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Fluctuating asymmetry as bioindicator of stress and developmental instability in *Perna viridis* (Asian Green Mussel) from the coastal areas in Northern Mindanao, Philippines

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Abstract

Perna viridis also known as Asian Green Mussels are ecologically, commercially and aesthetically important. However, populations often experienced stress. They are edible species of saltwater clam and have been cultivated, and commercially harvested in the Philippines also worldwide. Fluctuating Asymmetry (FA) is apopular tool to estimate the stress, quality, health of individuals and populations and used to measure developmental stability or the organism's ability to buffer environmental and genetic perturbations. This study demonstrated the use of FA for monitoring developmental stability of bivalve species *P. viridis*. It investigated the differences of FA of three different populations from Baliangao, Misamis Occidental; Mukas and Tubod, Lanao del Norte. Thirteen anatomical and mathematical landmarks were used and subjected to Procrustes superimposition and Principal Component Analysis (PCA) using "Symmetry and Asymmetry in Geometric Data" (SAGE) program. Results yield significant evidence of FA for all the populations examined. High significant values for FA were observed in the three sampling sites which were environmentally disturbed due to anthropogenic activities such as residential pollutants or wastes from ship terminals. Mukas showed the highest value for FA as there are fluvial vehicles present in the site. The three sites also exhibited significant directional asymmetry (DS) which suggests that variation in size or left-right side of each individual could be a product of genotype-environment interaction. Thus, the study demonstrates the potential of FA as an indicator for environmental stress.

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Introduction

Aquatic organisms experience environmental stresses including temperature fluctuation, salinity shift, oxygen deprivation and pollution as well as disease-causing biotic stressors such as bacteria, virus, fungi and parasites. Mussels are important ecologically, commercially and aesthetically and often experience stress. They play a key role in food chains, consuming plankton and other filter food and being consumed by fish, birds, marine mammals, other vertebrates, and various invertebrates. They also help to filter water, being natural water purifiers, and are indicators of water pollution. Hence, mussels are most commonly used organisms as pollution biomonitors of the marine environment around the world (Acosta and Lodeiros, 2004; Azizi *et al*., 2018). *Perna viridis* or known as Asian Green Mussel naturally occurs throughout tropical Asia. It is a large species of mussel which ranges from 8-16 cm in length. The shell tapers in size as it extends to the anterior (Rajagopal *et al*., 2006). The ventral margin (hinge) of the shell is long and concave. The periostracum, a thin outer layer, covers the shell. In juveniles, the periostracum has a bright green colour. As the mussel matures to adulthood, the periostracum fades to a dark brown colour with green margins. It is an invasive species and its rapid growth causes biofouling on ships, industrial plants and other man-made structures and can cause clogging of pipes and intakes. *P. viridis* is a good sample for indicating any environmental stress.

In this respect, Fluctuating asymmetry (FA) is considered a good descriptor of developmental stability, in that it reflects both genetic and environmental stresses and this has been a prominent paradigm in evolutionary biology for decades (Carchini *et al*., 2000). Because all life forms are more or less symmetrical, FA may be observed in all taxa (Graham *et al*., 1993). 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 (Waddington, 1942; Carpentero and Tabugo, 2014; Ducos and Tabugo, 2014).

It is notable that FA is important in that it reflects a population's state of adaptation, coadaptation, as well as fitness and individual quality. FA increases under both environmental and genetic stress (Waddingon, 1942; Graham *et al*., 1993). For that reason, one could see and compare developmental instabilities of invertebrates and vertebrates and attempt to decipher the underlying causal stress.

Fluctuating asymmetry is used by biologists to study the susceptibility of organisms to changes in environmental quality for decades now. As a sensitive indicator for environmental stress, it is a legitimate tool particularly when studies verify the biological relevance of stressors for the study organism and is particularly advantageous due to the case in identifying optimal levels (i.e. perfect symmetry) compared to physiological biomarkers (De Anna *et al*., 2013). Bioindicators are defined as functional measures of exposure to various stressors and can serve as an early warning system declines in environmental quality and population health (Adams *et al*., 2001).

Moreover, Fluctuating asymmetry (FA) appears to be a good bioindicator of the state of environmental quality. This is an easy way to do and inexpensive way of determining if the environment is capable of sustainable development (Angtuaco and Leyesa, 2004).

