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Heart beat rate adaptations to varying salinity of two intertidal Mediterranean bivalves: The invasive *Brachidontes pharaonis* and the native *Mytilaster minimus*

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Abstract
Changes in heart beat rates (HBR) of *Brachidontes pharaonis* (an invasive Lessepsian species) and *Mytilaster minimus* (a native Mediterranean species) in response to changes in salinity values from brackish (20) to extreme hyper-saline (75) were investigated both *in situ* and in the laboratory. The two species displayed different responses to varying salinity, with clear differences in HBR observed between groups investigated at different salinities and between groups originating from different environments. The native species, adapted to narrow salinity changes as those observed in the superficial waters of the Mediterranean Sea, showed signs of stress at salinities slightly above 37 (in the range 37–45). On the other hand, the invasive species displayed clear signs of stress only at salinities over 45 and exhibited heart activity even under hyper-saline conditions (i.e. salinity = 75). These results indicate that the Lessepsian species has the potential to invade most of the transitional environments across the entire Mediterranean basin. We therefore stress the need of including *Brachidontes pharaonis* in all coastal monitoring programmes assessing the actual and potential spreading of alien species in the Mediterranean region.

Keywords: *Bivalves,* Brachidontes pharaonis, Mytilaster minimus*, salinity, Mediterranean Sea*

Introduction

Since the opening of the Suez Canal in 1869, many species from the Red Sea and the Indian Ocean have entered the Mediterranean Sea and established dense populations in several coastal areas across the entire basin (Galil 2007). Most of these 'alien' species have spread across the Mediterranean Sea through human transportation (e.g. ballast waters), and reached sites a considerable distance from the Suez Canal. The bivalve *Brachidontes pharaonis*, for instance, penetrated the western Mediterranean Sea about 35 years ago (e.g. Eastern Sicily; Di Geronimo 1971). In the following years, *B. pharaonis* has entered other suitable habitats characterised by environmental conditions similar to those encountered in Red Sea Egyptian salt lakes (*sensu* Safriel et al. 1980): among those habitats, the ephemeral coastal pools and the salt ponds of the western Sicily (South Mediterranean Sea) have been consistently invaded (Sarà et al. 2003). These ponds, which are real hot spots characterised by very high densities of this bivalve, also function as concentration nuclei for further growth and spreading into the whole western basin of the Mediterranean Sea, as far as the Straits of Gibraltar (*sensu* Ruiz et al. 2000). Later, during the last seven years, *B. pharaonis* has increasingly been observed in many intertidal habitats of the Southern (Sarà & Buffa 2004; Sarà et al. 2007) and Northern Tyrrhenian Seas (French lagoons; Antoine Carlier, personal communication). In these areas, located at latitudes higher than those of the original site, *B. pharaonis* is establishing beds on several natural and artificial hard substrata, out-competing indigenous bivalve species such as *Mytilaster minimus* and *Mytilus galloprovincialis* (Safriel et al. 1980; Safriel & Sasson-Frostig 1988; Sarà et al. 2007). The ability of *B. pharaonis* to colonise different intertidal patches of the Mediterranean Sea allows the hypothesis that this species could have a high metabolic plasticity, which, in turn, would enable this bivalve to replace some of the native bivalve

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species (namely *M. galloprovincialis* and *M. minimus*; Safriel et al. 1980). To date, very scant information exists about the limits of tolerance to changes in salinity of the invasive species compared with that of the potentially threatened autochthonous species, *Mytilaster minimus* (Sarà et al. 2000, 2008).

In this study we measured, for the first time, variations in the heart beat rates (HBR) of two bivalves, commonly found in the Mediterranean Sea, in response to changes in salinity. The two species selected for the study are the small native *Mytilaster minimus*, the most frequent and common intertidal benthic species in the southern Mediterranean Sea, and the invasive Lessepsian species *Brachidontes pharaonis*, which is assumed to be the best candidate to replace *M. minimus* in the intertidal zone of the entire Mediterranean basin, thus posing a potential threat to the biodiversity of this region.

The aim of the present study, carried out by means of transplanting experiments, is to test the hypothesis that *B. pharaonis* and *M. minimus* exhibit different physiological responses to a wide range of salinities from those typical of brackish waters to those hypersaline conditions typical of salt production basins. The rationale behind this hypothesis is that the Lessepsian species could own a wider ability to sustain salinity stress as that typically encountered in most transitional environments along the coasts of the Mediterranean Sea, thus threatening the survivorship of the native mussels.

