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RNA/DNA ratio of crabs as an indicator of mangrove habitat quality

VALTER AMARAL[a,b,*], GIL PENHA-LOPES[a] and JOSÉ PAULA[a]
[a]*Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal*
[b]*Marine Biological Association of the UK, Citadel Hill, Plymouth, PL1 2PB, UK*

ABSTRACT

1. Pollution of mangrove ecosystems puts their future and that of local communities at risk. Only the use of informed and integrative approaches will successfully maintain and restore these valuable ecosystems.
2. Biochemical indicators of organism physiological condition have been widely used to evaluate habitat quality and for early detection of the impact of stressors. Mangrove crabs may be useful bioindicators of the quality of mangrove habitats, as they are characteristic and ecologically important organisms in mangrove environments.
3. The physiological condition (evaluated by the RNA/DNA ratio) of *Perisesarma guttatum* (Grapsidae) and *Uca annulipes* (Ocypodidae) was assessed to determine its potential as an indicator of habitat quality in one polluted and two relatively unpolluted Mozambican mangroves, during the rainy and dry seasons.
4. Both species showed seasonal effects on RNA/DNA ratio, but only *U. annulipes* was significantly affected by pollution. RNA/DNA ratio of *U. annulipes* may thus be a useful indicator of pollution and seasonality in mangrove habitats. There was a synergistic negative effect of the rainy season and pollution on the RNA/DNA ratio of *U. annulipes*. Due to higher DNA, rather than lower RNA contents, the RNA/DNA ratio of *P. guttatum* was always significantly lower than that of *U. annulipes*.
5. The knowledge gathered in this study can be used in integrative strategies, policies and programmes aiming to sustain, maintain and restore mangrove areas, and for the evaluation of mangrove habitat quality.

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KEY WORDS: biochemical indicators; environmental conditions; nucleic acid; Mozambique; *Perisesarma guttatum*; *Uca annulipes*

INTRODUCTION

Over the past 25 years, at least one-third of the world's mangrove forests are estimated to have been lost (Alongi, 2002; Duke *et al.*, 2007). The persistence of mangrove ecosystems, especially those near urban centres, is at serious risk, jeopardizing essential ecological functions and associated resources, and the future health of local communities (Alongi, 2002; Kathiresan and Qaim, 2005; Duke *et al.*, 2007). Consequently, there is a need for effective actions to sustainably protect and restore these valuable wetland ecosystems (Duke *et al.*, 2007).

Pollution is perhaps the most widespread driver of mangrove destruction and degradation, including several forms of sewage and solid waste, exposure to hot-water out-flows, toxic heavy metals, pesticides and oil, gas and petroleum production and accidents (Alongi, 2002; Mohammed, 2002; Duke *et al.*, 2007; Hogarth, 2007; Kruitwagen *et al.*, 2008). Several effects of pollution on mangrove-associated fauna and flora and human communities have already been quantified (Alongi, 2002). For example, abnormal growth and unilateral anophthalmia have been reported in mudskippers (Kruitwagen *et al.*, 2006), oxidation stress and lipid membrane damage have led to reduced growth in young seedlings of *Bruguiera gymnorrhiza* (Zhang *et al.*, 2007), and waterborne diseases such as cholera, diarrhoea and gastroenteritis have been directly linked to municipal sewage discharges (Mohammed, 2002). Although nutrients associated with sewage discharges may, up to a certain level, create favourable conditions for mangrove flora and fauna,

*Correspondence to: V. Amaral, Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal. E-mail: vlamaral@fc.ul.pt

generalized pollution usually has the opposite outcome (Meziane and Tsuchiya, 2002; Hogarth, 2007).