In this regard, this study was done to investigate the potential of FA as a bioindicator of environmental stress and determine developmental instability in *P. viridis* (Asian Green Mussel). Thus, results of the study would imply that individual FA can be used as a measure of genetic and/or environmental stress and at the same time provide useful information on the nature and variation of *P. viridis*.

Materials and methods

Sampling area, collection and preparation of bivalves

Perna viridis bivalve samples were taken from the marshes of Baliangao, Misamis Occidental (08°40′N 123°36′E), Mukas, Lanao del Norte (8° 5' 24" N 123° 51' 11" E) and Tubod, Lanao del Norte (08°03′N 123°48′E). Fig.1 shows the location of the three sites. A total of 90 samples of *P. viridis* were collected in which one population is composed of 30 samples coming from each site. The samples were cleaned off from their soft tissues before the shells were sun-dried. Photographs of the inner and left valves were then taken using a digital camera and used for testing fluctuating asymmetry.

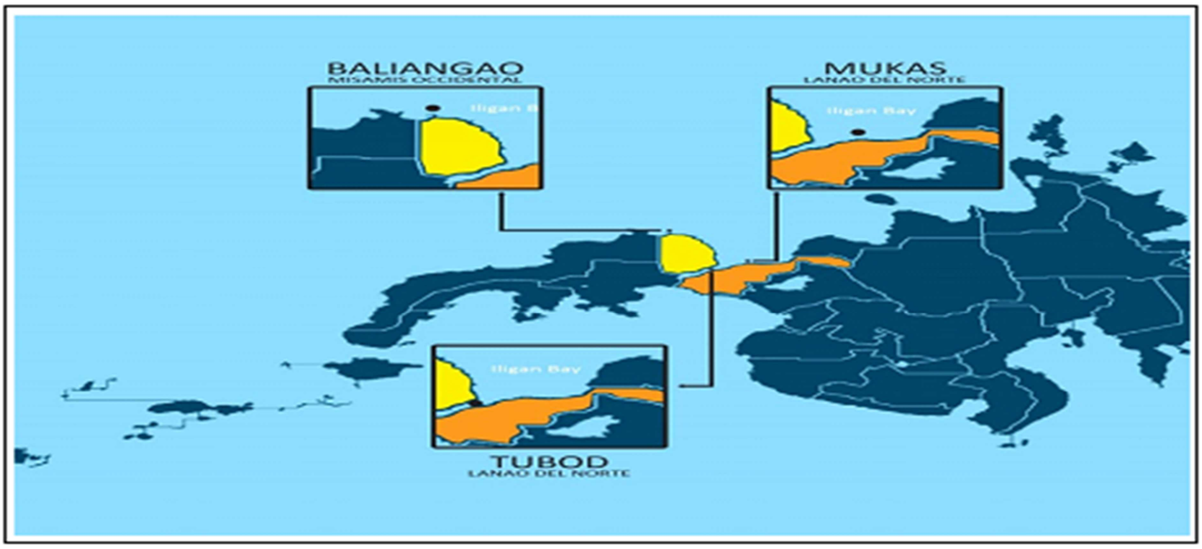

Fig.1. The three sampling sites: Baliangao, Misamis Occidental, Mukas, Lanaodel Norte and Tubod Lanao del Norte.

Landmark assignment

Anatomical and mathematical landmarks were assigned in the inner valves of *Perna viridis* with a total of 13 landmark points used which composed of 6 anatomical landmarks since the species lacks anterior adductor muscle, two landmarks were considered as mathematical, thus, a total of 7 mathematical landmarks (Fig. 2 and Table 1). The most biologically informative landmarks are anatomical landmarks which are points that are biologically homologous between organisms. Mathematical landmarks are generally less informative than anatomical landmarks, these are defined by some mathematical or geometric property such as points of maximum curvature or extreme points (Dryden and Mardia, 1998; Carpentero and Tabugo, 2014).

The right and left valves were oriented in the horizontal plane and then the anterior and posterior retractor landmarks were rotated to a horizontal line. Building of tps files and landmark assignment were done using tps Util and tps Dig version 2.12 (Adams *et al.*, 2004). Land-marking per specimen was done in triplicates to quantify and minimize measurement error.

