INSULA	Florianópolis	Nº 36	75-94	2007

FLUCTUATING ASYMMETRY AS A USEFUL BIOMARKER OF ENVIRONMENTAL STRESS: A CASE OF STUDY WITH *AVICENNIA SCHAUERIANA* STAPF & LEECHM. EX MOLDENKE (ACANTHACEAE)

ASSIMETRIA FLUTUANTE COMO UM BIOMARCADOR DE ESTRESSE AMBIENTAL: UM ESTUDO DE CASO COM *AVICENNIA SCHAUERIANA* STAPF & LEECHM. EX MOLDENKE (ACANTHACEAE)

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ABSTRACT

In bilaterally symmetrical organisms, fluctuating asymmetry (FA) provides a measure of environmentally induced departures from an ideal developmental program. FA should increase with increasing stress, and it should be exhibited at lower levels than those that could impact life history features. From the conservation point of view, it would be valuable to be one step ahead and detect the detrimental effect even earlier. We used the FA of stomata and salt-glands from leaves of *Avicennia schaueriana* species of two protected and two urbanized mangroves in Santa Catarina (Southern Brazil). We assess whether FA is sensitive enough to reflect the stresses in individuals exposed to varying environmental conditions. In order to do these measurements (counts), the leaves of *Avicennia schaueriana* were copied on acetate casts. Under the microscope, the leaf casts proved to be very clear, hence FA counts on the leaf casts were carried out perfectly. The highest FA values in both stomata and salt-glands were found in the central and landward sites of both urbanized mangroves. However, although the urbanized mangroves exhibited some high FA values, and the protected ones have some low ones, this was not always the case. Individual cases are discussed. Nevertheless, the results suggest that FA of stomata and salt-glands of *Avicennia schaueriana* leaves could be used as biomarker of environmental stress.

Key words: environmental stress, mangrove, leaf, stomata, salt-glands

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RESUMO

Em organismos com simetria bilateral, a avaliação da assimetria flutuante (FA) possibilita uma medida dos desvios induzidos pelo ambiente no seu desenvolvimento ideal. A FA deve aumentar com o aumento do estresse ambiental e, por sua vez, pode ser melhor percebida em locais onde os impactos não promovem mudanças nas características da história de vida dos organismos. Sob a perspectiva da conservação, esta ferramenta pode ser valiosa para a avaliação nos estágios iniciais de uma perturbação ambiental. Neste estudo foi determinada a FA de estômatos e glândulas de sal de folhas de *Avicennia schaueriana* em dois manguezais protegidos e dois manguezais impactados pela urbanização em Santa Catarina (sul do Brasil), verificando se a FA representa um método sensível o suficiente para refletir o estresse em indivíduos expostos a diferentes condições ambientais. Para realização das medidas (contagens), as folhas de *Avicennia schaueriana* foram copiadas em acetato e os moldes das folhas foram analisados sob microscopia. Os maiores valores de FA, tanto para estômatos como para glândulas de sal, foram encontrados na porção central e mais interna dos dois manguezais urbanizados. Embora manguezais impactados tenham exibido os maiores valores e os manguezais protegidos tenham mostrado os menores valores de FA, foram observadas variações no referido padrão. Contudo, os resultados sugerem que a FA de estômatos e glândulas de sal em folhas de *Avicennia schaueriana* pode ser usada como um biomarcador para a avaliação do estresse ambiental em áreas de manguezal.

Palavras-chave: estresse ambiental, manguezal, folha, estômato, glândula de sal

INTRODUCTION

Biomarkers are useful tools that have been developed for the monitoring of ecosystem health. However, for a biomarker to be a good ecological indicator, it has to be more sensitive than fitness components, so as to provide an early warning signal before inhabitant organisms are highly affected or lost (Mal *et al.* 2002). Physiological changes in body functions, such as genes, body fluids, enzymes activities, cell division, etc., occur before life history features are impacted, or even before morphological aberrations are produced. Fluctuating asymmetry (FA) can be used as a biomarker to indicate when an organism's physiological processes has resulted in the disruption of precise development, the loss of resilience or ability to regulate its development (Palmer 1994).

Due to the ease and precision with which measures can be carried out, and to the vast array of methods nowadays available, FA has often been used as a method of assessing environmental quality. Although contradictory evidence also exists (Anne *et al.* 1998; Hogg *et al.* 2001), indices obtained in experimental (Freeman *et al.* 1999; Kozlov *et al.* 1996; Andalo *et al.* 2000; Chapman & Goulson 2000) and field studies (Lempa *et al.* 2000; Martel *et al.* 1999; Møller & Lope 1998; Zvereva *et al.* 1997; Møller 1995) have repeatedly showed increased level of FA before life-history features are affected.

Fluctuating asymmetry (FA) is a pattern of bilateral variation in morphogenesis, which is random with respect to side and is caused as a product of distress in an organism internal cellular development or the external environment, causing long-term, detrimental change to organisms (Palmer 1994; Møller & Swaddle 1997). As the mean level of asymmetry in a sample of individuals fluctuates between a mean of zero, and variation is normally distributed around that mean, therefore FA is a property of the population (Palmer 1994; Møller & Swaddle 1997).

