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Fluctuating Asymmetry as bioindicator for stress in *Hippocampus* spp. (seahorse) found in Danajon Bank, Philippines

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Abstract

Seahorses (*Hippocampus* spp.) are considered as flagship species of the marine environment owing to the varied habitats they occupy. They are among the many genera whose life histories rendered them vulnerable to overfishing or other disruptions such as habitat damage. Thus, there is a need to monitor status of populations especially in Danajon Bank. This study assessed the potential of fluctuating asymmetry (FA) as a popular tool to estimate the quality, health and as an indicator for developmental instability and environmental stress in this vulnerable and endangered species in Danajon Bank. Four species of seahorse were assessed namely *H. comes, H. kuda*, *H. histrix*and *H. spinosissimus*. Sixteen anatomical landmarks were used and subjected to Procrustes superimposition and Principal Component Analysis (PCA) using "Symmetry and Asymmetry in Geometric Data" (SAGE) program. Results showed significant FA in four species of *Hippocampus* spp. both male and female. It is hypothesized that significant high FA for male samples accounts for their high vulnerability especially while providing post-zygotic care for offsprings by brooding embryos in their ventral surface. The underlying reason behind high FA may be attributed to stress of environmental origin. Significant FA somehow implies inability of these organisms to buffer stress and would mean developmental instability and have implications on species fitness, adaptation, quality of individuals and vulnerability in the marine environment. Data obtained on the nature and population status of seahorses may help in establishing tailor-made conservation programs in the Philippines.

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Introduction

Seahorses are not only charismatic creatures but also vulnerable species. Nowadays, seahorse populations had been confronted with threats due to habitat destruction and overexploitation because of its importance to aquarium trading, curios and traditional Chinese medicine (TCM). With much concern is that in the Pacific, Danajon bank (double barrier reef) spanning 97 miles along the islands of Cebu, Bohol, mainland Leyte and Southern Leyte, is the only well-documented seahorse sanctuary in the Philippines and was considered as an area where marine animals thought to have first evolved. It is considered as a home of over 200 threatened species including species of *Hippocampus* (seahorse) (Pichon, 1977; White and Cruz-Trinidad, 1998; Armada *et al.*, 2009). To date, Danajonbank, experienced very high fishing pressure and habitat disturbance. Accordingly, across Danajon Bank, coral reefs and resources known for their economic and cultural value, are in decline due to human activities (such as land-based sources of pollution and sedimentation, overfishing) and climate change (Diaz *et al.*, 2012). Along this line, monitoring seahorses is vital hence, the importance of this study. Protecting the seahorses would mean protecting diverse habitats including all marine life (Foster and Vincent, 2004) because they are often found on varied habitats which include seagrass beds, mangroves, coral reefs, sponge gardens and estuaries thus, considered as flagship species of various ecosystems (Loh *et al.*, 2014; Foster and Vincent, 2004).

For biomonitoring, the use of fluctuating asymmetry (FA) as a predictor for stress related changes have been promoted for conservation purposes. This is line with the thought that in bilateral symmetrical individuals, small random deviations from the perfect symmetry are commonly known as Fluctuating Asymmetry (FA) and it may sometimes occur. It offered an estimate of developmental "noise" (Waddington, 1942) that has been used as a measure of developmental stability (DS) and to assess the influence of environmental and genetic stress on development (Palmer and Strobeck, 1986).

Developmental stability refers to an organism's ability to buffer environmental and genetic perturbations experienced during its ontogeny (Waddington, 1942; Zakharov, 1989; Clarke, 1993). FA is considered as a reliable factor for measuring developmental instability (DI), because it reflects both genetic and environmental stresses and this has been an important theory in evolutionary biology for decades (Parsons, 1990; Ryazanova and Polygalov, 2013). FA is crucial because it reflects a population's state of adaptation and coadaptation. To add up, it is said to increase under both environmental and genetic stress (Graham *et al.*, 2010). Thus, there is a direct relationship between FA and DI. The conditions that are identified to increase FA consist of such environmental factors as temperature, nutrients, and light, as well as such genetic factors as mutation, hybridization, and inbreeding that contribute to developmental alteration (Ho´dar, 2002). A number of cases have shown a correlation between FA and several fitness components or genetic characteristics, in particular, mating success and individual level of heterozygosity (Carchini *et al.*, 2000). In this respect, the ubiquity of symmetry led to the utilization of FA over all other measures of developmental instability. FA could also provide major advantage over other indicators of stress because FA is easy to measure and cost-effective (Clarke, 1993; Tataro and Tabugo, 2015). Deviations from perfect symmetry may be measured as variances of linear dimensions, shape variation involving landmarks, or as continuous symmetry measures (Graham *et al.,* 2010; Carpentero and Tabugo, 2014). 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 (Valen 1962; Palmer 1994; Gangestad and Thornhill, 1999). Thus, FA analysis was considered in this study as an efficient tool in determining developmental instability. An assumption is that a stressful environment would result in higher FA levels than those observed in optimum environments (Parsons, 1961, 1962, 1990, 1992; Van Valen, 1962; Palmer and Strobeck, 1986; Velickovic, 2004; Leong *et al.,* 2013).

