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BIOCHEMICAL STUDIES OF THE LIMPET *Patella depressa* PENNANT, 1777 FROM MARINAS AND ROCKY SHORES OF THE PORTUGUESE COAST: A MARKER APPROACH

Dissertação para a obtenção do grau de mestre em Estudos Marinhos e Costeiros – Gestão Ambiental de Zonas Costeiras

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O conteúdo desta dissertação é da inteira responsabilidade da sua autora

ABSTRACT

Coastlines of many regions in the world are being transformed and becoming increasingly more subjected to anthropogenic changes such as the increase of man-made structures including marinas. Thus, it is important to understand the role of artificial structures in sustaining natural biochemical processes. Intertidal limpets such as *Patella depressa* Pennant are dominant grazers on artificial (i.e. marinas) and natural rocky shores on the Portuguese coast, playing a key role in structuring intertidal algal communities. Limpets have been mainly studied in ecological and taxonomic studies while biochemical descriptions are scarce. Moreover, limited knowledge exists on differences in biochemical responses of limpets living in artificial and adjacent natural rocky shores. The use of informative biological marker tools such pollution biomarkers and fatty acid trophic markers have also been overlooked. However, such tools provide information pertinent to the future sustainable exploitation and management of both natural and artificial ecosystems.

The present work encompassed two main aims. The first half of the work aimed to assess and compare gender and spatial variation of pollution biomarkers in the limpet *P. depressa* collected from marinas and rocky shores of Portuguese central and south coast, and also to examine the importance of biotic and abiotic factors (body size, sex and water temperature) in determining the outcome of biomarker analysis. The measured biomarkers were metallothionein (MT) concentration and acetylcholinesterase (AChE) enzymatic activity and indicators of physiological status (shell length, wet tissue weight, condition index and protein content) were also measured. Inhibition of AChE activity reflects exposure to neurotoxic compounds and MT content is used to indicate exposure to metals. The second main aim of the present work was to determine the importance of gender and habitat in explaining gonad fatty acid profiles using univariate and multivariate statistical analyses. Furthermore, a study of the *P. depressa* diet from marinas and rocky shores of the south Portuguese coast was made using a fatty acid trophic marker approach in order to assess dietary intakes and trophic relationships between limpets and their food sources at both artificial and natural substrata.

In the present study, an important result was the effect that gender had on biochemical responses of limpets. AChE and MT levels were higher in females. Higher

amounts of total lipid contents were also observed in female gonads. Males had higher concentrations of polyunsaturated (PUFA) fatty acids while females had higher concentrations of saturated (SFA) and monounsaturated (MUFA) fatty acids suggesting a higher degree of energetic storage in females and structural storage in males. This difference may be related to selective retention and sex reproductive strategies in lipid storage originated from diet. A FATM approach was also used to investigate *P. depressa* diet. This species was characterized by a 22:6(n-3)/20:5(n-3) ratio lower than 1, a 18:1(n-7)/18:1(n-9) ratio higher than 1, along with a high content of the acid 20:5(n-3), thus suggesting a more generalist herbivorous diet, richer in diatoms, bacteria and algae (mainly red algae). Dietary ingestion is thought to be a major route of metals uptake and accumulation in invertebrates. Hence, differences in storage and selective retention of specific fatty acids associated with different metal uptakes from diet were suggested to be the major causes for limpet biochemical responses and gender differences found in the present study.

Multivariate analyses showed significant differences in the fatty acid signature between habitats, however such habitat effect was only detected in males. Males from marinas showed higher concentrations of 20:5(n-3) than males from rocky shores most likely indicating that red algae and diatoms could be an important component of the male diet in marinas. Hence, limpets might be able to adjust their feeding behaviour in response to food availability. Despite the influence of biotic and abiotic factors on both pollution biomarker results, a general artificial/natural trend was observed. AChE activity levels varied significantly with locations in the Portuguese coast. An increasing gradient of activity from the central to the south locations and from artificial to natural rocky shores was observed suggesting that water temperature and neurotoxic contaminants could be responsible for these values. MT levels also revealed location differences with an unexpectedly elevated concentration in natural rocky shores compared to artificial (i.e. marinas) rocky shores. These differences were suggested to be related to shell length, body weight and metal detoxification mechanisms.

Both these results indicated that AChE activity as well as MT concentration could be good early indicators of contamination in *P. depressa* only if biotic and abiotic factors are taken into consideration when applying these biomarkers. The overall findings of the present work suggest that gender should be a factor that needs to be

taken into consideration when studying biochemical responses of limpets. The use of *P. depressa* as a sentinel species for biomonitoring potential toxic effects *in situ* appears feasible in a multi-biomarker approach, thus allowing examining relationships between biomarkers, contaminants and physiological status indicators of limpets. A FATM approach and multivariate statistical analyses of the Fatty Acid (FA) profiles proved to be useful tools for exploring and demonstrating differences in diet with gender and habitat and also to determine what FAs, and therefore what dietary items, were responsible for those differences. The integration of these findings on our current knowledge of the limpet species and of biochemical and marker studies was made in the final discussion chapter.

RESUMO

As orlas costeiras em todo o mundo tem vindo a sofrer alterações e a tornar-se progressivamente mais sujeitas à intervenção humana nomeadamente no que diz respeito à construção de infra-estruturas como marinas e portos. Deste modo, torna-se essencial estudar e comparar respostas bioquímicas de organismos comuns em ambientes naturais e intervencionados pelo homem de forma a gerir, planear e explorar de forma sustentável a zona costeira. As lapas da espécie *Patella depressa* Pennant são organismos abundantes em praias rochosas e substratos rochosos artificiais como portos e marinas ao longo da costa Portuguesa. Estes organismos desempenham um papel fundamental na estruturação das comunidades de algas em ambos os ecossistemas e apesar de exaustivamente estudados a nível ecológico, são escassos os conhecimentos ao nível da descrição e comparação das respostas bioquímicas desta espécie em substratos artificiais (marinas) e naturais (praias rochosas).

Dois tipos de marcadores bioquímicos foram usados no presente estudo: biomarcadores de exposição e marcadores associados à dieta através da análise de perfis de ácidos gordos. Numa primeira abordagem avaliaram-se as diferenças entre machos e fêmeas e compararam-se espacialmente as respostas bioquímicas de lapas provenientes de substratos naturais e artificias da costa Portuguesa. Analisou-se igualmente a importância e influência de alguns factores bióticos e abióticos nos resultados obtidos em cada um dos biomarcadores de exposição. Foi medida a concentração da proteína metalotioneina (MT) e a actividade da enzima acetilcolinesterase (AChE), biomarcadores de exposição a metais e compostos neurotóxicos, respectivamente. Foram igualmente avaliados alguns indicadores do estado fisiológico das lapas tais como o comprimento da concha, peso dos tecidos, o índice de condição e o conteúdo proteico dos tecidos. Numa segunda fase foi avaliada a diferença entre sexos e determinada a importância do habitat no perfil de ácidos gordos das gónadas das lapas. Nesta abordagem foi usada uma análise estatística multivariada dos dados que permitiu a análise simultânea de vários ácidos gordos. Com o intuito de avaliar a dieta de lapas provenientes de marinas e praias rochosas do litoral sul português foram igualmente usados marcadores específicos, analisando os perfis de ácidos gordos das gónadas das lapas.

Diferenças significativas entre machos e fêmeas foram observadas em todos os marcadores bioquímicos analisados neste estudo. As fêmeas apresentaram maiores valores de actividade de AChE e de concentração de MT. Valores mais elevados de conteúdo lipídico total foram igualmente observados nas gónadas das fêmeas. Os machos apresentaram maiores concentrações de ácidos gordos poli-insaturados (PUFA), enquanto que nas fêmeas dominaram os ácidos gordos saturados (SFA) e mono-insaturados (MUFA), sugerindo um maior armazenamento de ácidos gordos energéticos por parte das fêmeas enquanto que os machos acumulam mais ácidos gordos estruturais. Estas diferenças poderão estar relacionadas com diferentes estratégias reprodutivas adoptadas por machos e fêmeas no que diz respeito ao armazenamento de lípidos a partir da dieta. De forma a estudar a dieta desta espécie foram utilizados marcadores tróficos específicos. Esta espécie apresentou um rácio 22:6 (n-3) / 20:5 (n-3) inferior a 1, 18:1 (n-7) / 18:1 (n-9) superior a 1, e um alto teor do ácido gordo 20:5 (n-3), sugerindo uma dieta herbívora generalista, rica em diatomáceas, bactérias e algas (especialmente algas vermelhas). Estudos em invertebrados indicaram que a dieta é a principal via de acumulação de metais nestes organismos. Deste modo as diferenças obtidas neste estudo ao nível dos marcadores bioquímicos analisados podem estar relacionadas com a acumulação selectiva de ácidos gordos e consequentemente de diferentes metais provenientes da dieta ingerida pelas lapas.

A análise multivariada dos perfis em ácidos gordos das gónadas das lapas revelou diferenças significativas entre habitats, no entanto, estas diferenças só foram detectadas nas gónadas dos machos. Os machos provenientes das marinas apresentaram maiores concentrações de 20:5 (n-3) comparativamente aos machos das praias rochosas, podendo este facto estar associado a uma dieta mais rica em algas vermelhas e diatomáceas. Este resultado sugere deste modo uma possível adaptação do comportamento alimentar das lapas ao alimento disponível em cada um dos habitats. No que diz respeito aos biomarcadores de exposição, apesar da influência de factores abióticos e bióticos nos níveis de AChE e de MT obtidos, foi possível observar diferenças entre marinas e praias. A actividade da AChE variou significativamente ao longo da costa Portuguesa, observando-se um aumento de actividade da enzima do centro para o sul da costa portuguesa e das marinas para às praias. Diferenças na temperatura da água e/ou a presença de contaminantes poderão ser os factores responsáveis por estes gradientes. No entanto, as concentrações de MT foram mais

elevadas em substratos naturais comparativamente aos substratos artificiais (marinas). Estas diferenças poderão estar relacionadas com o comprimento da concha, com o peso corporal da lapa ou ainda associadas a mecanismos específicos de desintoxicação de metais.

Ambos os resultados demonstram a importância da medição de AChE e MT em *P. depressa* se factores como o sexo, o estado de maturação das gónadas, o tamanho e comprimento dos organismos e a temperatura da água do mar forem levados em consideração aquando da aplicação destes biomarcadores. Analisando todos os resultados obtidos pode-se concluir que a diferença entre sexos é um factor muito importante a ter em conta no estudo das respostas bioquímicas das lapas. A utilização da lapa *P. depressa* como espécie sentinela para a biomonitorização de potenciais efeitos tóxicos *in situ* é viável, se forem analisadas as inter-relações entre biomarcadores, contaminantes e indicadores do estado fisiológico das lapas. A utilização de um conjunto de marcadores específicos associados à dieta e de uma abordagem multivariada na análise dos perfis de ácidos gordos provaram ser instrumentos úteis na exploração e demonstração das diferenças existentes entre sexos e habitats. Permitiu igualmente determinar quais os ácidos gordos responsáveis por essas diferenças e consequentemente extrapolar possíveis dietas.

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ABBREVIATIONS:

AChE – acetylcholinesterase;

Ag – silver;

ARA – arachidonic acid, 20:4(n-6);

Cd – cadmium;

CI – condition index;

Cu – copper;

DHA – docosahexaenoic acid, 22:6(n-3);

EPA – eicosapentaenoic acid, 20:5(n-3);

FA – fatty acid;

FAME – fatty acid methyl esters;

FATM – fatty acid trophic markers;

Hg – mercury;

HUFA – highly unsaturated fatty acids;

MT – metallothionein;

MUFA – monounsaturated fatty acids;

PAH – polycyclic aromatic hydrocarbons;

PCB – polychlorinated biphenyls;

PT – protein content;

PUFA – polyunsaturated fatty acids;

SFA – saturated fatty acids;

TBT – tributyltin;

TL – total lipid content;

Zn – zinc.

CHAPTER 1

General Introduction

CHAPTER 1: GENERAL INTRODUCTION

1.1. Why study marinas and rocky shores?

Intertidal rocky shores are heterogeneous environments that support a wide variety of living forms and occur throughout the world coastlines (Thompson *et al.* 2002). In these systems, organisms are spatially distributed in a particular way, occurring at specific levels along a height axis, from the lower to the upper shore (Stephenson & Stephenson 1949; Lewis 1964; Underwood 1981; Raffaelli & Hawkins 1996; Thompson *et al.* 2002). This distribution is strongly driven by two major environmental gradients: (1) the sea-land vertical gradient along which species are distributed according to their physiological tolerance to physical factors such as aerial exposure, temperature and desiccation and (2) the horizontal gradient of exposure to wave action along which intertidal organisms develop morphological, physiological and behavioural adaptations to cope with different regimes of hydrodynamic conditions (Raffaelli & Hawkins 1996). Besides these environmental forces, biological factors such as competition, recruitment, behaviour and predation (Menge & Sutherland 1976; Raffaelli & Hawkins 1996) are also important structuring forces of intertidal assemblages on rocky shores.

Coastlines of many regions in the world are being transformed and becoming progressively more subjected to anthropogenic changes (Reilly *et al.* 1996; Gray 1997; Thompson *et al.* 2002). Progressive expansion of human populations and increasing urban development in coastal areas is leading to a reduction and fragmentation of natural habitats and to the proliferation of artificial hard structures including breakwaters, pier-pilings and jetties, floating pontoons, seawalls, harbours and marinas. These essentially represent artificial rocky shores, providing substrata for many

intertidal and subtidal benthic organisms (e.g. Reilly *et al.* 1996; Gray 1997; Chapman 2003; Chapman & Bulleri 2003; Bulleri & Chapman 2004; Airoldi *et al.* 2005; Bulleri 2005a; Bulleri *et al.* 2005). Loss of habitat is the most critical threat to marine biodiversity (Gray 1997) and as a consequence of global climate changes such as the rise in the sea level (Cabanes *et al.* 2001) and frequency of storms (Thompson *et al.* 2002), a further increase in the number of artificial coastal defence structures is be predicted in the next few decades (Reilly *et al.* 1996; Thompson *et al.* 2002; Alves *et al.* 2007). Therefore, there is a considerable challenge in distinguishing anthropogenic change from natural variability on these structures, and a comprehensive understanding of the potential impacts of these human disturbances on natural assemblages of organisms is essential in order to plan useful strategies of conservation and management of natural habitats (Thompson *et al.* 2002).

The comparison of assemblages between artificial and natural habitats is a fundamental step to assess if there is real loss and fragmentation of natural habitats. Recent studies evaluated the ecology of artificial habitats to assess the extent to which they can be considered as surrogates for natural habitats, and to identify changes caused to natural assemblages by artificial structures (Glasby 1999a,b; Chapman & Bulleri 2003; Bulleri & Chapman 2004; Bulleri 2005a; Bulleri *et al.* 2005). Current knowledge suggests that at high- and mid-shore levels, assemblages on artificial rocky shores differed from those on natural ones, but this was not the case at low-shore levels (Chapman & Bulleri 2003; Bulleri & Chapman 2004; Bulleri *et al.* 2005; Benedetti-Cecchi & Osio 2007). In general, a similar suite of species live on artificial and natural rocky shores, but vary in their relative abundances and frequencies of occurrence according to height on the shore and location (e.g. Hawkins & Hartnoll 1983; Chapman 2003; Chapman & Bulleri 2003; Bulleri & Chapman 2004; Bulleri 2005a,b; Bulleri *et*

al. 2005). Also according to these studies, fewer *taxa* are found on artificial than on natural rocky shores, suggesting that the former type of surface may not provide suitable intertidal habitat to support the full diversity of intertidal species, including those that are relatively rare (Chapman 2003; Bulleri & Chapman 2004; Moreira *et al.* 2006).

Differences between natural and artificial rocky shores can be in the: (i) nature, orientation and features (e.g. weathering, heterogeneity and inclination of the substratum and extent of intertidal habitat) (Archambault & Bourget 1996; Bulleri *et al.* 2005); (ii) light exposure and degree of shading (Glasby 1999a,b; Blockley & Chapman 2006); (iii) ecological processes such as recruitment, settlement, mortality, competition and/or predation (Chapman 2003; Bulleri 2005a,b; Bulleri *et al.* 2005; Rule & Smith 2005) and; (iv) wave-exposure and patterns of water flow and turbulence (Southward & Orton 1954; Glasby 1999a; Bulleri & Chapman 2004). Many of these differences were found to be related to the different outcome of biological processes on the two types of substrates, as a direct consequence of their intrinsic physical and chemical characteristics.

Marinas are important recreational and economic resources in coastal environments. They have been, however, set as primary causes of environmental disturbances such as: (i) changes in oceanographic patterns; (ii) changes in natural assemblages which involve the destruction of prior habitats and the subsequent introduction of artificial habitats, with these providing new surfaces for colonisation by organisms able to take advantage of the new physical and biotic conditions and; (iii) the establishment and subsequent spread of alien and invasive species due to boating activities (Turner *et al.* 1997; Bulleri & Chapman 2004; Davenport & Davenport 2006; Benedetti-Cecchi & Osio 2007). Marinas also tend to be hotspots of water and

sediments contamination by hydrocarbons due to oil combustion originated by an intense maritime traffic, sewage and antifouling paints compounds (Tributyltin - TBT and copper-Cu) (Crowe *et al.* 2000; Rilov *et al.* 2000; An & Kampbell 2003, Roméo *et al.* 2003).

The Portuguese coastline has an extension of more than 900 km and a high diversity of coastal types, altering particularly between rocky coasts and sandy shores. At present, the Portuguese coastal zone is strongly affected by coastal erosion, with a strong demographic pressure and human activities associated, being a threat to the delicate balance of coastal ecosystems. Approximately 60% of the Portuguese population lives close to the coastline and during the summer the population usually rise, due to an increase of tourism (Cravo *et al.* 2004; Taveira Pinto 2004; Alves *et al.* 2007). A large fraction of the Portuguese economy is therefore situated near or at the coastal zone. The rate of change of natural coastal areas into artificial ones is being faster than the increase of population density (Taveira Pinto 2004; Alves *et al.* 2007) and, in many European coastal zones like the Portuguese, the percentage of artificial areas on the coast is more than 45% of the total area (Taveira Pinto 2004; Alves *et al.* 2007). This trend does not show any marks of slowing down, increasing the level of vulnerability for coastal population and settlements, reinforced by an accelerated sea level rise due to climate change (Taveira Pinto 2004; Alves *et al.* 2007).

The consequences of the development of new structures that replace and fragment natural habitat in coastal environments have not been however, widely explored as useful tools for monitoring environmental change. It is important to understand the role of artificial structures such as marinas in maintaining natural biodiversity, ecological and biochemical processes, in order to make ecologically sensitive decisions about managing the development of man made structures in coastal

environments. This information is crucial to improve the design of future artificial structures that more closely mimic natural rocky habitats they may replace, and to potentially mitigate some effects of loss and fragmentation of coastal habitats in urban areas (Bulleri 2005a).

1.2. Why study biochemical responses in limpets?

Limpets are among the most abundant mobile organisms on rocky shores in Portugal (Guerra & Gaudêncio 1986; Boaventura *et al.* 2002a), and many studies from different geographical areas have shown that they play a major role in structuring intertidal assemblages in natural (Underwood 1980; Underwood & Jernakoff 1981; Hawkins & Hartnoll 1983; Boaventura *et al.* 2002b) and artificial habitats (Bulleri *et al.* 2000). The limpet *P. depressa* is one of the numerically dominant grazer, common in both natural and artificial (within marinas and harbours) rocky shores along the entire coast of Portugal. It is distributed throughout the intertidal zone and has a key role in the functioning of rocky shore communities (see Guerra & Gaudêncio 1986; Boaventura *et al.* 2002a,b). *Patella depressa* is a southern species, which extends from North Africa, along the Atlantic coasts of Europe to southwest England and Wales (Orton & Southward 1961; Fretter & Graham 1962, 1976; Guerra & Gaudêncio 1986). This makes it an ideal species to study as a potential indicator of environmental conditions, since an established biochemical method to analyse its biochemical components can then be applied on a broad scale.

Limpet spawning, resulting in the shedding of eggs and sperm directly into the sea, is apparently related to the occurrence of high wind speed under optimum conditions of air temperatures (Brazão *et al.* 2003a). After a period in the plankton, the short-lived free-swimming larvae (planktotrophic veliger larvae) settle lower on the shore or in damp crevices. As they grow, they slowly move upshore and inhabit

different shore levels (Fretter & Graham 1962, 1976; Branch 1985; Guerra & Gaudêncio 1986; Little & Kitching 1996). Despite the fact that the reproductive behaviour of this species changes along the Portuguese coast, *P. depressa* is considered a summer breeder (Guerra & Gaudêncio 1986; Brazão *et al.* 2003a). Sexes are separate throughout life, this species shows a gradual gonad development, beginning in September/October and extending up to February-April and an asynchronous breeding is typical (Brazão *et al.* 2003a).

Studies from different geographical areas suggested that populations of limpets might differ in abundance, size and reproductive output between artificial and natural rocky shores (Chapman 2003; Bulleri & Chapman 2004; Guerra-Garcia *et al.* 2004; Bulleri *et al.* 2005; Moreira *et al.* 2006). Some studies have shown smaller densities of limpets on artificial rocky shores (Chapman 2003; Bulleri & Chapman 2004; Bulleri *et al.* 2005), while others revealed greatest densities and smaller mean sizes limpets for the same habitat (Guerra-Garcia *et al.* 2004). Populations of limpets associated with artificial rocky shores were also found to contribute to a lesser extent to the total reproductive output (in terms of numbers and sizes of egg masses) than natural rocky shore-associated populations (Moreira *et al.* 2006). Bulleri *et al.* (2004) also found differences in the movement and homing behaviour of the limpet *Cellana tramoserica* (Sowerby) between natural and artificial structures on the north-west coast of Italy. According to this study, the orientation of limpet movements remained random on both kinds of structures but limpets on artificial structures showed longer distances movements and tended not to retain their original positions, suggesting that long-term dispersal in this species could be altered by the replacement of rocky shores with artificial structures (Bulleri *et al.* 2004). Nevertheless, all studies above strongly suggest a potential problem in the dynamics and sustainability of populations of limpets living

on artificial habitats, highlighting the importance of further research to understand the ecological and biochemical processes on artificial structures.

The organisms more commonly used as bioindicators are those belonging to the bivalve class, and more specifically the mussels *Mytilus* spp. (e.g. Langston *et al.* 1998; Cajaraville *et al.* 2000; Lehtonen *et al.* 2006; Schiedek *et al.* 2006). However, several others organisms such as limpets have also been proposed as sentinel organisms (Navrot *et al.* 1974; Nöel-Lambot *et al.* 1980; Campanella *et al.* 2001; Bebianno *et al.* 2003; Hamed & Emara 2006; Nakhlé *et al.* 2006). Patellid limpets are considered suitable organisms for comparative studies because they meet the most important requirements of the ideal biomonitor: (1) a relative high abundance in natural and artificial habitats; (2) well known biology; (3) wide geographical distribution; (4) sedentary state; (5) easy identification and sampling at all times of the year; (6) large enough to provide sufficient tissue for analysis; (7) detectable AChE activity and MT concentration; (8) tolerance to contaminated environments and physicochemical variables such as salinity and; (9) high capacity to tolerate, accumulate and regulate wide ranges of contaminant concentrations in their tissues in contaminated environments (Nöel-Lambot *et al.* 1980; Jones & Baxter 1985; Campanella *et al.* 2001; Bebianno *et al.* 2003; Hamed & Emara 2006; Nakhlé *et al.* 2006). Additionally, *P. depressa* forms an important part of the diet for various intertidal predators including crabs and fish (Cannicci *et al.* 2002; Monteiro *et al.* 2005; Silva *et al.* 2008), and have also regional economic importance as seafood in Portugal. All this features make this species a potentially suitable bioindicator organism for long-term coastal biomonitoring programs.