Biochemical indicators are valuable tools in the evaluation of organism health and environmental quality of its associated habitat (Dahlhoff, 2004; Gilliers *et al*., 2004; Amaral *et al*., 2008). Biochemical changes usually occur before those on slower processes, such as growth or reproduction, and thus may facilitate early detection of stress effects (Linton and Warner, 2003). Crabs are considered potentially good indicators of environmental habitat quality (Smith *et al*., 1991; Lohrer *et al*., 2004; Pagliosa and Barbosa, 2006). Ocypodid and grapsid crabs are among the most conspicuous and important organisms in mangrove intertidal environments (Smith *et al*., 1991; Skov *et al*., 2002; Kathiresan and Qaim, 2005; Hogarth, 2007). Nevertheless, little is known about the indicator potential of the physiological condition of mangrove crabs for the evaluation of habitat quality and the potential impact of seasonality and pollution.

The RNA/DNA ratio has been especially useful as an estimator of physiological condition of marine invertebrates and fish (Wright and Hetzel, 1985; Gilliers *et al*., 2004; Amaral *et al*., 2008). RNA content correlates with the synthesis of new proteins, which is usually interpreted as beneficial to the organism, and reflects active metabolic rates related to growth and reproduction. As DNA content remains relatively constant in an individual, as a function of chromosome number, RNA/DNA ratio is expected to increase when environmental conditions are favourable (Dahlhoff, 2004).

A concurrent study revealed that the RNA/DNA ratios of two ocypodid mangrove crab species (*Uca annulipes* and *Uca inversa*) might be more affected by climatic season than by sewage discharges, with higher ratios during the less rainy season and both species coping well in several vegetation and sewage combinations (Amaral *et al*., in press). However, the study was conducted in field mesocosms, where the sewage applied was being phyto-remediated, which means that the real effects of sewage were not evaluated (Amaral *et al*., in press). Furthermore, only ocypodid crabs were considered. To the authors' knowledge, no other study has focused on the effects of environmental variables, namely pollution and climatic season, on the RNA/DNA ratio of mangrove crabs in a real-life situation. In this context, this study investigated the bioindicator potential of the physiological condition of the grapsid *Perisesarma guttatum* (A. Milne Edwards, 1869) and of the ocypodid *Uca annulipes* (H. Milne Edwards, 1837), to evaluate the quality of mangrove habitats in terms of seasonality and pollution. It was hypothesised that the RNA/DNA ratio of crabs should be higher in unpolluted mangroves and during the dry season. Another objective was to provide baseline information on the physiological condition of those species. RNA/DNA ratio in crabs of each species was assessed in polluted and relatively unpolluted mangroves at Mozambique, during the rainy and dry seasons.

METHODS

Sampling sites

This study was conducted in three different mangrove systems located in southern Mozambique, East Africa: Costa do Sol, at Maputo Bay, on the mainland continent; and Saco and Ponta Rasa, at Inhaca Island (Figure 1). All systems are located at similar latitudes within Maputo Bay, in a transitional region of tropical to warm subtropical climate, characterized by hot and rainy (September to March) and warm and dry (April to August) seasons, and where the tidal regime is typically semidiurnal (Kalk, 1995).

Costa do Sol mangrove is characterized by a small and shallow seawater swamp located ~7 km north of Maputo city (25°55′ S, 32°35′ E). Maximum tidal amplitude is ~3.5 m, and average water temperatures and salinities (all salinity values presented in this study are based on the Practical Salinity Scale of 1978) vary from 20 to 30°C and 20 to 32, respectively (Litulo, 2005; PUMPSEA, 2007). The small seasonal river Quinhenganine discharges into the swamp. *Avicennia marina* and small patches of *Rhizophora mucronata* dominate the vegetation (PUMPSEA, 2007). The mangrove is bordered by a residential area, and has received domestic sewage, aquaculture residuals and solid dumps from various sources throughout recent decades (PUMPSEA, 2007).