These random variations are a direct result of the inability of individuals to resist or buffer the disruption of precise development (Freeman *et al.* 2003), caused by two opposing set of processes: developmental stability and developmental instability. The former, is a set of processes that tend to resist or buffer the disruption of precise development, and the latter, a set of processes that tends to disrupt it (Palmer 1994). Hence, an increase in FA could be produced due to an increase in developmental instability or a decrease in developmental stability, or both (Palmer & Strobeck 1992). Basically, the level of asymmetry that individuals within a population tend to display is a reflection of how well their genotype can express the ideal phenotype under the given conditions, which are identical for both sides of a paired trait (Møller & Swaddle 1997).

Developmental instability in a population can be caused by abiotic factors, such as temperature, nutrition, chemical, noise, and biotic ones such as herbivory, population density (Møller & Swaddle 1997). Developmental stability, on the other hand, could be reduced by genetic stress resulting from the disruption of coadapted gene complexes, including loss of genetic variation, hybridisation and the incorporation of a new mutant with major effects (Palmer & Strobeck 1986; Leary & Allendorf 1989). Therefore, developmental stability is high when organisms developed in optimal conditions, and is low when the conditions are suboptimal or adverse (Freeman *et al.* 1994).

Similarly, if asymmetry is an attribute of the genome alone, then it can be suggested that optimal genotypes are able to buffer environmental stress better than less fit genotypes, consequently being more symmetrical. This implies that FA may be an indicative of loss of genetic quality (Møller & Swaddle 1997). It could be, however, that those individuals were reared in a stress-free environment, resulting in more symmetrical phenotype. Notwithstanding, under undisturbed environmental conditions, even poor genotypes should be able to produce a stable and symmetrical phenotype (Andalo *et al.* 2000). To avoid erroneous assumptions, Andalo *et al.* (2000), suggests that FA is a reliable method for measuring individual genetic quality, in terms of resistance to developmental instability, Here, the FA of any trait should be assessed in a stressed environment versus a non-stress one, and the phenotype that increases FA the most, should have a poorer genotype. Therefore, deviations from perfect symmetry in a given trait can be used as an indicator of a poor or perturbing environment, before it directly affect the viability of a population. From the conservation point of view, it would be valuable to be one step ahead and detect the detrimental effect even earlier.

Because it reveals both genetic and environmental stress, FA could be used as an average measure of the population condition or fitness and the level of stress experienced by organisms in a given environment (Møller & Swaddle 1997; Thomson 1999), even at moderate polluted levels (Eeva *et al.* 2000), as opposed to life-history features (Hogg *et al.* 2001). In animals, several studies have demonstrated positive relationships between one or both types of stresses and the level of FA of the studied trait (Eeva *et al.* 2000; Chapman & Goulson 2000). However, as not all measurable traits are equally sensitive (Leamy 1993), the degree of asymmetry shown by an organism may not be correlative with stress (Freeman *et al.* 1999).

For plants, however, if leaves are to be symmetrical, then cell division by meristematic cells ought to be coordinated, implying that the actions carried out at various distances and potentially different environments (at the tip and along the leaf margins), may involve genetic and epigenetic factors (Freeman *et al.*, 1999). As FA provides a measure of population condition or fitness, slight deviations from the ideal developmental program, which derives from a priori knowledge of perfect symmetry, should be detected (Palmer & Strobeck 1986). In other words, it provides us with an indication of how well or poorly the genetic program for development has been followed. In brief, symmetry is thought to represent the optimal phenotype for a variety of traits (Freeman *et al.* 1999).

As Møller (1995), found in his Bumblebee-flower relationship study, there is a direct relationship between flower symmetry, fitness and bumblebee preference, meaning that the generation and breaking of symmetry have profound effects on the functioning of plants. Likewise, in a study relating leaf FA of white birch and herbivory, Lempa *et al.* (1999), investigated how FA was influenced by an abiotic factor, such as a moisture gradient. They found that trees exhibiting the highest leaf FA were the ones more environmentally stressed (eg. growing in the wettest sites) and, in turn, preferred by herbivorous, as opposed to flowers, but negatively affecting the plant, nevertheless. Another study conducted by Lempa *et al.* (2000), on the FA of birch leaves, he found that the trees exhibiting the highest FA on their leaves were, again, preferred by herbivores, presumably due to contain high concentrations of foliar gallotanins, a precious chemical for *Epirrita autumnata* larvae. Another study carried out by Møller & Lope (1998), on the effects of increase herbivory in stone oak trees, he found that after a year of intense herbivory, the leaves were more asymmetric, and resulted in an even higher predation. He also found that the more stressed (eg. highest herbivory) were the plants, the higher were their FAs, and as a result, the more likely to die they were.

Similarly, Møller (1998) found that different plant parts of three plant species show increase FA under radiation stress. He used the leaves of *Robinia pseudoacacia* and *Sorbus aucuparia* and the petals of *Matricaria perforata* to assess whether their respective FAs varied at different distances from the radiation source in Chernobyl, Ukraine. He found that the FA of the three species increased with decreasing distance from Chernobyl, concluding that the plants, due to the radiation, had lost the ability to control their development. In a study of how electromagnetic fields (EF) affect the soybean leaf FA, Freeman *et al.* (1999), found that soybean grown underneath a high-voltage power line (675 kV), exhibit a higher leaf FA than soybeans grown at 50 or 100 metres from the same power line, suggesting that EF affected the development of soybean plants.

Likewise, increase FA due to metal stress have been studied and demonstrated in several organisms. Mal *et al.* (2002), for example, found that as the shoot length, number and length of leaves, as well as its biomass decreased, the FA of the wetland species, *Lythrum salicaria*, increased in lead polluted environment versus a non-polluted one. Kozlov *et al.* (1996), in addition, found that the FA of silver birch, *Betula pendula* and two subspecies of *B. pubescens* (ssp. *B. pubescens* and spp. B. *tortuosa*) decreased hyperbolically with increasing distance from two copper-nickel smelters in Finland and Russia. He found that, as well as FA in their leaves were positively correlated to foliar nickel concentrations, FA was highest around the polluters which produced more emissions.