Meanwhile, stressors of interest in Danajon bank, include pollutants, changes in natural environmental parameters such as temperature, humidity, density, and shifts in resource use induced by global warming, habitat defragmentation and habitat loss, which often leads to detrimental consequences or loss of inhabitant organisms (Whiteman and Loganathan, 2001). In this regard, determining an easily measured bioindicator of stress would be of great importance in order to address conservation status of seahorses. Herewith, the main objective of this study is to determine fluctuating asymmetry (FA) of *Hippocampus* spp. in Danajon bank. FA is utilized as a potential indicator of DI and stress in populations of seahorses, which are considered vulnerable and endangered species in the Philippines.

Thereby, FA as a conservation tool has been raised, in line with its potential to predict future stress-mediated changes in fitness. There is growing evidence from various researches that FA, can act as a universal measure of developmental stability (DS) and predictor of stress-mediated changes in fitness (Graham *et. al.*, 2010). This study will provide knowledge and 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.

Materials and methods

Study area, specimen collection, identification and image processing

The study area was in Danajon bank, a double barrier, in the Pacific, spanning 97 miles along the islands of Cebu, Bohol, mainland Leyte and Southern Leyte (Fig. 1).

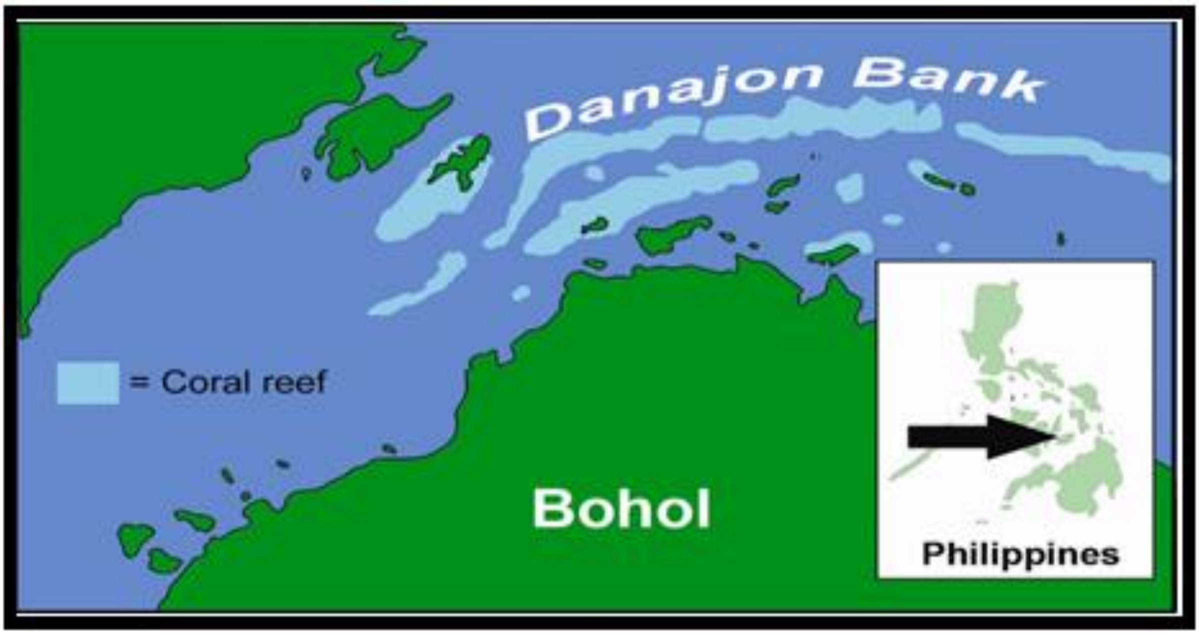

Fig. 1. Study site: Map of Danajon bank, a double barrier reef.