Measurement of fluctuating asymmetry

Difference in the fluctuating asymmetry (FA) indexes of *P.viridis*from three (3) different sites (Baliangao, Mukas, Tubod) portraying different environmental conditions were assessed and the possibility of FA as a tool to determine ecological stress was determined. The right and left valves of *P. viridis* collected from Baliangao, Mukas and Tubod, were assessed through Procustes method. The levels of FA for the inner valves of *Perna viridis* were obtained using the "Symmetry and Asymmetry in Geometric Data" (SAGE) software, version 1.0 (Marquez, 2006). This software analyzed the x- and y-coordinates of landmarks per individual using a configuration protocol. Procrustes superimposition analysis was performed with the original and mirrored configurations of the valves simultaneously using the SAGE program. The software analyzed the coordinates of the landmarks per individual, using a configuration protocol for both valves. The least squares Procrustes consensus of set of landmark configurations and the relabeled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry (Klingenberg *et al.*, 1998; Marquez *et al.*, 2010). 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, a Procrustes ANOVA was used. The advantage of the mixed-model design is that one can estimate directional asymmetry and measurement error simultaneously. Sides (directional asymmetry; DA), Individual x sides (fluctuating asymmetry; FA), and their respective error were included as effects. The ANOVA used most frequently for fluctuating asymmetry is a two-way, mixed-model ANOVA with replication. The main fixed effect is *sides* (*S*), which has two levels (left and right). The block effect is *individuals* (*I*), which is a random sample of individuals from a population. The *sides byindividuals interaction* ($S \times I$) is a mixed effect. Finally, an error term (*m*) represents measurement error (replications within *sides by individuals*). The effect called *sides* is the variation between the two sides; it is a measure of directional asymmetry. Meanwhile, 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 error term is the measurement, and is a random effect. Only Individual × Sides interaction denotes fluctuating asymmetry (FA) (Palmer and Strobeck, 1986; Graham *et al.*, 2010; Parés-Casanova and Kucherova, 2013; Carpentero and Tabugo, 2014).

Principal Component Analysis (PCA)

Moreover, to detect the components of variances and deviations, Principal Component Analysis (PCAs) of the covariance matrix associated with the component of FA variation were also performed for the samples to carry out an interpolation based on a thin-plate spline and then visualize shape changes as landmark displacement in the deformation grid (Marquez, 2006).

Results and discussion

Measurement of fluctuating asymmetry

Fluctuating asymmetry (FA) is considered a good descriptor of developmental stability and appears to be a good bioindicator of the state of environmental quality (Carchini *et al.*, 2000; Angtuaco and Leyesa, 2004).

Table 1. Position of the Thirteen (13) landmarks selected in the interior valve of Pernaviridis.

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

Table 2 shows the two-way, mixed model ANOVA with expected mean squares. It shows the Procrustes ANOVA results for *Perna viridis* (Asian green mussel) from three different locations, Baliangao, Mukas and Tubod respectively.

In this respect, the F values of "individual × sides" suggested highly significant FA for the three sites: Baliangao with 3.0901; Mukas with 18.91913; and Tubod 7.2272, as indicated by low mean square value of measurement error compared to the individual by sides mean square values.

The three sites have also shown significant scores on "individual" and "side" effects.

Table 2. Procrustes ANOVA results for *Perna viridis* (Asian green mussel) from three different locations

Effects	SS	dF	MS	F	p	Remarks
1. Baliangao						
Individual	0.34644	638	0.00054301	1.1226	0.072181	ns
Sides	0.055459	22	0.0025209	5.2116	3.67E-13	******
Individuals × sides	0.3086	638	0.0004837	3.0901	0	******
Measurement Error	0.41324	2640	0.00015653			
2. Mukas						
Individual	0.26258	638	0.00041157	1.3895	1.71E-05	******
Sides	0.10336	22	0.004698	15.8611	0	******
Individuals ×sides	0.18897	638	0.00029619	18.9193	0	******
Measurement Error	0.041331	2640	1.57E-05			
3. Tubod						
Individual	0.17573	638	0.00027543	2.7644	0	******
Sides	0.023553	22	0.0010706	10.7451	0	******
Individuals × sides	0.063568	638	9.96E-05	7.2272	0	******
Measurement Error	0.036396	2640	1.38E-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 effect called "sides" which refer to the variation between the two sides, a measure of directional asymmetry (DA) herewith, significant for both populations and were of the same level. A high FA and significant DA leads to generation of phenotypes interacting with the perturbed ambient. Thus, may indicate interplay of genotype and environment under more stressful environment (Trono *et al.*, 2015).

Table 3. Variance explained by first two principal components between the three populations of *Perna viridis* (Asian green mussel).