Anne *et al.* (1998), on the other hand, exposed soybean plants to a gradient of salinity, ranging from 0 to 9g NaCl l^{-1}. They found that although life history features such as growth, survival and reproduction of all plants receiving more than 3g NaCl l^{-1}, were being negatively affected, FA in the soybean leaves did not differ. FA in this study clearly failed to reflect the various salt concentrations and the plants died. As well as FA measures, however, they also tested for the standard error in allometric relationships between the regression coefficients of the cumulative internodes length versus node number. In this case there was a significant increase under increasing salinity. Allometric relationships, unlike FA, report important functional changes in organisms that FA may not detect (Freeman *et al.* 1999).

As primary producers, and common end-point for anthropogenic contaminants, mangroves can be used as first level indicator of environmental stress cause by pollution. In this study, we investigated the leaf FA of four mangrove forests located at different distances from urban centres, evaluating the differences in the number of stomata and salt-glands between the left and right midrib sides of *Avicennia schaueriana* Stapf & Leechm. ex Moldenke leaves. In this study, two questions arise.

The first, is FA sensitive enough to reflect the environmental stress of anthropogenic origin within and between mangroves? The second, can FA also reflect anthropogenic upset alone (eg. within protected areas and far from urban centres)?

MATERIAL AND METHODS

Sampling sites - This investigation was carried out on the floral FA of four mangrove forests in the state of Santa Catarina, Southern Brazil. More specifically, the leaf FA of the mangrove species *Avicennia schaueriana*. Three of the mangroves (Itacorubi, Tavares, and Ratones) are located along the Western coast of the Santa Catarina Island (27°34'14" to 27°35'31"S and 48°30'07" to 48°31'33"W) and the other (Aririú - 27°34'51" to 27°35'09"S and 48°32'04" to 48°32'33"W) in the continental side (Fig. 1).

Mangroves were selected on the basis of their reported level of pollution (Pagliosa *et al.* 2004; 2005; 2006a; and 2006b) and, their proximity to urban areas and the level of governmental protection they receive. The estuaries, when more than one was present within a mangrove, were again chosen in relation to their assumed pollution level, similarity (length from beginning to end) to the other estuaries, so as to try to standardize them, and to ease of access.

Two of the mangroves are located immediately next to urban areas and receive little (Itacorubi) or no effective protection (Aririú), hence, under high anthropogenic stress. The other two are located some distance away from (heavily) populated areas and enjoy semi-total (Tavares) or total (Ratones) protection. Three sampling sites were established on each mangrove: in the high salinity region, close to the river mouth; in the low salinity region, close to the limit of the tidal influence zone; and in the intermediate region, between the two. Some of their history, as well as a description of their present status is described below:

Itacorubi mangrove – This mangrove is located in the north bay of the Santa Catarina Island and fringed by two Northern neighborhoods (Itacorubi and Santa Monica) of the city of Florianópolis, capital of the Santa Catarina State. This mangrove is dissected by a motorway, and receives domestic and industrial discharge at various points. The Itacorubi mangrove is drained by the Sertão and Itacorubi Estuaries and covers and area of 1.62km^2 (Soriano-Sierra & Sierra de Ledo 1998). In 1978, this mangrove was declared a protected area, and it is currently under the supervision of the Santa Catarina Federal University. Before then, however, this mangrove received a daily input of rubbish of 92 tonnes in the purposefully built landfill (Soriano-Sierra & Sierra de Ledo 1998). Due to this, and that it is surrounded by houses and roads, and that it is rather poorly controlled by the university, the state of this mangrove is unfortunate, although it is recovering, it has to be added. The study in this mangrove was carried out in the Itacorubi estuary. The landward and most Eastward side of this