Materials and methods

Sampling and experimental design

Specimens of *Brachidontes pharaonis* and *Mytilaster minimus* were collected from a marine intertidal area (Capo Plaia, Cefalù; 38° 01.44' N; 13° 56.44' E) where they co-habit in waters with a mean salinity of ~37.4. These specimens were used to measure heart beat rates determined by using a non-invasive cardio-plethysmographich technique (Depledge & Andersen 1990), which has been successfully employed to investigate the ecological aspects of many intertidal benthic organisms, such as gastropods (De Pirro et al. 1999; Santini et al. 1999), and crabs (DeFur & Mangum 1979) under different levels of exposure to pollutants (Curtis et al. 2000; Halldórsson et al. 2008). In summer 2009, about 100 specimens of *B. pharaonis* and 100 of *M. minimus* were collected and immediately screened for HBR in marine conditions and in the air (see below). Infra-red sensors were glued externally to the left valve, close to the heart position. Signals obtained from mussel hearts were amplified and filtered by using a special amplifier card and then detected by means of a portable oscilloscope (Fluke™ 125) connected to a laptop computer equipped with the Fluke View 3.0 software. HBR (beat s^{-1}) were recorded at 5-min intervals during a 1-h experimental session. HBR values reported here are the average of 12 measurements per hour. After that, the mussels were brought to the laboratory and sorted by size (about 18.0 ± 2.0 mm and about 12.0 ± 1.0 mm for *B. pharaonis* and *M. minimus*, respectively). Thirty-six sorted individuals from each species were thus transplanted at five different salinities (20, 37, 45, 60 and 75; water temperature 22.0 ± 0.5°C) in five different steps (3 days acclimation and 1 day for measurement at each salinity). In addition to these, another experimental group comprised 36 individuals of *B. pharaonis* collected from the hyper-saline Marsala lagoon (Western Sicily: 37°51.5 N; 12°28.6 W) with an annual mean salinity of 40 where this species forms dense beds (Sarà et al. 2000, 2003; Sarà 2006, 2007). All experimental groups were left to acclimate for three days in the new conditions of salinity. Before the acclimation, infra-red sensors were glued to valves of each experimental specimen to avoid the possible interference of hand manipulation just before the measurements. HBR measurements were carried out on a total of 18 randomly selected specimens from each experimental group (i.e. level of salinity).

Statistical analyses

Either native or invasive species would have a degree of phenotypic plasticity and genetic variation in coping environmental stresses. In this study the invasive species *B. pharaonis* were collected from a pond and a marine intertidal rocky shore, while the native species *M. minimus* were collected from the rocky site only (*Mytilaster* is not present in the pond due to too high salinity). Because of this unbalanced experimental design, we were not able to ascertain the natural variability (plasticity) of *M. minimus* collected from different (marine and pond) sites. To cope with this possible bias, we used a series of partial two-way analysis of variance (ANOVA; Kelaher et al. 1998) contrasting *Mytilaster* (marine) vs. *B. pharaonis* (pond), *Mytilaster* (marine) vs. *B. pharaonis* (marine); *B. pharaonis* (marine) vs. *B. pharaonis* (pond) to test the hypothesis that HBR varied as a function of salinity (Salinity, 6 fixed levels). Two groups of specimens (Group, 2 levels) for each salinity were treated as random factors, with $n = 9$ as a combination of factors. For all analyses, the heterogeneity of variance was tested using Cochran's C test prior to the ANOVA, and the Student–Newman–Keuls (SNK) test allowed the appropriate means comparison.

ANOVA and SNK tests were carried out using GMAV rel 5.0 (University of Sydney).

Results and discussion

Heart beat rate in bivalves, which present the valve gaping modulation (Maire et al. 2007), should reach minimum values (or even the total arrest of heart contraction; Bakmet et al. 2005) when valves are closed to prevent stress (i.e. during emersion at low tides or when they are exposed to lethal levels of pollutants; Halldórsson et al. 2008). On the other hand, highest HBRs are reached when the bivalve's heart compensates for stressful conditions. However, along this minimum-to-maximum range of HBR, any single bivalve shows its own metabolic optimum, which corresponds to the trade-off between a low metabolic expenditure and the maximum gain in terms of energy intake guaranteed by food. Thus, the metabolic optimum is, on the one hand, species-specific and, on the other, variable as a function of acclimation ability. In the present study we therefore assumed that the higher the metabolic optimum as evidenced by HBR, the wider the ability to face wide varying environmental conditions (i.e. to colonise large numbers of habitats; *sensu* Bayne 1973). Results from the present study showed that the Mediterranean native species *Mytilaster minimus* and the Lessepsian invasive species *Brachidontes pharaonis* displayed different behavioural heart responses to varying salinity. HBR measured at different conditions were overall significantly different between groups, and varied as a function of origin of organisms (Table I).