Inhaca is a small island at ~32 km east of Maputo (26°S, 32°55′ E), lying between Maputo Bay and the Indian Ocean: the west and south coasts are sheltered, while the east coasts are exposed to the Indian Ocean (Kalk, 1995; Paula *et al*., 2003) (Figure 1). Maximum tidal amplitude is ~3.7 m, and average water temperatures and salinities vary from 19 to 28°C and 20 to 35, respectively (PUMPSEA, 2007). No rivers are present, and freshwater supply results from diffused groundwater flow and rainfall. There are few people in the south of the island, and the absence of industry contributes to the persistence of relatively unpolluted areas in relation to those on the mainland (Kalk, 1995). Saco mangrove covers an area of ~2.1 km^2 and is located in a small, enclosed and shallow bay in the south of the island (Figure 1). *Avicennia marina*, bordering the entire bay, and *R. mucronata*, *Ceriops tagal* and *Bruguiera gymnorrhiza*, lining channel banks and creeks, dominate the vegetation (PUMPSEA, 2007). Ponta Rasa mangrove is the smallest on the island, covering ~0.2 km^2, and is located on the south-west coast, facing Maputo Bay (Figure 1). The creek is densely bordered by *R. mucronata*, sparser patches of *C. tagal* and *B. gymnorrhiza* dominate higher areas, and the uppermost more sandy zone is characterized by *A. marina* (Paula *et al*., 2003).

Several pollution indicators have been evaluated in the three mangroves (PUMPSEA, 2007). Higher concentrations of nutrients, especially nitrates and nitrites, characterize Costa do Sol in relation to Saco and Ponta Rasa mangroves (Table 1). As a direct result, the benthic microalgae community is significantly more abundant in Costa do Sol. The level of phosphate was similar among mangroves, as well as those of pH and total organic content (Table 1). Costa do Sol mangrove also presents much higher abundance of coliform bacteria (*Escherichia coli*, *Vibrio cholerae* and *Salmonella* spp.), frequently occurring at levels above those recommended (PUMPSEA, 2007). Altogether, these conditions allow discrimination between the more polluted Costa do Sol, and the relatively unpolluted Saco and Ponta Rasa mangroves.

Field sampling

The physiological condition of *P. guttatum* and *U. annulipes* was evaluated by RNA/DNA ratio in both rainy (February)

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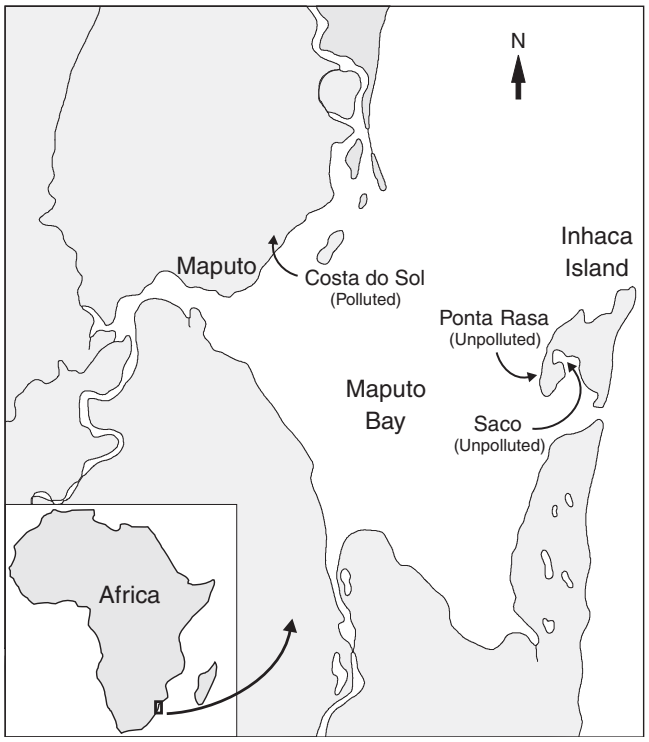

Figure 1. Location of the three different Mozambican mangrove systems sampled in this study (unpolluted — Ponta Rasa and Saco; polluted — Costa do Sol).