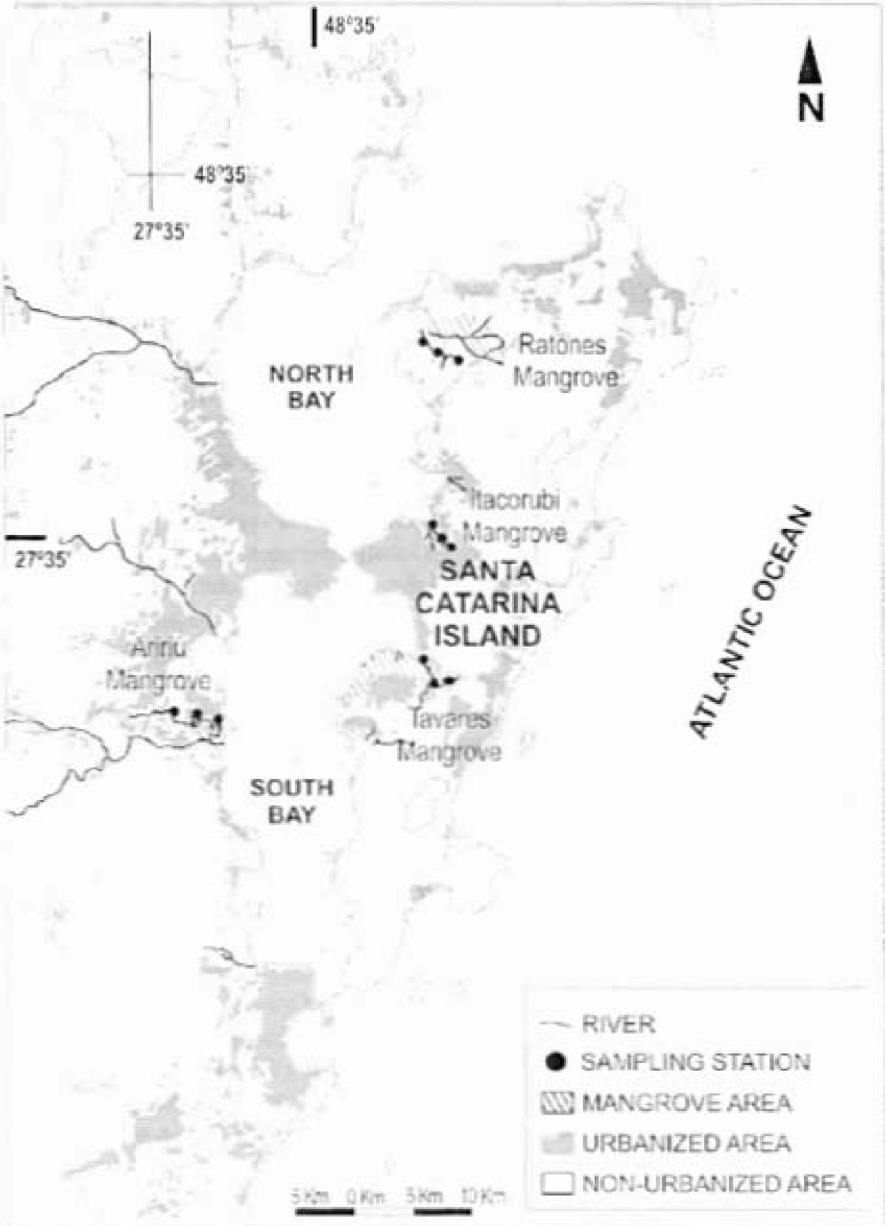

Figure 1. Map of Santa Catarina Island, Southern Brazil, and the studied mangroves.

estuary (site A), is bordering a double carriageway road and the Itacorubi neighborhood. The central part of the estuary (site B), is close to where it used to be the rubbish landfill, although now it seems to be recovering. The seaward and most westward part of the estuary (site C) is separated from the other two sites by a motorway. This site does not receive any domestic discharge or suffer any disturbance other than the one caused by the motorway. We classified this mangrove as an impacted one.

Aririú mangrove – This mangrove is located between the Southern outskirts of the city of Palhoça, Santa Catarina State, and the North-Eastern limits of the Serra do Tabuleiro State Park. The park covers and area of $900km^2$, out of which $1.3km^2$ is the mangrove itself. As the Aririú Estuary runs in a South-Easterly direction to the sea, and the city of Palhoça is spreaded all along the Northern side of it, the estuary itself was made the park's limits on that side, meaning that any area of mangrove remaining between the estuary and the growing city is being lost to human expansion and impact. As a result of that, nowadays most of its original area, on the northern margin of it, is already built-up, and the level of pollution and anthropogenic impact is the highest of all, with sewage channels as well as waste widespread all over it. This mangrove presents large areas without any mangrove trees (or even any type of vegetation). The sampling sites (A, B and C) in this mangrove were all located between the national motorway going to the south (site A, North-West) and the sea (site C, South-East), covering a distance of about 4km. Although site A and C along the estuary are relatively close to urban areas, the most heavily populated one along it is site B, in the middle. We classified this mangrove as an impacted one.

Tavares mangrove – This mangrove is located in the Southern bay of the Santa Catarina Island. In the past, this mangrove suffered many invasions and alterations, such as the construction of the Air Force Base and the Florianópolis International Airport, together with their respective roads leading to them. In 1992, however, this mangrove was taken under the supervision of the Brazilian Environmental Agency, and came to take part of the Pirajubaé Marine Sustainable Reserve. The only activity allowed in this mangrove is that of education and sustainable fishing by license holders (usually people living in the immediate neighboring areas). As a result of this, nowadays this mangrove is one of the better preserved. This mangrove is drained by the Tavares and Fazenda estuaries, and covers and area of 1444ha. This study was carried out in the Tavares Estuary, which covers a distance of about 3.5km. Site C (North) in this mangrove is relatively close (500m) to the busy road linking the South-East and South-West of the island, site B is in a non-disturbed area, and site C is at the seaward side, just before the road linking the city of Florianópolis to the airport and Air Force. We classed this mangrove as a non-impacted or controlled one.

Ratones mangrove – This mangrove is located in the north bay of the Santa Catarina Island, at about 25km north of the city of Florianópolis. Due to land reclamation for agriculture, some urban development and road construction, by 1987, this mangrove had already lost 62,3% of its original area . However, as in 1987, this mangrove was declared an ecological station, its remaining 6.25km² are very well-preserved. However, although the levels of pollution in this mangrove are rather low, and the only allowed activity is that of education, some wood (site C) and sand (site A and B) extraction if often carried out by villagers, just opposite the estuary at site C. This mangrove is drained by the Ratones and Verissimo estuaries. This study was carried out in the Verissimo estuary, which is about 3km. Sites A, B and C are, again, located in the landward, middle and seaward side of the estuary respectively, being site A (South) and C (North) the ones which suffer some type of anthropogenic disturbance. However, as in overall this mangrove is very well preserved, we classed it as a non-impacted one.