Patellid species have seldom been used in ecotoxicological studies however some biomarker studies are available including AChE and MT measurements (Noël-Lambot *et al.* 1980; Bebianno *et al.* 2003; Brown *et al.* 2004; Douhri & Sayah 2009).

Some studies have already dealt with limpets sensitivity and ability to regulate and accumulate different metals in their soft tissues (Noël-Lambot *et al.* 1980; Campanella *et al.* 2001; Cubadda *et al.* 2001; Hung *et al.* 2001; Bebianno *et al.* 2003; Conti & Ceccheti 2003; Brown *et al.* 2004; Cravo *et al.* 2004; Cravo & Bebianno 2005; Hamed & Emara 2006; Nakhlé *et al.* 2006) and shells (Foster & Chacko 1995; Cravo *et al.* 2002, 2004), but also polychlorinated biphenyls (PCBs) (Tena & Montelongo 1999) and polycyclic aromatic hydrocarbons (PAHs) (Peña-Méndez *et al.* 2001). This ability may be responsible for the tolerance of this genus to contaminated environments (Howard & Nickless 1977; Alyakrinskaya 2002).

Metallothioneins (MTs) were already identified and well characterised in the whole soft tissues, viscera and foot of limpets (Howard & Nickless 1977; Noel-Lambot *et al.* 1980; Langston *et al.* 1998; Bebianno *et al.* 2003). In Patellids, MT was initially classified as class I MT due to their similarity with the mammalian MT (Fowler *et al.* 1987), but more recently this classification was replaced by the MT family 2, i.e. mollusc MT (Binz & Kagi 1999). MTs in limpets have at least two isoforms and are similar to mussels, oysters and clams (Howard & Nickless 1977; Noel-Lambot *et al.* 1980; Bebianno & Langston 1991, 1992a; Bebianno *et al.* 1993; Langston *et al.* 1998).

Previous studies in limpets demonstrated that Cu, a metal very common in antifouling paints in marinas, has the capacity to affect the respiratory and cardiac physiology of limpets when present in seawater at high concentrations (Marchán *et al.* 1999; De Pirro *et al.* 2001; Brown *et al.* 2004; Chelazzi *et al.* 2004). In these studies, acute exposure to waterborne Cu was followed by a reduction in heart rate (bradycardia) in different species of gastropod limpets, and it was hypothesized that Cu-induced bradycardia could be an adaptive mechanism by which these molluscs are able to reduce

blood flow and uptake of metal across the gills in polluted environments (Marchán *et al.* 1999; Chelazzi *et al.* 2004).

Food supply is thought to be a major route of uptake for metals in invertebrates (Bryan 1984; Depledge & Rainbow 1990). Limpets are herbivorous grazers that remove, almost continuously, the microbial films that coats worldwide intertidal rocky surfaces (Hill & Hawkins 1991; Jenkins & Hartnoll 2001; Jenkins *et al.* 2001; Thompson *et al.* 2004, 2005). This biofilm is mainly composed of unicellular microbes, algal germlings, diatoms and detritus, which limpets apparently unselectively consume during feeding excursions around their home scars (Fretter & Graham 1962, 1976; Branch 1981; Steneck & Watling 1982; Della Santina *et al.* 1993, 1995; Jenkins & Hartnoll 2001; Jenkins *et al.* 2001; Thompson *et al.* 2004, 2005). Consequently, there is a considerable variation in their diets (Fretter & Graham 1962, 1976; Branch 1981).

The limpet *P. depressa* was chosen in the present study because biochemical studies (including AChE activity, MT concentration and fatty acid analysis) of limpet species, particularly of this genus, have scarcely been done (but see Noël-Lambot *et al.* 1980; Bebianno *et al.* 2003; Brazão *et al.* 2003b; Morais *et al.* 2003; Brown *et al.* 2004; Douhri & Sayah 2009). No information is also available regarding limpet biochemical responses to artificial and natural rocky shores conditions, particularly taking into consideration gender differences. Additionally, a FATM approach was used to explore habitat (marinas vs. rocky shores) influence and feeding strategies of the limpet *P. depressa*. Because the operation of marinas in coastal areas is certain to continue, and possibly expand, it is necessary to gain an understanding of the impact of activities in marinas on grazer intertidal populations. Without understanding how grazers use different types of artificial habitats (e.g. marinas, harbours) in comparison to natural habitats, the effects of the introduction of artificial structures cannot be fully

appreciated. How changes to artificial habitats might affect limpets biochemical responses remains, therefore, unexplored.

1.3. Biochemical Markers Analysis

The present study included specific biochemical markers such as pollution biomarkers (acetylcholinesterase-AChE and metallothionein-MT measurements), physiological status indicators (condition index, tissue protein contents and biometric measurements) and fatty acid trophic markers. All these biochemical parameters have been chosen to investigate gender and spatial variation in biochemical responses of the limpet *P. depressa*, and to evaluate the specificity of the responses to natural and anthropogenic changes. Biomarkers indicating exposure to pollutants and their effects have been increasingly applied to assess the health of marine ecosystems. Among the biomarkers that are currently used to monitor the quality of the marine environment, AChE and MT are part of a well-established core suite of biomarkers used routinely in marine biomonitoring programmes and recognized at European level (see Cajaraville *et al.* 2000). AChE activity is an important enzymatic biomarker involved in the synaptic transmission of nerve impulses and its inhibition reflects neurotoxicity (Payne *et al.* 1996; Cajaraville *et al.* 2000; Soreq & Seidman 2001). MT content is used to indicate exposure to metals (Bebianno & Langston 1989; Roesijadi 1992, 1996; Langston *et al.* 1998; Cajaraville *et al.* 2000; Bebianno *et al.* 2003; Amiard *et al.* 2006). Trophic markers are useful tools to provide information on the dietary intake and identify nutritional sources and feeding strategies (Dalsgaard *et al.* 2003).

1.3.1. Pollution Biomarkers

A large amount and great diversity of chemical compounds have been and are still being introduced into aquatic ecosystems as a direct consequence of a wide variety

of anthropogenic activities. Such contamination represents a serious threat to the overall health of aquatic ecosystems (Gray 1997; Cajaraville *et al.* 2000; Picado *et al.* 2007). Marine pollution has been traditionally studied in terms of chemical concentrations of contaminants, mainly metals. However, in the last decade considerable efforts have been made to validate measurements of biological parameters to complement the information given by the chemical analysis of contamination (Cajaraville *et al.* 2000; Picado *et al.* 2007). As defined by Depledge (1993) *in* Amiard *et al.* (2006), a biomarker is "a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)". Thus, the recent development of biomarkers with warning prognostic capability based on the study of biological responses of organisms to pollutants, has provided the biochemical tools essential to the implementation of programs for monitoring contaminants effects (Cajaraville *et al.* 2000; Picado *et al.* 2007). Biomarkers provide information on the sensitivity of organisms in terms of uptake, biotransformation and detoxification patterns (Cajaraville *et al.* 2000; Galloway *et al.* 2002), allowing an integrated measurement of bioavailable pollutants (Damiens *et al.* 2007). In order to apply biomarkers for assessing pollution effects in such variable ecosystems, the natural variability of biomarkers has to be estimated.

1.3.1.1. Acetylcholinesterase (AChE) Activity

The discovery of the first neurotransmitter — acetylcholine — was soon followed by the discovery of its hydrolysing enzyme, AChE, regarded as a "true" and "specific" cholinesterase (ChE) (Soreq & Seidman 2001). AChE is an enzyme vital to the correct transmission of nervous impulses and it is found in all animal phyla,

including molluscs (Payne *et al.* 1996; Cajaraville *et al.* 2000; Soreq & Seidman 2001). It is also recognized as one of the oldest environmental biomarkers (Payne *et al.* 1996). Apart from AChE, a number of other cholinesterases collectively termed as pseudocholinesterases have also been evaluated as biomarkers (Butyrylcholinesterase – BChE, Propionylcholinesterase – PChE). Hence, based on substrate specificity and inhibitor sensitivity, it is possible to distinguish between the 'true' and 'pseudo' forms of cholinesterase (Forget & Boquené 1999).

AChE is responsible for the rapid hydrolysis of the neurotransmitter acetylcholine (ACh) into the inactive products choline and acetic acid in neuronal synapses and neuromuscular junctions (Soreq & Seidman 2001). These are reabsorbed and used as raw materials for the continued production of ACh. When AChE is inactivated by neurotoxic compounds, the enzyme is no longer able to hydrolyse acetylcholine. Thus, ACh is not broken and accumulates within nerve synapses, causing a continuous and excessive stimulation of the nerve/muscle fibres, along with an interruption of the nervous transmission. Therefore, AChE-inhibiting neurotoxic compounds can cause progressive deterioration of cognitive, autonomic and neuromuscular functions in aquatic organisms, for example movement disturbances, poor attachment to the substratum, reduced feeding activity, impaired respiratory functions and reduced ability to respond to environmental stimuli, tetanus and eventually paralysis and death (Payne *et al.* 1996; Bocquené & Galgani 1998; Kirby *et al.* 2000; Fulton & Key 2001). Hence, the toxicity is the result of excessive stimulation of cholinergic nerves and is dependent upon the ability to inhibit AChE.

It has been demonstrated, in both field and laboratory studies, that AChE activity is inhibited by organophosphorus (irreversible inhibitor) and carbamate (slowly reversible inhibitor) neurotoxic compounds, widely used in agriculture as pesticides

(Burgeot *et al.* 1996; Bocquené & Galgani 1998; Forget & Bocquené 1999; Kirby *et al.* 2000; Bebianno *et al.* 2004; Matozzo *et al.* 2005; Glusczak *et al.* 2006; Roméo *et al.* 2006; Douhri & Sayah 2009). Although organophosphorus (OPs) and carbamate (Cs) are relatively non-persistent in the aquatic environment, their potency is such that their use remains a concern since they have the potential to exhibit neurotoxic activity even at low concentrations (Kirby *et al.* 2000). Directly released into the environment, these toxic compounds can ultimately reach the sea (Mora *et al.* 1999). Because OPs and Cs have a relatively short half-life and are water soluble, the detection of AChE and other ChE inhibition (through reduced enzyme activity) in target organisms has been used for years as a sensitive and specific biomarker of exposure to organophosphate and carbamate pesticides in wild life, in order to monitor the effects of contaminants on living organisms even when they are no longer detectable in solution (Payne *et al.* 1996; Bocquené & Galgani 1998; Kirby *et al.* 2000; Fulton & Key 2001).

Nevertheless, recent studies provide evidence that AChE activity not only responds to specific neurotoxic compounds but also to other contaminants including metals, domestic effluents and petroleum, hydrocarbons and detergents, algal toxins and complex mixtures of pollutants (Payne *et al.* 1996; Bocquené & Galgani 1998; Solé *et al.* 2000; Lehtonen *et al.* 2003). Therefore, a more general use of this biomarker has been suggested for the assessment of environmental quality and as a general physiological stress response of aquatic organisms (Damiens *et al.* 2007). Numerous laboratory and field studies have demonstrated the potential value of AChE activity measurement in both vertebrates and invertebrates (e.g. Burgeot *et al.* 1996; Bocquené & Galgani 1998; Forget & Bocquené 1999; Kirby *et al.* 2000; Matozzo *et al.* 2005; Moreira & Guilhermino 2005; Glusczak *et al.* 2006; Roméo *et al.* 2006; Douhri &

Sayah 2009). Consequently, AChE has been an extensively used biomarker in coastal biomonitoring programs (Cajaraville *et al.* 2000; Dellali *et al.* 2001; Picado *et al.* 2007).

In the marine environment, the choice of a monitoring target species has mainly concerned vertebrates (e.g. Payne *et al.* 1996; Kirby *et al.* 2000; Minier *et al.* 2000; Glusczak *et al.* 2006) and bivalve molluscs, such as the mussels *Mytilus* spp. (Mora *et al.* 1999; Moreira & Guilhermino 2005; Pfeifer *et al.* 2005; Lehtonen *et al.* 2006; Magni *et al.* 2006; Schiedek *et al.* 2006), or the oysters *Crassostrea gigas* Thunberg and *Ostrea edulis* Linnaeus (Bocquené *et al.* 1997; Solé *et al.* 2000; Valbonesi *et al.* 2003; Damiens *et al.* 2004; Tanguy *et al.* 2005), essentially because of their capacities to accumulate contaminants. Indeed, the use of sessile molluscs has been highly recommended within the framework of biosurveillance programmes in the Mediterranean (UNEP 1999) (Valbonesi *et al.* 2003). Clams have also been proposed as possible bioindicators of aquatic pollution in areas where mussels and oysters are not available (see Mora *et al.* 1999; Solé *et al.* 2000; Dellali *et al.* 2001; Valbonesi *et al.* 2003; Pérez *et al.* 2004; Matozzo *et al.* 2005; Lehtonen *et al.* 2006). Less information is available on AChE activity in gastropod molluscs (but see Bevelaqua *et al.* 1975; Brown *et al.* 2004; Romèo *et al.* 2006; Gharbi-Bouraoui *et al.* 2008; Douhri & Sayah 2009) of which limpets are part.

AChE exhibit genetic and molecular polymorphism and their distributions and physiological roles differ among species, often resulting in a highly variable degree of inhibition associated with toxicity (Soreq & Seidman 2001; Galloway *et al.* 2002). The data on tissue and species specific AChE expression, the detection of its various isoforms, its cholinergic and non-cholinergic functions and its wide neuronal and non-neuronal cellular distribution on vertebrates and invertebrates, has made it the focus of intense research for much of the past century (Soreq & Seidman 2001).

Knowledge about structure, roles, function and distribution of AChE remains limited in the case of aquatic macro-invertebrates (Dauberschmidt *et al.* 1997; Mora *et al.* 1999). This reflects the generally low endogenous enzyme activities levels reported in molluscs compared with enzymes from other species, mainly vertebrates (Payne *et al.* 1996; Bocquené & Galgani 1998; Mora *et al.* 1999; Kirby *et al.* 2000; Minier *et al.* 2000; Glusczak *et al.* 2006). In vertebrates, the presence of AChE has been demonstrated in a variety of tissues of marine organisms including nervous tissues, brain, gills, red blood cells and muscles (Payne *et al.* 1996; Kirby *et al.* 2000; Minier *et al.* 2000; Glusczak *et al.* 2006). In molluscs, despite the relative tissue-specific levels of AChE reported in the literature, it is usually best measured in the haemolymph, gills, digestive glands and adductor muscles, which thus offer the potential for monitoring AChE activity (e.g. Bocquené & Galgani 1998; Mora *et al.* 1999; Leiniö & Lehtonen 2005; Matozzo *et al.* 2005).

An ideal biomarker would be expected to show little variation in response to environmental and/or biotic factors and vary only in response to pollutant exposure. However, this is hardly the case in reality. Biotic (sex, size, age, gonadal maturity, trophic bioavailability or starvation physiological conditions) and abiotic (temperature, pH, oxygen, salinity) parameters are susceptible to interact with contaminants on biomarker responses, and natural variability in responses can also occur even in the absence of toxicant exposure (Vidal *et al.* 2002; Robillard *et al.* 2003; Pfeifer *et al.* 2005; Binelli *et al.* 2006). In order to distinguish between natural and pollution caused alterations in the AChE activity is it necessary to understand the natural enzyme variability and to determine the biotic and abiotic factors that may bias it. In most marine adult organisms, the natural variability in the activity of AChE was found not to be directly linked to age, sex or reproductive period (Bocquené & Galgani 1998).

Instead, the environmental water temperature emerges as the most important natural factor affecting AChE activity (Bocquené & Galgani 1998; Dellali *et al.* 2001; Pfeifer *et al.* 2005; Matozzo *et al.* 2005). Indeed, in poikilothermic organisms, such as fish, crustaceans and molluscs, ambient temperature is one of the major factors driving physiological and biochemical processes. Temperature can directly affect the function of enzymes by changing their physical structure thereby changing their catalytic efficiency or binding capacity (Pfeifer *et al.* 2005).

The measurement of this parameter has been published as a reference method by ICES (Bocquené & Galgani 1998), and it is an useful component in a panoply of biomarkers since it is a marker of the early effect of complex chemicals on marine organisms (Cajaraville *et al.* 2000; Magni *et al.* 2006). In Portugal, the measurement of this biomarker in molluscs remains limited (Bebianno *et al.* 2004; Moreira & Guilhermino 2005). Moreira & Guilhermino (2005) measured AChE activity in the mussel *Mytilus galloprovincialis* Lamarck along the northwest Portuguese coast, while Bebianno *et al.* (2004) measured AChE activity in different tissues of the clam *Ruditapes decussatus* Linnaeus, in laboratory conditions and under environmental stresses in a perspective of a multibiomarker approach to assess environmental changes.

1.3.1.2. Metallothionein (MT) concentration

Much of the work with metals in the area of biochemical biomarkers has focused on MTs. MTs are a class of heat-stable, sulphur-rich, low molecular weight (6–10 kDa) cytosolic proteins of non-enzymatic nature, characterised by a unique amino acid composition with very high cysteine and metal contents and no aromatic amino acids. The thiol groups (–SH) of cysteine residues enable these soluble proteins to bind several metals, bringing them to a non-toxic form, thus reducing their deleterious effects (Roesijadi 1992; 1996; Langston *et al.* 1998; Nordberg 1998; Cajaraville *et al.* 2000;

Amiard *et al.* 2006). Hence, MTs are able to bind extremely high concentrations of essential metal cations, such as Cu and zinc (Zn), or metals without a known biological function, such as cadmium (Cd), mercury (Hg) and silver (Ag) (Roesijadi 1992, 1996; Langston *et al.* 1998; Nordberg 1998; Cajaraville *et al.* 2000; Amiard *et al.* 2006).

The biological functions of metallothioneins are still under debate. Given the metal-binding capacity of MTs, it is generally recognised that the primary role of these proteins is related to the metabolism and homeostatic control of essential trace metals (Cu and Zn) for cell growth and development, as they represent essential metal stores able to fulfil enzymatic and other metabolic demands (Roesijadi 1992, 1996; Nordberg 1998). But MTs are also involved in the detoxification of excess amounts of both essential and nonessential toxic metals (Cd, Hg, Ag), allowing organisms to tolerate metal-contaminated environments (Roesijadi 1992, 1996; Langston *et al.* 1998; Nordberg 1998; Bebianno *et al.* 2003; Amiard *et al.* 2006). Binding of MT to excess of essential or pollutant metals protects the organism against toxicity by limiting the availability of these cations at undesirable sites (Roesijadi 1992, 1996; Langston *et al.* 1998; Nordberg 1998; Cajaraville *et al.* 2000). MTs also seem to have other important functions including transport and storage of metals, protection of cells against intracellular oxidative damage or free radical scavengers and protection of organisms from a variety of stress conditions (Roesijadi 1992, 1996; Nordberg 1998).

MTs are widely distributed in many vertebrates (Kirby *et al.* 2000; Dang *et al.* 2001; Amiard *et al.* 2006) but also in several species of marine invertebrates, mainly molluscs and crustaceans (Bebianno & Langston 1991, 1992a,b; Roesijadi 1992, 1996; Bebianno & Serafim 1998; Langston *et al.* 1998; Amiard *et al.* 2006). Among molluscs, MTs are identified and fully characterized in bivalves, such as mussels, oysters and clams (Bebianno & Langston 1991, 1992a; Langston *et al.* 1998; Bebianno *et al.* 2000;

Tanguy *et al.* 2003; Damiens *et al.* 2007), but also in gastropods, including limpets (Howard & Nickless 1977; Noel-lambot *et al.* 1980; Bebianno & Langston 1992b; Bebianno *et al.* 2003; Brown *et al.* 2004). High levels of these proteins have been identified in molluscs tissues directly involved in uptake, storage and excretion of metals such as digestive glands and gills (Bebianno *et al.* 1993; Mouneyrac *et al.* 1998; Bebianno *et al.* 2000).

Metallothioneins have been subdivided into three different classes (I, II and III), depending on the structural and biochemical similarities with mammalian metallothioneins (class I) (Fowler *et al.* 1987). Class I comprises all proteinaceous MTs with locations of cysteine closely related to those in mammals. Some molluscan MTs belong to this class, such as those characterized in mussels (Mackay *et al.* 1993), oysters (Unger & Roesijadi 1996), clams (Bebianno *et al.* 2000) and limpets (Howard & Nickless 1977, Noel-Lambot *et al.* 1980). According to a new MT classification proposed by Binz & Kagi (1999), which takes into account phylogenetic features as an additional classification criterion, molluscan MTs belong to family 2 whereas crustacean MTs belong to family 3 (Amiard *et al.* 2006). Multiple isoforms with different physiological roles have been identified and isolated from vertebrates and invertebrates, and polymorphism appears to be particularly important in invertebrates compared to mammals (Langston *et al.* 1998; Tanguy *et al.* 2003; Amiard *et al.* 2006). Molecular biological techniques continue to elucidate the biology of metallothioneins, in the identification of different isoforms, their different induction properties and ultimately subtle differences in their physiological roles. However, according to Amiard *et al.* (2006), some questions still remain: does each MT isoform have a different specific role? What are the turnover rates and ultimate fates of MTs and their metal loads?

Induction of MTs represents a specific biological response to high availabilities of metals in the environment (Bebianno & Langston 1991, 1992b; Roesijadi 1992, 1996; Bebianno & Serafim 1998; Brown *et al.* 2004). MT expression generally increases with elevation of tissue concentrations of MT-inducing metals, biologically essential or not (Zn, Cu, Cd, Hg and Ag) to the organisms (Roesijadi 1992, 1996). MT induction has been demonstrated in both vertebrates and invertebrates organisms from polluted populations in several *in situ* studies or following laboratory exposure to specific metals (e.g. Bebianno & Langston 1991, 1992a,b; Roesijadi 1992, 1996; Bebianno & Serafim 1998; Mourgaud *et al.* 2002; Brown *et al.* 2004). More and more *in situ* studies combine the quantification of several biomarkers, of which MT is only one (Carajaville *et al.* 2000; Downs *et al.* 2001; Chèvre *et al.* 2003). Induction of MTs by metals, however, is highly variable, specific and metal-dependent and also depends on species, tissue and history of metal exposure (Roesijadi 1992, 1996; Bebianno *et al.* 1993; Langston *et al.* 1998). Such variation is intra- and interspecific, and it is down to a variety of environmental and physiological reasons. However, the induction of these proteins has been proposed as "early warning marker" for the detection of metal exposure (Cajaraville *et al.* 2000; Picado *et al.* 2007).

