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Effect of Mussel's Gender and Size on a Stress Response Biomarker

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Abstract In mussels, stress signals such as heat, osmotic shock and hypoxia lead to the activation of the phosphorylated p38 mitogen activated protein kinase (pp38-MAPK). This stress activated protein has been efficiently used as a biomarker to several natural and anthropogenic stresses. However, what has not been tested is whether differences in gender or size can affect the response of this biomarker. The present study tested whether there was variation in the expression of pp38-MAPK in mussels *Perna perna* of different gender and size classes when exposed to natural stress conditions, such as air exposure. The results show that gender does not affect the expression of pp38-MAPK. However, size does have an effect, where mussels smaller than 6.5 cm displayed significantly ($p<0.05$) lower levels of pp38-MAPK when compared to those larger than 7 cm. Mussels are one of the most used bioindicator species and the use of biomarkers to determine the health status of an ecosystem has been greatly increasing over the years. The present study highlights the importance of using mussels of similar size classes when performing experiments using stress-related biomarkers.

Keywords *Perna perna* · Shell length · Male and female · pp38-MAPK · Hypoxia · Protein expression

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The phosphorylated p38 mitogen activated protein kinase (pp38-MAPK) is activated particularly by stress signals, such as heat, osmotic shock, endotoxins, etc (Canesi et al. 2002). In mussels they have been used as a biomarker to several natural and anthropogenic stresses such as osmotic stress, hypoxia and petroleum derivatives, among others (Canesi et al. 2002; Burlando et al. 2006; Hamer et al. 2008). Although it has been efficiently used to determine the physiological response of mussels to different impacts, no information is available on whether these stress responses are variable between genders or among size classes. For example, most studies do not mention if mussels used in their experiments were male or female (e.g., Burlando et al. 2006; Cheung et al. 2006; Hamer

et al. 2008). One drawback of having possible gender differences in stress response is that they can only be differentiated through gonad's colors (Seed and Suchanek 1992) after the experiments have been made and mussels sacrificed and opened. Furthermore, some studies use mussels of a wide range of sizes (e.g., Malagoti et al. 2007; Tsangaris et al. 2008), while others not even mention this parameter in their studies (Burlando et al. 2006; Cheung et al. 2006). If the response to stress differs between genders or among size classes of mussels, studies may show confounding results due to intrinsic physiological differences rather than the treatment itself. Additionally, basic information on the effect of natural disturbances (e.g., air exposure during low tide) on any specific biomarker should be tested prior to anthropogenic pollution analyses (Alves de Almeida et al. 2007).

In order to test the effect of gender and size under natural stress conditions, the present study tested whether there was variation in the expression of a stress-activated protein (pp38-MAPK) in mussels of different gender and size classes when exposed to air. The brown mussel *Perna perna* was used, since it is the most economically important mussel in Brazil and has a great range in size (up to 12 cm; Marenzi and Branco 2005). A total of 54 organisms from 2.5 to 10.5 cm were collected at 'Centro de Estudos Ambientais e Desenvolvimento Sustentável' (CEADS) -UERJ (Ilha Grande, RJ, Brazil) and divided in two centimeter size classes. Natural stress was induced by exposing mussels to air for 6 h to simulate the effect of tidal fluctuation. After this period, mussels were sexed based on gonad's color, their gills fixed in RNAholder (Biosystems) and stored at −20°C. Total protein was extracted by homogenizing the gills in 1× Tris-buffered saline pH 7.5, 1 mM EDTA, protease inhibitor cocktail (1 tablet/10 ml; Roche), centrifuging at 13000 × *g* for 10 min at 4°C and then retrieving the clear supernatant. Protein concentration of each sample was determined by Bradford assay and the relative amount of pp38 was assessed by ELISA. 100 µl of the total protein extract (20 µg/well) was incubated in a 96-wells plate (Corning Costar) for 24 h at 4°C. After washing three times with PBS solution containing 0.05% Tween-20 (PBS/Tween), the plates were blocked with a 1% Bovine Serum Albumin (BSA; Sigma) in PBS (100 µl/well) overnight at 4°C. Afterwards, 100 µl of rabbit anti-pp38 antibody (1:2000; Santa Cruz Biotech) in PBS/BSA (0.3% BSA) was added to each well and incubated for 2:30 h at 29°C. After washing three times with PBS/Tween, the pp38 present was detected by a secondary anti-rabbit IgG (goat), coupled with horseradish peroxidase (1:10000; Santa Cruz Biotech) using 3,3,5,5-tetramethylbenzidine (TMB; Pierce) as a substrate. The reaction was stopped (typically after 10 min), by adding 1 M H_2SO_4 and read with a spectrophotometer (Teckhand Coulter DTX 800) at 450 nm (OD450). The OD450 corresponding to the expression of pp38 in each sample was compared among different gender and size classes with a Two-Way ANOVA. A One-Way ANOVA followed by a Tukey post hoc test was used to determine where differences were significant. All data was tested for normality and homoscedasticity prior to statistical analyses. Statistical tests were performed using the program Systat 10.2.