Table 1. Mean values (±SD) of biochemical water parameters in Ponta Rasa, Saco and Costa do Sol mangroves, from February to August 2006. Measurements were conducted during spring tides. TOC — total organic carbon. Adapted from PUMPSEA (2007)

Parameters	Ponta Rasa	Saco	Costa do Sol
pH	7.82±0.40	7.86±0.31	7.86±0.30
NO_3^- (μmol L^{-1})	0.70±0.06	0.80±0.08	3.90±0.76
NO_2^- (μmol L^{-1})	0.17±0.06	0.17±0.09	0.48±0.25
PO_4^- (μmol L^{-1})	0.44±0.12	0.40±0.06	0.43±0.14
TOC (%)	5.95±4.19	4.41±4.31	3.49±2.33

and dry (August) seasons, in the three mangroves. Crabs of each species are mainly active during low tides, usually foraging for food around their burrows, to which they quickly return when threatened by human presence (Skov *et al.*, 2002). Only active individuals with dimensions corresponding to adult stages, were considered in this study, i.e. *P. guttatum* and *U. annulipes* of 15 to 22 and 13 to 16 mm carapace width (CW), respectively. At each season, the same habitat was sampled at each mangrove: more landward zones characterized by the presence of *A. marina*, and where the different crab species form fairly monospecific assemblages throughout an intertidal fringe. Sampling consisted of haphazardly collecting 40 to 50 adult male crabs of each species from the intertidal zone during the morning low tides, of spring tides. As RNA/DNA ratio exhibit higher amplitude changes around moult events (Chang, 1995), only individuals in intermoult, as evaluated *in situ* by the hardness of the carapace, were considered.

A general feature of crabs is a larger claw in males than females, with *Uca* presenting extreme claw asymmetry. The muscle tissue of claws of male individuals of each species was used to determine the RNA/DNA ratio. After collection, the largest claw of each individual was removed and transported to the laboratory in cool boxes (~−10°C). Transportation time varied between 25 and 30 min. In the laboratory, claws were promptly freeze-dried and stored at −80°C until nucleic acid quantification was performed. Claws of seven individuals of each species, season and mangrove system combination were then randomly selected for biochemical analysis.

Biochemical analysis

Analyses were performed on 10 to 15 mg (freeze-dried weight) of claw white muscle tissue. RNA/DNA quantification was conducted following the Schmidt–Thannhauser method, according to the detailed procedures of Amaral *et al.* (2008). The sample was homogenized in 0.2N $HClO_4$ (PCA), incubated on ice and centrifuged (all centrifugations were at 6000*g* at 0–4°C for 13 min). The pellet was washed twice with PCA (0.2 N), resuspended in 0.3 N NaOH and incubated at 37°C. PCA (2.0 N) was added and the sample was incubated on ice and centrifuged. The supernatant containing the RNA fraction was measured for its absorbance at 260 nm. The pellet was washed twice with PCA (0.2 N), PCA (0.6 N) was added and the sample was incubated at 70°C. The sample was then incubated on ice and centrifuged. The supernatant containing the DNA fraction was measured for absorbance at 260 nm. Absorbance was determined in a NanoDrop® ND-1000 full spectrum spectrophotometer (NanoDrop, Wilmington, USA). Concurrently, nucleic acid extracts were inspected for contaminations by estimation of 260/280 and 260/230 nm

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ratios. Three readings were performed per sample. All metal, plastic and glassware were autoclaved prior to use.

Statistical analysis

Effects of species, season and mangrove system on the physiological condition of crabs, as evaluated by RNA/DNA ratio, were estimated using three-way analysis of variance (ANOVA). Cochran's tests revealed homoscedasticity of variances, and Shapiro–Wilk's tests revealed normality of residuals. Variance was low and no transformation of data was necessary. *A posteriori* comparisons were performed using Tukey's honest significant differences (HSD) tests.