Hormones and other endogenous factors were also found to induce biosynthesis of MT (Schiedek *et al.* 2006). Basal levels of MT in organisms have been shown to be influenced by several abiotic and biotic factors (e.g. water temperature, salinity, dissolved oxygen levels, season, reproductive state, growth rate, size/age, weight, sex, food availability) (Mouneyrac *et al.* 1998; Leung *et al.* 2001; Serafim & Bebianno 2001; Leung *et al.* 2002; Bebianno *et al.* 2003). However, specific methodologies such as the estimation of condition index (indicator of the nutritional health status of an

individual) and the use of transplantation experiments have been proposed to correct for several of these confounding factors (Mourgaud *et al.* 2002; Damiens *et al.* 2007).

Direct measurement of MTs has been widely proposed as a sensitive biomarker of environmental metal contamination in marine environments, however, the choice of the best species for the assessment and monitoring of environmental quality and the selection of the most relevant organ for MT determination are subjects still under discussion (Amiard *et al.* 2006). Bivalves have been the best candidates as bioaccumulators of chemical compounds in so-called "Mussel Watch" biomonitoring programmes involving MT concentrations as biomarkers (Langston *et al.* 1998; Cajaraville *et al.* 2000). Despite the priority given to mussels (Bebianno & Langston 1991, 1992a) and oysters (Mouneyrac *et al.* 1998; Tanguy *et al.* 2003), when these selected specimens were not available, alternative organisms such as clams (e.g. Bebianno *et al.* 1993; Bebianno & Serafim 1998; Bebianno *et al.* 2000), dogwhelks (Leung *et al.* 2001, 2002), periwinkles (Bebianno & Langston 1992b) and limpets (Howard & Nickless 1977; Noel-lambot *et al.* 1980; Bebianno *et al.* 2003; Amiard *et al.* 2004; Brown *et al.* 2004; Amiard *et al.* 2006) were used.

MT is also part of a the suite of biomarkers recognized at European level and selected for the monitoring of the marine environment in the Mediterranean Action Plan of the United Nations Environment Program (UNEP/RAMOGE 1999), and examined in the framework of biological effect quality assurance in monitoring programmes (BEQUALM) (Amiard *et al.* 2006). In Portugal, several studies measured this biomarker in molluscs, mainly bivalves (Bebianno *et al.* 1994; Bebianno & Machado 1997; Bebianno & Serafim 1998; Bebianno *et al.* 2000; Bebianno *et al.* 2004), while studies on gastropods including limpets remain limited (Bebianno & Langston 1992b; Bebianno *et al.* 2003).

1.3.2. Fatty Acid Trophic Markers (FATM)

Lipids are major sources of metabolic energy and of essential materials for the formation of cell and tissue membranes (Sargent 1995). They are very important in the physiology and reproductive processes of marine animals and reflect the particular biochemical and ecological conditions of the marine environment (Dalsgaard *et al*. 2003; Bergé & Barnathan 2005). Lipids also provide energy for growth during conditions of limited food supply, when carbohydrate levels (the main energetic reserve in molluscs) are low (Gabbott 1983; Ruiz *et al.* 1992; Abad *et al.* 1995; Pazos *et al.* 1996, 1997).

The lipid composition of molluscs can be affected by external (exogenous) factors, such as fluctuations in the environmental conditions and qualitative and/or quantitative changes in food availability, or by internal (endogenous) factors such as sexual maturation (Gardner & Riley 1972; Simpson 1982; Bayne & Newell 1983; Gabbot 1983; Whyte *et al.*1990, Pazos *et al.* 1997; Galap *et al.* 1999). Accumulation and depletion of stored reserves in molluscs depends mainly on the stage of gonad development, environmental factors affecting metabolic activities and on the quantity and nutritional value of the food supply (Gabbott 1983; Whyte *et al.* 1990; Pazos *et al.* 1996). Glycogen is usually the major stored source of energy in molluscs (Barber & Blake 1981), while lipids are considered as the nutritive reserve product of the gonads (Wenne & Polak 1989). A strong correlation between the gonad lipid content and the phase of the reproductive cycle has been established in several bivalves and prosobranch species (Simpson 1982; Abad *et al.* 1995; Pazos *et al.* 1996, 1997).

Spatial and seasonal variations in lipid and fatty acid composition have been reported for several marine molluscs (Taylor & Venn 1979; Gabbott 1983; Kluytmans *et al.* 1985; Hayashi & Kishimura 1991; Ruiz *et al.* 1992; Abad *et al.* 1995; Pazos *et al.*

1996, 1997; Brazão *et al.* 2003b) and are generally related to the growth-maturation cycle: in the summer when the growth phase takes place, reserves of lipids are build up and stored, and these are later mobilised for gametogenesis in the maturation phase (often autumn or winter), being normally lost during spawning. However, the majority of publications concerning fatty acid composition in molluscs have focussed on bivalves and cephalopods, probably as a result of their great commercial importance and impact on public health.

The quality as well as the quantity of algal lipids is very important in the nutrition of marine animals as algae are main sources of the essential fatty acids that marine molluscs cannot synthesise *de novo* (Sukenik *et al.* 1993; Abad *et al.* 1995; Brown *et al.* 1997). There is a large amount of literature detailing the fatty acid composition of many species of marine algae (Chuecas & Riley 1969; Sargent *et al.* 1989; Sukenik *et al.* 1993; Brown *et al.* 1997). Availability and nutritional value of algal lipids are very important in the nutrition, growth and development of aquatic animals such as marine fish larvae, shrimps and molluscs (Sukenik *et al.* 1993). Certain fatty acids present in algae are considered nutritionally essential for most marine species, affecting their growth and condition (Pazos *et al.* 1997).

The majority of studies on the feeding habits and food sources of marine organisms are based mainly on the analysis of gut contents that provide information mainly on recent feeding (Santelices & Correa 1985; Hill & Hawkins 1991; Della Santina *et al.* 1993). A more complete technique using Fatty Acid Trophic Markers (FATM) can provide more information on the dietary intake and origin of lipid reserves generated over a longer period of time (Dalsgaard *et al.* 2003). FATM has been successfully applied in the last few decades to identify nutritional sources and feeding strategies of a wide variety of organisms including mammals, fish, echinoids, bivalves

and gastropods (Kharlamenko *et al.* 2001; Iverson *et al.* 2002; Grahl-Nielsen *et al.* 2003; Howell *et al.* 2003; Hughes *et al.* 2005).

The FATM concept is based on the main assumption that certain essential fatty acids cannot be produced by biosynthesis in animals and thus can only be derived from food intake. Such compounds are laid down from marine primary producers and generally remain intact through digestion, being conservatively transferred through aquatic food webs. Thus, they can be recognized in their primary consumer tissues with minimal modification from diet. These FA patterns can then be used as potential taxonomic markers regarding the presence and combination of certain FA characteristic of particular algal classes (Dalsgaard *et al.* 2003; Latyshev *et al.* 2004; Bergé & Barnathan 2005). Thus, a fatty acid approach can be useful to explore habitat influence and feeding strategies, since the relative proportion and composition of FA in marine organisms are characteristic for every species and genus (Dalsgaard *et al.* 2003; Bergé & Barnathan 2005).

According to Dalsgaard *et al.* (2003) an obstacle associated with the application of FATM has been the interpretation of the large data sets routinely produced in these types of analyses (typically arrays of more than 30 FA determined simultaneously from one or more samples). Since diet was found in previous studies to be an important factor in limpet fatty acid profiles, a FATM approach was used in the present study and multivariate statistical methods were applied to analyse the large FA data sets, in an attempt to investigate the diet of the limpet *P. depressa* collected from marinas and rocky shores of the Portuguese coast. In Portugal, studies on the biochemical composition, particularly in terms of fatty acid profiles in gastropods limpets remains scarce (but see Brazão *et al.* 2003b; Morais *et al.* 2003).

1.4. Overview and Aims

Although the ecology of limpets in intertidal rocky shores communities has been extensively studied across the world (Underwood 1979; Bowman 1985; Branch 1985), relatively little work has been done on biochemical responses of key marine grazers, particularly limpets, to the replacement of natural habitats by artificial structures (i.e. marinas). The primary aim of this dissertation was to investigate gender and spatial variation in biochemical responses of the limpet *P. depressa* from artificial (i.e., marinas) and natural rocky shores of the central and south Portuguese coast, using pollution biomarkers and fatty acid trophic marker approaches.

In this general introduction (Chapter 1) a briefly overview was done by reviewing what is already known on biochemical marker analyses, together with a summary of the current knowledge on limpets. Chapter 2 explored pollution related biomarker responses (AChE activity and MT concentration) of limpet foot and soft body tissues to establish the natural variability of biomarkers on artificial and natural rocky shores, in order to identify the effect of artificial substrata on limpet biochemical responses. The specific aims were to test: 1) differences in AChE activities, MT concentrations, target tissues total protein contents and condition indexes among gender and between limpets from different locations of the central and south coast of Portugal and; 2) to study the relationships between biomarkers and physiological status indicators. Data on the fatty acid composition of limpet gonad tissues, and comparisons between gender and habitats of the south Portuguese coast are investigated in Chapter 3 in an effort to assess the trophic relationships between limpets and their food sources at both artificial and natural substrata. The specific aims were to: (1) describe qualitatively the gonad fatty acid composition of *P. depressa* from different habitats; (2) examine gender and habitat differences in gonad fatty acid profiles (individual FAs and FAs groups) using

multivariate statistical analyses; (3) test for gender and habitat differences on the gonad total lipid content, FATM ratios and biometric measurements (shell length and gonad dried weight) and; (4) assess the relationships between gonad total lipids and biometric measurements. The general discussion (Chapter 4) focussed on the importance of the results obtained in this study to better understand the role of artificial structures in maintaining natural biochemical processes in limpets, and suggested future approaches.

CHAPTER 2

Influence of gender and location on AChE activity and MT concentration of the limpet *Patella depressa* Pennant, 1777 (Gastropoda: Prosobranchia)

Brazão S., Gomes T., Narciso L., Boaventura D., Bebianno M.J. Submitted to *Science of the Total Environment*

CHAPTER 2: Influence of gender and location on AChE activity and MT concentration of the limpet *Patella depressa* Pennant, 1777 (Gastropoda: Prosobranchia)

2.1. Introduction

A large amount and diversity of organic pollutants are continuously introduced into coastal zones as a result of anthropogenic activities, through discharges of domestic wastes, water and industrial effluents, runoffs from agricultural practices and also via harbour activities (Gray 1997; Cajaraville *et al.* 2000; Picado *et al.* 2007). Aquatic organisms can then be exposed to compounds of anthropogenic origin through water and sediments and/or via dietary path (Bryan 1984; Depledge & Rainbow 1990; Robillard *et al.* 2003).

The increasing urgency to detect and assess the negative effects of anthropogenic contaminants at low concentrations and/or in complex mixtures (whose interactions can create additive, synergistic or antagonistic effects) has led to the development of early warning signals or biomarkers (Cajaraville *et al.* 2000; Picado *et al.* 2007). These signals consist of biochemical and/or physiological sublethal changes in organisms exposed to contaminants that can be used in a predictive way, before a more detrimental damage becomes evident (Cajaraville *et al.* 2000). Contrary to the simple measurement of contaminant concentrations within body tissues of the bioaccumulator organism, biomarkers can offer complete and biologically more relevant information on the potential *in situ* pollutant toxic effects to the health of aquatic organisms (Cajaraville *et al.* 2000; Picado *et al.* 2007). They also provide information on species sensitivity in terms of uptake, biotransformation and detoxification patterns (Cajaraville *et al.* 2000; Galloway *et al.* 2002), and can be either specific (monitoring the presence/effects of specific chemical classes) or general (assessing a variety of

toxicological endpoints) (Kirby *et al.* 2000; Binelli *et al.* 2005). Evaluation of spatial variation in the natural levels of biomarkers for a particular organism collected in specific habitats constitutes a particularly important research strategy for comparative and conservation studies.

The use of biomarkers is conditioned by every physical and/or biological factor that can influence the biochemical responses of target organisms apart from pollutants. Biomarker responses are known to vary considerably with environmental factors including both biotic (age of the organism, sex, size, weight, reproductive cycle, growth and diet) and abiotic factors (season, temperature, salinity, pH, dissolved oxygen and turbidity), thus making it difficult to detect the specific effects of chemical pollutants (Bocquené & Galgani 1998; Vidal *et al.* 2002; Bebianno *et al.* 2003; Damiens *et al.* 2004; Amiard *et al.* 2006).

The two exposure biomarkers used in the present study, AChE activity and MT concentration are widely used in ecotoxicological studies and in coastal biomonitoring programs (Cajaraville *et al.* 2000; Dellali *et al.* 2001; Picado *et al.* 2007). Both biomarkers have been measured and validated in a restrict number of target species, mainly mussels and other bivalve molluscs (Cajaraville *et al.* 2000; Binelli *et al.* 2006; Lehtonen *et al.* 2006; Schiedek *et al.* 2006). AChE is an enzymatic biomarker of neurotoxicity involved in the synaptic transmission of nerve impulses (Payne *et al.* 1996; Cajaraville *et al.* 2000; Soreq & Seidman 2001). Its activity prevents continuous muscular contraction and is vital to the normal functioning of the sensorial-nervous and neuro–muscular systems representing also a prime target on which some pollutants can exert a detrimental effect (Payne *et al.* 1996; Kirby *et al.* 2000). Inhibition of the AChE enzyme results in an interruption of nerve impulse transmissions, producing acute toxic effects that can lead to the progressive deterioration of cognitive, autonomic and

neuromuscular functions in aquatic organisms (e.g., reduced feeding activity, impaired respiratory functions, poor attachment to the substratum, reduced ability to respond to environmental stimuli and eventual paralysis or even death) (Bocquené & Galgani 1998; Kirby *et al.* 2000).

Exposure of aquatic animals, including gastropod molluscs, to neurotoxic compounds (i.e. organophosphorous and carbamates pesticides widely used in agriculture) can be assessed by the measurement of AChE activity (Bevelaqua *et al.* 1975; Brown *et al.* 2004; Roméo *et al.* 2006; Douhri & Sayah 2009). Other important groups of contaminants such as metals, hydrocarbons, domestic effluents, detergents, algal toxins and complex mixtures of pollutants have also been shown in both field and laboratory studies to cause an inhibition of this enzyme (Payne *et al.* 1996; Bocquené & Galgani 1998; Solé *et al.* 2000; Lehtonen *et al.* 2003).

Metallothioneins (MT) are non-enzymatic proteins with low molecular weight and have been identified and fully characterized mainly in bivalves (Roesijadi 1992; Mackay *et al.* 1993; Roesijadi 1996; Langston *et al.* 1998), but also in gastropods, including limpets (Howard & Nickless 1977; Noël-Lambot *et al.* 1980). MTs are a specific biomarker of metal exposure that consist of sulphur-rich metalloproteins related to the metabolism and regulation of essential trace metals (e.g. Cu and Zn) for cell growth and development, but are also involved in the detoxification of excess amounts of both essential and nonessential metals such as Cd, Hg and Ag (Roesijadi 1992, 1996; Langston *et al.* 1998; Cajaraville *et al.* 2000; Amiard *et al.* 2006). Induction of MT represents a specific biological response to high availabilities of metals in the environment (Roesijadi 1992, 1996; Langston *et al.* 1998; Amiard *et al.* 2006), and has been demonstrated in both vertebrates and invertebrates organisms from polluted populations in several *in situ* studies or following laboratory exposure to specific metals

(e.g. Bebianno & Langston 1991, 1992a,b; Roesijadi 1992, 1996; Bebianno & Serafim 1998; Brown *et al.* 2004).

Patela depressa is a grazing gastropod common in both natural and artificial rocky shores of the coast of Portugal with a key role in the functioning of rocky shore communities (see Boaventura *et al.* 2002a). *Patella* limpets are considered suitable organisms for comparative studies due to its relative abundance, wide geographical distribution, sedentary state, easy identification and sampling at all times of the year, tolerance to contaminated environments and high capacity to accumulate and regulate wide ranges of contaminant concentrations in their tissues including metals, PAHs and PCBs (Noel-lambot *et al.* 1980; Tena & Montelongo 1999; Campanella *et al.* 2001; Peña-Méndez *et al.* 2001; Bebianno *et al.* 2003, Cravo *et al.* 2004; Nakhlé *et al.* 2006). Additionally, *P. depressa* forms an important part of the diet of various intertidal predators including crabs and fishes (Cannicci *et al.* 2002; Monteiro *et al.* 2005; Silva *et al.* 2008), and are thus potentially suitable as bioindicator organisms for long-term monitoring programs. The *Patella* genus has been used in several biomarker studies (e.g. Noël-Lambot *et al.* 1980; Bebianno *et al.* 2003; Brown *et al.* 2004; Douhri & Sayah 2009), nevertheless no information is available regarding AChE activities and MT concentrations in limpets taking into consideration gender differences.

In order to understand the spatial variability of biomarkers and to set natural levels for limpets originated from two different ecosystems of the Portuguese coast - artificial and natural rocky shores - two categories of biological parameters were estimated: (i) AChE activities and MT concentrations and (ii) physiological status indicators including condition index (CI), total protein content (PT), and biometric measurements such as wet tissue weight and shell length. This contributes to a better understanding of exposure biomarkers by confirming earlier responses. Hence, this

study aimed to test: 1) for differences in AChE activities, MT concentrations, target tissues total PT contents and CI between gender and locations of the central and south coast of Portugal and; 2) to study the relationships between biomarkers and physiological status indicators.

2.2. Methods

2.2.1. Sampling and sample preparation

Patella depressa was collected intertidally during low tide at six locations: three marinas (Vilamoura, Portimão and Cascais) and three natural rocky shores (Olhos d'Água, Vau and Cabo Raso) relatively close to each marina (between 3 and 6 km apart of each other) in the central and south Portuguese coast (see Figure 2.1, coordinates are given in Table 2.1). In order to test the biochemical responses of the selected species to more stressed environmental conditions, only marinas that had been established for at least 10 years and each supported more than 600 boats were selected. This study did not include temporal variability and the collection of the specimens was conducted during the summer (May and June 2006). This was because individuals were found in the most advanced stages of gonad development (Brazão *et al.* 2003a) and, thus avoided some of the possible confounding effects of abiotic and biotic factors on biomarker responses including reproductive cycle, diet and food availability and/or season.

The Portuguese coastal environment has been pressured by the development of touristic infrastructures including man-made structures such as marinas, and intense beach use especially during spring and summer months (Taveira Pinto 2004; Alves *et al.* 2007). Sampling locations were spread, taking into consideration the highly anthropized areas as potential point sources of contamination. The degree of human influence in artificial rocky shores was expected to be higher than in natural rocky

shores essentially due to contamination from urban sources and marina associated leisure and boating activities.

Table 2.1. Geographical co-ordinates of sampling sites.

Locations	Rocky Shore	Coast	Latitude (ºN)	Longitude (ºW)
Vilamoura	Artificial	South1	37º 04' N	08º 07' W
Portimão	Artificial	South2	37º 07' N	08º 31' W
Cascais	Artificial	Central	38º 41' N	09º 25' W
Olhos d'Água	Natural	South1	37º 05' N	08º 11' W
Vau	Natural	South2	37º06' N	08º34' W
Cabo Raso	Natural	Central	38º 42' N	09º 29' W

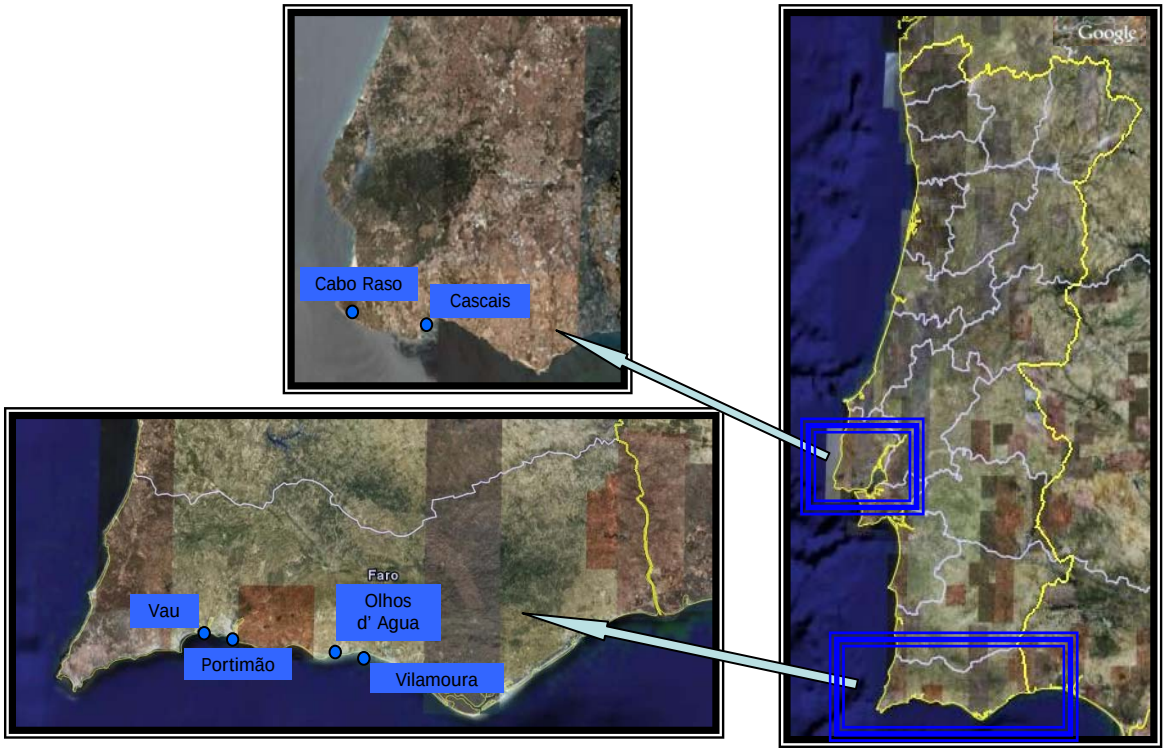

Figure 2.1. Map of the Portuguese Mediterranean and Atlantic coast showing the study sampling locations. Rocky Shores: Cabo Raso, Vau and Olhos de Água. Marinas: Cascais, Portimão and Vilamoura.

After collection, limpets were transported alive to the laboratory in bags and submerged in approximately 2 cm of seawater collected from the sampling site. Respectively for the analyses of AChE activity and MT concentration, the foot and soft

bodies (whole body without radula) tissues of *P. depressa* were dissected in the laboratory. The tissues were immediately frozen in liquid nitrogen to prevent enzyme deterioration, and stored at -80 ºC until analysis. Limpet shell length and target tissue wet weights were registered. To minimise the differences in the time taken to process each limpet, the dissection of limpets groups collected from different locations was always performed in the same order as the field sampling. Six adult male and female replicates were selected for tests, ranging in size between 20 and 50 mm and in the same reproductive condition (stage IV of gonad development). It is important to emphasize that the tests were made on animals representative of the natural populations, with individuals from natural rocky shores being on average smaller than those from artificial rocky shores.