Site	PC 1 (%)	PC 2 (%)	Overall (%)
Baliangao	42.37	18.97	61.35
Mukas	50.31	18.41	68.72
Tubod	30.77	18.41	49.19

A possible explanation for high levels of FA arises from the differences in genetic composition of the populations resulting in different tolerance to stress. Individuals 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. Possible sources of developmental noise include exogenous and endogenous stresses such as low habitat quality to low genetic heterozygosity among others (Utayopas, 2001). Moreover, 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.

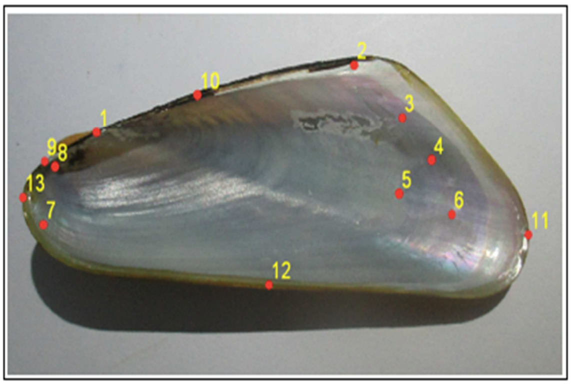

Fig. 2. Location of the thirteen (13) landmarks on bivalve interior of *Perna viridis*.

This study focuses on the three coastal areas in Northern Mindanao, Philippines with each sampling site showing different developmental noise which may be the possible cause of the different levels of high FA.

In Baliangao, Misamis Occidental, with a significant result for FA, 3.0901, a study from Metillo *et al.* (2004) indicates an examination of the character of various groups of stakeholders in the area revealed the relative weight of stakes that each group claimed on the coastal ecosystem and the pressure brought on the environment by the interplay of their activities. Some activities of these stakeholders served as examples of the heavy pressures exerted on the resources. Among these activities were: cutting of mangroves for fuel wood or for constructing the *bungsod*, a type of fishing gear; the burning of mangroves for big-time charcoal making; conversion of mangrove areas to fishpond areas; trampling of seagrass while gathering shellfish, use of destructive fishing methods and fishing gear, indiscriminate use of agrochemicals that pollute the coastal waters, implementation of inappropriate policies and irrelevant programs, inadequate knowledge of skills on biodiversity conservation; and many more, which all contributed to the destruction of several parts of the coastal ecosystem.

Meanwhile, Mukas, Lanaodel Norte has the highest significant result for FA, 18.91913, could be attributed by the presence of fluvial vehicles in the area. The main effects of these fluvial vehicles or marine transport operations on water quality predominantly arise from dredging, waste, ballast waters and oil spills. Dredging is the process of deepening harbor channels by removing sediments from the bed of a body of water. Dredging is essential to create and maintain sufficient water depth for shipping operations and port accessibility. Dredging activities have a two-fold negative impact on the marine environment. They modify the hydrology by creating turbidity that can affect the marine biological diversity.

The contaminated sediments and water raised by dredging require spoil disposal sites and decontamination techniques. Waste generated by the operations of vessels at sea or at ports cause serious environmental problems, since they can contain a very high level of bacteria that can be hazardous for public health as well as marine ecosystems when discharged in waters. Major oil spills from oil cargo vessel accidents are one of the most serious problems of pollution from maritime transport activities.

The third area, Tubod, Lanaodel Norte, which also has a significant FA level of 7.2272. The area is described as vulnerable to various impacts such as quarry activity, domestic wastes and other anthropogenic activities. All of the three sampling sites showed significant FA with different causes of developmental noise. Thus, FA can be used as an indicator of individual quality and adaptation thereby, also demonstrating the potential for FA as a biomarker of stress and developmental instability of populations.

Principal Component Analysis (PCA)

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 (Carpentero and Tabugo, 2014).

