies because wing veins which contain trachea, blood lacunae and nervous tissue are sensitive to developmental disturbances [18].

In this study, FA was investigated for *Aedes albopictus*, a mosquito of the family Culicidae [19], considered a vector of many viruses such as dengue fever and Eastern equine encephalitis virus [20]. They are mostly infected with type 1 dengue virus which continue to spread worldwide [21], especially in tropical areas. *Ae. albopictus* known as "Asian tiger mosquito" which was originated in southeast Asia was spread around the world via trading of used tires [22]. This type of mosquitoes bite during the day [23] and breed in all types of water receptacles and in all macro-habitats [24]. In a certain district in India, the researcher concluded that their number are scattered almost equally in rural and urban areas than *Aedes aegypti* [25]. A person gets infected by being bitten by an infected *Aedes* mosquito which causes a flu-like illness known as dengue fever. However, dengue fever illness will eventually become dengue hemorrhagic fever, a complicated and a severe form of dengue fever which is fatal [26].

Dengue is the most important arthropod-borne viral infection of human, affecting 2.5 billion of people worldwide and about 50 million infections occur each year [27]. As a continuing global threats [28], there is a need to increase monitoring capacity. A researcher for tropical medicine, Dr. Maria Rosario Capeding said that epidemics of dengue fever and dengue hemorrhagic fever occur every 3-4 years in the Philippines [29]. Since high incidence of dengue has been predicted in the country [30], this may prove that Philippines is one of those countries which are victims of this disease. Hence, there is the need of the country's monitoring capacity to increase and to improve against the outbreak. In 2014, there were 59,943 dengue cases recorded nationwide in which 10.47% out of it were coming from Region X (WHO, 2014; DOH-10). The Department of Health-Northern Mindanao (DOH-10) has recorded lower death rate of dengue cases across the region compared to that of the previous year. Iligan city as being part of the said region has 452 from 229 cases in year 2013 with zero deaths [31].

In this regard, this study aims to determine developmental stability using fluctuating asymmetry (FA) in the wings of *Ae. albopictus* from selected barangays in Iligan City. The barangays are: Tambacan, Tipanoy and Del Carmen respectively. The wings were investigated because it is said to have contributed much to the unparalleled success of winged insects. In the recent years, much interest has also has been devoted to the determination and examination of FA as an indicator of individual quality. Here, a hypothesis assumes that fluctuating asymmetry may reflect quality of individuals. Thus, this study provides information on the nature and variation of populations of *Ae. albopictus* found in selected barangays in Iligan City. Results of FA in the wings may imply the state of adaptation and coadaptation of *Ae. albopictus.* Data obtained on the population status and nature of *Ae. albopictus* may contribute to information and knowledge for implementation and disease control initiatives of government agencies in the Philippines.

2. Materials and Methods

2.1. Specimen Collection and Processing

Adult mosquitoes were collected from any vegetation, housing unit and grassy places in Del Carmen, Tipanoy and Tambacan, Iligan City. Collection of adult mosquito samples was done using aerial nets. The adult mosquitoes that were trapped inside the net were transferred into plastic bottles for easy transport to the laboratory. Sampling was done early in the morning and late afternoon (months of April to May 2014 and Dec. to Feb. 2015). The collected adult mosquitoes were sorted and identified in the laboratory using a stereoscope and digital microscope. Only female *Ae. albopictus* adults (N=60) per site were utilized in this study. Identification of *Ae. albopictus* was performed through existing protocols on illustrated keys presented for identification of female (*Aedes*) mosquitoes.

The wings of identified *Ae. albopictus* were gently detached from the thorax using long-handled forceps, placed on a glass slide and secured with another glass slide to cover. Wings were photographed under a dissecting microscope with consistent magnification, and the digital images were kept on file for data analysis.

2.2. Landmark Assignment and Digitization

For morphometric analysis, eighteen landmarks in the wings of female *Ae. albopictus* were used. TPS files were created using TP Sutil and landmarks were digitized using TPSdig software. Descriptions of identified landmarks are presented in Table 1. The landmarks were placed at intersections of wing veins or intersections of veins and the wing margin (Fig. 1).

Table 1: Description of assigned landmarks on *Ae. albopictus* wings.