This is the only well-documented seahorse sanctuary in the Philippines and was considered as an area where marine animals thought to have first evolved. Danajon Bank located off northern Bohol Island, is the only double barrier reef in the Philippines and is 1 of only 3 such sites in the Indo-Pacific. This double barrier reef consists of 3 large reefs and spread across almost 130 kms. Based on dive surveys and local information from fisher folks, four species of seahorse out of seven that thrive in the Philippines are found in Danajon (Pichon, 1977; White and Cruz-Trinidad, 1998; Armada *et al.*, 2009). *Hippocampus* spp. adult seahorse specimens were in courtesy of iseahorse, phils., ZSL. Specimens were bycatch samples of fishermen in the area. Identification of samples was done through illustrated keys, Guide to the identification of Seahorses (Lourie *et al.*, 2004) and consultation of experts.

Microhabitats associated with species of seahorses were noted based on iseahorse underwater surveys (through SCUBA diving) undertaken in the area. Photographs were taken for all the samples then processed for image analysis. Digital images of the left and right lateral sides of the adult seahorses were taken using standard procedure and landmarked assignment was done using tpsDig2 software. Landmarking per specimen was done in triplicates in order to quantify and minimize measurement error. Sixteen anatomical landmarks in the lateral sides of seahorses were used. Descriptions of identified landmarks are presented in Table 1 and Fig. 2.

Fluctuating Asymmetry Analysis (FA) and Principal Component Analysis (PCA)

Fluctuating Asymmetry (FA), directional asymmetry (DA), and antisymmetry (AS) are three types of deviations from perfect bilateral symmetry. FA measures the variance in left-right differences and corresponds to a random variation, thus, can be used to measure developmental instability, whereas DA and AS are considered to be inappropriate as descriptors of developmental stability because both are developmentally controlled and are probably adaptive as asymmetries.

FA levels were assessed using the "Symmetry and Asymmetry in Geometric Data" (SAGE) program, version 1.0 (Marquez, 2006). The software analyzed the x and y coordinates of the landmarks per individual, using a configuration protocol for both left and right lateral sides of *Hippocampus* spp. (seahorses). Procrustes superimposition analysis was performed (Fig. 3) with the original and mirrored configurations of the right and left lateral sides simultaneously. The least squares Procrustes consensus set of landmark configurations and their relabelled mirror images is a perfectly symmetrical shape, while FA is the deviation from perfect bilateral symmetry (Klingenberg *et al.,* 1998; Marquez, 2006). 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 Analysis of Variance (ANOVA) was used.

Sides (DA), *individual x sides* (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 *sides* (*S*) has two levels (left and right). The block effect *individuals* (*I*) is a random sample of individuals from a population. The *sides by individuals interaction* (*S x 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. 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 x 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 (Samuels *et al.,* 1991; Palmer and Strobeck, 2003; Graham *et al.*, 2010). Moreover, to detect the components of variances and deviations, Principal Component Analysis (PCA) 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; Albarran-Lara *et al.,* 2010).

Results and discussion

Seahorses are classified as vulnerable and endangered species. They are among the many genera that have been vulnerable to overfishing and habitat destruction. The suitability of specific habitat where seahorses thrive is essential for their survival. It should provide all necessary factors to ensure survival and reproduction. The areas surrounding Danajon bank, a home for seahorses are composed of coral reefs, mangroves and seagrass beds, habitats in shallow and relatively well-protected areas. The reef is a source of livelihood and food supplying Cebu, Leyte, Southern Leyte and Bohol.

Table 1. Position of the sixteen anatomical landmarks selected on the lateral sides of *Hippocampus* spp.