Bivalves of the two species acclimated at the sea conditions or exposed to air (prior to transplantation) showed similar HBR values (ANOVA, $p > 0.05$; Figure 1), whereas *B. pharaonis* collected from the pond showed HBR significantly higher than those measured in marine individuals of the two species (Figure 1). These results indicate that *B. pharaonis* living in the pond showed a higher metabolism than the marine specimens and suggests that the tidal emersion – coupled with dramatic changes in the environmental conditions surrounding the bivalves – might be a relevant factor enhancing the metabolism of *B. pharaonis*. The fact that the HBR of organisms of different species inhabiting the same habitat share similar physiological responses has only rarely been highlighted across the current literature. Investigations carried out on other mussels and chitons (*Mytilus edulis* and *Katherina tunicata* from Alaska coasts) showed, for instance, that phylogenetically distant species can have very different heart beat responses to stepwise varying salinities (in the range 10 species 30; Stickle & Sabourin 1979). Under brackish conditions

Table I. Post-hoc comparison SNK outcome to verify intra-specific/origin differences as a function of varying salinities (Bp-SN = *Brachidontes pharaonis* from S. Nicola intertidal marine conditions; Myt-SN = *Mytilaster minimus* from S. Nicola intertidal marine conditions; BP-Ett = *B. pharaonis* from Ettore pond; [*= $P \le 0.05$; **= $P \le 0.01$; ***= $P \le 0.001$; ns = no significant difference ($P > 0.05$)].

Bp-SN (sea)	Air	20‰	37‰	45‰	60‰	75‰
Air	–					
20‰	ns	–				
37‰	ns	ns	–			
45‰	*	*	*	–		
60‰	*	*	*	*	–	
75‰	*	*	*	*	*	–
Myt-SN (sea)						
Air	–					
20‰	ns	–				
37‰	ns	ns	–			
45‰	*	*	*	–		
60‰	*	*	*	*	–	
75‰	*	*	*	*	–	–
Bp-Ett (pond)						
Air	–					
20‰	*	–				
37‰	ns	*	–			
45‰	*	*	ns	–		
60‰	*	*	*	*	–	
75‰	*	*	*	*	*	–

(salinity equal to 20), both species, independently from their origin, showed no significant differences in HBR, which were also relatively low (Table I; Figure 1). Since both species are typical of marine habitats and have been observed only occasionally in some brackish Mediterranean sites at higher latitudes (e.g. in the Po river Delta) (De Min & Vio 1997; Marchini & Marchini 2006), we could infer that the significant decrease in HBR of *B. pharaonis* from the pond could be interpreted as a brachycardia-like response to brackish salinity.

The physiological divergence between the marine and pond species was again evident at the typical seawater salinity (i.e. 37), where the HBR of the *B. pharaonis* from the pond was significantly higher than those of the two marine groups (Figure 1; Table I). At higher salinities, all of the three experimental groups showed significant HBR behaviours. *M. minimus* showed the highest HBR at a salinity of 45, when *B. pharaonis* specimens from the marine and pond habitats showed an increase in HBR, reaching values not significantly different each other (ANOVA, $p > 0.05$). At a salinity of 60, *M. minimus* exhibited a clear decline in HBR values, which continued at a salinity of 75 and indicated a clear cardiac sufferance as highlighted by a noticeable brachycardia. At the salinity of 60 specimens belonging to the