RESULTS

Contamination of extracted nucleic acids was negligible as both 260/280 and 260/230nm ratios were higher than 1.9, at all times, for both RNA and DNA. ANOVA analysis (Cochran's $C = 0.245$, $P = 0.49$) revealed significant effects of species, season and mangrove system, and of all interactions on the RNA/DNA ratios (Table 2). In the unpolluted Ponta Rasa and in the polluted Costa do Sol mangroves the RNA/DNA ratios of both crab species were higher in the dry than in the rainy season (HSD $P < 0.01$, in all cases), while in the unpolluted Saco mangrove there were no differences among seasons (HSD $P > 0.98$, in all cases) (Figure 2).

Table 2. Results of three-way ANOVA analyses on the effects of species, season and mangrove system on RNA/DNA ratio of *Perisesarma guttatum* and *Uca annulipes*

Source of variation	df	MS	*F*	*P*
Species (A)	1	42.34	841.66	< 0.001
Season (B)	1	7.56	150.27	< 0.001
Mangrove (C)	2	4.62	91.85	< 0.001
A × B	1	0.57	11.33	< 0.01
A × C	2	2.88	57.20	< 0.001
B × C	2	1.27	25.22	< 0.001
A × B × C	2	0.10	2.04	< 0.001
Error	72	0.05		

Overall, the RNA/DNA ratio was significantly lower in *P. guttatum* than in *U. annulipes* (1.2 and 2.6, respectively, HSD $P < 0.001$) at all times (HSD $P < 0.05$, in all cases) (Figure 2). The overall average of RNA content in samples of freeze-dried muscle was similar in both species (Student's $t = 1.57$, $df = 82$, $P > 0.12$, Figure 3). DNA overall average content was, however, significantly higher in *P. guttatum* than in *U. annulipes* (Student's $t = 7.72$, $df = 82$, $P < 0.001$, Figure 3). Therefore, the overall lower RNA/DNA ratio in *P. guttatum* resulted not from lower RNA, but from relatively higher DNA content of the muscle tissue in relation to that of *U. annulipes*.

Regarding the effects of mangrove system, there were no significant differences in the RNA/DNA ratio of *P. guttatum* among mangroves (HSD $P > 0.20$). In contrast, the RNA/DNA ratio in *U. annulipes* was significantly lower in the polluted Costa do Sol than in the unpolluted Ponta Rasa and Saco mangroves (HSD $P < 0.001$, in both cases), which showed no differences among them (HSD $P = 1.00$) (Figure 2).

DISCUSSION

The RNA/DNA ratio can be a useful index for the effects of environmental constraints on protein synthesis, growth rate

