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Fluctuating asymmetry as bioindicator of stress and developmental instability in *Gafrarium tumidum* (ribbed venus clam) from coastal areas of Iligan Bay, Mindanao, Philippines

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Abstract. Fluctuating asymmetry (FA) is any deviation from bilateral symmetry which is usually quantified as numerically unequal differences between the absolute of the right and left sides and is a useful trait to monitor developmental stability and ecological stress. This study sought to evaluate developmental stability of *Gafrarium tumidum* (ribbed venus clam) by the use of FA from two marine coastal areas of Mindanao, Philippines (Lugait, Misamis Oriental and Buruun, Lanao del Norte). Analysis was based on the Procrustes method and makes comparison of FA indices of homologous points and identifies the level of developmental stability of the bivalve species. Using landmark method for shape asymmetry, anatomical and mathematical landmarks were used and analyzed using Symmetry and Asymmetry in Geometric Data (SAGE) program. Thirteen landmarks were tested for samples for both populations. Procrustes ANOVA results showed variation and significant evidence of FA for both populations and no indication of Directional Asymmetry (DA). Possible explanation for significant FA for populations mean varying level of stress as experienced by populations, with Lugait relatively higher than Buruun, suggesting that there is a significant variation between the left and right side of each individual induced by the environment. 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, adaptation and quality of individuals.
Key Words: *Gafrarium tumidum,* bilateral symmetry, developmental instability, deviation of symmetry, ecological stress.

Introduction. Fluctuating asymmetry, (FA) is the variance in subtle differences between the left and the right sides in bilaterally symmetrical organisms or parts of them, and it provides a measure of how well an individual can buffer its development against internal genetic and external environmental stress during ontogeny (Van Valen 1962; Palmer 1994). The organism has the ability to buffer environmental and genetic perturbations experienced during its ontogeny and it is referred to as developmental stability (Waddington 1942; Zakharov 1989; Clarke 1998). It is assumed that it is influenced by genotype, environment and/or genotype by environment interactions (Van Dongen & Lens 2000). FA is the most commonly used tool for measuring developmental instability herewith, a direct relationship between FA and developmental instability. An assumption of FA 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. Noteworthy, is that FA reflects a population's state of adaptation, coadaptation, fitness and individual quality. It increases under both environmental and genetic stress (Waddingon 1942; Graham et al 1993). According to studies, it is hypothesized that both genomic and environmental changes can increase FA, which represents a possible deterioration in developmental homeostasis of the animal. This is apparent in adult morphology. Such genetic perturbations include intense directional selection and linked to certain specific genes (Vollestad et al 1999). Meanwhile, environmental perturbations include temperature extremes in particular, audiogenic stress, protein deprivation and exposure to pollutants (Mpho 2000; Velickovic 2004). The use of FA as an indicator of developmental stability and a measure of ecological stress (Parsons 1961, 1962, 1990, 1992; Van Valen 1962; Palmer & Strobeck 1986; Leary & Allendorf 1989) is based on the assumption that a stressful environment would result in higher FA levels than those observed in optimum environments.

FA variance of populations and absolute FA values of individuals has been found to increase with stress (Leung & Forbes 1996). One possible mechanism of relations between FA and stress is that organisms require energy to compensate for stress. This should reduce energy for growth and reproduction (Koehn & Bayne 1989), which may eventually influence populations. Stress may also reduce the energy available to maintain developmental precision (Sommer 1996). Thus, FA should increase with stress. Fluctuating asymmetry could provide advantages over other bioindicators of stress because FA is cost-effective and easy to measure (Clarke 1993). Also, FA has been related to quality of organisms; therefore a change in FA should be biologically relevant (Sommer 1996).

Meanwhile, the intertidal zone is one of the most important habitats for bivalves where it experiences large and sometimes rapid fluctuations caused by tidal actions, rain and freshwater run-off (Shumway 1977). Owing to this, intertidal bivalves are mostly exposed to multiple stressors including periodic hypoxia, hyposalinity, temperature fluctuations and pollution (Ivanina et al 2012). *Gafrarium tumidum* Röding, 1798 is commonly found on some of coastal shores, especially on sandy areas in calm lagoons near seagrasses of intertidal shores with coarse sand. It is a small sturdy clam with a ribbed pattern. This clam is a facultative mobile suspension feeder bivalve which is just is one of the molluscan species that are considered delicious and a good source of protein rich food among the sea foods. This species is a cheap important food source in many countries of the Indian Ocean (Nayar & Rao 1985; King et al 1990; Jagadis & Rajagopal 2007) and South-East Asia (Nielsen 1976; Purchon & Purchon 1981; Davy & Graham 1993; Toral-Barza & Gomez 1985).