2.2.2. Acetylcholinesterase (AChE) activity and Total Protein Content (PT) of the foot muscle

Foot muscle tissues of limpets were examined for AChE enzyme activity and total protein content. Dissection and freezing procedures were conducted at 0–4 °C. Foots were homogenized (1:4 tissue weight/buffer volume) in ice-cold (to minimize any temperature increase) Tris-HCl buffer (100 mM, pH 8.0) containing Triton X100 (1%) for 1 minute using an Ultra-Turrax. The homogenates were then centrifuged for 30 minutes at 12000*g* at 4 ºC and aliquots of the supernatant (called S9 fraction) used for AChE measurements. Measurements of AChE activity were performed using the method of Ellman (Ellman *et al.* 1961), through the colorimetric reaction between acethylthiocholine (ATC) and the reagent dithiobisnitrobenzoate (DTNB). All assays were performed in duplicate and at room temperature. Buffer blanks (50 µl) were incubated for 5 minutes with 100 µl of DTNB (1 mM, Sigma-Aldrich) in Tris–HCl buffer (100 mM, pH 8.0). The reaction was started by the addition of 100 µl of ATC (10

mM, Sigma-Aldrich). Samples were incubated for 5 min at room temperature. Changes in absorbance at 405 nm were then recorded for 1 min on an UV Visible Spectrometer Spectronic Unicam. Total protein concentrations in the different S9 fractions were determined according to Lowry *et al.* (1951), using bovine serum albumin (BSA) as a standard. All enzymatic activities were expressed as nmol of acethylthiocholine hydrolyzed per minute per gram of total protein (nmol min^{-1} mg^{-1} total protein).

2.2.3. Metallothionein (MT) concentration and Total Protein content (PT) of soft body tissues

Metallothionein (MT) is a well known biomarker of the effect of metal contamination (Bebianno & Langston 1989; Roesijadi 1992, 1996; Langston *et al.* 1998; Cajaraville *et al.* 2000; Bebianno *et al.* 2003; Amiard *et al.* 2006). The MT quantification was performed on the whole soft tissues of *P. depressa.* Tissues were homogenised in three volumes of a Tris-HCl buffer (20 mM, pH=8.6), and centrifuged at 30000*g* for 45 minutes (4ºC). The soluble fraction was heat-treated at 80ºC for 10 minutes, and centrifuged at 30000*g* for another 45 minutes (4ºC). Aliquots of the heat denatured cytosol were used to quantify MT concentrations by Differential Pulse Polarography, according to the method developed by Bebianno & Langston (1989). The standard addition method was used for calibration of MT concentrations with rabbit liver MT (10 $mg.l^{-1}$, MT-I from Sigma-Aldrich), due to the absence of a limpet MT standard. MT concentrations in the whole soft tissues of *P. depressa* were expressed as $mg.g^{-1}$ dry weight (dw) of tissue initially homogenised. Total protein concentrations (PT) in the soft body of the limpets were determined according to the method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as a standard.

2.2.4. Determination of the condition index (CI)

A condition index (CI; % not unit-based) for general physiological condition of the mollusc was calculated for each individual according to Nakhlé *et al.* (2006) as the ratio: (dry mass of soft tissues / shell weight) x 100. For CI determination, 12 individuals, 6 males and 6 females from each location were measured and dissected. The shell of limpets was individually sized and weighted. Soft tissues were carefully separated from shells and washed in distilled water to remove dirt. Soft entire bodies were dried at 80 ºC for 48 h and then weighted to determine dry tissue mass.

2.2.5. Statistical analysis

A two-way mixed model of ANOVA was used to investigate the effects of gender and location on the biomarker responses (AChE activities and MT concentrations), and also on total protein contents and condition index. The factors tested were "sex" (fixed, orthogonal, 2 levels: male and female) and "location" (fixed, orthogonal, 6 levels), with 6 replicates. Cochran's C-test was used to verify homogeneity of variance. Where this assumption was violated, the appropriate transformations were used (Underwood 1997; Underwood & Chapman 1998). Tests of homogeneity, ANOVA and SNK (Student–Newman–Keuls) *a posteriori* comparison tests were done using GMAV5 for Windows Statistical Software (Institute of Marine Ecology, Sydney, Australia).

The relationship between biomarker responses and biometric measurements was examined by principal component analysis (PCA), using Plymouth Routines in Multivariate Ecological Research (PRIMER) v6 (Clarke & Warwick 2001; Clarke & Gorley 2006). Two data matrixes of 4 variables each were used (AChE Activity, Foot Muscle Total Protein content, Shell Length and Foot Muscle Tissue Weight) and (MT Concentration, Soft Body Total Protein Content, Shell Length and Soft Body Tissue Weight). Due to scale differences between variables, the analysis was done on

transformed and normalised residuals. Pearson correlation coefficients (*r*) were calculated to investigate statistical relationships and find out the possible influences of physiological status indicators (PT, Shell Length, Tissue Weight and CI) on AChE activity and MT concentration for pooled gender data, males and females across all sampled locations. For all statistical analysis, the significance level was $p < 0.05$.

2.3. Results

Mean levels of AChE activity, MT concentrations, total protein contents, condition index and biometric measurements recorded from *P. depressa* specimens for each gender and location are shown in Table 2.2.

2.3.1. AChE activity and Total Protein content (PT) of the foot muscle

Mean AChE activities varied from 4.18 to 7.64 nmol min^{-1} mg^{-1} total protein in males and 4.75 to 8.45 nmol min^{-1} mg^{-1} total protein in females (Table 2.2), reflecting the natural individual differences in AChE activity. Foot muscle total PT contents varied from 19.89 to 39.94 $mg.g^{-1}$ in males and from 16.11 to 36.77 $mg.g^{-1}$ in females (Table 2.2).

Two-way ANOVA results showed effects of gender and location on these parameters and are presented in Figure 2.2. Significant differences were found at both measurements between sexes (AChE: $F_{1,60}=22.01$, $p<0.001$; PT: $F_{1,60}=13.91$, $p<0.001$) and locations (AChE: $F_{5,60}=27.76$, $p<0.001$; PT: $F_{5,60}=86.02$, $p<0.001$). Female limpets showed higher significant AChE activities levels (6.37 ± 0.26 nmol min^{-1} mg^{-1} total protein) than males (5.42 ± 0.23 nmol min^{-1} mg^{-1} total protein), while the foot total PT contents were higher in males (29.96 ± 1.35 $mg.g^{-1}$) than for females (27.21 ± 1.41 $mg.g^{-1}$).

Table 2.2. Summary of biomarkers (AChE and MT) and indicators of physiological status of limpets from six locations (artificial and natural rocky shores) of the Portuguese coast.

Gender	Location	Condition Index	AChE Activity (nmol min^{-1} mg^{-1} total protein)	Shell Length (mm)	Foot Muscle Wet Weight (g)	Foot Muscle Total Protein Concentration ($mg.g^{-1}$)	MT Concentration ($mg.g^{-1}$ dry weight)	Shell Length (mm)	Soft Body Wet Weight (g)	Soft Body Total Protein Concentration ($mg.g^{-1}$)
Male	Vilamoura	22.79±0.80	5.39±0.83	46.31±1.73	1.67±0.18	19.89±1.08	4.27±0.36	45.00±2.87	5.30±1.00	35.55±1.92
	Portimão	23.83±2.09	4.90±0.40	45.46±1.28	1.21±0.09	28.01±1.13	4.30±0.26	44.85±1.89	5.73±1.06	36.70±2.95
	Cascais	17.31±0.70	4.18±0.46	47.48±4.76	1.56±0.40	39.94±2.46	6.56±0.96	47.19±4.42	4.82±1.23	35.54±4.07
	Olhos d'Água	20.26±4.48	7.64±0.36	33.42±0.93	0.55±0.14	24.26±1.37	11.36±0.78	29.83±0.96	1.29±0.14	28.89±0.93
	Vau	20.05±1.21	5.73±0.46	29.53±0.82	0.45±0.04	28.23±2.95	13.53±1.66	26.64±0.63	0.89±0.11	27.00±2.33
	Cabo Raso	15.05±0.47	4.65±0.31	33.6±0.72	0.46±0.05	39.40±1.06	8.82±0.80	32.23±1.38	1.37±0.16	27.54±1.11
Female	Vilamoura	24.77±1.05	7.34±0.49	44.27±2.27	1.32±0.12	16.11±1.70	5.00±0.19	45.34±2.90	5.58±1.32	46.09±3.95
	Portimão	24.21±1.00	5.93±0.41	47.43±2.87	1.22±0.12	25.83±1.23	5.89±0.66	42.37±1.40	3.86±0.40	48.96±4.76
	Cascais	16.67±1.10	4.75±0.45	49.07±1.88	1.39±0.23	36.77±2.43	7.13±0.69	44.95±1.50	4.51±0.67	46.60±1.75
	Olhos d'Água	22.79±3.53	8.45±0.68	32.85±2.40	0.57±0.11	22.13±1.87	14.39±2.33	30.94±1.23	1.18±0.21	34.01±0.30
	Vau	20.73±3.98	6.87±0.54	30.15±1.47	0.45±0.07	24.23±1.54	16.53±1.93	25.87±0.58	0.88±0.03	34.19±2.55
	Cabo Raso	13.58±0.91	4.86±0.34	34.69±1.47	0.42±0.09	38.19±1.61	9.86±0.60	30.65±0.65	1.17±0.15	36.94±3.16

Data (n=6) are shown as mean ± standard deviation (SD)

Olhos d'Água showed the highest mean AChE activities (8.05±0.29 nmol min^{-1} mg^{-1} total protein), followed by Vilamoura (6.36±0.44 nmol min^{-1} mg^{-1} total protein), Vau (6.30±0.29 nmol min^{-1} mg^{-1} total protein) and then by Portimão (5.42±0.25 nmol min^{-1} mg^{-1} total protein), Cabo Raso (4.76±0.16 nmol min^{-1} mg^{-1} total protein) and Cascais (4.47±0.23 nmol min^{-1} mg^{-1} total protein) (Figure 2.2). No significant differences in AChE activity were found between Vilamoura and Vau, and between Portimão, Cabo Raso and Cascais ($p>0.05$) (Figure 2.2). Cabo Raso and Cascais showed the highest mean foot muscle total protein contents (38.80±0.68 and 38.36±1.26 $mg.g^{-1}$, respectively), followed by Portimão (26.92±0.65 $mg.g^{-1}$) and Vau (26.23±1.27 $mg.g^{-1}$), then by Olhos d´Água (23.20±0.85 $mg.g^{-1}$) and finally by Vilamoura (18.00±0.89 $mg.g^{-1}$) (Figure 2.2). No significant interaction between the two factors (sex x location) was observed.

2.3.2. Relationship between AChE activity and biometric measurements

Principal Component Analysis (PCA) was used to explore any differences in the AChE activity and total Protein content of the limpet foot muscle as a function of the biometric measurements (Figure 2.3). Both PC 1 (50.7%) and PC 2 (41.4%) together explained 92% of total variance. In PC 1, the main differences were explained by biometric measurements, i.e., shell length and foot muscle weight. These variables were positively correlated (Pearson's r =0.9103, p<0.0001). In PC 2, the major variables causing differences were AChE activity and total Protein content of the foot muscle. AChE activity was negatively related to the total PT content of the foot muscle (Pearson's r =-0.6862, p<0.0001). The plot PC 2 versus PC 1 showed a clear geographical pattern (Figure 2.3). Limpets from the south coast showed higher AChE activities and lower total PT contents than limpets from the central coast of Portugal,

while limpets from artificial rocky shores showed higher shell length and foot muscle weight.

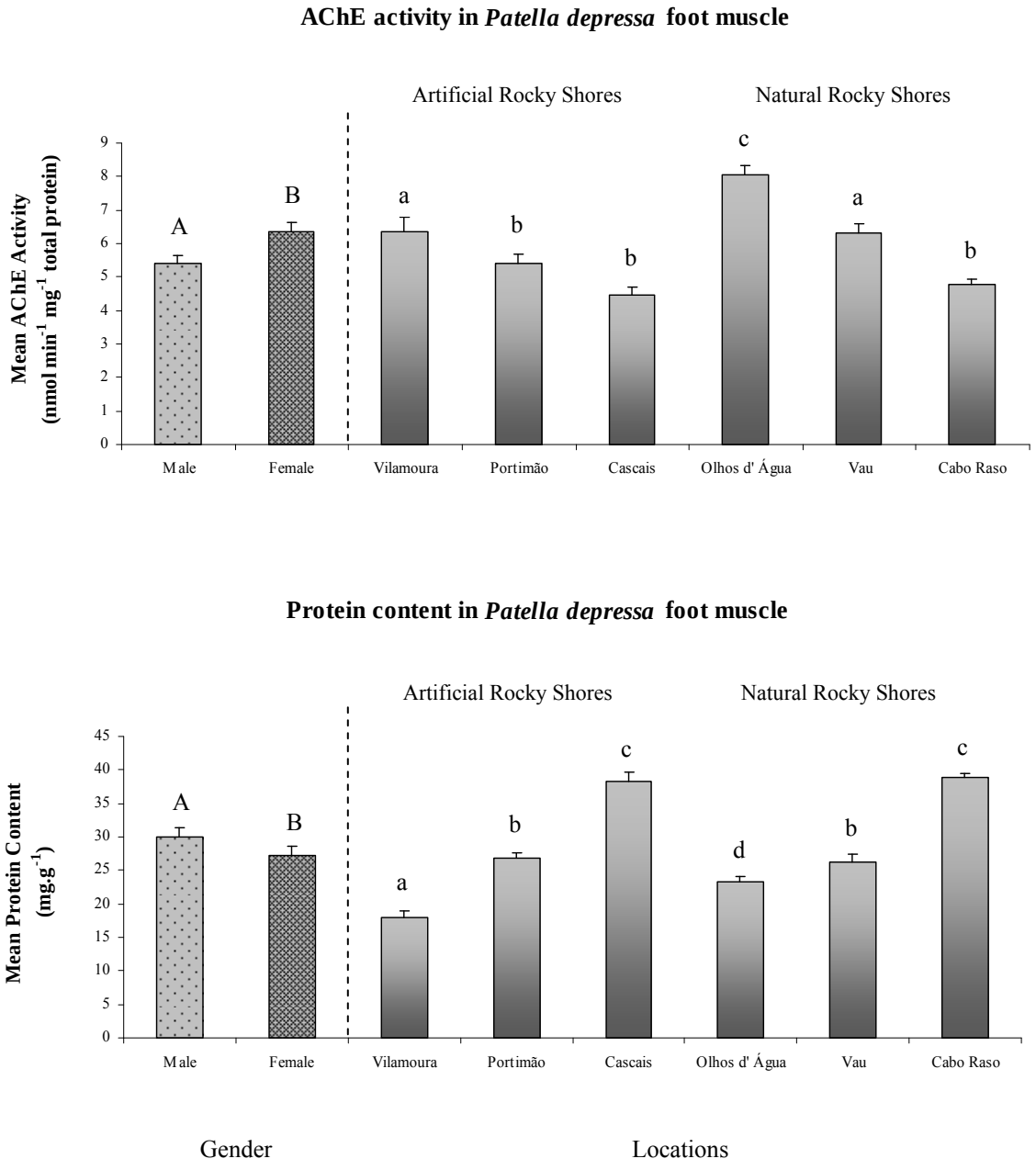

Figure 2.2. Mean AChE activity and protein content of *P. depressa* foot muscles from six locations of the Portuguese Coast. Histogram bars represent means ± SE. The bars with the same superscript are not significantly different ($p>0.05$); small letters were used for locations and capital letters for gender.

Despite the larger average size of shell and foot muscle weight on artificial rocky shores limpets (Table 2.2), AChE activities and total PT contents were negative and weakly correlated with size of limpet (Pearson's r =-0.2835, p=0.0079 and r =-0.0384, p=0.3744, respectively) and foot muscle weights (Pearson's r = -0.1892, p=0.0557 and r=-0.1936, p=0.0516, respectively).

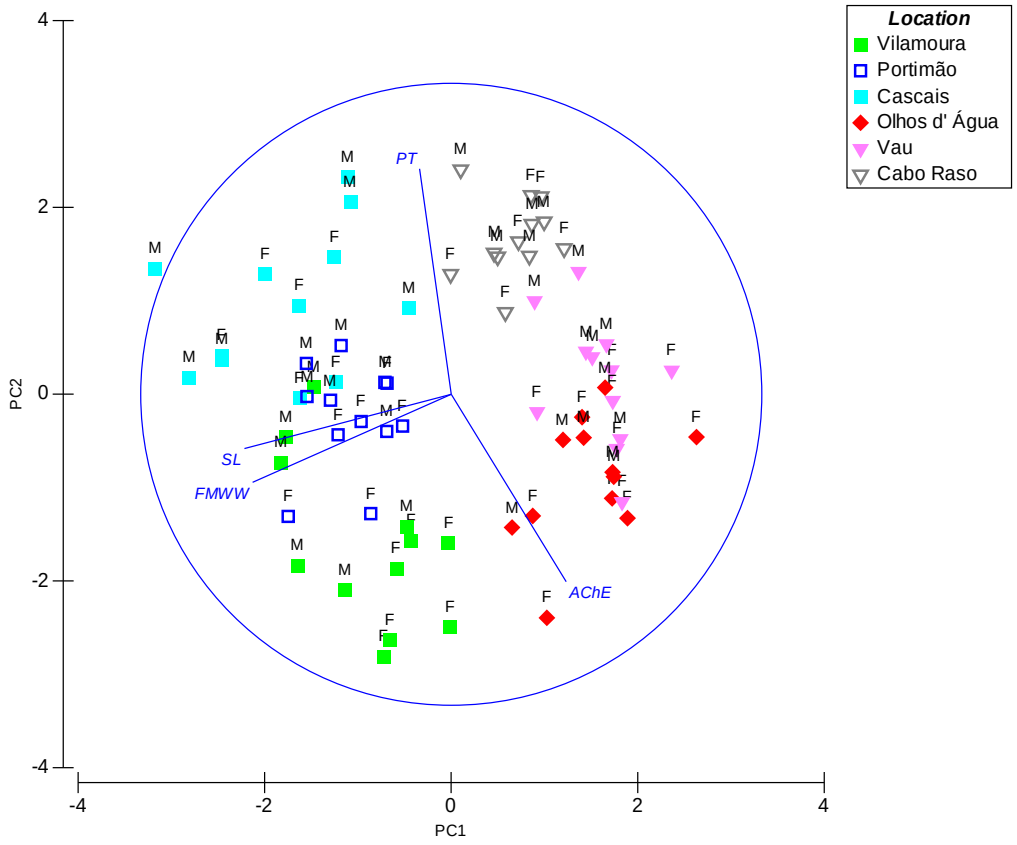

Figure 2.3. PCA analysis of AChE activity, total protein content, shell length and foot muscle wet weight of the limpets collected from marinas and rocky shores of the Portuguese coast (F=female, M=male, SL=Shell Length, FMWW=Foot Muscle Wet Weight, AChE= Acetylcholinesterase activity and PT= total Protein content).

2.3.3. MT concentration and Total Protein content (PT) of soft body tissues

Mean MT concentrations varied from 4.27 to 13.53 mg.g^{-1} dw in males and 5 to 16.53 mg.g^{-1} dw in females, while total PT content varies from 27 to 36.70 mg.g^{-1} in males and from 34.01 to 48.96 mg.g^{-1} in females (Table 2.2).

Two-way ANOVA results showing effects of gender and location on these parameters are presented in Figure 2.4. Significant differences were found at both measurements between sexes (MT: $F_{1,60}$=17.85, p<0.001; PT: $F_{1,60}$=65.41, p<0.001) and locations (MT: $F_{5,60}$=80.34, p<0.001; PT: $F_{5,60}$=16.07, p<0.001). Female limpets showed higher significant MT concentrations (9.80±0.81 $mg.g^{-1}$ dw) and soft body total PT contents (41.13±1.34 $mg.g^{-1}$) than males (MT: 8.14±0.64 $mg.g^{-1}$ dw; total PT: 31.87±0.96 $mg.g^{-1}$) (Figure 2.4). Vau showed the highest mean MT levels (15.03±0.97 $mg.g^{-1}$ dw), followed by Olhos d'Água (12.87±0.95 $mg.g^{-1}$ dw), Cabo Raso (9.34±0.37 $mg.g^{-1}$ dw), Cascais (6.85±0.41 $mg.g^{-1}$ dw) and finally by Portimão (5.10±0.34 $mg.g^{-1}$ dw) and Vilamoura (4.64±0.18 $mg.g^{-1}$ dw) (Figure 2.4). Portimão, Cascais and Vilamoura showed higher mean foot muscle total protein contents (42.83±2.64, 41.07±2.24 and 40.82±2.17 $mg.g^{-1}$, respectively) than Cabo Raso, Olhos d'Água and Vau (32.24±1.81, 31.45±0.84 and 30.60±1.59 $mg.g^{-1}$, respectively) (Figure 2.4). No significant interaction between the two factors (sex x location) was observed.

2.3.4. Relationship between MT concentration and biometric measurements

Principal Component Analysis (PCA) was made to explore any differences in the MT and total protein content of the limpet soft body as a function of the biometric measurements (Figure 2.5).

Both PC 1 (78.6%) and PC 2 (15.8%) together explained 94.3% of total variance. In PC 1 the main differences were explained mostly by the MT levels and biometric measurements, i.e., shell lengths and soft body weights. Biometric measurements variables were positively correlated (Pearson's r =0.9725, p<0.0001), while MT concentration was negatively related to shell length (Pearson's r =-0.8381, p<0.0001) and soft body wet weight (Pearson's r =-0.8459, p<0.0001).

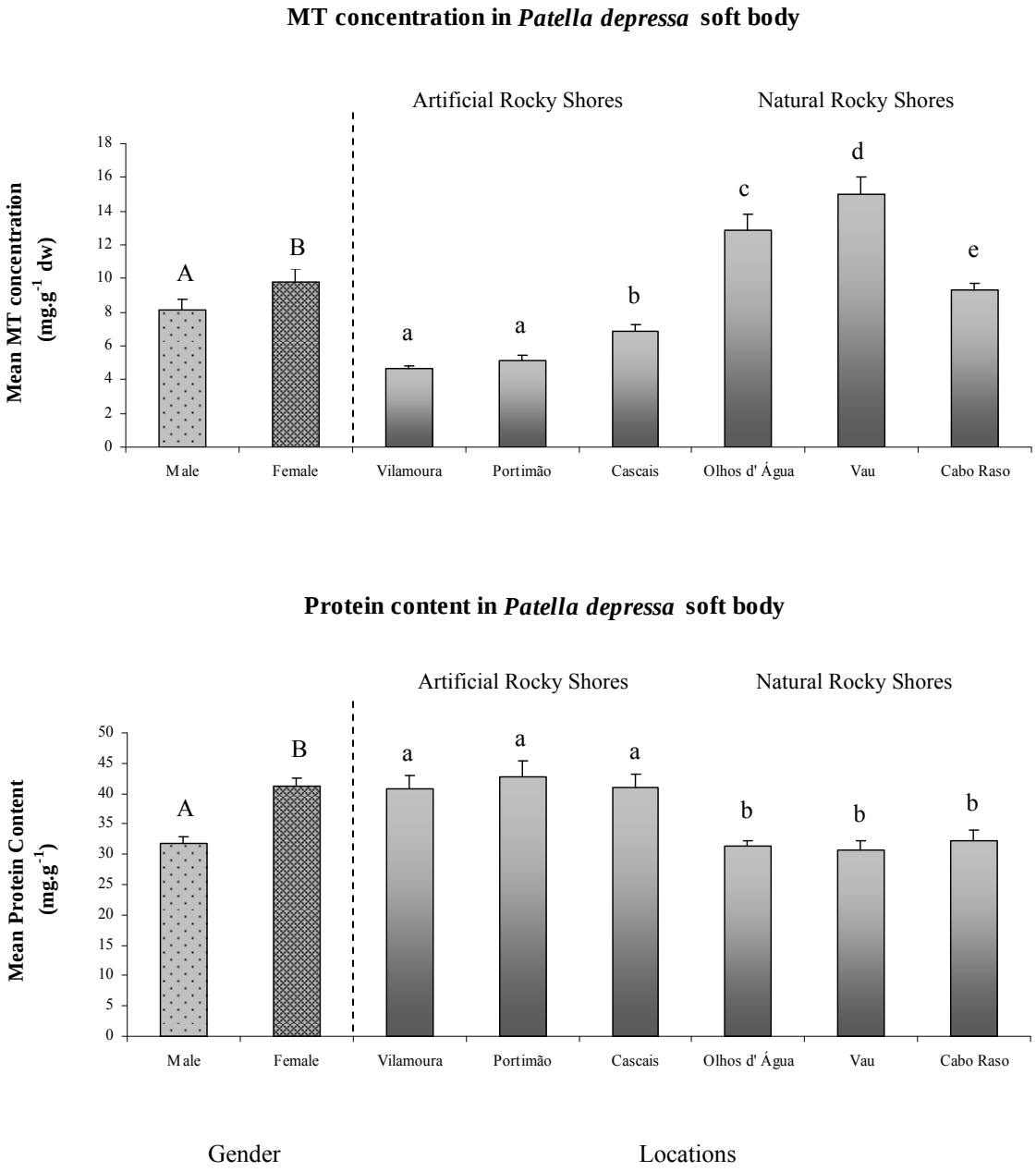

Figure 2.4. Mean MT concentration and protein content of *P. depressa* soft bodies from six locations of the Portuguese Coast. Histogram bars represent means ± SE. The bars with the same superscript are not significantly different (p>0.05); small letters were used for locations and capital letters for gender.