Leaves Sampling Procedures - In a given organism, developmental stability can be expected to be influence only within the time the organism is undergoing development (Freeman *et al.* 1994). In plants, as opposed to animals, development does not involve cell migration, instead they grow by adding cells to previous cells (Freeman *et al.* 1999). Therefore, as developmental stability requires the constant generation of the same phenotype under certain environmental conditions, it should be expected that adverse conditions experienced during leaf development be reflected in leaf FA (Freeman *et al.* 1999).

The leaf collection of *Avicennia schaueriana* was carried out between the end of December 2002 and the end of January 2003. The collected leaves were always leaves that received sun, and came always from heights of 1.5-2.5mts above the ground.

As an individual tree fluctuating asymmetry (Møller & Swaddle 1997), one mature and fully developed leaf (only) was taken per individual. As a measure of the population-level fluctuating asymmetry, 30 trees were sampled per site, and that was repeated at every site (3) in every mangrove (4), making a total of 360 leaves in the four mangroves.

The reason for having collected only one leaf per tree, was to follow the recommendations on meristic traits (Palmer 1994), where their numbers are fixed early in development, and are easily spotted and counted. However, to minimize the error in every sample, five counts were made in both sides of every leaf (see leaf gland count below).

Leaf-casting and counting - Due to the natural leaf decomposition process, after having taken them from the trees, the leaf-casting part of the work was always carried out immediately after collection, avoiding the casting of degraded leaves. Once in the lab, in order to get rid off salt particles and anything else attached to them that could potentially hinder the casting, the leaves were carefully washed and dried. Once dried, they were laid up abaxial surface on a lab bench, and a copy cast was taken from midrib both sides of every leaf. In order to do this, a 1cm² piece of acetate was soaked for 3-5 seconds in an acetone solution, until it was soften by the reagent effect. Then, using a pair of forceps, the piece of acetate was drawn out of the solution and placed flat on the abaxial surface of the leaf, 0.5 cm from the midrib in the middle region of the leaf. Immediately after that, a 4 cm^2 plastic sheet (not soluble in acetone) was placed on top of the acetate and, with the intention of taking any air bubbles out, the plastic sheet was pressed down on a sideways manner. The leaf, together with the acetate and plastic sheet were then left to dry for around 10 minutes. Once the acetate sheet had been allowed to dry and was solid, using the forceps, the acetate was peeled off.

Stomata and salt-glands counts were carried in the 0.307mm^2. Five counts were carried out in both sides of leaf midrib. For the counts, a light microscope, Zeiss, model Axiostar plus was used (magnification of 100x). The number of individual glands per count was then added up and a single value calculated per site (signed asymmetry).

FA index- The absolute asymmetry \R-L\ was calculated by the difference between the number of stomata or salt-glands in the two sides of midrib leaf. As recommended by Palmer and Strobeck (1986), FA indexes were obtained for each site, as the sum of the differences squared between the number of them in the right (R) and the left (L) of the same leaf, divided by the number of specimens (N):

$$A = \Sigma(R - L)^2/N$$

Statistical analysis - Using the absolute asymmetry per individual (unsigned), as recommended by Palmer (1994), due to not being so sensitive to small departures from normality, overall site variances were checked for homogeneity using Levene's test. In addition to this, Hartley's *Fmax* test was also used to check for homogeneity but, as opposed to the former one, Hartley's *Fmax* was used on the index, no on the absolute asymmetry. Since the index is a variance, Hartley's *Fmax* is the most powerful test for the significance of the difference between two samples (Palmer, 1994). This test is performed by obtaining the ratio of the larger index value over the smaller, and the result is compared against a table of critical values. In this study, the *Fmax* critical value at 0.05, when comparing sites within mangroves (A, B or C) was 2.40, and when comparing similar sites (either all A, B or C) across mangroves the four mangroves, was 2.61. An index is said to be significantly non-homogenous when the value obtained in the ratio is larger than the critical value.

RESULTS

When examined under the microscope, the leaf casts, copied from the abaxial surface of *Avicennia schaueriana* leaves, clearly showed the morphological features of both stomata and salt-glands found in the epidermis of the leaves (Fig. 2). Due to their distinct morphology and to the leaf casts' quality, the percentage error in the gland count was minimal.

Urbanized mangroves - The highest FA values for both stomata and salt-glands of *Avicennia schaueriana* leaves between the four mangroves, was found in the central site of Aririú mangrove. The FA of stomata and salt-glands were found to be 219.86, and 44.43 respectively (Tab. 1). Site A and C of Aririú mangrove, were also found to exhibit a high FA for salt-glands (30.1 for site A and 16.7, for site C) at their respective sites, although that was not so for stomata glands at neither of those sites (Tab. 1).

The central site of Itacorubi mangrove, although not as high as Aririú mangrove, was also found to exhibit high FA values for both stomata and salt-glands. The FA values for both stomata (126.63) and salt-glands (21.4) at this site, were higher than any of the FA values found at any of the protected mangroves' sites (Tab. 1). In Itacorubi mangrove, the site C shows the lowest FA value for both stomata (74.96) and salt-glands (9.86).

Protected mangroves - Tavares mangrove, site A, was found to exhibit the highest FA for stomata (118.33), and the lowest for salt-glands (10.86) at that site (Tab. 1). Tavares mangrove site B, was also found to exhibit the lowest FA value for stomata (92.43), although the FA of salt-glands at that site, exhibited a relative average value (Tab. 1). Site C of this mangrove, was found to exhibit relatively intermediate values for salt-glands and stomata (Tab. 1).