Landmark #	Position of anatomical landmark
1	top of coronet
2	topmost antero-lateral of part of the eye
3	junction immediately in front of the eye (bump in front of eye)
4	upper tip of the snout
5	lower tip of the snout
6	base of snout
7	junction in proximity to the cheek
8	base of the operculum
9	eye (lateral proximal end)
10	eye (lateral distal end)
11	dorso-lateral tip of first trunk ring
12	dorso-lateral tip of fourth trunk ring
13	antero-lateral tip of dorsal fin
14	mid-lateral point of dorsal fin
15	postero-lateral tip of dorsal fin
16	ventro-lateral tip of second to the last trunk ring

It provided protection to the islands from typhoons and storms also. The decline of Danajon Bank, was due to illegal fishing and over-fishing which compounded with the use of sodium cyanide. Sedimentation has increased in recent years as land use has intensified, resulting to marginal fishers catching fewer fish every year. Habitat destruction was evident due to overfishing and densely populated coastal islands. This leads to decline of biodiversity (Diaz *et al.*, 2012). With such threat, monitoring populations is vital hence, this study. For biomonitoring, the use of fluctuating asymmetry (FA) as a predictor for stress related changes have been promoted for conservation purposes. In this study, this method was adapted to monitor seahorse populations in Danajon bank. FA is directly related to developmental instability (DI), hence, a tool in investigating DI. An underlying hypothesis for FA analysis is that the development of the two sides of a bilateral 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; Gangestad and Thornhill, 1999). Moreover, in this study FA was used as a tool because of its potential to predict future, stress-mediated changes in fitness (Cairns *et al.*, 1993; Clarke, 1993). Fluctuating asymmetry is largely a population parameter (Van Valen, 1962).

Measurement of FA levels

FA levels were assessed using the coordinates of the tangential space including the product of the coordinates of the left and right homologous points. The final result is provided in Table 2. A Procrustes two-way, mixed-model ANOVA with replication was used with expected mean squares. The expected mean squares consist of variance components for each effect and the appropriate coefficients. The effect called *sides(S)* is the variation between two sides which is a measure of DA which has two levels (left and right). The effect called *individuals (I)* is the variation among individual which can be interpreted as size/shape variation; the individual mean square is a measure of total phenotypic variation and it is a random sample of individuals from a population. The *individual x sides* interaction *(S x I)* is the failure of the effect of individuals to be the same from side to side; it is a measure of FA and antisymmetry. It is a mixed effect.

Table 2. Procrustes ANOVA results for the four species of *Hippocampus* spp. (seahorses) found in Danajon bank.

Effects	SS	DF	MS	F	P	Remarks
H. comes female						
Individuals	0.772	196	0.0039386	2.2244	1.72E-08	******
Sides	0.0049	28	0.00017533	0.099022	1	ns
Individual x sides	0.347	196	0.00117706	5.3097	0	******
Measurement Error	0.2988	896	3.3346E-04	--	--	
H. comes male						
Individuals	0032278	56	0.000594	1.3743	0.11865	ns
Sides	0.0060051	28	0.000214	0.49598	0.997706	ns
Individual x sides	0.024215	56	0.000432	32.3687	0	******
Measurement Error	0.004489	336	1.34E-05	--	--	
H. kuda female						
Individuals	0.040831	28	0.001458	2.8113	0.003977	***
Sides	0.0045605	28	0.000163	0.314	0.99846	ns
Individual x sides	0.014524	28	0.000519	38.5841	0	******
Measurement Error	0.003011	224	1.34E-05	--	--	
*H. kuda*male						
Individuals	0.006895	28	0.000246	0.63307	0.8837	ns
Sides	0.005728	28	0.000205	0.52588	0.95266	ns
Individual x sides	0.010892	28	0.000389	4.834	5.91E-12	******
Measurement Error	0.018026	224	8.05E-05	--	--	
H. histrix male						
Individuals	0.004013	28	0.000143	0.73833	0.7864	ns
Sides	0.008291	28	0.000296	1.5253	0.135	ns
Individual x sides	0.005436	28	0.000194	8.8321	0	******
Measurement Error	0.004924	224	2.20E-05	--	--	
H. spinosissimus male						
Individuals	0.007517	28	0.000268	1.2101	0.30861	ns
Sides	0.011646	28	0.000416	1.8749	0.051022	ns
Individual x sides	0.006212	28	0.000222	3.9917	2.41E-09	******
Measurement Error	0.012449	224	5.56E-05	--	--	

*Note: side = directional asymmetry; individual x sides interaction = fluctuating asymmetry; *significant, $p < 0.05$, ns – statistically insignificant; significance was tested with 99 permutations, ***** = highly significant.

The error term *(m)* is the measurement error (replications within *sides by individuals* and it is a random effect (Parés-Casanova and Kucherova, 2013). Only *individual x sides* interaction denotes FA (Samuels *et al.*, 1991; Palmer and Strobeck, 2003; Carpentero and Tabugo, 2014).