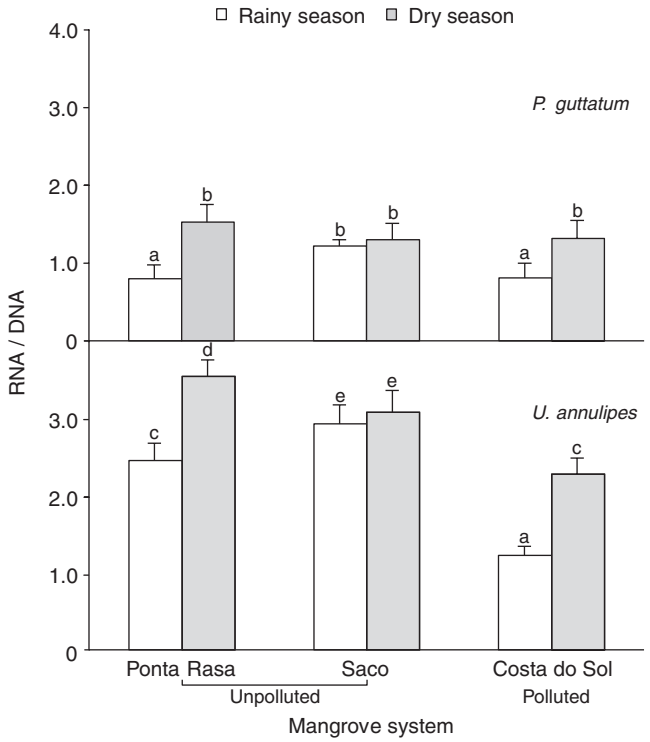

Figure 2. Mean RNA/DNA ratio of *Perisesarma guttatum* and *Uca annulipes* in three different Mozambican mangrove systems (unpolluted — Ponta Rasa and Saco; polluted — Costa do Sol), in the rainy and dry seasons. Bars that do not share any letters are significantly different at $P < 0.05$ (Tukey's HSD test). $N = 42$. Error bars: ± s.e.

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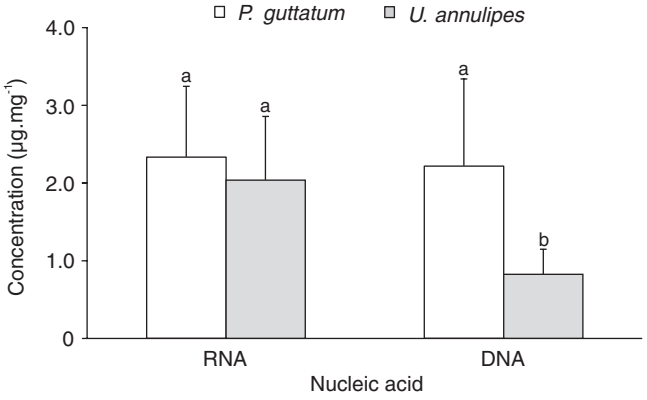

Figure 3. Mean overall RNA and DNA contents (µg mg^{-1}) in freeze-dried muscle tissue of *Perisesarma guttatum* and *Uca annulipes*. Bars that do not share any letters are significantly different at $P<0.001$ (Student's *t* test), and comparisons were only performed within each nucleic acid. $N=42$. Error bars: ±s.e.

and physiological health of fish (Buckley *et al*., 1999; Gilliers *et al*., 2004) and marine invertebrates (Wright and Hetzel, 1985; Dahlhoff and Menge, 1996; Parslow-Williams *et al*., 2001; Buckley and Szmant, 2004), including crabs (Wang and Stickle, 1986; Mayrand *et al*., 2000; Amaral *et al*., 2008). While high RNA levels are usually interpreted as beneficial, reflecting active metabolic states and elevated protein production for growth and reproduction, increased protein synthesis might sometimes reflect a stress response (Buckley and Szmant, 2004; Dahlhoff, 2004), invalidating, in such cases, the bioindicator potential of the RNA/DNA ratio.

The RNA/DNA ratios of *P. guttatum* and *U. annulipes* were sensitive to climatic season and mangrove pollution, but showed different interspecific patterns. The exception was the unpolluted Saco mangrove, where no seasonality was observed in either species. Nevertheless, in general, those ratios were more affected by seasonality than by pollution, with lower ratios in the rainy season in each species. High responsiveness of the RNA/DNA ratio of two *Uca* species to seasonality was recently reported in a field-mesocosm study conducted in Tanzania (Amaral *et al*., in press).

Optimum temperatures usually favour metabolic activities, translated into enhanced growth and reproductive output (Ota and Landry, 1984; Wagner *et al*., 2001; Clemmesen *et al*., 2003; Melzner *et al*., 2005). In Mozambique, the lower temperature and humidity characterizing the dry season may be more favourable to crabs. The exception to this pattern was the unpolluted Saco mangrove, where the enclosed character of the site and strong influence from the Indian Ocean (Kalk, 1995; Paula *et al*., 2003) may attenuate the impact of seasonal climatic fluctuations, resulting in the lack of marked seasonal differences in the RNA/DNA ratio of each species. This continuum of climatic conditions, may also explain why the RNA/DNA ratios in the unpolluted Saco mangrove were intermediate, for each species, to those from the rainy and dry seasons in the other unpolluted mangrove studied, Ponta Rasa. It is suggested that season effects on the RNA/DNA ratios of mangrove crabs may only be discernable in systems experiencing marked seasonal climate fluctuations.