Landmark #	Description of Landmark
1	intersection of Costa (C)
2	distal end of the Radius (R)
3	radial branch 2
4	radial branch 3
5	distal end of radial branches 4 & 5
6	distal end of M1 & 2
7	distal end of M3 & 4
8	distal end of cubital vein 1
9	distal end of cubital vein 2
10	Anal vein
11	origin of cubital 1
12	midpoint branch of cubital 3
13	medio-cubital cross vein
14	midpoint branch of medial vein
15	radio-sectoral vein
16	radio-medial cross vein
17	midpoint branch of radial vein
18	origin of radius branches 2 & 3

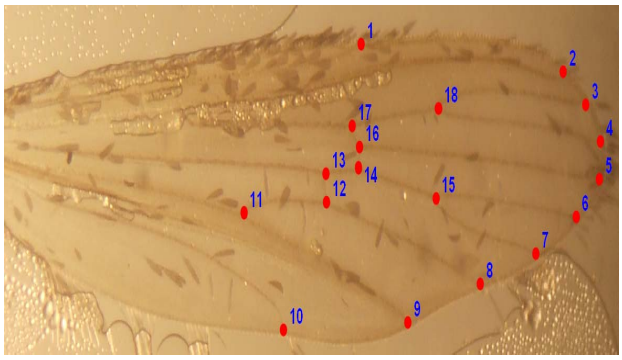

Fig 1: *Ae. albopictus* wing with eighteen landmarks.

2.3. Measurement of Fluctuating Asymmetry

The levels of FA were measured using the "Symmetry and Asymmetry in Geometric Data" (SAGE) program, version 1.0. This software analyzed the x- and y-coordinates of landmarks per individual. Procrustes superimposition analysis was performed with the original and mirrored configurations simultaneously. The least squares Procrustes consensus of set of landmark configurations and their relabelled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry. The squared average of Procrustes distances for all specimens is the individual contribution to the FA component of variation within a sample. To detect the components of variances and deviations, Procrustes ANOVA was used [32, 33].

Principal Component Analysis (PCA) as an important step in geometric morphometrics was performed in this study as a tool to understand overall patterns of shape variation and as a means for producing mathematically uncorrelated shape variables to use in subsequent analyses of wings. PCAs of the covariance matrix associated with the component of FA variation were performed for each population to carry out an interpolation based on a thin-plate spline to visualize shape changes as landmark displacement in the deformation grid [32, 34].

3. Results and Discussion

Most of the *Ae. albopictus* collected were found on wet and grassy land where coconut trees were abundant. *Ae. albopictus* mosquitoes from Tipanoy and Tambacan were both collected from areas where coconut shells were scattered on the vegetation and grassy land while *Ae. albopictus* mosquitoes from Del Carmen were collected from scrubland and on the mountainside. It was found out that male *Ae. albopictus* fly as a group while, females were often found solitary. Only female individuals were utilized for the study since they are associated with blood meal.

It was observed that *Ae. albopictus* vary in length from 2 to 10 mm [35] which has a single silver longitudinal dorsal stripe and legs with white bands. A white spot was found on the base of the Costa of its transparent wings [36]. Males were smaller than females but they were morphologically very similar, however antennae of male in comparison to female was brushier and tarsus of the hind leg was more silvery. Female lays her eggs not directly into the pool or stagnant water but only near to it. Breeding sites were likely to be close to where mosquito was found because they had short flight range Females lay eggs above the surface of the water in tree holes, coconut holes, tires or other containers capable of holding water and relying on rainfall to raise the water level and inundate the eggs for hatching wherein 150 to 250 eggs were laid per oviposition [37].

Procrustes method was used to assess the Fluctuating asymmetry (FA) value of the right and left wings of female mosquitoes. Procrustes ANOVA results yield highly significant FA in the wings of the specimens collected from Tipanoy, Del Carmen and Tambacan. Fluctuating asymmetry, directional asymmetry and antisymmetry are the three types of symmetry. Fluctuating asymmetry is characterized by small random deviations from perfect bilateral symmetry. These small random deviations result in a normal or leptokurtic distribution of asymmetry around a mean of zero. Directional asymmetry is characterized by a symmetry distribution that is not centered around zero but is biased significantly, towards larger traits either on the left or the right side. Antisymmetry is characterized by being centred around a mean of zero. Directional symmetry and antisymmetry are developmentally controlled and therefore likely to have adaptive significance while fluctuating asymmetry is not likely to be adaptive as symmetry is expected to be the ideal state. An underlying assumption of fluctuating asymmetry analysis is that the development of the two sides of a bilaterally symmetrical organism is influenced by identical genes and, therefore, non-directional differences between the sides must be environmental in origin and reflect accidents occurring during development [38, 16, 39]. Only *individual x sides* interaction denotes fluctuating asymmetry (FA) (Table 2).

Table 2: Procrustes ANOVA results of *Ae. albopictus* from three different barangays.