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 which indicates a significant FA for all populations from Danajon bank. F values for "*individuals x sides*" effect indicating fluctuating asymmetry (FA) for all four species were highly significant and not significant for the effect *'sides'* which denotes directional asymmetry (DA). Highest significant FA was in *H. kuda* females but could be attributed also as size/shape variation among individuals aside from environmental origin or pure FA effect since it was found also to be significant for '*individuals*' effect. Male populations showed high FA (*individual x sides*) that could be highly attributed to environmental origin since it showed not significant for both '*individuals*' and '*sides*' effect. Highest significant FA was recorded for *H. comes* males. It is hypothesized that significant high FA for male samples accounts for their high vulnerability especially while providing post-zygotic care for offsprings by brooding embryos in their ventral surface. The underlying reasons behind high FA may be attributed to stress of environmental origin considering that Danajon bank have experienced habitat degradation.

Table 3. Presence of localized trait fluctuating asymmetry in the populations of *Hippocampus spp*.

Character	*H. comes*		*H. kuda*		*H. histrix*	*H. spinosissimus*
	F	M	F	M	M	M
1. top of coronet	*	**	*	***	ns	**
2. topmost antero-lateral of part of the eye	*	**	**	*	**	**
3. junction immediately in front of the eye (bump in front of eye)	**	**	**	ns	**	***
4. upper tip of the snout	*	**	***	ns	ns	**
5. lower tip of the snout	*	***	***	ns	**	**
6. base of snout	*	**	ns	ns	ns	**
7. junction in proximity to the cheek	ns	*	*	*	**	***
8. base of the operculum	*	**	ns	*	**	ns
9. eye (lateral proximal end)	*	**	ns	**	*	**
10. eye (lateral distal end)	ns	**	**	**	*	ns
11. dorso-lateral tip of first trunk ring	*	**	***	*	**	**
12. dorso-lateral tip of fourth trunk ring	*	***	***	*	**	ns
13. antero-lateral tip of dorsal fin	*	ns	**	ns	**	***
14. mid-lateral point of dorsal fin	*	**	**	ns	*	***
15. postero-lateral tip of dorsal fin	*	***	**	*	ns	ns
16. ventro-lateral tip of second to the last trunk ring	*	**	**	*	ns	ns

***highly significant, *ns*-not significant; $p<0.05$ is significant.

In the light of the results, 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 in the marine environment.

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 (Graham *et al.*, 1993).

In this respect, results suggest that populations of *Hippocampus* spp. present in this area have poor developmental homeostasis, thus high developmental instability (DI). Hence, may reflect the quality of individuals.

The possible cause of developmental instability were well studied and it include a range of environmental factors (e.g. nutrients, light, deviant climatic conditions, food deficiency, parasitism, pesticides) and genetic factors (e.g. inbreeding, hybridization, novel mutants).

Other environmental perturbations include temperature extremes in particular, protein deprivation, audiogenic stress, and exposure to pollutants (Mpho *et al.,* 2000). Hence, the environment plays a very significant role especially in Danajon bank.

In a study by Lin *et al.*, 2006, both abiotic and biotic factors are important including the availability of food types. It demonstrated the effect of different food types and temperature on the development and hatching time of *H. kuda*. In addition, generally for fishes, Kwarnemo, 1994 stated in her study that there is an influence of temperature on the relative reproduction rates of males and females as measured based on aquarium experiments, with naturally changing as well as constant temperatures over the entire reproductive season.

Reproductive rates increased with increasing water temperature in both sexes, but more strongly among males. Limitations such as temperature, food, size and availability of nests remain (Clutton-Brock and Parker, 1992). At this point, FA is used as an indicator of individual quality and as a bioindicator tool for environmental monitoring and conservation biology (Tomkins and Kotiaho, 2001).