In Verissimo mangrove, only one site (site C, landward) exhibited the highest value for stomata (114.73). This mangrove, was also found to have the lowest FA value for stomata (86.03) at site A. Site B for both glands, and site A and C for salt-glands, were found to have relatively intermediate values (Tab. 1).

General analysis - Levene's test on the squared (to eliminate the sign) variances, revealed that no individual mangrove, in either the stomata or salt-glands exhibited significant non-homogenous variances between sites (Tab. 2). However, when the data were compared between specific sites (A, B or C), across all mangroves, sites A and B from Aririú mangrove (urbanized) were found to exhibit significantly non-homogeneous variances (P = 0.008, for site A and P = 0.027, for site B) for salt-glands. This was not the case for stomata (Tab. 3).

Using Hartley's *F*max test, on the index data directly (a ratio of the larger over the smaller), the Aririú mangrove, site B was found to be significantly non-homogenous for both stomata and salt-glands ($F_{2,40} = 2.66$, for salt-glands and $F_{2,40} = 2.59$, < 0.05 for stomata, Tab. 4). Moreover, when the sites' indices, were compared across specific sites (A, B or C) between the four mangroves, site A and B from Aririú mangrove were significantly non-homogenous for salt-glands ($F_{2,61} = 2.77$, for site A and $F_{2,61} = 3.1$, < 0.05, Tab. 5). Conversely, no specific site (A, B or C) was found to be significantly non-homogenous for stomata (Tab. 5).

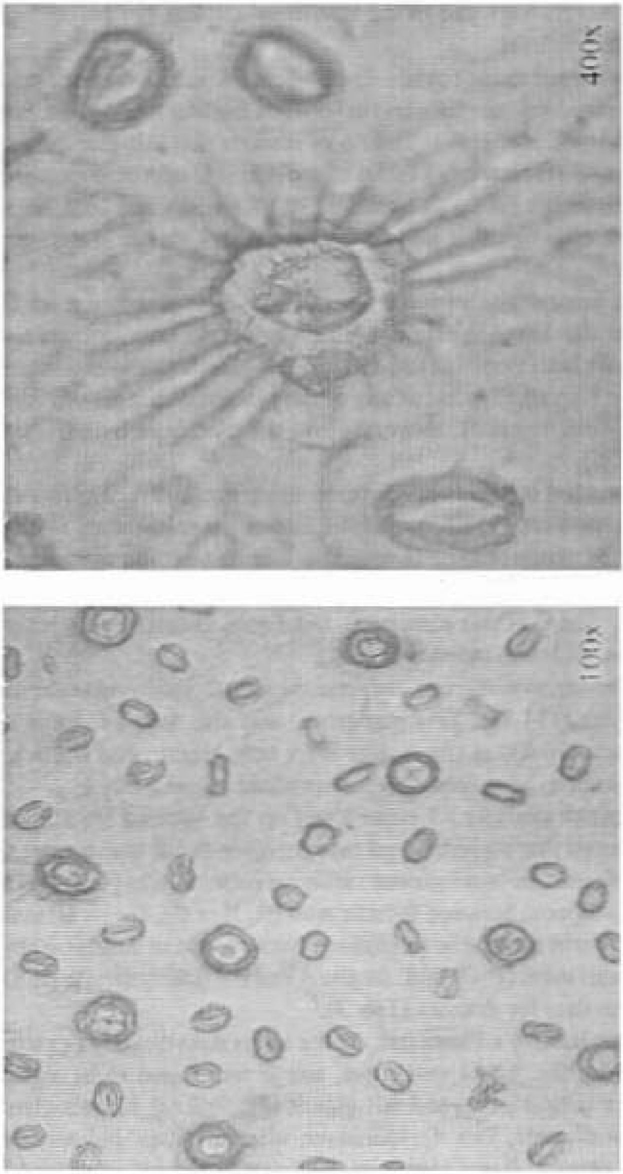

Figure 2. Light micrographs of abaxial surface of leaf (acetate) casts, exhibiting both stomata (elongated) and salt-glands (round) on the mangrove species, *Avicennia schaueriana*, from Tavares mangrove. The casts were unstained.

Table 1. FA index of stomata and salt-glands of *Avicennia schaueriana* leaves from sites in urbanized and protected mangroves in Santa Catarina, Southern Brazil.

Plant structure/site	Mangrove			
	Urbanized		Protected	
	Aririú	Itacorubi	Tavares	Ratones
Stomata				
Site A (landward)	110.56	97.6	118.33	86.03
Site B (central)	219.86	126.63	92.43	96.2
Site C (seaward)	84.7	74.96	85.53	114.73
Salt-gland				
Site A (landward)	30.1	13.33	10.86	14.43
Site B (central)	44.43	21.4	14.33	18.3
Site C (seaward)	16.7	9.86	15.6	12.23

n = 30

Table 2. Levene test on the FA of stomata and salt-gland of *Avicennia schaueriana* leaves from urbanized and protected mangroves in Santa Catarina, Southern Brazil.

Plant Structure	Mangrove			
	Urbanized		Protected	
	Aririú	Itacorubi	Tavares	Ratones
Stomata	P = 0.227	P = 0.384	P = 0.490	P = 0.577
Salt-gland	P = 0.073	P = 0.068	P = 0.432	P = 0.197

P = 0.05

Table 3. Levene test on the FA of stomata and salt-gland of *Avicennia schaueriana* leaves from mangrove sites in Santa Catarina, Southern Brazil.