Most previous FA studies have been interested in developmental stability per se, as a measure of individual quality or of environmental and genetic stress to which organisms are subjected (Moller & Swaddle 1997). Accordingly, this study determine developmental stability via fluctuating asymmetry of the bivalve species *G. tumidum* obtained from two different marine coastal locations of Iligan Bay, Mindanao at the same time provide information on the nature and variation of *G. tumidum*. This study opens a new direction of investigation, where a wide field of applications at the interface of evolutionary and developmental biology still awaits exploration.

Material and Method. Both Lugait (8°20’39.57N,124°15’ 29.07E) and Buruun (8°11’28.3”N, 124°10’33.59”E) are coastal areas in Iligan Bay which are identified as direct depository of some industrial wastes/effluents. Iligan Bay is located at the southern part of Mindanao Sea, east of Panguil Bay and west of Macajalar Bay. It is bounded at the northeast by the coastal areas of Gitagum, Misamis Oriental and at the northwest by Plaridel, Misamis Occidental. The southern part is bounded by Clarin, Misamis Occidental at the west and Maigo, Lanao del Norte at the east. Iligan Bay lies approximately between 8° 30’ 31” north latitude, 123° 43’ 15” east longitude. The mouth of Iligan Bay is approximately 350 miles. It has an estimated area of about 2000 km^2 (Camarao et al 1983) (Figure 1).

The samples were cleaned, and then soft tissues were removed. Photographs of the inner right and left valves were taken using a DSLR camera and used for testing fluctuating asymmetry. Each sample image was processed in triplicate yielding a total of 300 images (75 individuals) from Lugait and 340 images (85 individuals) from Buruun, respectively. Morphometric analysis was based on unambiguous and repeatable anatomical marks of the clam. A total of 13 anatomical and mathematical landmarks were used and digitization was done using the TpsDig program (Table 1 & Figure 2).

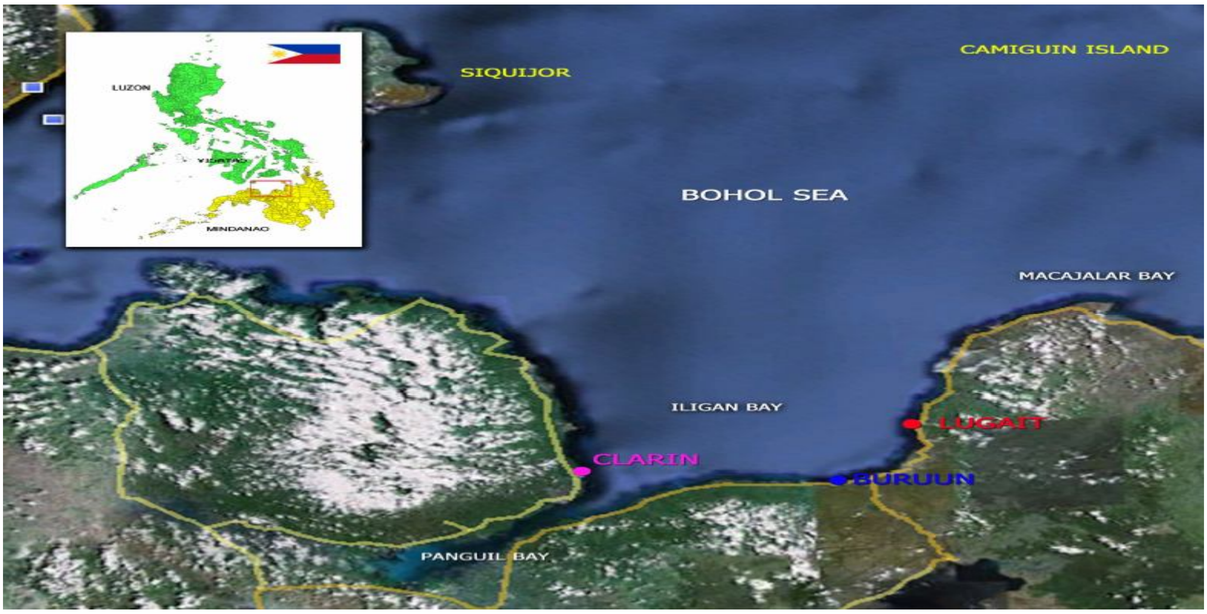

Figure 1. Map of the sampling locations: Lugait (8°20’39.57N, 124°15’ 29.07E), Misamis Oriental and Buruun (8°11’28.3”N, 124°10’33.59”E), Iligan City, Lanao del Norte, Mindanao, Philippines.