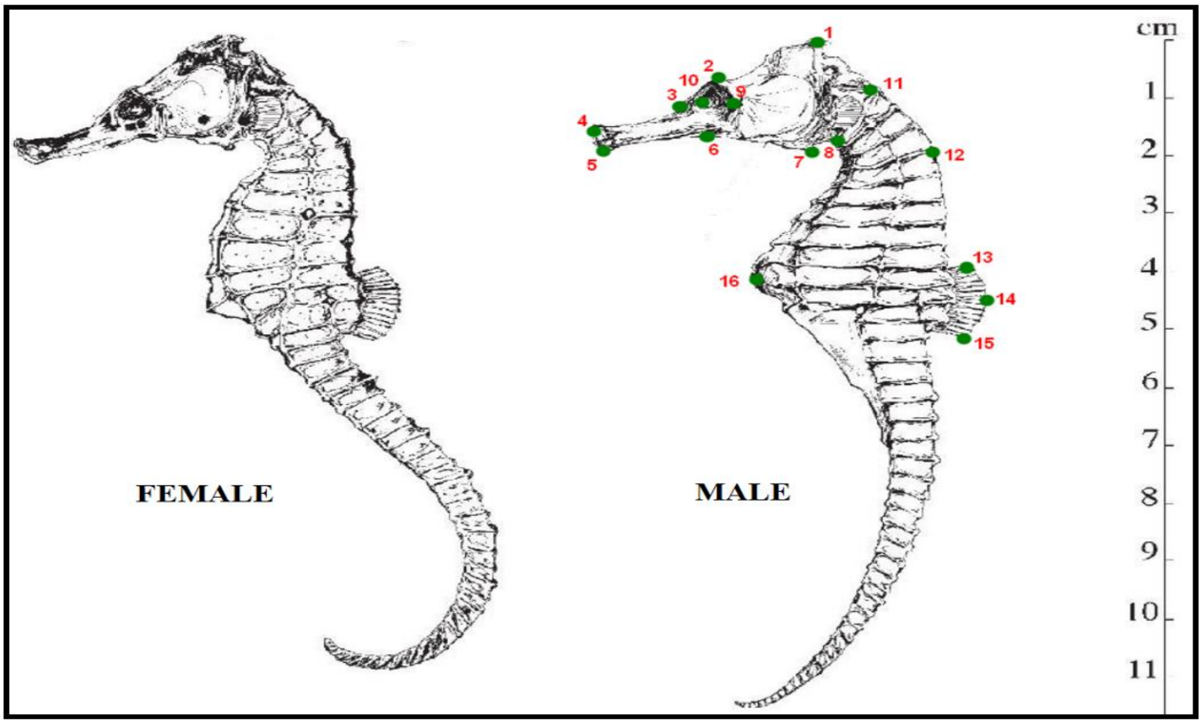

Fig. 2. Location of the 16 landmarks on the lateral sides of *Hippocampus* spp.

The individual's failure to buffer the stress leads to deviation in its relative symmetry. In this respect, it is perceived that there is a direct relationship between FA and developmental instability (Graham *et al.,* 2010). Here, a high significant FA would mean developmentally unstable populations and indicative of vulnerability of seahorses in the marine environment in Danajon bank. Noteworthy, is that FA is pertinent because it reflects a population's state of adaptation, coadaptation, fitness and reflects individual quality.

It increases under both environmental and genetic stress (Waddington, 1942; *Graham et al.*, 1993). Based on the results, this will pose a challenge especially on implementation of conservation measures.

Localized Trait Fluctuating Asymmetry

Accordingly, based on the sixteen bilateral traits examined separately, the results identified that all four species of *Hippocampus* exhibited FA in localized traits respectively (Table 3).

Generally, for localized trait fluctuating asymmetry it was evident on the topmost antero-lateral of part of the eye and the dorso-lateral tip of first trunk ring. Apparently, for *H. comes* it was evident on the lower tip of the snout, dorso-lateral tip of fourth trunk ring and postero-lateral tip of dorsal fin. For *H. kuda,* it was observed on the top of coronet, eye (lateral distal end), dorso-lateral tip of first trunk ring and dorso-lateral tip of fourth trunk ring.

Meanwhile, for *H. histrix* variations were on the topmost antero-lateral of part of the eye, junction immediately in front of the eye (bump in front of eye), junction in proximity to the cheek, base of the operculum, dorso-lateral tip of first trunk ring, dorso-lateral tip of fourth trunk ring, and antero-lateral tip of dorsal fin.

Moreover for *H. spinosissimus* variations were on the junction immediately in front of the eye (bump in front of eye), junction in proximity to the cheek, antero-lateral tip of dorsal fin and mid-lateral point of dorsal fin.