Effects	SS	dF	MS	F	P	Remarks
Tipanoy						
Individuals	0.10851	608	0.000178	0.79712	0.99738	Ns
Sides	0.008343	32	0.000261	1.1645	0.24719	Ns
Individuals x sides	0.13613	608	0.000224	10.4138	0	Highly significant
Measurement Error	0.05504	2560	2.15E-05	--		
Del Carmen						
Individuals	0.44498	608	0.000732	0.89406	0.91617	Ns
Sides	0.017156	32	0.000536	0.65494	0.92924	Ns
Individuals x sides	0.49771	608	0.000819	8.6462	0	Highly Significant
Mesurement Error	0.24237	2560	9.47E-05	--		
Tambacan						
Individuals	0.21967	608	0.000361	1.2456	0.003427	Ns
Sides	0.005049	32	0.000158	0.54396	0.98174	Ns
Individuals x sides	0.17635	608	0.00029	11.9624	0	Highly Significant
Measurement Error	0.062073	2560	2.42E-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; Ns-not significant

The mean square of the interaction of "*sides*" and "*individuals x sides*" effects revealed a high value compared to the low value of mean square measurement error. Thus, F values for "*individuals x sides*" effect for all sites were significant. However, Tambacan has relatively higher F value compared to the other two sites. A higher F value would mean smaller P value (*P<0.001 is significant) and significant FA. It could denote more stress experienced by the population in the area. Only *individual x sides* interaction denotes fluctuating asymmetry (FA). Directional asymmetry (DA) ('sides') was not significant in all samples. Significant FA and increase FA present inability of species to buffer stress in its developmental pathways hence, would mean developmental instability and have implications on species fitness. Hypothesis assumes that fluctuating asymmetry has costs, reflects the quality of individuals and the level of genetic and environmental stress experienced by individuals or populations during development. In this case, stresses including pollution, changing temperature, predation, competition and human activity such as fogging system, as observed from the sampling sites were believed to be the possible reasons why all the population yielded a high significant FA [40].

Another way to examine the variability of landmark points in tangent space is to run a principal component analysis (PCA) on the tangent coordinates derived from Procrustes analysis. This method may be more reliable for visualizing variation in landmarks than superimposition methods (Table 3 and Fig. 2). Based on the percentage of overall variation exhibited by PC1 and PC2, the population from Del Carmen exhibited more variation compared to Tambacan and Tipanoy. PC 1 accounts for most of the variation.

Table 3: Variance explained by first two Principal Components of *Ae. albopictus* from selected barangays.

Barangay	PC 1 (%)	PC 2 (%)	Overall (%)
Tipanoy	27	21	48
Del Carmen	41	19	60
Tambacan	37	21	58