Table 1

Position of the thirteen landmarks selected in the interior valve of *Gafrarium tumidum*

Landmark #	*Position*
	Anatomical Landmarks
1	Umbo
2	End of ligament
3	Junction of posterior retractor and posterior adductor
4	Junction of posterior adductor and pallial sinus
5	Inside of pallial sinus
6	Outside of pallial sinus
7	Junction of anterior adductor and pallial line
8	Junction of anterior retractor and anterior adductor
	Mathematical Landmarks
9	Near umbo
10	Dorsal margin maxima
11	Posterior margin maxima
12	Ventral margin maxima
13	Anterior margin maxima

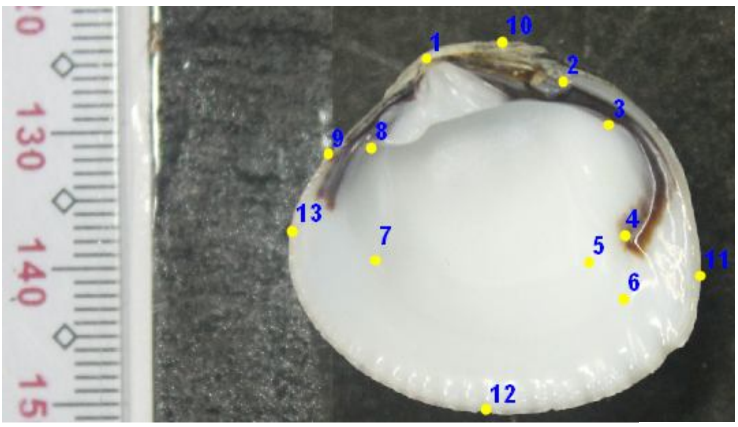

Figure 2. Location of the 13 landmarks on bivalve interior of *Gafrarium tumidum.*

In order to obtain the individual FA levels of the bivalves, the ''Symmetry and Asymmetry in Geometric Data'' (SAGE) program, version 1.0 (Marquez 2006) was used. This software analyzed the x- and y-coordinates of the 13 landmarks per individual, using a configuration protocol that divides both sides of the bivalve by considering the anatomical and mathematical landmarks as the symmetry axis (Mardia et al 2000; Klingenberg et al 2002). The configuration protocol considered 13 paired landmarks to estimate FA level. Procrustes superimposition analysis was performed with the original and mirrored configurations simultaneously. The least squares Procrustes consensus of the set of landmark configurations and their relabeled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry (Klingenberg & McIntyre 1998; Klingenberg et al 2002). The squared average of Procrustes distances for all specimens is the individual contribution to the FA component of shape variation within a sample (Zelditch et al 2004).

Deviations from symmetry are often rather small therefore, measurement errors (ME) can be particularly important in fluctuating asymmetry measures (Palmer 1994; Merila & Biorklund 1995). The measurement error was evaluated for each trait by conducting two-way analysis of variance (ANOVA) with "Side" and "Individual" as random factors. To detect the components of variances and deviations, a Procrustes ANOVA was used. The method also tested the presence of directional asymmetry (DA), the significant of asymmetry on trait size, and the presence of non-directional asymmetry which is related to FA or antisymmetry (Palmer & Strobeck 1986; Palmer 1994). Shape asymmetry data were analyzed with 99 permutations using SAGE software (Marquez 2006). The DA (directional asymmetry) "sides" and the "individuals x sides" interaction (FA), and their respective error were included as effects. The squared average of Procrustes distances for all specimens is the individual contribution to the FA component of variation within a sample. The effect called "sides" is the variation between the two sides; it is a measure of directional asymmetry. The effect called *individuals* is the variation among individual genotypes; the "individuals" mean square is a measure of total phenotypic variation and it is random. Meanwhile, 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 thus, a mixed effect. The measurement error is a random effect. Only Individual x Sides interaction denotes fluctuating asymmetry (FA) (Samuels et al 1991; Palmer & Strobeck 1986, 2003; Carpentero & Tabugo 2014).