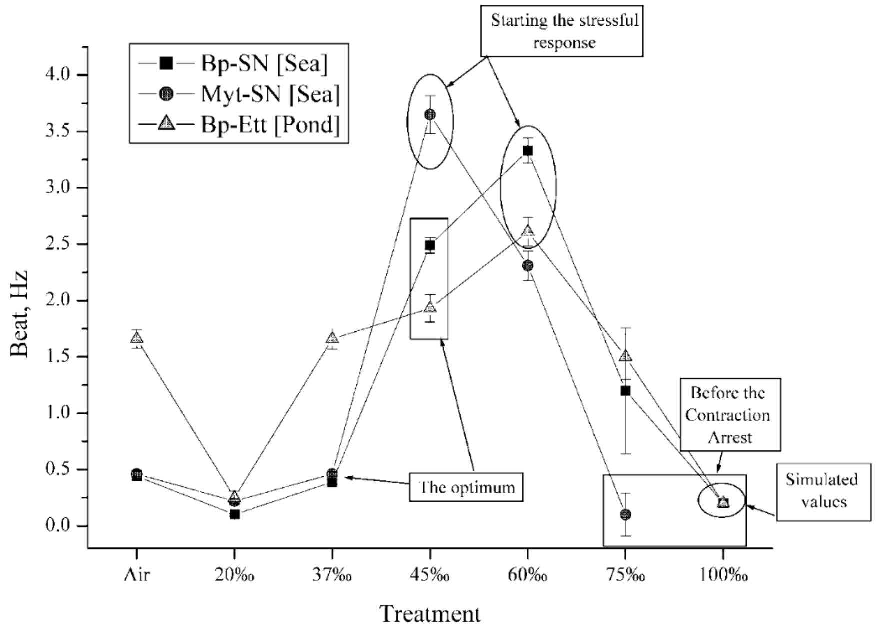

Figure 1. HBR of investigated species at different levels of salinities (Bp-SN = *Brachidontes pharaonis* from marine site, S. Nicola; Bp-Ett = *B. pharaonis* from ponds; Myt-SN = *Mytilaster minimus* from marine site, S. Nicola). HBR values at 100 are simulated.

two groups of the invasive species showed their highest HBR (i.e. tachycardia), which declined considerably only at the highest salinity of 75. Although, unfortunately, we did not measure HBR of the invasive species under salinity higher than 75, we can hypothesise that the brachycardia, which should anticipate the total arrest of heart contraction (Bakmet et al. 2005), would occur for salinities (probably > 100) much higher than those measured in this experiment. This hypothesis is corroborated by the fact that *B. pharaonis* forms extended beds also in the Red Sea salt lakes, where salinity can be as high as 90 (Cohen et al. 1977). Previous studies carried out to test the effect of salinity on the metabolic adaptation of bivalves have dealt primarily with the adaptation of mussels (particularly *M. edulis*) to variable salinity across a range from 5 to 37 (Stickle & Sabourin 1979; Widdows 1985; Bakmet et al. 2005). Those studies were mostly aimed at analysing the effects of increasing salinities over the typical values encountered by *Mytilus edulis* in Northern European seas including estuaries and intertidal habitats (Bayne 1973; Stickle & Sabourin 1979; Widdows et al. 1985; Bakmet et al. 2005). In this study, for the first time, we have provided experimental evidence of significant differences in the modulation of heart beat rate by bivalves living in the warm and salty Mediterranean Sea and nearby brackish and hyper-haline habitats. Overall, the results of the present study highlight that two investigated species can adapt differently to varying salinities and, in particular, that the native species *M. minimus* does not show any stress response until the salinity remains below 45, whereas the invasive species *B. pharaonis* shows a clear response to salinity stress at a threshold well above 45. The results of the present study allow also inferring that the ability to colonise new, harsh environments by *M. minimus* and *B. pharaonis* primarily depend upon physical and chemical factors such as salinity or temperature (Sarà et al. 2008), which also determine their geographical distribution at the large scale. Unfortunately, we were unable to analyse the relationships between HBR and respiration. Nevertheless, it is well known that HBR and oxygen consumption are correlated positively (e.g. Bayne 1973). At the same time, the higher the oxygen consumption, the lower the Scope for Growth (i.e. the difference between energy intake and metabolic output) (Bayne et al. 1976; Stickle & Sabourin 1979; Widdows 1985), and in turn, it depends upon the quality and quantity of food availability (Gosling 2004; Sarà & Pusceddu 2008). Based on these assumptions the HBR of the investigated species to varying salinity could also be coupled with the trophic availability, which could change considerably under the different salinity conditions. Therefore, a further step in evaluating the spreading potential of the invasive species in the Mediterranean basin under an increasing salinity scenario and the eventual coupled decline or replacement of the native one will be the assessment of the physiological responses of the two species under scenarios of variable food availability. Since the present climate changes of the Mediterranean Sea might produce significant variations in the production of the entire basin (Danovaro et al. 2004), this step appears extremely urgent.

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