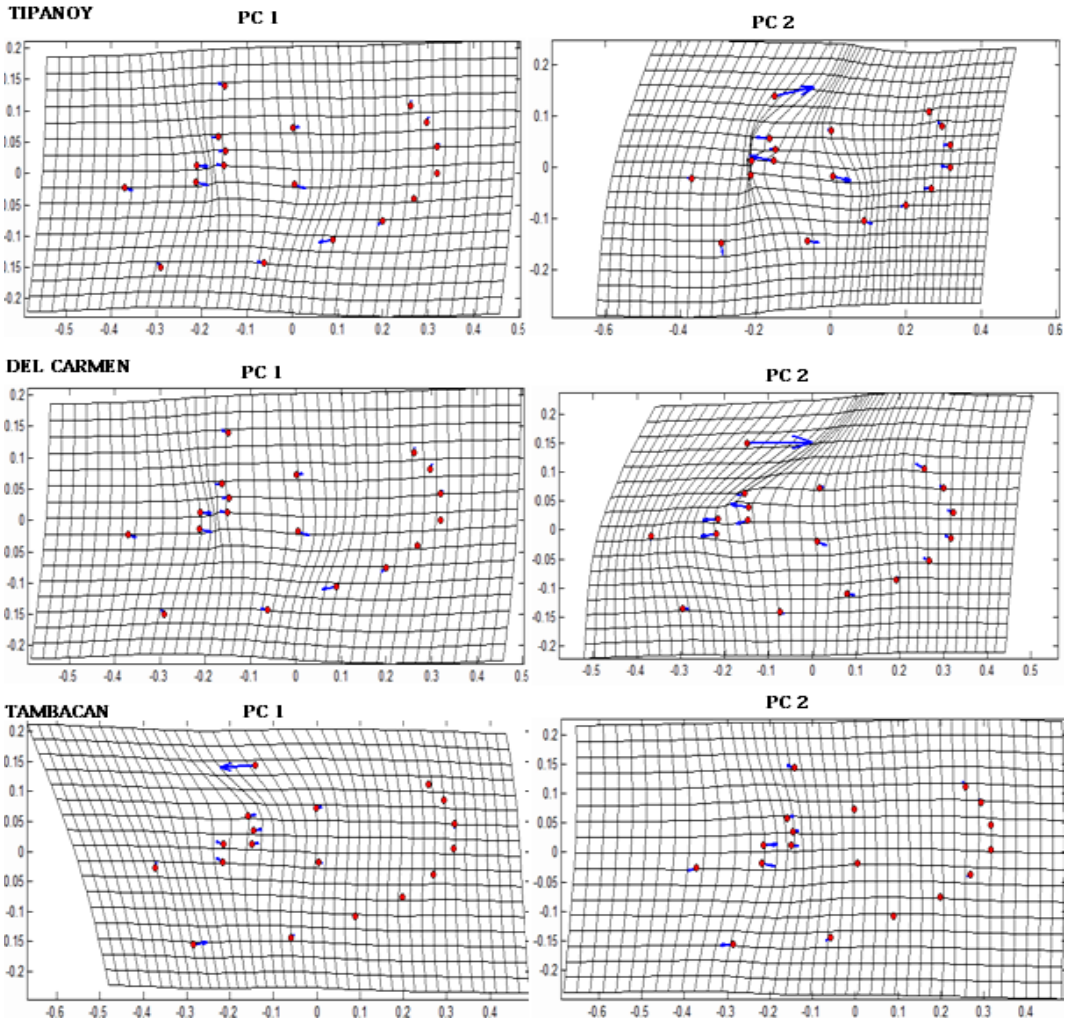

Fig 2: PCA implied deformation for *individual x side* interaction of fluctuating asymmetry of *Ae. albopictus* in three barangays (see magnitude of blue arrows)

Noteworthy, is that the population from Del Carmen were often exposed to fogging yet it seems that their number remains the same and still aggressive, despite the fact that among the selected barangays it had the highest variation. This may imply that they were able to adapt to the said stress in the environment. This situation may support the study of Rodhain and Rosen (1997) that some *Ae. albopictus* had the ability to maintain its flexibility in habitats and hosts, making it prone to faster morphological change [41]. In addition, this case may be explained by a phenomenon known as plasticity wherein some environmental elements may exert strongly influence the wing size [42, 43]. It is believed that one of the environmental elements that affect their morphology may include fogging activity implemented by the barangay due to the dengue cases

history. Fogging is believed to be the contributor as an environmental stress to a high significant FA in this site.

Meanwhile, all the mosquito samples from Tambacan, were collected near the dumpsite and river. Diverse insects were also observed in this area. The presence of different species of insects and frogs or toads imply that they were often prone to predation as indicated by the fact that some of the mosquitoes collected had impaired wings. One study reported that predation risk could impose physiological stress to an individual. The presence of dumpsite also serves as environmental stress that may contribute to the increase of developmental instability of the *Ae. albopictus* in this area [44]. It was noted that the sampling site was also on the river bank with abundant Nipa palm. Baisas *et al*. (1960) in his study stated that Nipa palm serve as a breeding site and nursery for *Aedes* mosquitoes [45]. This setting may prove the existence of different types of *Aedes* mosquito species allowing competition. Fluctuating asymmetry indicates levels of competition. Competition increases the developmental instability of a species [46]. Since population from this site yielded high significant FA, this indicates that developmental instability of the species in this area could be attributed to extensive competition. Hence, in general, the stresses that caused a high significant FA in Tambacan population were pollution, predation and competition.

Meanwhile, Tipanoy population yielded the least variation among the three because the site wherein the samples were collected was least disturbed due to the fact that the site was far from the housing unit. However, results exhibited a high significant FA for the population in this area. The sampling site selected had many coconut shells scattered on the ground. Coconut shells serve as an ideal breeding site for *Ae. albopictus* [47]. However, coconut shells could serve as a breeding site not just for *Aedes* mosquitoes but also for all types of mosquitoes. This implies that competition was also present in this area which caused elevated FA value.

Since results yielded high FA value for all the samples from the three sites, it may represent that *Ae. albopictus* as main dengue vector found in these barangays were perceived as developmentally unstable. In general, they are not fit enough to buffer against any stresses in Iligan City and that it may be easily eradicated by some preventive measure implemented by the community.

4. Conclusion

Developmental stability is defined as the ability of an organism to moderate its development against genetic or environmental conditions and produce the genetically determined phenotype. In this study, Fluctuating asymmetry (FA) was obtained using Procrustes ANOVA as a measure of developmental stability and there is an inverse relationship between developmental stability and FA. Results showed that *Ae. albopictus* found in all sampling sites barangays: Tipanoy, Del Carmen and Tambacan have high FA values, with Tambacan population having relatively high FA compared to the other sites. It could be that *Ae. albopictus* found in these areas has poor developmental homeostasis in stressful environments thus affecting their developmental stability. Samples exhibit low developmental stability. Hence, control measures can easily be implemented and eradication is plausible considering that the populations are developmentally unstable in nature. As long as some preventive measures are implemented by the community partnered by aggressiveness and persistence, eradication of dengue can be achieved. Data obtained on the nature and population status of *Ae. albopictus* may help in establishing tailor-made vector control plan.

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