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 to visualize shape changes as landmark displacement in deformation grids (Marquez 2006).

Results and Discussion. An underlying hypothesis of FA analysis is that the development of the two sides of a bilaterally symmetrical organism is often influenced by identical genes and thus, non-directional differences between the sides must be environmental in origin and reflect accidents occurring during development (Palmer 1994; Van Valen 1962; Gangestad & Thornhill 1999). Fluctuating asymmetry of the right and left valves of *G. tumidum* collected from Buruun and Lugait, were assessed through Procrustes method using SAGE software. FA is directly related to developmental instability (DI), hence, a tool in investigating DI. The ubiquity of symmetry is a major advantage of FA over other measures of developmental instability. One could compare developmental instabilities and examine the underlying cause. Here, FA refers to small random deviations from perfect symmetry in bilateral paired structures (i.e. right and left valves) and it is thought to reflect an organism's ability to cope with genetic and environmental stress during development. Its utility as a bioindicator of such stresses is based on the assumption that perfect symmetry is an *a priori* expectation for the ideal state of bilateral structures. It may reflect a population's average state of adaptation and coadaptation (Graham et al 2010; Parsons 1990; Galbo & Tabugo 2014). Also, it is thought to increase under both environmental and genetic stress (Graham et al 2010).

FA was determined using the coordinates of the tangential space including the product of the coordinates of the left and right homologous points in formula which provided the final result of the Procrustes ANOVA (Table 2). 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. F values for "individuals x sides" effect for both sites were significant. However, Lugait has relatively higher F value than Buruun. A higher F value would mean smaller P value (*P<0.001 is significant). Only Individual x Sides interaction denotes fluctuating asymmetry (FA). Directional asymmetry (DA) ("sides") was not significant in all samples.

Table 2

Procrustes ANOVA results of *Gafrarium tumidum* from Buruun, Iligan City, Lanao del Norte and Lugait, Misamis Oriental, Mindanao, Philippines

Effect	*SS*	*dF*	*MS*	*F*	*Remarks*
			Buruun		
Sides	0.0047361	22	0.00021528	1.02170	ns
Individual x sides	0.38939	1848	0.00021071	1.1398*	significant
Measurement error	0.69139	3740	0.00018486	-	-
			Lugait		
Sides	0.0055874	22	0.00025397	1.2111	ns
Individual x sides	0. 34139	1628	0.0002097	1.603*	significant
Measurement error	0.43169	3300	0.00013081	-	-

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 higher the F value the greater the stress is. It was noted that coastal areas in Lugait are lined with many industries. Thus, wastes and effluents from industries and fluvial vehicles that go directly to the coastal waters may contribute to ecological stress, especially, organisms inhabiting the intertidal zones.

In this regard, stress in this field can be clearly manifested as high levels of asymmetry. High FA would mean high developmental instability, the inverse of developmental stability. A possible explanation for high levels of FA detected may rise from the differences in genetic composition of the populations resulting in different tolerance to stress. Also individuals in their respective locations might have experienced developmental perturbations/noise early in life (exogenous and endogenous stresses such as low habitat quality to low genetic heterozygosity) which resulted to the observed deviations from bilateral symmetry based on the trait examined (Utayopas 2001). Other factors include genetic factors (e.g. inbreeding, hybridization, novel mutants) and environmental factors (e.g. food deficiency, deviant climatic conditions, parasitism, pesticides). Hence, FA can be used as a bioindicator of individual quality and adaptation thereby, also demonstrating the potential for FA as a biomarker of stress and developmental instability of populations of bivalves (Mpho 2000).

Moreover, Principal Component Analysis (PCA) was also performed in order to visualize the covariance shape change for each principal component and to see the general direction and magnitude of the fluctuation for each landmark. The red dots represent the morphological landmarks used in the study while the blue arrows indicate the direction as well as the magnitude of the fluctuation. The percentage values of PCA represent the level of variability in the data. Here, the amount of overall variation exhibited by PC1 and PC2 of samples from Lugait exhibited more percentage of variation than samples from Buruun (Table 3 & Figure 3). Also, higher FA was exhibited by the samples from Lugait.