In PC 2, the most explanatory variable was soft body total protein content. Total PT content was positively related to shell length (Pearson's r =0.5403, p<0.0001) and soft body wet weight (Pearson's r =0.5212, p<0.0001), and was negatively related to the

MT concentration (Pearson's r =-0.4789, p<0.0001). The plot PC 2 versus PC 1 showed a clear location pattern (Figure 2.5). Limpets from natural rocky shores (Olhos d'Água, Vau and Cabo Raso) showed higher MT concentrations than limpets from artificial rocky shores (Vilamoura, Portimão and Cascais), but lower total PT content, soft body weights and smaller shell length.

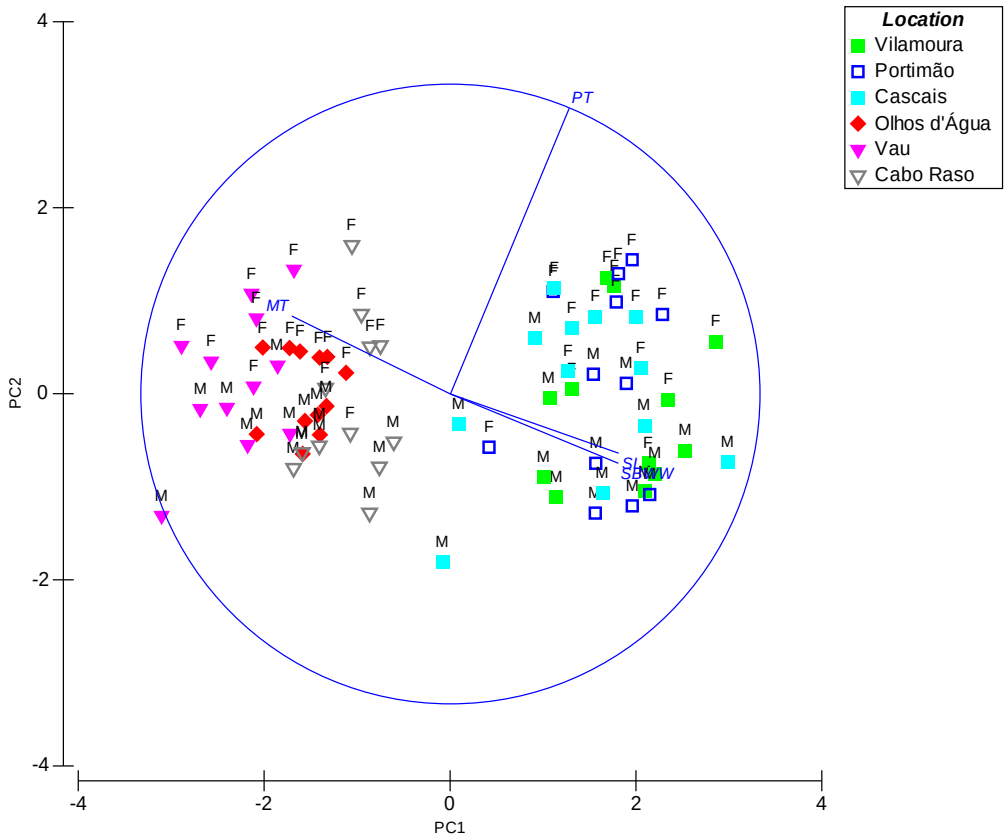

Figure 2.5. PCA analysis of MT concentration, total protein content, shell length and soft body wet weight of the limpets collected from marinas and rocky shores of the Portuguese coast (F=female, M=male, SL=Shell Length, SBWW=Soft Body Wet Weight, MT= Metallothionein concentrations and PT= total Protein content).

2.3.5. Condition index (CI)

Mean condition indexes were calculated for each location as an indication of the nutritional health status of limpets (Table 2.2). Two-way ANOVA results showing effects of gender and location on this parameter are presented in Figure 2.6. No significant differences were found between gender ($F_{1,60}$=0.40, p=0.5283), suggesting

that sex was not a significant factor in intra-or inter-location differences. However, significant differences were found between locations ($F_{5,60}$=11.93, p<0.001) (Figure 2.6): Cabo Raso (14.31±0.41%) and Cascais (16.99±0.45%) showed lowest CI values than Vau (20.39±1.41%), Olhos d'Água (21.53±1.96%), Vilamoura (23.78±0.54%) and Portimão (24.02±0.78%), suggesting geographical (central-south direction) differences rather than natural vs. artificial rocky shores differences.

Pearson's correlation analysis of AChE activity levels, MT concentrations and Condition Index for pooled gender data across all sampled locations revealed a weak but significant relationship between these measurements (AChE, CI: r =0.3250, p=0.0027 and MT, CI: r =-0.2295, p=0.0262).

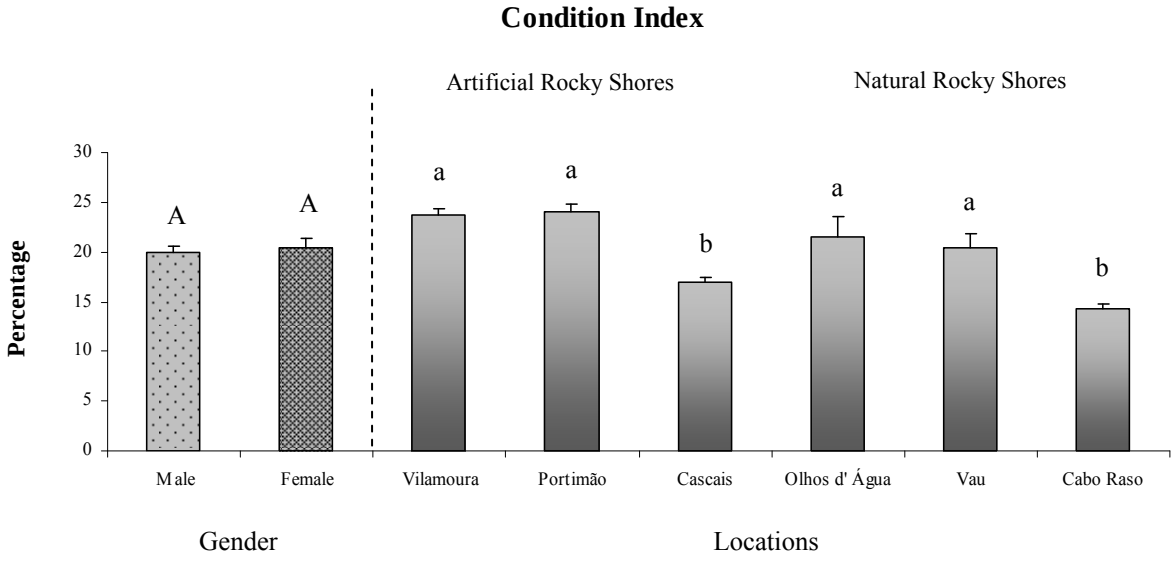

Figure 2.6. Condition Index (%) of the limpet *P. depressa* collected from six locations of the Portuguese Coast. Histogram bars represent means ± SE. The bars with the same superscript are not significantly different (p>0.05); small letters were used for locations and capital letters for gender.

2.4. Discussion

Gender was respectively directly related to AChE and MT levels in *P. depressa* foot muscle and soft body. AChE activity and MT concentration were higher in females. There is no available literature explaining this gender differences in gastropod molluscs, however differences in hormonal control, metabolic and intake rates, or even differences in animal movement may be indirectly related to contamination. Nevertheless, further research is needed to understand them. Previous biochemical studies on the fatty acid composition of the soft body and gonads of *P. depressa* also revealed gender differences and these were suggested to be related to sex reproductive strategies in lipid storage from diet (Brazão *et al.* 2003b; Morais *et al.* 2003).

2.4.1. Evaluation of AChE activities in the limpet *P. depressa*

To our knowledge, this is the first published work with AChE biomarker in limpets having in consideration gender differences. Female limpets showed higher significant AChE activities levels than males, while the foot total PT contents were higher in males comparing to females. This observation is not in agreement with those previously reported for other organisms (e.g. fish, polychaete), where the natural variability in the activity of AChE was not directly related to gender (Payne *et al.* 1996; Bocquené & Galgani 1998; Kirby *et al.* 2000; Scaps & Borot 2000; Flammarion *et al.* 2002). Very few published values are available concerning AChE activity in gastropod molluscs; particularly limpets (see Table 2.3). The obtained values of this study range from approximately 4.2 to 7.7 in males and 4.8 to 8.5 nmol min^{-1} mg^{-1} total protein in females.

Table 2.3. Summary of AChE activities of the limpet *P. depressa* (present work) and in other Mollusc species from other geographical locations.

Class	Species	Location	ACHE activity (nmol min^{-1} mg^{-1} protein)	Tissue	Reference
Gastropod	*Patella depressa*	Portugal	4.2-7.7 (♂) / 4.8-8.5 (♀)	Foot muscle	This work
Gastropod	*Patella vulgata*	UK	332.5 (a)	Haemolymph	Brown *et al.*, 2004
Gastropod	*Patella vulgata*	Morocco	0.51-2.04 (a)	-	Douhri & Sayah., 2009
Gastropod	*Aplysia californica*	US	10	Haemolymph	Bevelaqua *et al.*, 1975
Gastropod	*Murex trunculus*	Tunisia	7.3-13.5	Digestive gland	Gharbi-Bouraoui *et al.*, 2008
Gastropod	*Murex trunculus*	Tunisia	58.79	Digestive gland	Roméo *et al.*, 2006
Gastropod	*Murex trunculus*	Tunisia	33.71	Muscle	Roméo *et al.*, 2006
Bivalve	*Mytilus edulis*	Baltic Sea	22.6-19.3	Gill	Leiniö & Lehtonen 2005
Bivalve	*Mytilus edulis*	Baltic Sea	19-79	Gill	Schiedek *et al.*, 2006
Bivalve	*Mytilus edulis*	Baltic Sea	19.2-25.9	Gill	Lehtonen *et al.*, 2006
Bivalve	*Mytilus galloprovincialis*	France Mediterranean Sea	27.6-58.5	Gill	Bodin *et al.*, 2004
Bivalve	*Macoma balthica*	Baltic Sea	14.7-16.9	Foot muscle	Leiniö & Lehtonen 2005
Bivalve	*Macoma balthica*	Baltic Sea	17.7-21.8	Foot muscle	Lehtonen *et al.*, 2006
Bivalve	*Scrobicularia plana*	Spain	3.0-6.1	Digestive gland	Pérez *et al.*, 2004
Bivalve	*Crassostrea gigas*	France	24.3 (b)	Mantle	Bocquené *et al.*, 1997
Bivalve	*Crassostrea gigas*	France	28.1 (b)	Gill	Bocquené *et al.*, 1997

(a) µmol ACTC $min^{-1}mg^{-1}$ protein; (b) nmol AcSCh hydrolyzed/min/mg of protein

Table 2.4. Summary of MT concentrations of the limpet *P. depressa* (present work) and in other Mollusc species from other geographical locations (dw = dry weight; ww= wet weight).

Class	Species	Location	MT concentration ($mg.g^{-1}$ dw)	Tissue	Reference
Gastropod	*Patella depressa*	Portugal	4.27-13.53 (♂), 5-16.53 (♀)	Whole soft tissue	This work
Gastropod	*Patella aspera*	Portugal	4.9-9.8	Whole soft tissue	Bebianno *et al.*, 2003
Gastropod	*Patella vulgata*	UK	≈30 (a)	Whole soft tissue	Brown *et al.*, 2004
Gastropod	*Nucella lapillus*	Scotland	0.1-0.5 (b)	Gland of Leiblein	Leung *et al.*, 2001
Gastropod	*Nucella lapillus*	France	400-900 (c)	Whole soft tissue	Amiard *et al.*, 2004
Gastropod	*Littorina littorea*	France	400-600 (c)	Whole soft tissue	Amiard *et al.*, 2004
Bivalve	*Mytilus galloprovincialis*	Portugal	3.9-13.0	Whole soft tissue	Bebianno & Machado, 1997
Bivalve	*Mytilus galloprovincialis*	Portugal	2.5	Gills	Bebianno & Serafim 1998
Bivalve	*Mytilus edulis*	UK	2.75	Whole soft tissue	Bebianno & Langston 1991
Bivalve	*Mytilus edulis*	Baltic Sea	338-359 (a)	Gills	Leiniö & Lehtonen 2005
Bivalve	*Mytilus edulis*	Baltic Sea	303-426 (a)	Digestive gland	Lehtonen *et al.*, 2006
Bivalve	*Ruditapes decussatus*	Portugal	2.2	Gills	Bebianno & Serafim 1998
Bivalve	*Ruditapes decussatus*	Portugal	2.05	Whole soft tissue	Bebianno *et al.*, 2000
Bivalve	*Ruditapes decussatus*	Portugal	2.45	Digestive Gland	Bebianno *et al.*, 2000
Bivalve	*Macoma balthica*	Baltic Sea	521-460 (a)	Foot muscle	Leiniö & Lehtonen 2005
Bivalve	*Macoma balthica*	Baltic Sea	392-460 (a)	Digestive gland	Lehtonen *et al.*, 2006

(a) $\mu g.g^{-1}$ ww; (b) $mg.g^{-1}$ ww; (c) mg Kg^{-1} ww

Similar AChE activities (considering sexes together) were found in the haemolymph of the marine gastropod *Aplysia californica* (between 2 and 10 nmol min^{-1} mg^{-1} total protein) (Bevelaqua *et al.* 1975), and in the digestive gland of the gastropod *Murex trunculus* (9.4 nmol min^{-1} mg^{-1} total protein) (Gharbi-Bouraoui *et al.* 2008). Brown *et al.* (2004) and Douhri & Sayah (2009) also reported specific activities in the limpet *Patella vulgata* Linnaeus (respectively, around 300 µmol ACTC $min^{-1}mg^{-1}$ protein and 2.04 µmol $min^{-1}mg^{-1}$ protein).

Despite the inherent variability among species and tissues, the level of activity of AChE found in the foot tissue of *P. depressa* was quite low compared with other gastropods (Roméo *et al.* 2006) and bivalve molluscs such as mussels (Bodin *et al.* 2004; Leiniö & Lethonen 2005; Lehtonen *et al.* 2006; Schiedek *et al.* 2006), oysters (Bocquené *et al.* 1997) or even clams (Leiniö & Lethonen 2005; Lehtonen *et al.* 2006) (see Table 2.3). However, direct comparison is often confounded by differences between sample preparation methods and by heterogeneity of enzymatic units used in literature to describe data. The reason why *P. depressa* AChE activity is so low is difficult to explain, and it was impossible to further clarify this point. The lower values AChE activities in limpets may be a result of the application of test methods not originally designed and adequately characterized for measuring enzymatic activities in these species, and thus not taking into account several species specificities, such as optimal enzyme reaction temperature (Dauberschmidt *et al.* 1997).

In bivalves, AChE is usually best measured in gills (e.g. Bocquené & Galgani 1998; Bodin *et al.* 2004; Leiniö & Lethonen 2005; Lehtonen *et al.* 2006; Schiedek *et al.* 2006) but, in this study, foot muscle tissue was used exclusively, since it offers some logistical advantages over gills, such as ease of collection and a larger quantity of material. Nevertheless, a methodology improvement is needed for this species. Foot

tissue has already been shown to be a good alternative over small gills in previous studies working with bivalve molluscs (Leiniö & Lehtonen 2005; Lehtonen *et al.* 2006).

The survey has shown that it is possible to significantly discriminate levels of AChE activity in *P. depressa* between locations in the Portuguese coast. Despite the lack of an appropriate reference point or pristine site exhibiting ‘‘baseline’’ levels of activity, the hypothetically more impacted locations showed lower levels of AChE. A tentative ranking of neurotoxic contamination, in terms of reduced muscle AChE activities, was established as (most contaminated first): Cascais, Cabo Raso, Portimão > Vau, Vilamoura > Olhos d’Água. This gradient shows a general geographical trend in the Centre-South direction, possibly more responsive to differences in seawater temperatures (around 22ºC in the south and 16ºC in the centre Portuguese coast during the summer), rather than exposure to chemical pollution.

In poikilothermic organisms, ambient temperature is one of the major factors driving physiological and biochemical processes and can also influence the sensitivity of different organisms to toxicants (Chapman *et al.* 2006). Biochemical reaction rates, metabolic rates, and nearly all other rates of biological activity increase exponentially with temperature (Chapman *et al.* 2006). Lower AChE activities caused by decreasing temperature in the locations from the central coast can then be explained by a general reduction of the limpet metabolic rate. This hypothesis is in agreement with Bocquené & Galgani (1998) that claimed water temperature as the most important natural factor affecting AChE activity. Indeed, it has been demonstrated that AChE activities in molluscs clearly varies according to seasons, increasing during the summer, along with elevations in water temperature (Damiens *et al.* 2004; Leiniö & Lehtonen 2005; Pfeifer *et al.* 2005). Thus, the sampling central locations chosen in the present study may

represent an area where the effect of temperature on AChE activity is more pronounced than the effect of neurotoxic substances.

Despite this geographical trend, biomarker results were in accordance to the expected pollution gradient, i.e., lower AChE activities in limpets at artificial rocky shores (Vilamoura, Portimão and Cascais) compared to natural geographical associated rocky shores (Olhos d'Água, Vau and Cabo Raso). It can be concluded that the observed intra- and inter-location differences could be in part neurotoxin-mediated. These results may suggest that rocky shores located within marinas may be more subjected to integrated effects of several classes of contaminants such as PAHs from maritime traffic and sewage and ships' antifouling compounds (Tributyltin and Cu), rather than organophosphorous and carbamates pesticides, since sampling locations are not directly influenced by agricultural wastes. Similar lowest AChE activities were found in mussels collected close to a harbour in the Baltic and Mediterranean Sea (Damiens *et al.* 2007; Schiedek *et al.* 2006).

Pearson correlation analysis revealed a weak negative relationship between AChE activity and shell length and no correlation with foot weight, despite the morphological differences between limpets from artificial and natural rocky shores. In the absence of other influences (all the specimens were in the same maturation stage), the lowered activity may be a product of water temperature and/or the result of neurotoxic contamination. Similar results have been reported in other studies, where natural variation in AChE activity was related to water temperature (Damiens *et al.* 2004, Leiniö & Lehtonen 2005; Pfeifer *et al.* 2005), and weakly negative dependent to size (Burgeot *et al.* 1996; Flammarion *et al.* 2002).

The total protein content of the foot of *P. depressa* was negatively related to AChE activity. Similar results were reported in the foot of the bivalve *M. balthica*

(Leiniö & Lehtonen 2005) where the protein content of the foot of *M. balthica* decreased in parallel with a slight elevation in the activity of AChE. Increased AChE activities in the limpet *P. vulgata* also occurred in accordance with significantly lower haemolymph protein content (Brown *et al.* 2004).

2.4.2. Evaluation of MT concentrations in the limpet *P. depressa*

Metals are among the major contaminants reaching the marine environment and the food web chain is believed to be a major route of uptake for metals in invertebrates (Bryan 1984; Depledge & Rainbow 1990). In the present study, significant gender differences were found in MT levels of limpet soft bodies. MT concentration and total PT content were higher in females. To our knowledge, there is no data in the literature taking into consideration gender differences in MT determination in limpets. Nonetheless, this observation is not in agreement with those reported by Bebianno *et al.* (2000) and Bebianno *et al.* (2004) for the clam *R. decussatus* of the south coast of Portugal, where MT levels were found not to differ significantly between sexes. Although in molluscs MT levels are tissue specific and generally higher in tissues directly involved in metal uptake, storage and excretion such as gills and digestive glands (Bebianno & Langston 1991; Bebianno *et al.* 1993, 1994; Bebianno *et al.* 2000; Amiard *et al.* 2006), in the present study, soft body tissues were used due to their easy dissection and larger size.

Taking into account the fact that the concentrations are expressed as either wet weight or dry weight and inter-organ differences are generally found, comparison with other species may be difficult. However, in order to compare the present data with those published by other authors, metallothionein levels were express on dry tissue mass basis (see Table 2.4). The range of MT levels observed in limpets from artificial and natural rocky shores of the Portuguese Coast (4.27-13.53 $mg.g^{-1}$ dw in males and 5-16.53

mg.g^{-1} dw in females) are in agreement with the values found for *Patella aspera* (Röding) (4.9 to 9.8 mg.g^{-1} dw) (Bebianno *et al.* 2003) and for the mussel *M. galloprovincialis* (3.9 to 13.9 mg.g^{-1} dw) (Bebianno & Machado 1997) also in the Portuguese coast (considering sexes together). On the other hand, lower MT values were found in the clam *R. decussatus* (2.05 mg.g^{-1} dw) also in the Portuguese coast (Bebianno *et al.* 2000). According to Brown *et al.* (2004), there is evidence to suggest that the MT metal binding capacity in the genus *Patella* is lower than for oysters or mussels, restricting the ability of limpets to regulate metals.

Based on the assumption that metals induce MT, it would be expected that tissues with the highest accumulated metal concentrations should have the highest MT concentrations. Unexpectedly, limpets from artificial rocky shores showed lower MT concentrations than those from natural rocky shores, despite the fact that the organisms collected at artificial rocky shores were probably more exposed to hydrocarbons and metals from the maritime traffic and marina associated activities (e.g. increasing concentration of Cu in antifouling paints), especially during the summer when sampling occurred. With respect to levels of potential metal contamination exhibited in the limpet soft bodies from the different locations, the following ranking was found (the most contaminated first): Vau> Olhos d' Água> Cabo Raso> Cascais> Portimão, Vilamoura. Given the lack of a 'reference' site in the present study, the results obtained from different locations were only compared location by location, thus it is complex to determine at to what extent the response of this biomarker may have resulted from contaminant exposure or to natural environmental variability.