The RNA/DNA ratio for *U. annulipes* was sensitive to mangrove pollution, being significantly lower at the polluted peri-urban Costa do Sol mangrove than at the unpolluted Ponta Rasa and Saco mangroves in both seasons. This contrasts with the lack of pollution sensitivity of the RNA/DNA ratio for *P. guttatum*. Several studies conducted in different organisms support the usefulness of the RNA/DNA ratio as an indicator of pollution (Wo *et al*., 1999; Dahl *et al*., 2006). However, the opposite has also been reported, especially under good feeding conditions (Houlihan *et al*., 1994).

Fiddler crabs, such as *U. annulipes*, feed mainly on microphytobenthos and organic matter associated with sediment surface (Skov *et al*., 2002), which are usually increased in polluted habitats (Ashton *et al*., 2003; Lim and Heng, 2007) such as Costa do Sol mangrove. Stable isotope signatures of fiddler crabs have revealed a clear diet change in *Uca* crabs between unpolluted mangroves (Ponta Rasa and Saco and Saco), where a broad range of food sources was identified, and polluted mangroves (Costa do Sol), where microphytobenthos was almost exclusively the only food source (PUMPSEA, 2007). Although an increase in food availability would theoretically be favourable, it may be of poor quality due to pollution-association, negatively affecting synthetic or metabolic pathways and rates of fiddler crabs, and resulting in a generally lower physiological condition (Meziane and Tsuchiya, 2002).

The RNA/DNA ratio of *U. annulipes* showed an average decrease of ~64% in the polluted Costa do Sol mangrove during the rainy season, while showing reductions of only ~17 and ~43% when considering separately season and pollution, respectively. This suggests that pollution may act synergistically with climatic season, leading to serious deleterious effects on fiddler crabs. Although this combination is a relevant and, unfortunately, widespread scenario, few studies have so far addressed its effects on crabs (Walther *et al*., 2002; Lannig *et al*., 2006).

The RNA/DNA ratio of *U. annulipes* was significantly higher than that of *P. guttatum* at all times. Interestingly, this resulted from higher DNA rather than lower RNA contents in the muscle tissue of *P. guttatum*. Naturally higher cellular DNA content among closely related species has been documented in several animal taxa, including crustaceans (Hartley, 1990; Gregory *et al*., 2000; Boulesteix *et al*., 2006). On the other hand, the larger claws of *Uca* males are supported by larger muscle fibres (Govind *et al*., 1986; Rhodes, 1986), which may be originated by larger muscle cells (Penney *et al*., 1983; Nader, 2007). Therefore, a similar weight of freeze-dried

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claw muscle tissue may contain fewer cells, and thus lower DNA content, in *U. annulipes* than in *P. guttatum*. Only histological and genetic studies will be able to clarify this issue.

CONCLUSIONS

This study provides statistical evidence that the RNA/DNA ratio of *U. annulipes* varies both with season and habitat pollution level, supporting its usefulness as an indicator of the environmental quality of mangrove habitats, especially in systems with marked climatic seasons. This study furthermore suggests a synergistic and negative effect of the rainy season and pollution on the physiological condition of *U. annulipes*. The RNA/DNA ratio of *P. guttatum*, despite being affected by season, does not seem to be a good indicator of environmental quality of mangrove habitats. Future research should focus on mangrove crab responses to other environmental stressors, so they can be used as sentinel species in integrative management and conservation programmes.

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