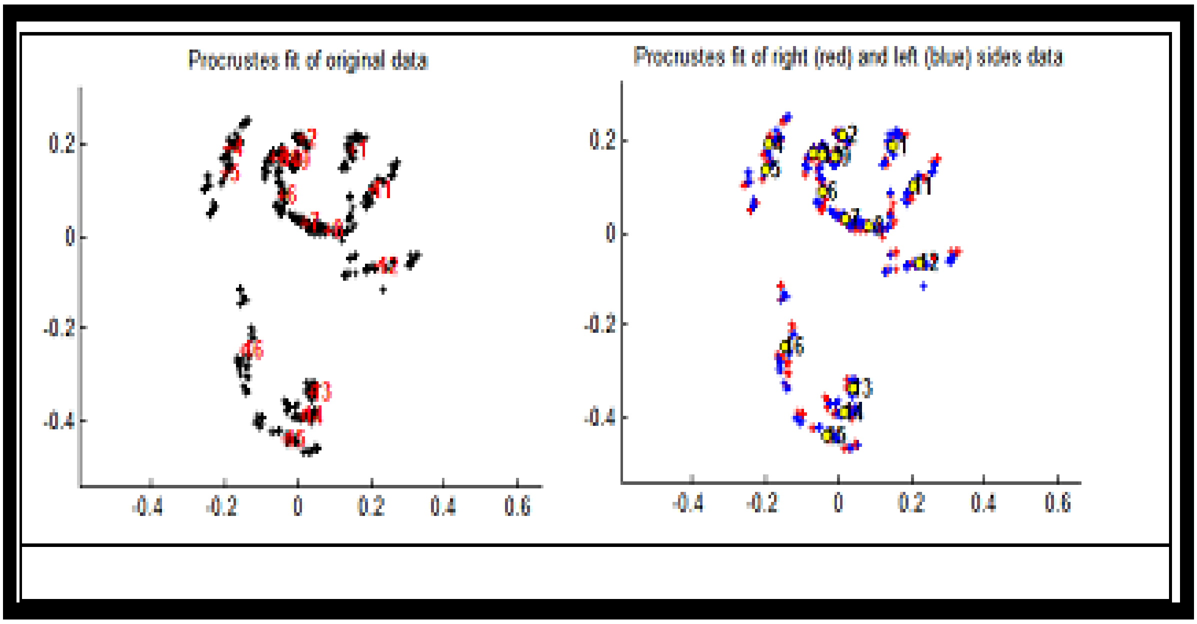

Fig. 3. Procrustes fitted image of *Hippocampus* spp. done by SAGE software.

Principal Component Analysis

In addition, PCA from Procrustes analysis may serve as a reliable tool in visualization of variations in landmarks (Galbo and Tabugo, 2014). It was used to determine 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 in Fig.4 represent the morphological landmarks used in the study while the blue arrows indicate the direction as well as the magnitude of the fluctuation for PC 1 only that shows majority of the percentage of variation. Herewith, the degree of variation as reflected by the magnitude of fluctuation for each landmark was evident on *H. comes* and *H. kuda* species that also exhibited high significant FA.

The significant FA and variation may imply the effect of environmental origin. Both species mostly inhabit shallow waters less than 10m. Where, *H. comes* occupy coral reefs, sponge gardens, and sargassum while, *H. kuda* occupy coastal bays, lagoons, seagrass, floating seaweed, sandy sediments and rocky littoral zones.

However, rare recorded sighting of *H. comes* was found on maximum depth of 20m and *H. kuda*was found on maximum depth of 55m. Coastal areas and shallow areas are more vulnerable to anthropogenic disturbances.

In Danajon bank, habitat destruction was evident due to overfishing and densely populated coastal islands.