However, the above gradient shows the existence of a clear artificial/natural substrata trend and it can be deduced that, in the absence of other abiotic and biotic influences, the lowered concentration could be indeed a product of site-specific

differences in shell length, body weight and/or metals contamination. Unfortunately, due to technical limitations, no detailed analysis of all the contaminants possibly causing—individually or in combination— any biological effects are available in the present study for tissues or marine environments, to support the latter hypothesis.

Bebianno *et al.* (2003) and Cravo *et al.* (2004) working on the limpet *P. aspera* of the south coast of Portugal found lower Cd but higher Zn and Cu concentrations in limpets collected in areas directly influenced by high ship traffic in a marina. On the other hand, Noël-lambot *et al.* (1980), working on *P. vulgata* collected from a polluted environment found that Cd bound to thioneins, but this was not the case for Zn and Cu. Following exposure to Cu, Brown *et al.* (2004) also found no MT induction in the limpet *P. vulgata.* Additionally Bebianno *et al.* (2003) also detected no significant relationship between MT and Cu in the soft tissues of *P.aspera* and, when Cu body burdens were greater than 5 $mg.g^{-1}$ MT levels decreased.

In invertebrates there are two well documented mechanisms of intracellular detoxification of different metals that have been related to increase metal tolerance and even genetic resistance of organisms in polluted environments: (1) metal-binding to intracellular proteins including metallothioneins (or metallothionein-like proteins) and (2) insolubilisation of metals in the form of granules which are stored for life or regularly eliminated (Amiard *et al.* 2004, 2006). Depending on the species, the relative importance of these two detoxification mechanisms varies considerably (Amiard *et al.* 2004, 2006). Insolubilisation of metals (mainly Cu used in antifouling paints) in the form of granules may be the most important detoxification strategy adopted by limpets and responsible for the lower MT concentrations found in limpets from marinas in the present study. Further studies are essential to understand the detoxification mechanisms in limpets.

Additionally, it is well known that body weight influence metal concentrations in aquatic molluscs (Noël-lambot *et al.* 1980; Langston *et al.* 1998; Leung *et al.* 2001; Bebianno *et al.* 2003). As mentioned before, the tests were carried out on experimental animals representative of the natural populations with smaller individuals from natural rocky shores by comparison with those from artificial rocky shores, and this fact may have influenced limpets biomarker responses. These size differences may be due to variations in type and food availability, predation pressure, wave action and/or genotype.

In the present study, correlation analysis showed strong negative links between MT levels, shell length and soft body weight. These results are not in agreement with that observed by Bebianno *et al.* (2003) for *P. aspera* from the south coast of Portugal, where positive relationships were found between MT levels and shell size or soft body weight. In opposition, several studies with mussels (de Lafontaine *et al.* 2000) and oysters (Mouneyrac *et al.* 1998) showed higher MT concentrations in small individuals.

Indices such as CI are known to correlate well with measured biomarker values. In this study, the CI showed significant but weak correlations with AChE and MT (AChE, CI: r =0.3250, p=0.0027 and MT, CI: r =-0.2295, p=0.0262 for all locations). The highest condition index values were recorded in the south coast, possibly indicating that food availability and diet quality are better and/or nutrient inputs are more stable at this geographical area, where in the summer months water temperature is higher. This aspect needs to be more fully studied, as poor environmental conditions may not always correspond to low physiological responses in marine organisms.

2.5. Conclusion

In summary, this work stresses the importance of considering gender whenever differences between field locations in AChE and MT levels are to be interpreted.

Despite the influence of abiotic and biotic factors in the levels of both studied biomarkers, a general trend was observed between limpets from artificial and natural rocky shores, and these differences may be in part mediated by several classes of contaminants. The measurement of biomarkers in adult limpets (which are not subjected to seasonal and sexual variations and having gender in consideration) may thus constitute a useful tool to assess the modifications of the environment due to anthropogenic influences. The results obtained here also underline the importance of identifying the potential interfering abiotic and biotic factors (e.g. water temperature, body size and weight), as well as the direction and magnitude of their impacts on the biomarker signals observed in wild populations. Further research is needed using *in vitro* or *in vivo* laboratorial assays to evaluate the effects of specific classes and mixtures of environmental contaminants on the enzymes of this species in order to validate the field results. Use of control specimens collected at a pristine site of the studied ecosystem, the use of depurated organisms at laboratory conditions and/or the use of recovery techniques (when a suitable control site is not available) would be valuable tools in the interpretation of future results. The use of *P. depressa* as a sentinel species for biomonitoring potential toxic effects *in situ* appears feasible using a multi-biomarker approach, where the relationships between biomarkers, contaminants and physiological status indicators of limpets can be examined. As demonstrated here, spatial variability and natural ranges in the responses must be carefully taken into account when comparing biomarker responses in different areas.

CHAPTER 3

Influence of gender and habitat (marinas vs. rocky shores) on gonad fatty acid composition of the limpet *Patella depressa* Pennant, 1777 (Gastropoda: Prosobranchia) of the Portuguese south coast

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CHAPTER 3: Influence of gender and habitat (marinas vs. rocky shores) on gonad fatty acid composition of the limpet *Patella depressa* Pennant, 1777 (Gastropoda: Prosobranchia) of the Portuguese south coast

3.1. Introduction

In similarity with other intertidal gastropods, limpets are widespread on rocky shores and other hard substrates on many coastlines throughout the world. Moreover, they play an important role in structuring intertidal algal communities and thus in littoral marine food webs (Underwood 1980; Underwood & Jernakoff 1981; Hawkins & Hartnoll 1983; Jenkins *et al.* 2001; Boaventura *et al.* 2002b; Thompson *et al.* 2004). Patellid limpets have been described as being generalist microphagous grazers that remove the epilithic microbial film (Hill & Hawkins 1991; Jenkins & Hartnoll 2001; Jenkins *et al.* 2001). This is mainly composed of bacteria, diatoms, cyanobacteria, settling stage of invertebrates and protozoa, with associated micro and macroalgae propagules and detritus, which limpets apparently unselectively consume during feeding excursions in the vicinity of their home scars (Fretter & Graham 1962, 1976; Branch 1981; Steneck & Watling 1982; Della Santina *et al.* 1993, 1995; Jenkins & Hartnoll 2001; Jenkins *et al.* 2001; Thompson *et al.* 2004, 2005). Consequently, there is a considerable variation in their diets.

The majority of studies on the feeding habits and food sources of limpets are based on the analysis of gut contents (Santelices & Correa 1985; Hill & Hawkins 1991; Della Santina *et al.* 1993). This analysis provides information mainly on recent feeding, however, a more complete technique using fatty acid trophic markers (FATM) can provide information on the dietary intake and origin of lipid reserves generated over a longer period of time (Dalsgaard *et al.* 2003). FATM has been successfully applied in the last few decades to identify nutritional sources and feeding strategies of a wide

variety of organisms including mammals, fish, echinoids, bivalves and gastropods (Kharlamenko *et al.* 2001; Iverson *et al.* 2002; Grahl-Nielsen *et al.* 2003; Howell *et al.* 2003; Latyshev *et al.* 2004; Hughes *et al.* 2005). The main assumption of this technique is that certain essential fatty acid (FA) cannot be produced by biosynthesis in animals and thus can only be derived from food intake. Such compounds are laid down from marine primary producers consisting of phytoplankton (comprising both microalgae and photoautotrophic bacteria) and macroalgae, and generally remain intact through digestion, thus being conservatively transferred through aquatic food webs. Hence, they can be recognized in their primary consumer tissues with minimal modification from diet. These FA patterns can then be used as potential taxonomic markers regarding the presence and combination of certain FA characteristic of particular algal classes (Dalsgaard *et al.* 2003; Latyshev *et al.* 2004; Bergé & Barnathan 2005). Thus, a fatty acid approach can be useful to explore habitat influence and feeding strategies, since the relative proportion and composition of FA in marine organisms are characteristic for every species and genus, reflecting the special biochemical and ecological conditions of the marine environment (Dalsgaard *et al.* 2003; Bergé & Barnathan 2005).

The fatty acid composition of marine molluscs is known to be related to external factors such as fluctuations in environmental conditions (mainly water temperature), quantity and nutritional value of the food supply (Bayne & Newell 1983; Gabbot 1983; Whyte *et al.* 1990, Pazos *et al.* 1997), or by internal factors, such as sexual maturation and nutritional state of the animal (Gardner & Riley 1972; Gabbot 1983; Galap *et al.* 1999). In the present study, the fatty acid composition of the gonad was used to explore gender and habitat differences, and also to analyse diet and trophic interactions of *P. depressa*. Having in mind that in Patellid gastropods lipids are the major food reserve and nutritive storage product of the gonads (Blackmore 1969), and that this tissue may

fill much of the body cavity of limpets (gonad stage IV), gonad tissues were chosen for the determination of fatty acid profiles, thus allowing the examination of limpets diet and trophic interactions. Topics, which are currently of particular concern, are the manner in which the fatty acid profile in an animal is influenced by its diet and/or by the conditions under which it lives. To date, mainly ecological and taxonomic studies have been made with limpets, while comparative biochemical research on the occurrence and distribution of FA in limpet gonads has been very limited (but see Kawashima *et al.* 2002; Brazão *et al.* 2003b; Morais *et al.* 2003; Kawashima & Ohnishi 2006; Kawashima *et al.* 2008).

Man-made structures are proliferating in coastal urban areas and they are known to cause fragmentation and loss of natural habitats (Chapman 2003; Chapman & Bulleri 2003; Airoldi *et al.* 2005; Bulleri 2005a; Bulleri *et al.* 2005). However, artificial habitats can also provide suitable surfaces for the colonization of diverse assemblages of marine algae and invertebrates (Bulleri 2005a,b). Many marine organisms including limpets can persist in artificial structures where natural habitat is extensively replaced by urban structures (Bulleri *et al.* 2000; Chapman 2003; Bulleri *et al.* 2004). More recently, patterns of distribution and abundance of organisms have been compared between artificial and natural habitats in urban environments, in order to understand the ecology and the effects of artificial habitats on natural assemblages of organisms and to provide a sustainable management (see Glasby 1999b; Chapman & Bulleri 2003; Bulleri & Chapman 2004; Bulleri 2005a, Bulleri *et al.* 2005). Results from these studies indicated that mid-shore assemblages on artificial habitats tend to be different from those on natural habitats, primarily due to differences in the relative abundances of the species present (Chapman & Bulleri 2003; Bulleri & Chapman 2004; Bulleri *et al.*

2005). Fewer studies have investigated differences in the biochemical composition of the tissues of organisms living in both natural and artificial habitats.

The present study aimed to investigate the influence of gender and habitat on gonad fatty acid profiles using multi and univariate statistical analyses. Furthermore, it aimed to study the diet and trophic interactions using a fatty acid trophic marker approach to make a comparative analysis between limpets collected from marinas and relatively adjacent rocky shores. The specific aims were to: (1) qualitatively describe the gonad fatty acid composition of *P. depressa* from different habitats; (2) examine gender and habitat differences in gonad fatty acid profiles (individual FAs and FAs groups) using multivariate statistical analyses; (3) test for gender and habitat differences on the gonad total lipid content, FATM ratios and biometric measurements (shell length and gonad dried weight) using univariate statistical analyses and (4) assess the relationships between gonad total lipids and biometric measurements. Ecological interpretation of the fatty acid composition of gonad samples was considered at two levels. Firstly, using the entire profile of fatty acids as a fingerprint (individual FA and FA groups) and, secondly, focusing on particular compounds (FATM) that can be traced to a specific food source in order to estimate its contribution to the diet. This study also aimed to investigate the application of multivariate statistical methods and a fatty acid trophic marker approach to the FA profile data to determine to what extent these analyses are useful to clarify nutritional and environmental adaptive strategies of limpets; these methods have scarcely been used in such studies.

3.2. Methods

3.2.1. Sampling

Patella depressa is considered a summer breeder and is the dominant intertidal limpet throughout the mid intertidal zone along the entire Portuguese coast (Guerra &

Gaudêncio 1986; Boaventura *et al.* 2002a; Brazão *et al.* 2003a). Because there was a constraint related to the number of limpets necessary to perform the fatty acid analysis only two locations per habitat (marinas vs. rocky shores) were sampled. Therefore, the study was conducted in two marinas (also denominated as artificial rocky shores) and two natural rocky shores, each relatively close to each marina (between 3 and 6 km apart of each other) in the south Portuguese coast, where average sea temperatures in late spring are close to 17ºC. Four locations were selected: two marinas, Vilamoura (37º 04' N; 08º 07' W) and Portimão (37º 07' N; 08º 31' W) and two rocky shores, Olhos d' Água (37º 05' N; 08º 11' W) and Vau (37º06' N; 08º34' W) (Figure 2.1). In order to test the responses of the selected species to more stressed environmental conditions, only marinas that had been established for at least 10 years and each supported more than 600 boats were selected.

Limpets were collected during May 2006 since at this time *P. depressa* populations have been found to be in advanced stages of gonad development (stages IV and V) (Brazão *et al.* 2003a,b; Morais *et al.* 2003), which are more suitable for fatty acid analysis, and also avoid seasonal variation in limpets biochemical composition. Based on visual observation, marinas and rocky shores differed markedly in the number and variety of species of algae present, with natural shores offering a higher variability of algae species in contrast with marinas in which the most common algae were red algae (e.g. *Corallina elongata* J.Ellis & Solander, *Gelydium* sp., *Caulacanthus* sp.). Each location was sampled during spring low tides in the mid shore zone, thus ensuring that the limpets were at the same stage of their feeding activity.

Fatty acid analysis of both males and females of similar shell lengths (between 20 mm and 50 mm) was performed on the gonad stage IV, which was found in a preliminary assessment to be the most abundant in the population. Its is important to

emphasize that the tests were carried out on experimental animals representative of the natural populations, with individuals from natural rocky shores being in general smaller then those from artificial rocky shores. Thus, a small sample size of stage IV gonads was obtained from rocky shore limpets and 4/5 individuals of the same sex and rocky shore were pooled in each replicate. Six replicates per sex and location were used for fatty acid analyses. Thus, biochemical analyses were conducted on 48 individuals of *P. depressa* providing gonad profiles that may be compared statistically.

3.2.2. Laboratorial procedure and fatty acid analysis

The shell length of each limpet was measured and the sex and gonad stage assessed by cutting away the foot from the visceral mass and shell, and flipping it forward. Sex was determined by macroscopic examination through colour, the male gonad being pinkish white or cream and the female brownish or dark green (Orton *et al.* 1956; Orton & Southward 1961). The gonad stage of development was macroscopically identified according to the stages defined by Orton *et al.* (1956) for *P. vulgata*. Gonads were dried weighted and then analysed to determine the fatty acid composition.

Male and females gonads were removed from the shell and stored in pre-weighted 'Eppendorf'- type tubes, placed on iced for a few minutes and then frozen at -80ºC for posterior fatty acids analysis. After freeze-drying in a Savant VP100® (24 h), samples were ground in a Potter homogenizer with chloroform–methanol–water (2:2:1.8), according to the procedure of Bligh & Dyer (1959). After saponification and esterification of the lipid extracts (Metcalfe & Schmitz 1961), the fatty acid methyl esters (FAME) were injected into a capillary column (30 m fused silica, 0.32 I.D.) installed in a Varian Star 3400CX gas–liquid chromatograph. Helium was used as carrier gas, at a flow rate of 1 ml/min; oven temperature was 180ºC for 7 min and then

200ºC (with a temperature gradient of 4ºC/min) over a period of 71 min. Both the injector and the FID detector were set at 250ºC. Gas–liquid chromatography (GLC) data acquisition and handling was done through a Varian integrator 4290 connected to the GLC. Peak quantification was carried out with a Star Chromatography workstation. Peak identification was carried out using as reference well-characterised cod liver oil chromatograms (Christie 1982).

3.2.3. Fatty Acid Trophic Markers (FATM)

According to Dalsgaard *et al.* (2003), the presence and combinations of certain FA can be associated to particular algal classes and thus act as potential markers. A variety of FATM were analysed for particular food sources in order to gather information on dietary composition and feeding performance (Table 3.1). Some important ratios are: (1) 18:1(n-7)/18:1(n-9) that has been proposed as a relative measure to distinguish carnivores from herbivores diet in marine invertebrates; (2) 16:1(n-7)/16:0 and (3) 22:6(n-3)/20:5(n-3) (DHA/EPA) which allows differentiation between flagellate - or diatom -based diets and; (4) 20:5(n-3)/20:4(n-6) (EPA/ARA) used as a marker of red calcareous algae input to the animal diet (Fleurence *et al.* 1994; Dalsgaard *et al.* 2003 and references therein).

3.2.4. Statistical analysis

3.2.4.1. Multivariate Analysis

Non parametric multivariate techniques were used to examine gender and habitat differences in gonad fatty acid profiles using Plymouth Routines in Multivariate Ecological Research (PRIMER) v6 & PERMANOVA + ß 17 software (Anderson 2001a,b; Anderson 2005; Anderson & Gorley 2007; Anderson *et al.* 2008). The

PERMANOVA tests were chosen because it allows testing of complex experimental designs using multivariate data and interaction terms.

Table 3.1. Fatty acids and fatty acids ratios used as markers for food sources in the present study (Fleurence *et al.* 1994; Dalsgaard *et al.* 2003 and references therein).

Fatty acid or ratio	Marker for:	Fatty acid or ratio	Marker for:
20:5n-3 16:1(n-7)/16:0 ≥1 16:1(n-7) 22:6(n-3)/20:5(n-3) (DHA/EPA) <1	Diatoms	20:5(n-3)/20:4(n-6) (EPA/ARA) >10 20:5(n-3) 16:0 20:4(n-6)	Red algae
18:1(n-7) 16:0 16:1(n-7) 18:1(n-7)/18:1(n-9) >1 14:0 17:0	Bacteria	18:1(n-9) 18:2(n-6) 18:3(n-3)	Brown algae
		16:0 18:3(n-3) 18:1(n-7) 16:4(n-3)	Green algae
18:1(n-9) 18:1(n-7)/18:1(n-9) <1 22:6(n-3)	Zooplankton		
		20:4(n-3)	Protozoa

To eliminate quantitative differences between the analysed samples and better visualise dissimilarities in the proportion of the different fatty acids, the FA concentrations were expressed in relative terms as a percentage of the total Fatty Acid Methyl Esters (FAME). Euclidean distances measures of similarity were used to examine the data matrices which were left untransformed (Clarke 1993).

Two permutation tests (PERMANOVA) were undertaken on 47 individual FAs as well as 7 groups (sums) of FA selected based on the presence in the compiled data set (summarised in Table 3.2), to assess the significance of the FA profiles among the considered sources of variation. The original design consisted of three factors: sex (fixed with 2 levels, male and female), habitat (fixed with 2 levels, marina and rocky shore) and location (2 levels, random, nested in habitat). The test statistic was computed

under 9999 permutations. When there were too few possible permutations to obtain a test with reasonable power in the multivariate analysis, a p-value was calculated using a Monte Carlo random sample from the asymptotic permutation distribution (see Anderson & Robinson 2003).

Non-metric multidimensional scaling (nMDS) ordination and Principal Coordinate analysis (PCO) were used to graphically visualize similarity patterns in the data. These plots produce a 2-dimensional arrangement of data in a way that best reflects fatty acid compositional similarities/dissimilarities (Clarke 1993; Clarke & Gorley 2006; Anderson *et al.* 2008). In this ordination, individuals with similar fatty acid signatures are grouped together while dissimilar data is set apart. Differences in the fatty acid signatures with sex and habitat were explored using the SIMPER (similarity percentages) procedure (Clarke 1993; Clarke & Warwick 2001; Clarke & Gorley 2006; Anderson & Gorley 2007; Anderson *et al.* 2008). Bubble plots were displayed showing the relative percentage of the most important fatty acids selected by SIMPER procedures. The relationship between gonad total lipids and biometric measurements measured in limpets was examined by principal component analysis (PCA) using a data matrix of 3 variables (Gonad Total Lipids - TL, Shell Length – SL and Gonad Dried Weight – GDW). Pearson correlation test was used to assess the association of biometric measurements (SL and GDW) with biochemical composition (GTL) of limpets. Due to scale differences between variables, the analysis was based on transformed and normalised residuals.

3.2.4.2. Univariate Analysis

A three-way mixed model ANOVA was used to test for gender and habitat differences on the gonad total lipid content and FATM ratios (summarized in Table 3.1). The same design was used for biometric measurements of limpets (shell length and

gonad dried weight). The factors tested were "sex" (fixed, orthogonal, 2 levels: male and female), "habitat" (fixed, orthogonal, 2 levels: marina and rocky shore) and "location" (random, nested within habitat, 2 levels). Cochran's C-test was used to check homogeneity of variance. Where this assumption was violated, the appropriate transformations were used (Underwood 1997; Underwood & Chapman 1998). Tests of homogeneity, ANOVA and SNK (Student–Newman–Keuls) *a posteriori* comparison tests were done using GMAV5 for Windows Statistical Software (Institute of Marine Ecology, Sydney, Australia). Differences were accepted as significant at $p < 0.05$.

3.3. Results

3.3.1. Gonad Lipid content and biometric measurements

Differences in Gonad Total Lipids (GTL), Shell Length (SL) and Gonad Dried Weight (GDW) between limpets collected from marinas and rocky shores of the Portuguese coast are shown in Figure 3.1. Results from the statistical analysis on total lipid content of the gonads revealed significant differences among sexes ($F_{1,2}$=168.57, p=0.006), while no differences were found between marinas and rocky shores ($F_{1,2}$=0.15, p=0.738). The total lipid content was significantly higher in females (24.60 ± 0.97% dw) than males (7.72± 0.46% dw) (Figure 3.1A). Conversely, significant differences were found in biometric measurements between habitats (SL: $F_{1,2}$=40.85, p=0.024; GDW: $F_{1,2}$=59.17, p=0.017) and no significant differences were found between sexes (SL: $F_{1,2}$=24.18, p=0.0710; GDW: $F_{1,2}$=9.57, p=0.0905). Limpets from marinas had larger shell lengths (46.61 ± 0.69 mm, Figure 3.1B) and gonad weights (0.22 ± 0.02 g, Figure 3.1C) than those from rocky shores (SL: 30.27 ± 0.55 mm; GDW: 0.08 ± 0.005 g).