The results show that stressed mussels smaller than 6.5 cm displayed significantly ($p<0.05$) lower levels of pp38 when compared to those larger than 7 cm (Tables 1 and 2, Fig. 1). In contrast, no significant differences in the expression of pp38 were found between size classes 3–4 cm and 5–6 cm, and between 7–8 cm and 9–10 cm (Table 2, Fig. 1). Additionally, no differences in the expression of pp38 were observed between male and female *P. perna* of all size classes (Table 1, Fig. 1).

Among marine invertebrates, mussels are one of the most used bioindicator species due to their sessile and filter feeding nature (e.g., Gaitanaki et al. 2004; Burlando et al. 2006; Cheung et al. 2006; Hamer et al. 2008). Studies using biomarkers of stress in mussels

Table 1 Result from the Two-Way ANOVA comparing the expression of pp38-MAPK (OD450) between gender and among size classes in the mussel *Perna perna*

Variables	df	F	p
Size	3	11.695	**0.000**
Gender	1	0.730	0.396
Size*Gender	3	0.377	0.770
Error	66		

In bold is the significant p value ($p<0.05$)

Table 2 Results from the Tukey post hoc test performed after the One-Way ANOVA comparing the expression of the pp38-MAPK among size classes. The matrix shows the p values for all multiple comparisons

	3–4 cm	5–6 cm	7–8 cm	9–10 cm
3–4 cm	1.000			
5–6 cm	0.577	1.000		
7–8 cm	**0.006**	**0.000**	1.000	
9–10 cm	**0.000**	**0.000**	0.988	1.000

In bold are the significant p values ($p<0.05$)

to determine the health status of an ecosystem has been increasing rapidly in the past few years, and this tendency is reflected on programs such as the 'Mussel Watch Program' all over the world (Goldberg 1975). The expression of pp38-MAPK as a stress response to air exposure has been observed in *Mytilus galloprovincialis* in the Mediterranean (Gaitanaki et al. 2004; Hamer et al. 2008). However, it was unknown if differences in gender and/or mussel's size also affected the response of stress-related biomarkers.

The present study found that gender does not affect the expression of pp38 when *P. perna* have been exposed to air. In contrast, the expression of pp38 was significantly different between the smaller and larger size classes. Mussels larger than 6.5 cm showed higher stress response in comparison to those smaller than 6 cm. Since all mussels were collected at the same locality and exposed to the same natural stress (hypoxia), the results of the pp38 expression should be due to physiological differences related to the size of these animals.

Differences related to size have been observed in blue mussels (*Mytilus edulis*). In this species, larger animals appeared to be more susceptible to mortality in response to air exposure than smaller ones (Sukhotin et al. 2003). This higher susceptibility of larger mussels to air may be due to their higher absolute values of metabolic rates compared to smaller mussels (Sukhotin et al. 2003). Higher metabolic rates could lead to higher oxidative stress induced by hypoxia, which results in the activation of antioxidant defense mechanisms to protect the organisms under such conditions (Gaitanaki et al. 2004).

In contrast, the higher stress response of larger mussels maybe due to age, since in the course of ageing many animals, including mussels, become more susceptible to oxidative stress (Canesi and Viarengo 1997). In addition to hypoxia, oxidative stress can also be achieved due to the presence of many xenobiotics including heavy metals (Livingstone 2001). Therefore, larger mussels may experience a relative higher stress due to natural and/or anthropogenic impacts, which can directly increase the phosphorylation of p38-MAPK.

To conclude, gender differences do not affect the response of the phosphorylated p38-MAPK. However, differences in size do have a significant effect on the expression of this stress-related biomarker. Therefore, this parameter should be taken into consideration when performing experiments using stress-related biomarkers, since it may affect the design and sampling strategies of future studies.

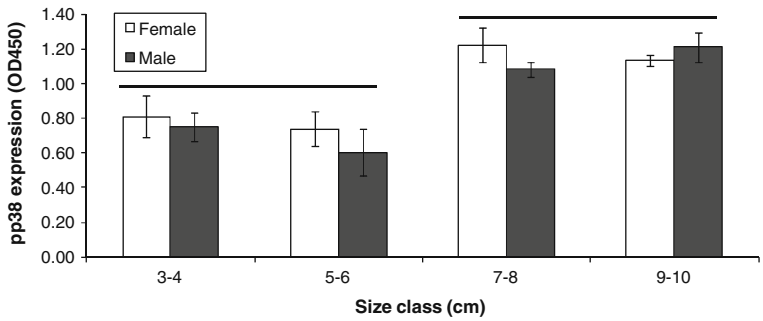

Fig. 1 Phosphorylated p38-MAPK (pp38-MAPK) from mussels of different genders and size classes. Significant differences in the expression of pp38-MAPK were found between smaller (3–4 cm and 5–6 cm) and larger (7–8 cm and 9–10 cm) size classes. Horizontal lines above bars group mussels where no significant differences ($p>0.05$) in pp38-MAPK were found. Bars are average ± standard errors

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