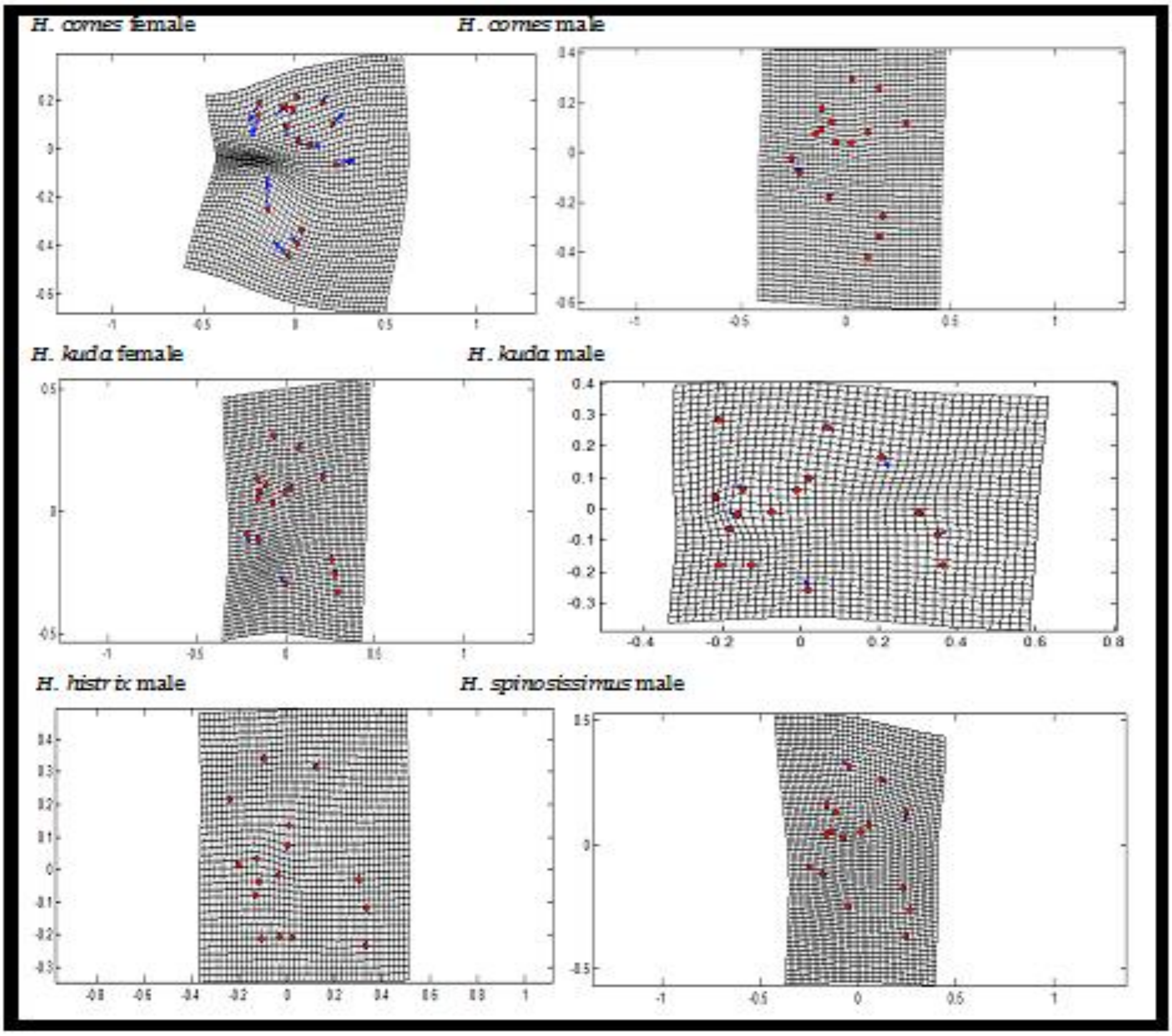

Fig. 4. PCA implied deformation for *individual x side* interaction of fluctuating asymmetry of *Hippocampus spp.*

Conclusion

This study takes precedence because it highly demonstrated the potential of FA as a tool for biomonitoring and its efficacy in measuring developmental instability (DI) in populations of *Hippocampus* spp. in Danajon bank. In this respect, FA was used as an indicator of individual quality and adaptation thereby, also demonstrating the potential for FA as a bioindicator of stress and developmental instability in populations of seahorses. Results yield significant FA for all populations of four species of seahorses from Danajon bank. Male populations showed high FA (individual × sides) effect that could be highly attributed to environmental origin since it showed not significant for both 'individuals' and 'sides' effect. It is hypothesized that significant high FA for male samples accounts for their high vulnerability especially while providing post-zygotic care for offsprings by brooding embryos in their ventral surface.

The underlying reasons behind high FA may be attributed to stress of environmental origin considering that Danajon bank have experienced habitat degradation. 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, quality of individuals and indicative of vulnerability of seahorses in the marine environment especially in Danajon bank. Based on the results, this will pose an immense challenge, especially on implementation of conservation measures on populations of seahorses in Danajon bank.

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