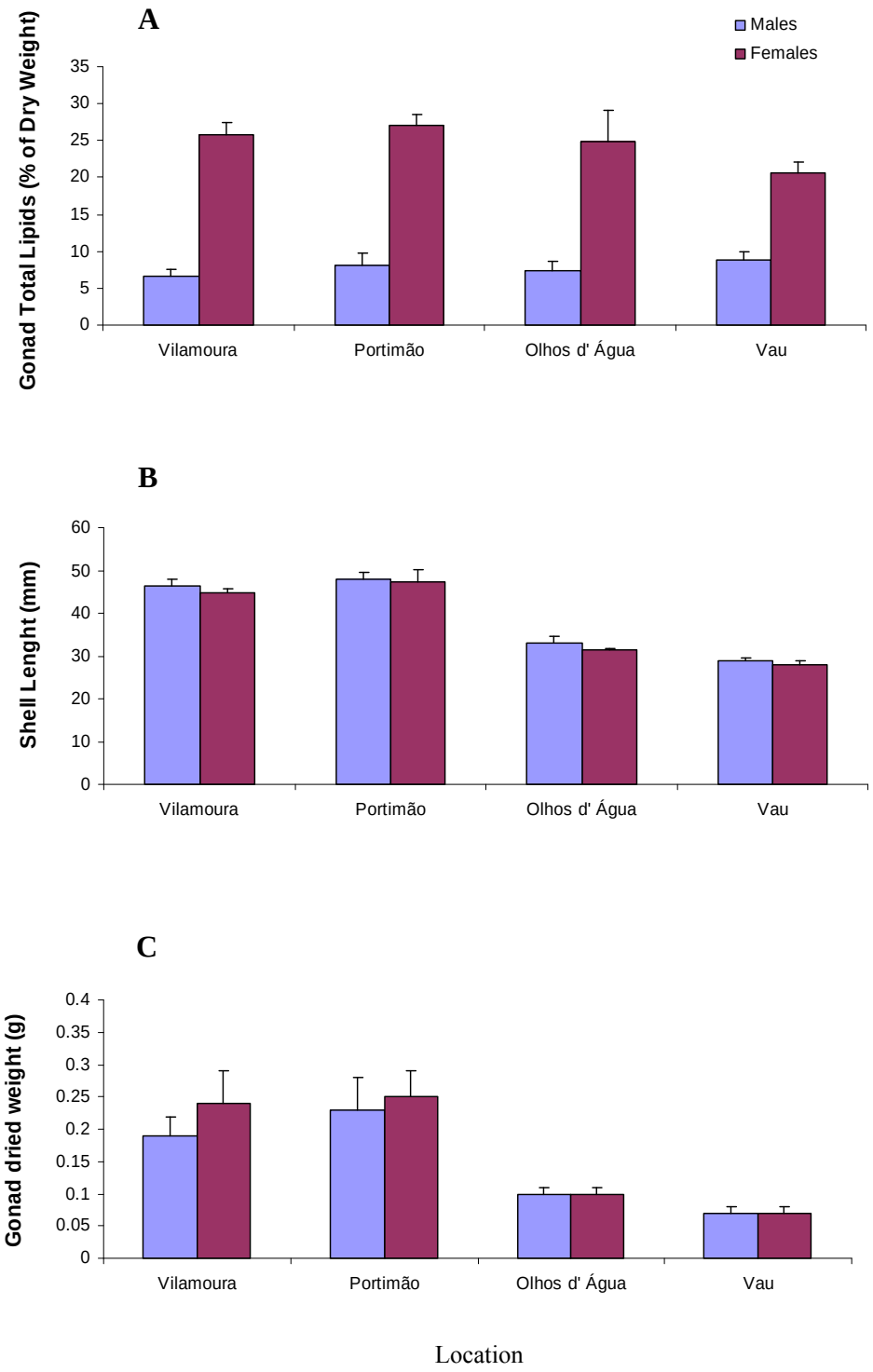

Figure 3.1. Gonad Total Lipids (A), Shell Length (B) and Gonad Dried Weight (C) of the limpets collected from marinas (Vilamoura and Portimão) and rocky shores (Olhos d'Água and Vau) of the Portuguese south coast.

A Principal Component Analysis (PCA) was used to explore differences in the Gonad Total Lipids of limpets as a function of the biometric measurements (Figure 3.2).

PC 1 (62%) and PC 2 (33.1%) explained together 95% of total variance. In PC 1 the main differences were from biometric measurements, i.e., shell lengths and gonad dried weights. These variables were positively correlated to each other (Pearson's r =0.8458). In PC 2, the major contributing variable was gonad total lipids. The plot PC 2 versus PC 1 showed a clear habitat and gender pattern (Figure 3.2). Limpets from marinas showed larger shell lengths and gonad dried weights, while female limpets showed higher gonad total lipids than males. Despite the larger average shell size of limpets from marinas, gonad total lipid content was not correlated with either size (Pearson's r = 0.0026) or gonad weights (Pearson's r = 0.1236) of limpets (Figure 3.2).

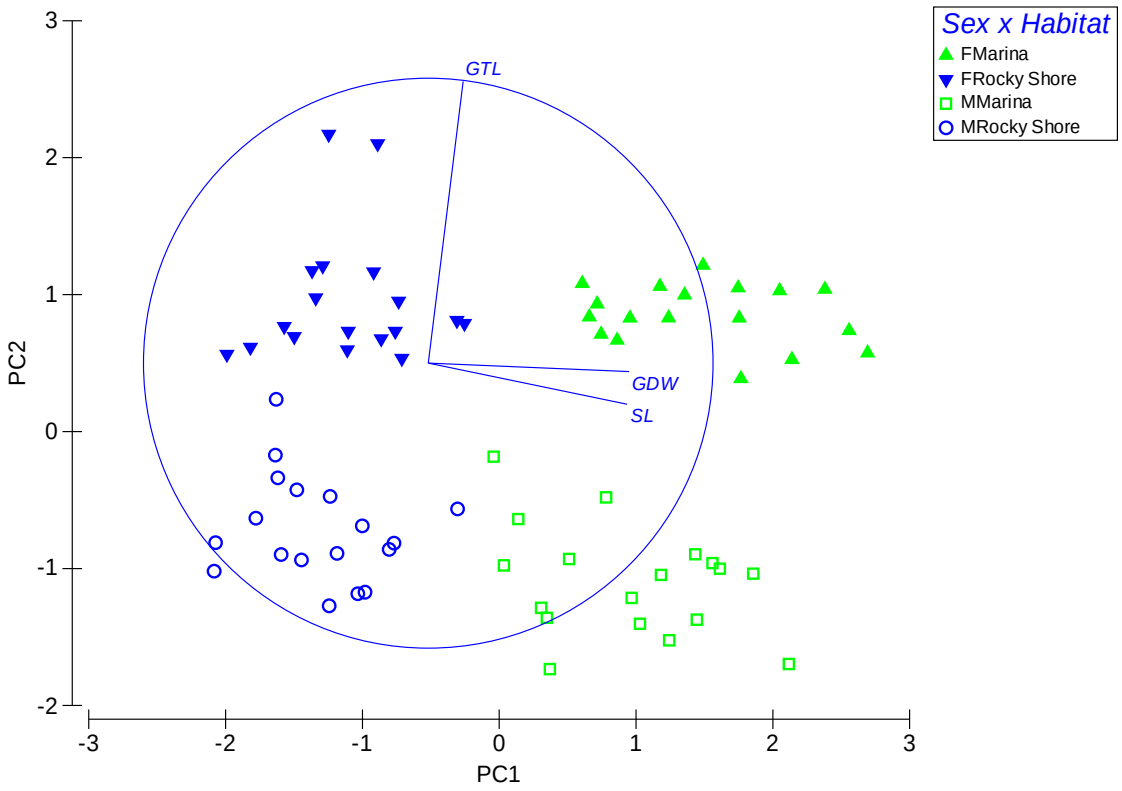

Figure 3.2. PCA analysis of gonad total lipids, shell length and gonad dried weight of the limpets collected from marinas and rocky shores of the Portuguese south coast (GTL= Gonad Total Lipids, SL=Shell Length and GDW= Gonad Dried Weight).

3.3.2. Fatty acid analysis

A detailed description of mean values of individual and groups of fatty acid percentages is summarized in Table 3.2. Forty-seven individual fatty acids ranging from 12 to 24 carbon atoms were originally identified in the gonad samples. They consisted of saturated (SFA), branched, monounsaturated (MUFA), polyunsaturated (PUFA) and highly unsaturated (HUFA) fatty acids.

Gonads of limpets were found to contain a range of SFA with chain lengths between C12 and C24. Among the SFA and, independently of the sex, palmitic (16:0) acid was the dominant fatty acid found in the gonad samples at all locations, with percentage values ranging between 11.04 and 25.33%. The FAs 18:1(n-7) and 18:1(n-9) dominated the MUFA (10.55 to 13.13% and 2.44 to 12.07%, respectively) and large amounts of the PUFA 20: 5(n-3) were also detected (6.07 to 33.31%). Lower amounts of the SFA 14:0, 17:0 and 18:0 (2.59 to 8.70%, 1.19 to 3.76% and 3.14 to 6.35%, respectively), the MUFA 16:1n-7, 20:1n-7 and 20:1n-9 (1.20 to 4.20%, 1.13 to 2.49% and 6.53 to 8.79%, respectively) and the PUFA 20:4n-3 (2.26 to 4.89%) were also found in the gonads of the limpets (Table 3.2). An interesting result was the presence of tracer amounts of 20:4n-6 (ARA, arachidonic acid) in both male and female limpets.

The biochemical data was examined with multivariate analyses of variance in order to determine whether significant differences among sex and habitat existed for the 47 individual fatty acids and for 7 FAs groups. Results from the PERMANOVA analysis of the individual fatty acids revealed that the factors sex and habitat, as well as the interaction term Sex x Habitat, significantly explained the variation in the data, indicating differences in gonad fatty acid composition between sexes that varied across habitats (Table 3.3A).

Table 3.2. Male and female gonad fatty acid composition (% of total FAME) of *P. depressa* collected from Marinas (Vilamoura and Portimão) and Rocky Shores (Olhos d'Água and Vau) of the Portuguese south coast. Values are mean ± standard error of mean where n=6. Groups of FAs are highlighted.

Fatty acids	**Vilamoura**		**Portimão**		**Olhos d' Água**		**Vau**	
% total FAME	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
12:0	0.05±0.02	0.08±0.01	0.13±0.02	0.12±0.03	0.04±0.03	0.08±0.01	0.00±0.00	0.10±0.01
13:0	0.02±0.02	0.09±0.05	0.01±0.02	0.05±0.01	0.14±0.08	0.06±0.01	0.00±0.00	0.08±0.02
14:0	2.75±0.23	8.21±0.43	2.59±0.31	8.21±0.46	4.19±0.78	8.70±0.23	4.15±0.20	8.60±0.30
15:0	0.65±0.03	0.54±0.08	0.67±0.03	0.50±0.04	0.89±0.08	0.69±0.06	0.85±0.05	0.70±0.11
16:0	11.46±0.65	25.33±1.25	11.04±0.55	24.34±0.73	12.06±0.75	24.60±1.46	12.88±0.35	24.67±0.85
17:0	3.42±0.27	1.19±0.11	3.36±0.35	1.11±0.09	3.76±0.43	1.34±0.07	3.62±0.56	1.33±0.11
18:0	6.35±0.30	3.74±0.27	6.09±0.25	3.14±0.19	5.84±0.21	3.40±0.19	5.98±0.27	3.39±0.11
20:0	0.00±0.00	0.22±0.05	0.12±0.17	0.21±0.05	0.00±0.00	0.34±0.03	0.00±0.00	0.26±0.12
22:0	0.01±0.01	0.13±0.03	0.01±0.01	0.08±0.01	0.00±0.00	0.11±0.02	0.00±0.00	0.15±0.04
Sum SFA	**24.71±0.57**	**39.53±2.08**	**24.01±0.93**	**37.76±1.07**	**26.91±1.33**	**39.32±1.97**	**27.48±0.28**	**39.27±1.24**
14:1(n-5)	0.00±0.00	0.29±0.05	0.00±0.00	0.35±0.04	0.00±0.00	0.25±0.04	0.00±0.00	0.25±0.03
16:1(n-9)	4.75±0.52	0.11±0.01	4.26±0.25	0.15±0.03	4.59±0.13	0.11±0.01	3.65±0.26	0.11±0.01
16:1(n-7)	1.20±0.26	4.20±0.28	1.84±0.55	3.65±1.01	1.78±0.18	3.71±0.21	2.38±0.27	3.86±0.21
16:1(n-5)	0.17±0.07	0.30±0.06	0.22±0.04	0.35±0.03	0.29±0.06	0.24±0.02	0.45±0.16	0.29±0.05
18:1(n-9)	2.44±0.22	11.36±0.53	3.15±0.28	11.86±0.50	4.57±0.75	12.04±0.74	5.68±0.95	12.07±0.92
18:1(n-7)	11.78±0.92	13.13±0.52	11.24±0.63	12.22±0.43	11.25±0.75	10.55±0.60	12.09±0.31	11.65±0.49
18:1(n-5)	0.45±0.06	0.34±0.05	0.54±0.06	0.42±0.06	0.55±0.05	0.31±0.03	0.63±0.08	0.35±0.02
19:1(n-10)	0.48±0.07	0.27±0.02	0.75±0.12	0.28±0.02	0.69±0.07	0.38±0.05	0.52±0.03	0.24±0.05
19:1(n-8)	0.21±0.03	0.46±0.11	0.31±0.09	0.53±0.13	0.25±0.04	0.42±0.15	0.17±0.08	0.35±0.14
20:1(n-9)	7.85±0.61	6.55±0.42	8.79±1.41	6.38±0.57	6.94±0.83	6.53±0.34	5.79±1.35	7.21±1.18
20:1(n-7)	1.59±0.16	2.49±0.10	1.94±0.28	2.22±0.15	1.60±0.18	2.29±0.11	1.13±0.18	2.21±0.19
20:1(n-5)	0.74±0.05	0.31±0.07	1.09±0.30	0.44±0.11	0.49±0.07	0.29±0.05	0.36±0.13	0.40±0.20
22:1(n-11)	0.00±0.00	0.53±0.08	0.07±0.06	0.43±0.05	0.08±0.07	0.41±0.01	0.06±0.09	0.65±0.16
22:1(n-9)	0.00±0.00	0.07±0.02	0.00±0.00	0.03±0.01	0.00±0.00	0.04±0.02	0.00±0.00	0.01±0.01
22:1(n-7)	0.00±0.00	0.14±0.05	0.00±0.00	0.05±0.03	0.00±0.00	0.10±0.00	0.00±0.00	0.11±0.04
24:1(n-9)	0.00±0.00	0.01±0.01	0.00±0.00	0.02±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Sum MUFA	**31.67±1.77**	**40.55±1.17**	**34.21±1.64**	**39.35±0.68**	**33.07±1.09**	**37.67±0.87**	**32.92±1.95**	**39.76±0.47**
Iso 15:0	0.12±0.02	0.15±0.07	0.09±0.04	0.08±0.03	0.17±0.02	0.13±0.04	0.26±0.11	0.15±0.05
Anteiso 15:0	0.11±0.00	0.05±0.01	0.11±0.03	0.03±0.01	0.18±0.02	0.04±0.01	0.11±0.07	0.05±0.02
Iso 16:0	0.22±0.04	0.32±0.08	0.25±0.04	0.28±0.04	0.39±0.04	0.37±0.04	0.34±0.04	0.39±0.05
Anteiso 16:0	0.71±0.06	0.04±0.01	0.69±0.07	0.05±0.01	0.99±0.10	0.04±0.02	0.80±0.19	0.10±0.02
Iso 17:0	0.52±0.09	1.00±0.21	0.53±0.08	0.66±0.07	0.69±0.05	0.93±0.04	0.62±0.05	1.01±0.09
Anteiso 17:0	0.24±0.07	0.32±0.03	0.22±0.03	0.32±0.05	0.30±0.05	0.41±0.06	0.39±0.12	0.28±0.05
Sum Branched	**1.91±0.25**	**1.89±0.35**	**1.90±0.27**	**1.42±0.11**	**2.73±0.25**	**1.92±0.122**	**2.53±0.16**	**1.99±0.18**
16:3(n-4)	0.48±0.09	0.31±0.08	0.48±0.09	0.31±0.05	0.42±0.04	0.31±0.03	0.52±0.06	0.42±0.05
16:4(n-3)	0.05±0.05	0.04±0.03	0.08±0.05	0.07±0.02	0.00±0.00	0.02±0.02	0.12±0.12	0.02±0.02
18:2(n-6)	0.40±0.07	1.18±0.20	0.46±0.06	1.57±0.21	0.37±0.04	0.65±0.07	0.48±0.09	0.77±0.13
18:3(n-3)	0.74±0.10	1.33±0.29	0.57±0.09	1.49±0.24	0.54±0.08	1.40±0.25	0.45±0.08	1.08±0.24
18:4(n-3)	1.49±0.17	0.48±0.06	1.15±0.34	0.64±0.07	1.81±0.20	0.87±0.11	1.69±0.26	0.86±0.20
20:2(n-6)	0.47±0.06	0.86±0.12	0.20±0.13	0.73±0.15	0.51±0.07	0.63±0.08	0.36±0.12	0.52±0.18
20:3(n-6)	0.00±0.00	0.04±0.01	0.03±0.03	0.20±0.16	0.07±0.06	0.04±0.03	0.06±0.06	0.06±0.04
20:4(n-6)	0.95±0.08	1.99±0.23	1.03±0.13	1.95±0.54	1.07±0.18	1.98±0.13	1.08±0.09	1.96±0.31
20:3(n-3)	0.27±0.04	0.72±0.13	0.26±0.05	1.02±0.11	0.29±0.02	0.57±0.08	0.18±0.16	0.52±0.08
20:4(n-3)	2.26±0.26	3.26±0.41	2.72±0.26	4.89±0.47	3.25±0.39	4.41±0.45	2.96±0.42	3.47±0.42
20:5(n-3)	33.31±1.41	6.07±1.36	31.96±2.23	6.72±0.73	28.15±1.74	8.42±0.84	27.46±2.28	6.87±0.56
21:5(n-3)	0.00±0.00	0.38±0.08	0.03±0.03	0.42±0.04	0.00±0.00	0.25±0.04	0.00±0.00	0.31±0.06
22:4(n-6)	0.05±0.05	0.24±0.08	0.00±0.00	0.26±0.05	0.00±0.00	0.17±0.03	0.00±0.00	0.12±0.04
22:5(n-6)	0.00±0.00	0.12±0.04	0.00±0.00	0.14±0.02	0.00±0.00	0.05±0.01	0.00±0.00	0.01±0.01
22:5(n-3)	1.10±0.13	0.69±0.20	0.87±0.16	0.71±0.10	0.76±0.07	0.84±0.09	0.52±0.16	0.60±0.09
22:6(n-3)	0.13±0.06	0.33±0.10	0.05±0.05	0.36±0.04	0.04±0.06	0.49±0.16	0.60±0.18	0.70±0.28
Sum PUFA	**41.71±1.42**	**18.03±2.69**	**39.89±2.19**	**21.47±1.47**	**37.29±1.61**	**21.09±2.02**	**37.08±1.99**	**18.98±1.48**
Sum HUFA	**38.54±1.42**	**14.69±2.42**	**37.15±1.83**	**17.39±1.28**	**34.15±1.54**	**17.84±1.69**	**33.82±2.10**	**15.84±1.53**
Sum (n-3)	**39.36±1.50**	**13.30±2.37**	**37.68±2.37**	**16.31±1.20**	**34.85±1.71**	**17.26±1.85**	**34.58±1.98**	**15.12±1.23**
Sum (n-6)	**1.87±0.15**	**4.42±0.42**	**1.73±0.28**	**4.85±0.56**	**2.01±0.23**	**3.52±0.18**	**1.98±0.31**	**3.44±0.40**
Total Lipids (TL) (% of Gonad Dry Weight)	6.58±1.05	25.83±1.60	8.07±1.69	27.03±1.51	7.41±1.18	24.84±4.28	8.82±1.11	20.72±1.47

Pairwise comparisons in the interaction term Sex x Habitat showed significant differences between males and females within marinas (t=20.003, p (MC) = 0.0009) and rocky shores (t=18.098, p (MC) = 0.0006), as well as significant differences between habitats which was only detected in males (t=3.4473, p (MC) = 0.006). Overall, although the factors sex and habitat interacted, the effect of sex was more important than the habitat to distinguish limpet gonad fatty acid compositions (values of their mean squares shown in Table 3.3A). The PERMANOVA analyses of the FAs groups only showed significance of the factor sex (p (MC) = 0.0013, Table 3.3B). No significant differences were found in the random factor location nested in habitat for both analyses.

PCO and MDS ordination plots based on relative presence of individual (Figure 3.3A) and groups of FAs (Figure 3.3B), also revealed a clear separation of gender that explained 94.8% of total variation in Figure 3.3A. Furthermore, the separation of habitats was not so clear (1.7% of total variation), despite the significant differences detected in males. A low stress value (0.01) emphasised a good representation of similarities in both 2-dimensional plots.

SIMPER analysis identified the most important fatty acids (i.e. contributing to ≥ 1% of percentage dissimilarity) in discriminating gender and habitat differences in the interaction term Sex x Habitat (Table 3.4). The eicosapentaenoic acid EPA 20:5(n-3) was the main differentiating FA (65.92 and 60.32% contribution to dissimilarity in marinas and rocky shores, respectively) due to its elevated presence in males at both habitats. The four other main differentiating variables were 16:0 (17.72 and 21.98% contribution), 18:1(n-9) (7.41 and 7.48% contribution), 14:0 (2.95 and 3.04% contribution) and 16:1(n-9) (1.85 and 2.36% contribution). Females contained a much

higher proportion of 16:0, 18:1(n-9) and 14:0 and the lowest proportion of 20:5(n-3) and 16:1(n-9) comparing to gonads of males (Table 3.4, Figure 3.4).

Table 3.3. Results of the three-factor PERMANOVA and pairwise comparisons testing habitat and sex differences in gonad individual fatty acids (A) and fatty acid groups (B) of the limpet *P depressa*. Analyses based on Euclidean distances dissimilarities on untransformed data from 47 (individual FA) and 7 variables (FA groups). p(MC) = p-value calculated by Monte Carlo method.