Plant structure	Sites within mangroves		
	A (landward)	B (central)	C (seaward)
Stomata	P = 0.720	P = 0.317	P = 0.616
Salt-gland	P = 0.008	P = 0.027	P = 0.446

P = 0.05

Table 4. Hartley F_{max} *test* on the FA of stomata and salt-glands of *Avicennia schaueriana* leaves from urbanized and protected mangroves in Santa Catarina, Southern Brazil.

Plant Structure	Mangrove			
	Urbanized		Protected	
	Aririú	Itacorubi	Tavares	Ratones
Stomata	$F_{2,40} < 2.59$	$F_{2,40} > 1.68$	$F_{2,40} > 1.38$	$F_{2,40} > 1.33$
Salt-gland	$F_{2,40} < 2.66$	$F_{2,40} > 2.17$	$F_{2,40} > 1.43$	$F_{2,40} > 1.49$

Table 5. Hartley's F_{max} test on the FA of stomata and salt-glands of *Avicennia schaueriana* leaves from mangrove sites in Santa Catarina, Southern Brazil.

Plant structure	Sites within mangroves		
	A (landward)	B (central)	C (seaward)
Stomata	$F_{2,61} > 1.37$	$F_{2,61} > 2.37$	$F_{2,61} > 1.53$
Salt-gland	$F_{2,61} > 2.77$	$F_{2,61} < 3.1$	$F_{2,61} < 1.69$

DISCUSSION

The counts for both stomata and salt-glands in *Avicennia schaueriana* leaves, showed higher FA in the two urbanized mangroves than in protected ones. Several factors, such as high concentrations of heavy metals and nutrients, as well as direct invasion and usage of the ecosystems' resources, found in the two impacted mangroves and absent in the protected ones, are evidence of the degree of localized environmental stress, and allow us to make some speculations regarding the increase/decrease in FA values.

Despite certain low FA values in the most impacted mangroves and some high values in protected ones, FA was greatest in the mangroves which received less protection or were closer to urban centres. While we cannot rule out the existence of an internal stress (genetic), the data are most consistent with the notion that environmental stress, in the form of toxicants and other human-induced impacts, is causative of high FA values in the non-protected sites.

Since developmental stability is the ability of a genotype to produce the ideal phenotype under a given set of conditions, it is hardly surprising that individuals inhabiting polluted sites are poorer at controlling their development than individuals inhabiting cleaner sites. Martin & Coughtrey (1981) found that even in an aerially polluted system, the major sink for all heavy metals emissions is the soil. Machado *et al.* (2001) moreover, in his study of metal accumulation in mangrove sediments, found that metal concentrations in the sediments were higher at the subsurface. Additionally, in aerially polluted sites, Kozlow *et al.* (1996) found that birch leaves' FA decreased hyperbolically with distance away from pollution sources (copper and nickel smelter).

In addition, most contaminated places are not only contaminated by one chemical, but by a mixture of them (Mal *et al.* 2002). Pagliosa (2004) and Pagliosa *et al.* (2006a) registered a higher concentration of Cu, Zn and Pb in the urbanized mangroves than in protected mangroves of Santa Catarina Island, and one additional increase in Zn concentrations belongs the last 10 years. Furthermore, although Cu and Zn are essential trace elements for plants, and can actually stimulate photochemical activities at low concentrations, uptake in excess can lead to negative effects in the Calvin cycle and inhibition of photosystem II activity (Giardi *et al.* 2001). Moreover, with the combination of the three metals, Pb, Zn and Cu, Peroxidase activity in *Avicennia* *marina* was shown to increase and photopigments decrease, resulting in phytotoxic effects (MacFarlane 2002).

Furthermore, as they reflect anthropogenic disposal into aquatic systems, the concentration of dissolved nitrogen compounds and other nutrients are important factors to verify pollution (Braga *et al.* 2000), nitrate and phosphate being typical of eutrophication problems. Sediment pollution in aquatic systems, moreover, has long been attributed to water quality (Gonçalves *et al.* 1992). In mangroves, this is due to physical and biogeochemical processes in the sediments, which immobilized and accumulate metals and other toxicants, acting as a sink and reducing their transport to other areas (Machado 2001).

In Santa Catarina, the disposal of domestic and industrial discharges to the sea is thought to be the most stressful factor in coastal ecosystems (Santa Catarina 1997). Different concentration of contaminants registered previously within and between the four studied mangroves reflects the localized input of anthropogenic wastes to these systems (Pagliosa 2004; Pagliosa *et al.* 2005, 2006a and 2006b). Essentially, as the seaward sites of all mangroves are influenced more by sea currents, tides and winds, which together promote turbulence and inputs of new water to the systems, than more landward ones, the concentrations of metals and nutrients (as well as dissolved O_2 and salinity) follows an increasing pattern from the sea to the land. This is particularly obvious in the non-protected mangroves, where the concentrations of nutrients were the highest in the landward and central sites and lowest in the seaward ones. The FA values we obtained from the seaward sites of the two urbanized and thus more impacted mangroves are the lowest in the two mangroves for stomata and salt-glands.

Then, the second highest FA value in the inner site of Aririú mangrove, presents a relatively high FA for stomata, and a very high for salt-glands, closely reflecting the high levels of environmental stress at that site. Similarly, the middle site of Itacorubi mangrove, presents the highest FA value in that mangrove for both stomata and salt-glands, reflecting, again, a high environmental stress level.