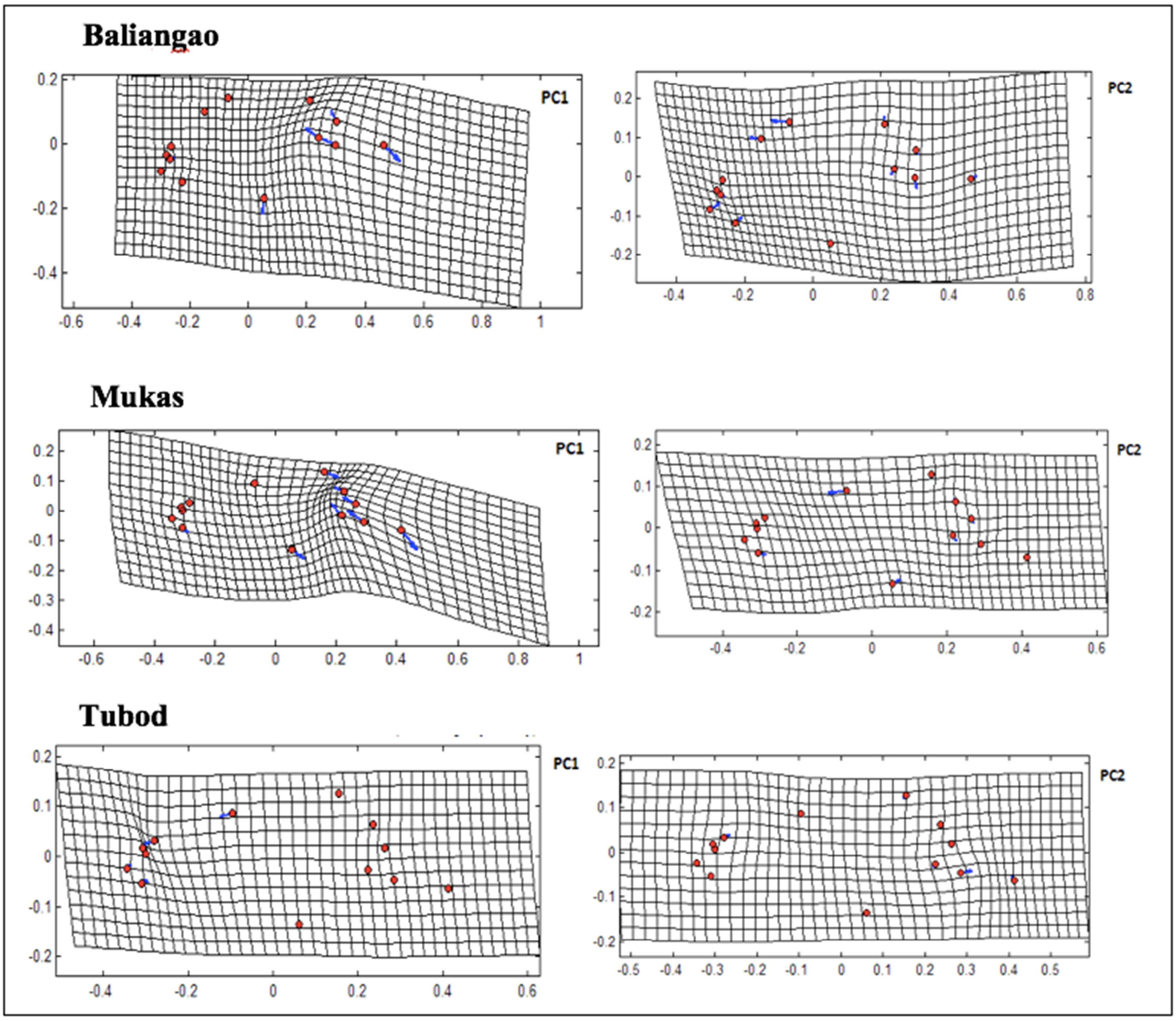

Fig.3. PCA implied deformation for *individual* × *side* interaction of fluctuating asymmetry from the three different locations.

The percentage values of PCA represent the level of variability in the data (Table 3 and Fig. 3). Here, the amount of overall variation exhibited by PC1 and PC2 of samples from Tubod was found to be intermediate from Baliangao and Mukas, with Mukas exhibiting the greatest percentage of variation (68.72%). Higher FA was also observed for Mukas. This could have been attributed by both genetic and, predominantly, of environmental stressors.

Conclusion

Highly significant values for FA were observed in the three sampling sites; Baliangao, Mukas and Tubod, which are environmentally disturbed due to anthropogenic activities such as residential pollutants or wastes from ship terminals. Mukas shows the highest value for FA as there are fluvial vehicles present in the site. The three sites also exhibited significant DS which suggests that variation in size or left-right side of each individual could be a product of genotype-environment interaction. Thus, the study demonstrates the potential of FA as an indicator for environmental stress. The relationships between homeostasis and FA can be further described as high FA results from poor developmental homeostasis hence, high developmental instability. Developmental instability, is believed to stem from various exogenous and endogenous stresses collectively referred to as developmental noise or perturbations.

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