Table 3
Variance explained by first two principal components between populations of *Gafrarium tumidum* from Buruun, Iligan City, Lanao del Norte and Lugait, Misamis Oriental

G. tumidum	*PC1 (%)*	*PC2 (%)*	*Overall (%)*
Buruun	19.8	18.1	37.9
Lugait	22.1	17.3	39.4

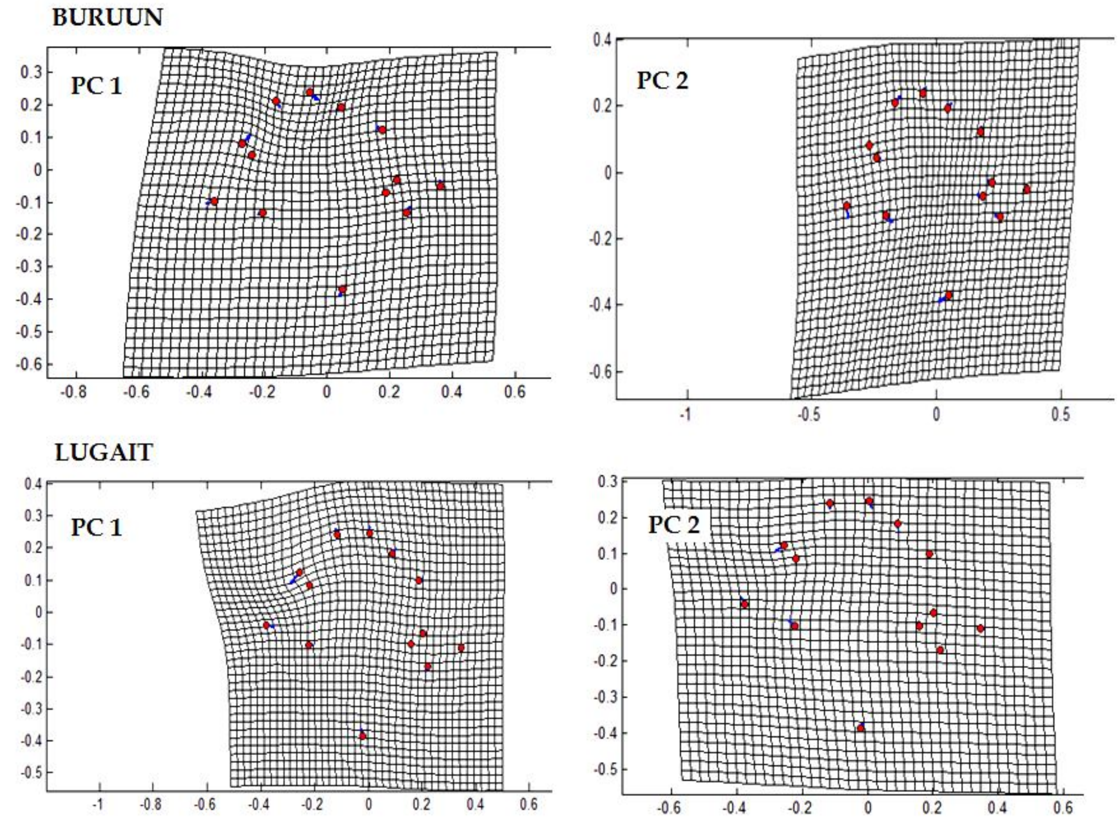

Figure 3. PCA implied deformation for *individual x side* interaction of fluctuating asymmetry of G*. tumidum* in Buruun and Lugait.

It has been argued that the degree of fluctuating asymmetry (FA) of morphological traits may provide a valuable indicator of environmental and/or genetic stress including the degree of such stress (Leary & Allendorf 1989). Thus, it has a potential use in conservation biology, where measurements of asymmetry can indicate that some populations are under some kind of stress. In this study, measurement of FA proved to be effective as an indicator of ecological stress and developmental instability.

Conclusions. This study demonstrates the potential of FA as a bioindicator of stress and its efficacy in measuring developmental instability in *G. tumidum.* Results yield that species from Lugait have higher FA than species from Buruun. The amount of overall variation exhibited by PC1 and PC2 of samples from Lugait (39.4%) was relatively higher than Buruun (37.9%) based on the Principal Component Analysis. A higher FA would mean lower developmental stability. Moreover, stress present could be attributed to pollution in general, and/or declination of habitat quality for both sampling sites. Thus, FA is indeed a good indicator of levels of developmental instability as well as a useful tool in detecting ecological stress in organisms/populations. An increase level of FA, have possible implications on species fitness and adaptation. Knowledge gained from various species and their population dynamics is vital in developing programs to preserve biological diversity especially, populations in coastal areas.

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