The highest FA values found for stomata and salt-glands in Aririú, however, were in the central mangrove site. Aririú estuary is located parallel to the outskirts of the city of Palhoça, with the middle site being immediately behind houses and next to a busy road, thus being used as the local dump (the city of Palhoça is neither turistic, nor rich, especially not the outskirts) and suffering constantly from human disturbance and intrusion. In this mangrove, the varying number as well as intensity of pollutants and anthropogenic disturbance, as well as the new nature of the stress (that part of the city has not been there for long, 10-20 years maximum, and houses are relatively new and growth towards the mangrove is still observed) may be producing the unusually high FA value for stomata and salt-glands. Andalo *et al.* (2000), for instance, conducted a manipulative experiment with *Lotus corniculatus* in which they reared them in environments of varying CO_2 concentrations and nitrogen availability. They found highest FA of both flowers and leaves in the elevated CO_2 and changing nitrogen treatments, showing that both the overloading of nutrient, as well as changing environment is stressful to plants.

In Itacorubi mangrove, on the other hand, the FA value obtained in the landward site for both stomata and salt-glands, reflects neither the high level of pollution in both heavy metals and nutrients (Pagliosa 2004; Pagliosa *et al.* 2005; 2006a; and 2006b) nor the direct anthropogenic disturbance caused by intrusion and by the busy road next to it. This mangrove site is located next to a dual-carriageway, behind which the Santa Monica neighborhood is settled, and from where all its contaminants come from. Here, moreover, neither the dual-carriageway, nor the neighborhoods have been built recently. Since they are in the island, in the middle of the city, capital of the Santa Catarina State, the environmental stress imposed to that site of the mangrove has been there for decades (maybe even the last century). Here, we propose that plants at this site, as a result of the increasing and prevailing anthropogenic stress (due to growing population, industries, volume of motorized traffic, etc.) during those years, have evolved some mechanism at cellular level that buffers the effect of those levels of stress, to the point that it is not longer stressfull. Queitsch *et al.* (2002) argues that heat-shock protein 90 (Hsp90), a protein induced by stress, in response to changing circumstances, profoundly affects developmental plasticity (especially in plants), in turn allowing morphogenetic variation. They argue that under specific pressures, the buffering capacity of Hsp90 could provide a way by which a population may evolve a different genotypic state (eg. one that allows a trait to dynamically act in response to the environmental change imposed to it over developmental time). Similar buffering or resistance have been suggested for the sheep blowfly, *Lucilia cuprina*, where the levels of FA in a pure breeding strain, resistant to diazinon insecticide, were the same as the one found in a non-resistant laboratory strain (Mackenzie & O'Farrell 1994; MacKenzie & Clarke 1998). They argue that as populations become used to being under certain type of stress, the level of FA decreases and ultimately returns to normal, suggesting that adaptations occurs.

In the protected mangroves, on the other hand, the FA value for both stomata and salt-glands was either low or at least constant between sites in individual mangroves, again reflecting the level of stress experienced by organisms in those mangroves. Tavares mangrove, for instance, except for stomata FA in the inner site, presents rather low FA values in all sites for stomata and salt-glands. The landward site in that mangrove is relatively closed (~500m) to a busy road. This may partially explain the asymmetry obtained in that site. However, although most of the FA values we obtained correlated between them (eg. If stomata FA was high at a given site, salt-gland FA was also high at that site), with all the work of no correlation between traits reported (Leamy 1993; Møller 1995; Andalo 2000; Møller & Swaddle 1997), it would not be much of a surprise to get uncorrelated FAs. However, in general terms, this mangrove's FA values, reflects the evenness of the chemical ones.

Ratones mangrove, again, exhibits a reasonably low FA for both glands, and a relatively constant one between sites. However, this mangrove, as opposed to the other protected (Tavares) and the two impacted ones (Aririú and Itacorubi), presents the highest FA values in the central (salt-gland) and the seaward site (stomata) of the mangrove. As previously pointed out, in the central and inner sites of this mangrove, activities such as sand and wood extraction are carried out by the fishermen living in the surround area. The consequences of this, moreover, are not only those of intrusion and structural damage of the trees (physical damage of the environment), but also the overload of silicate in the water (Pagliosa 2004; Pagliosa *et al.*, 2006b). We believe this is having an effect in the stability of the plants (eg. increasing developmental instability), and this, in turn, influencing FA in the leaf symmetry at those sites.

Nevertheless, in spite of certain contradictory findings which, to some extend, can be explained, both stomata and salt-gland FA exhibited greater values where the levels of anthropogenic disturbance and level of contaminants were higher, clearly as a result of the localized level of stress. Typically, this was the case for the non-protected mangroves, as in the case of the Aririú mangrove, or the one surrounded by the neighbourhoods, and cut through by a motorway (Itacorubi), illustrating the important role played by conservation units in maintaining a sustainable and healthy environment.

Although by using FA, one is not able to establish a specific or general pollution toxicity threshold, when the regulation of symmetry is broken, it is obvious that in the presencial a high concentration of any pollutant, or any other stressor (eg. audiogenic), developmental stability will be disrupted, although not the growth itself. Therefore, the use of stomata and salt-glands FA of *Avicennia schaueriana* leaves as biomarkers, can provide useful information about the level of environmental stress in biomonitoring studies. Moreover, as shown by the leaf micrographs, if the casts are treated with care, quality is not lost and measurements can be carried out at a later date. Due to this, and that the type of trait was meristic (as opposed to continuous), the amount of error in this study was minimum, making the observations even more robust.

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