A)

Source of variation	df	SS	MS	F	P	p(MC)
Sex = Se	1	9986.2	9986.2	724.69	-	**0.0001**
Habitat = Ha	1	103	103	4.1858	-	**0.033**
Location (Ha) = Lo(Ha)	2	49.216	24.608	1.3868	0.1875	-
SexHa	1	164.56	164.56	11.942	-	**0.0018**
SexLo(Ha)	2	27.56	13.78	0.77658	0.606	-
Residual	40	709.78	17.745	-	-	-
Total	47	11040	-	-	-	-
Pair-Wise Tests – Term SexHa			**t**		**P**	**p(MC)**
Females vs males within marinas			20.003		-	**0.0009**
Females vs males within rocky shores			18.098		-	**0.0006**
Marinas vs rocky shores within females			1.3743		0.1757	-
Marinas vs rocky shores within males			3.4473		**0.006**	-

B)

Source of variation	df	SS	MS	F	P	p(MC)
Sex = Se	1	16911	16911	231.82	-	**0.0013**
Habitat = Ha	1	125.16	125.16	4.7776	-	0.1065
Location (Ha) = Lo(Ha)	2	52.392	26.196	0.6378	0.5719	-
SexHa	1	217.06	217.06	2.9755	-	0.1824
SexLo(Ha)	2	145.9	72.949	1.7761	0.1634	-
Residual	40	1642.9	41.073	-	-	-
Total	47	19094	-	-	-	-

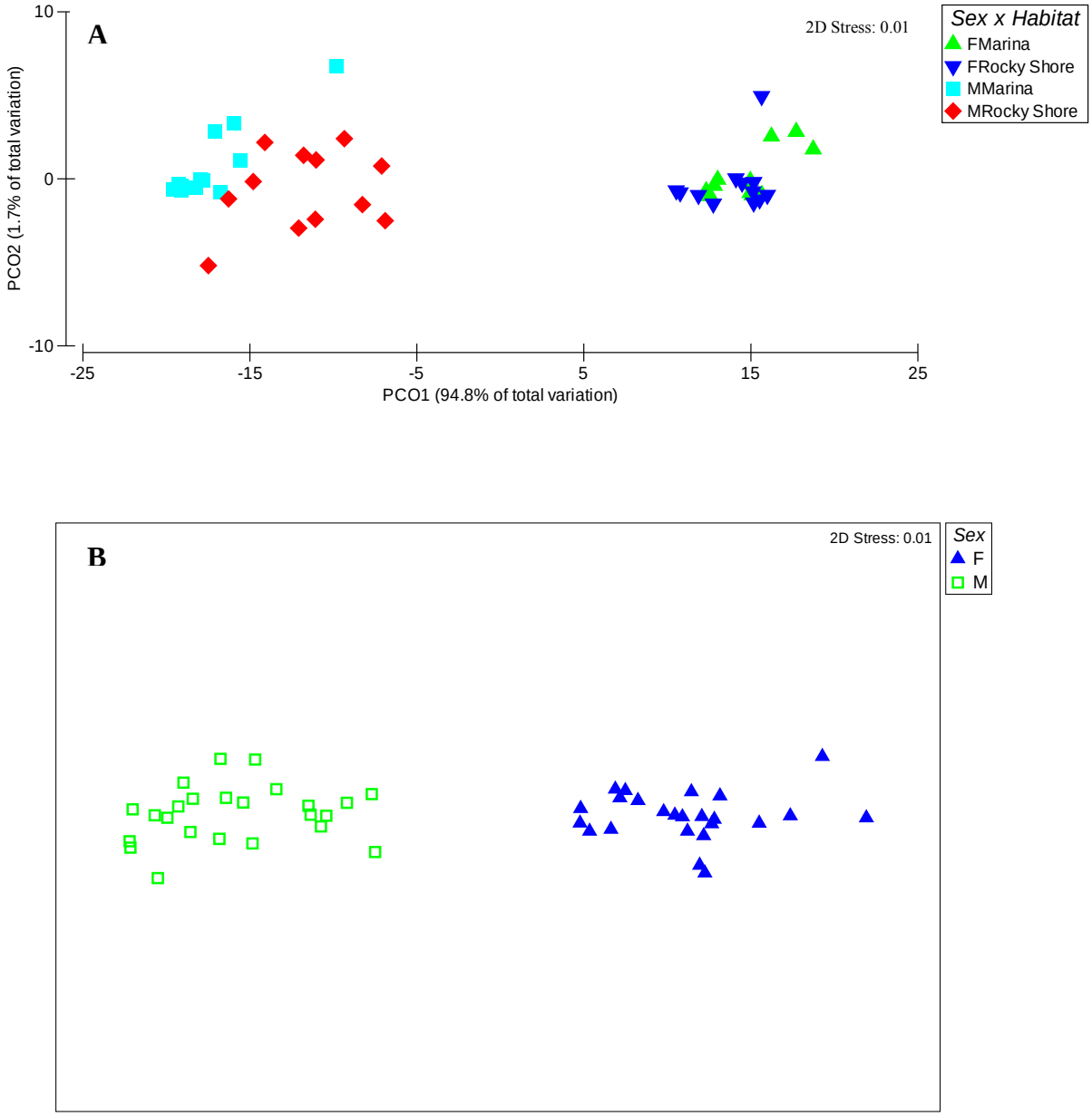

Figure 3.3. Two-dimensional PCO and MDS plots based on the results of PERMANOVA analyses on individual fatty acids (A) and groups of FA (B) data comparing gonad fatty acid compositions in male (M) and female (F) limpets between marinas and rocky shores of the Portuguese south coast.

Differences between habitats were only detected in males and the most important fatty acids contributing for these differences were: 20:5(n-3), 20:1(n-9), 18:1(n-9), 16:0, 14:0, 18:1(n-7), 16:1(n-7), 20:4(n-3), 17:0 and 16:1(n-9). It was found

that 20:5(n-3), 20:1(n-9) and 16:1(n-9) were in higher proportions in marinas, whereas the remaining fatty acids showed higher values in rocky shores (Table 3.4).

Table 3.4. One-way SIMPER analysis of the fatty acids contributing to the significant differences found in the interaction term Sex x Habitat. Cut off for low contributions = 95%.

Group	Marina			
	Female	Male		
Variable	Mean	Mean	Contribution%	Cumulative%
20:5(n-3)	6.4	32.6	65.92	65.92
16:0	24.8	11.2	17.72	83.65
18:1(n-9)	11.6	2.79	7.41	91.06
14:0	8.21	2.67	2.95	94.00
16:1(n-9)	0.128	4.51	1.85	95.85

Group	Rocky Shore			
	Female	Male		
Variable	Mean	Mean	Contribution%	Cumulative%
20:5(n-3)	7.64	27.8	60.32	60.32
16:0	24.6	12.5	21.98	82.30
18:1(n-9)	12.1	5.13	7.48	89.78
14:0	8.65	4.17	3.04	92.81
16:1(n-9)	0.109	4.12	2.36	95.18

Group	Males			
	Marina	Rocky Shore		
Variable	Mean	Mean	Contribution%	Cumulative%
20:5(n-3)	32.6	27.8	54.61	54.61
20:1(n-9)	8.32	6.37	13.29	67.90
18:1(n-9)	2.79	5.13	10.03	77.93
16:0	11.2	12.5	4.40	82.33
14:0	2.67	4.17	4.13	86.47
18:1(n-7)	11.5	11.7	3.36	89.83
16:1(n-7)	1.52	2.08	1.39	91.22
20:4(n-3)	2.49	3.11	1.32	92.53
17:0	3.39	3.69	1.24	93.77
16:1(n-9)	4.51	4.12	1.21	94.99

Table 3.5. One-way SIMPER analysis of the fatty acids groups contributing to the significant differences found between males and females gonads.

Variable	Mean			
	Female	Male	Contribution%	Cumulative%
n-3	15.5	36.6	31.25	31.25
HUFA	16.4	35.9	26.68	57.93
PUFA	19.9	39	25.90	83.83
SFA	39	25.8	12.36	96.19
MUFA	39.3	33	3.39	99.58
n-6	4.06	1.9	0.38	99.96
Branched	1.81	2.27	0.04	100.00

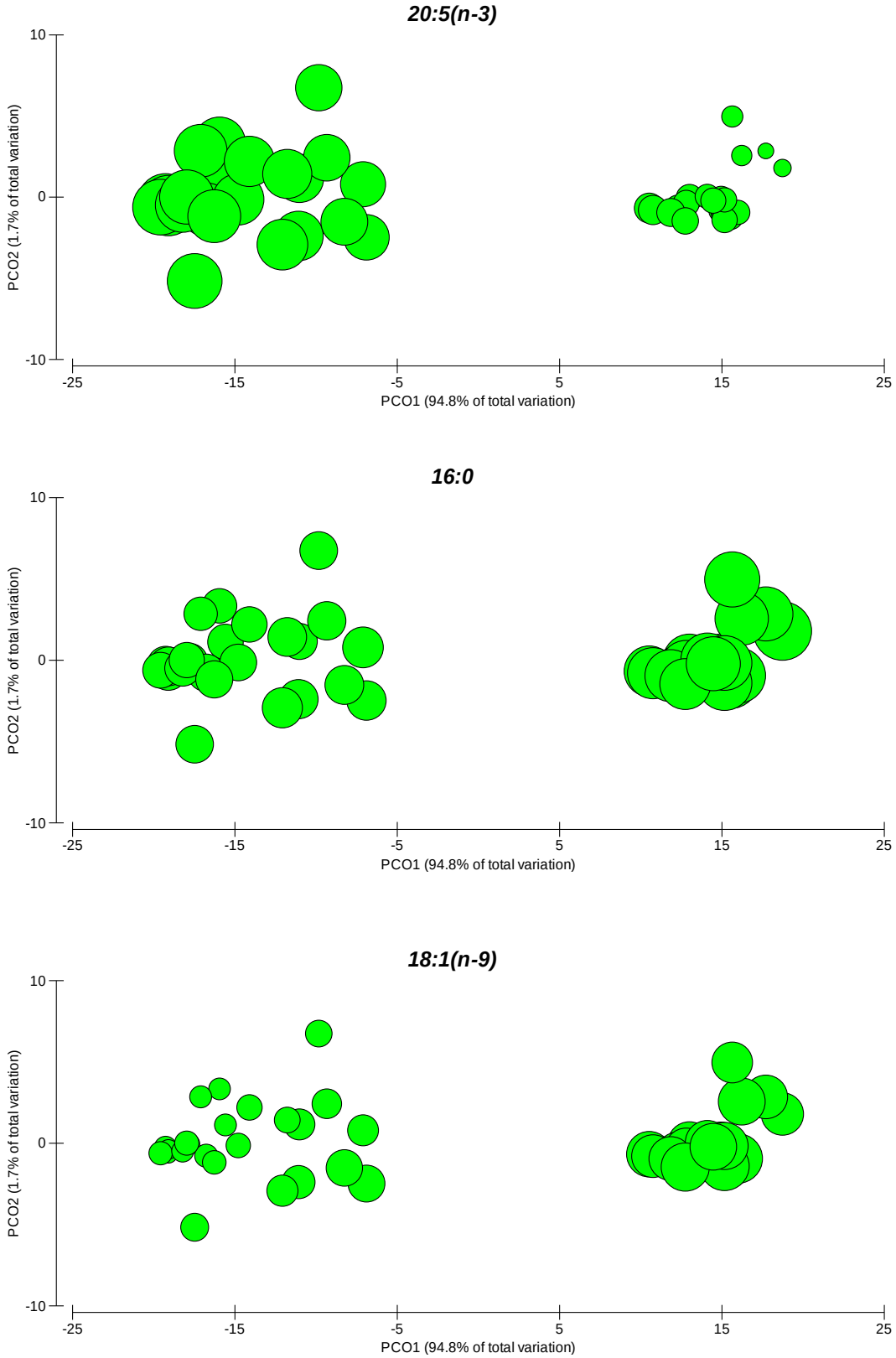

Figure 3.4. Bubble plots of the three most important individual FAs (20:5(n-3), 16:0 and 18:1(n-9)) selected by SIMPER analysis in discriminating gender and habitat differences in the interaction term Se x Ha.

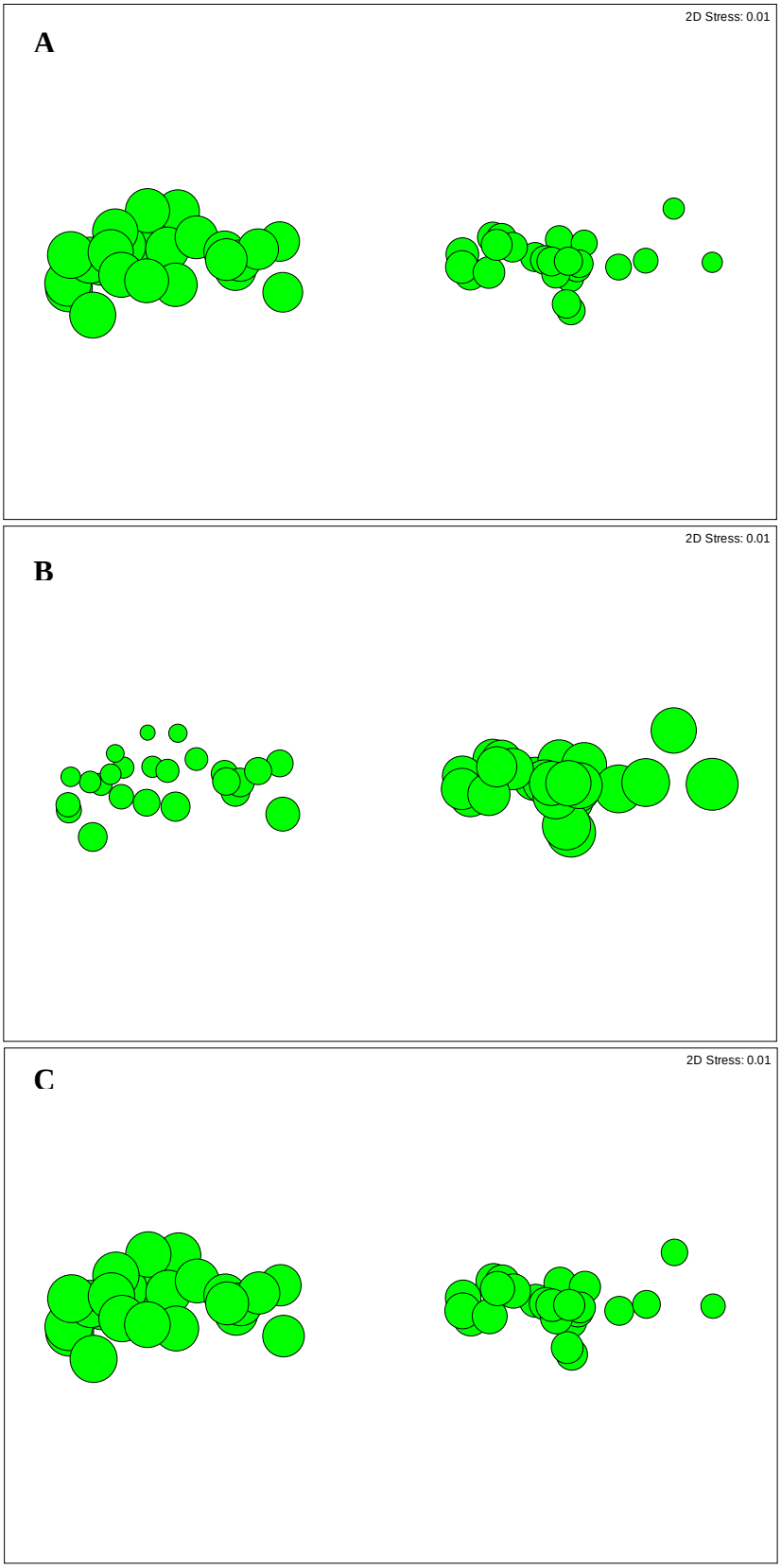

Figure 3.5. Bubble plots of three important FA groups selected by SIMPER analysis in discriminating males and females limpets: A) n-3, B) SFA and C) PUFA.

The groups of FA identified by SIMPER analysis as most contributing for gender differentiation were: n-3 (31.25% contribution to dissimilarity), HUFA (26.68% contribution), PUFA (25.90% contribution), SFA (12.36% contribution), MUFA (3.39% contribution), n-6 (0.38% contribution) and Branched (0.04% contribution). Males were characterised by presenting higher n-3, HUFA, PUFA and branched percentages. Female limpets, on the other hand, had a higher proportion of SFA, MUFA and n-6 than males. (Table 3.5, Figure 3.5).

3.3.3. Fatty Acid Trophic Markers (FATM)

FATM ratios (indicated in Table 3.1) were examined using analyses of variance, to obtain information on dietary composition and feeding performance.

The ratio 16:1(n-7)/16:0, commonly as an indicator of diatoms input to the animal diets, showed values lower than 1 (Figure 3.6) and no significant differences were found for this diatom marker between sexes ($F_{1,2}$= 0.07, p= 0.8199) or habitats ($F_{1,2}$= 1.08, p= 0.4077). However, the ratio 22:6(n-3)/20:5(n-3) (DHA/EPA), also an indicator of diatoms input to the animal diets, showed values lower than 1 (Figure 3.6) and significant differences were found between sexes ($F_{1,2}$= 96.74, p= 0.0102) and location nested in habitat ($F_{1,2}$= 5.76, p= 0.0063, Vau>Olhos d'Água).

The ratio 20:5(n-3)/20:4(n-6) (EPA/ARA), a common indicator of red calcareous algae input to the animal diets, showed values lower than 10 for females (6.05±2.57), but higher than 10 for males (31.01±1.88). Hence, significant differences were found between sexes ($F_{1,2}$= 53.35, p= 0.0182) while no significant differences were detected between habitats ($F_{1,2}$= 6.88, p= 0.1198) (Figure 3.6).

The ratio 18:1(n–7)/18:1(n–9), an indicator that allows the distinction between carnivores and herbivores diets, showed values higher than 1 and significant differences were found between sexes ($F_{1,2}$= 58.50, p= 0.0167). Males (3.39±0.27) had a

significantly higher amount of this indicator than females (1.02±0.03). However, it was also detected significant differences between location nested in habitat ($F_{2,40}$= 4.04, p= 0.0252, Vilamoura>Portimão) (Figure 3.6).

3.4. Discussion

To date, few studies have investigated the biochemical composition of limpet gonads (but see Kawashima *et al.* 2002; Brazão *et al.* 2003b; Morais *et al.* 2003; Kawashima & Ohnishi 2006; Kawashima *et al.* 2008). The experimental design and methodology used in this study was successful in testing the influence of gender and artificial habitats on the gonad fatty acid composition of limpets.

For gonad total lipids, males had values between 6.58 and 8.82% of dried weight and females had between 20.72 and 27.03%. The higher amount of total lipid content found in female limpets in the present study confirmed previous observations in which the lipid content of the testes was found to be lower than in the ovaries (Blackmore 1969; Simpson 1982; Kawashima *et al.* 2002; Brazão *et al.* 2003b; Morais *et al.* 2003). It was also detected that limpets from marinas had larger shell lengths and gonad weights than those from rocky shores. Despite this, gonad total lipid content was not correlated with limpet either size or gonad weights.

The fatty-acid profile of *P. depressa* gonads was characterised by high levels of the PUFA 20:5n-3, the SFA 14:0, 16:0 and the MUFAs 16:1n-7, 18:1n-7 and 18:1n-9, which is consistent with previous studies on limpets (Kawashima *et al.* 2002; Brazão *et al.* 2003b; Morais *et al.* 2003; Kawashima & Ohnishi 2006) and characteristic of molluscs in general (Johns *et al.* 1980; Go *et al.* 2002). A FATM approach was used to explore habitat influence and feeding strategies of the limpet *P. depressa* defined as a generalist grazer (Hill & Hawkins 1991; Jenkins & Hartnoll 2001; Jenkins *et al.* 2001).

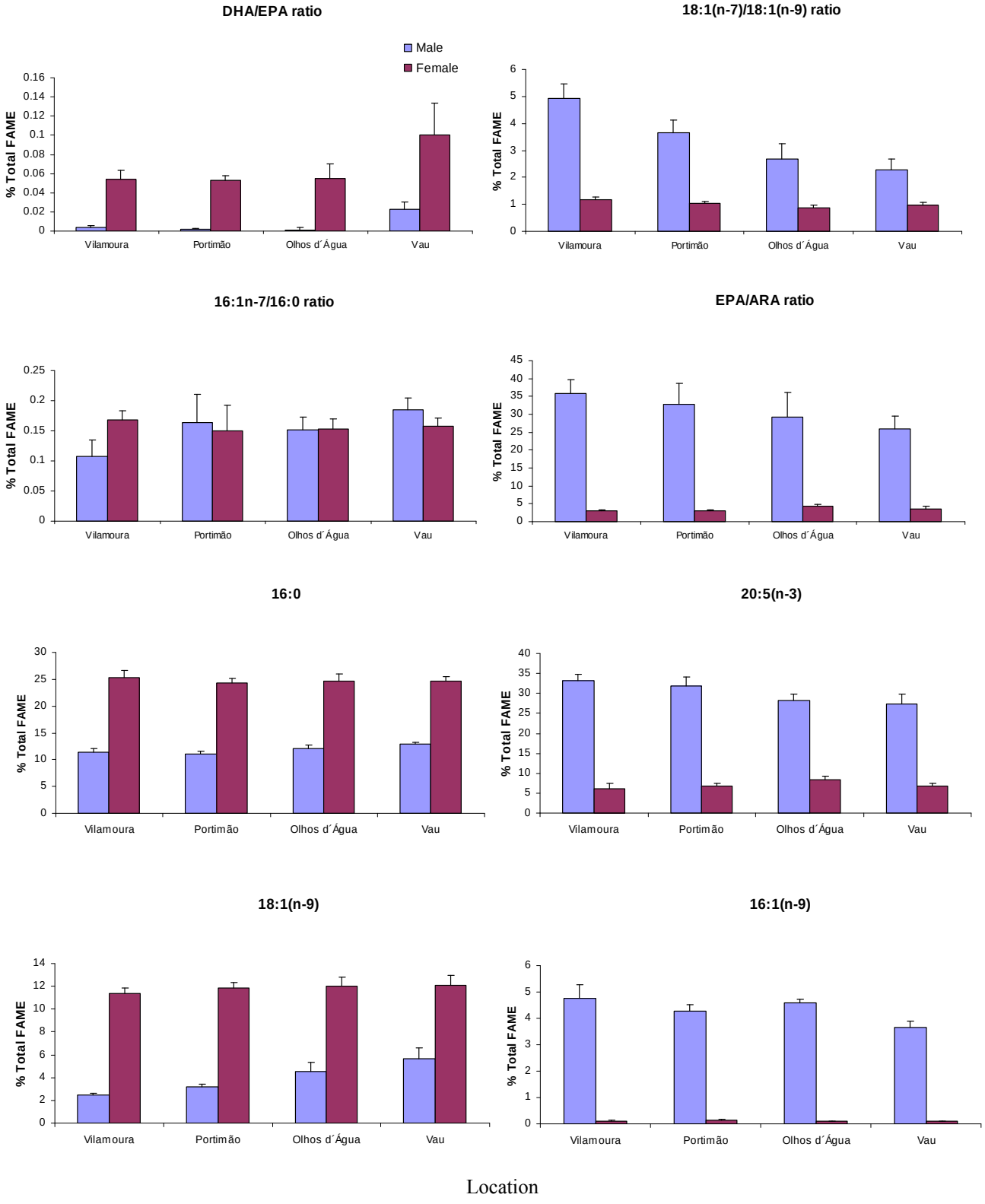

Figure 3.6. Relative composition (% of total FAME) of selected FATM in female and male *P. depressa* gonads, in marinas and rocky shores of the Portuguese south coast.

According to Dalsgaard *et al.* (2003) the FA composition of the tissues of an organism resembles the diet closely, being the similarities more obvious in animals ingesting more algae. Hence, the combination of high contributions of 20:5(n-3), 16.1(n-7) and a ratio 22:6(n-3)/20:5(n-3) lower than 1, is indicative of a diatom based diet (despite the ratio 16:1(n-7)/16:0 be lower than 1). On the other hand, combined high values of 14:0, 16:0, 16:1(n-7), 18:1(n-7) and a ratio 18:1(n-7)/18:1(n-9) higher than 1 also suggests bacteria as the most likely potential food sources available to the limpets at both habitats. Nevertheless, it has been suggested that the MUFA 18:1(n-7) and 18:1(n-9) (considered, respectively, bacterial and carnivorous feeding markers) are also major end products of the fatty-acid biosynthesis in some marine invertebrates, including limpets (Johns *et al.* 1980). Then, a particularly high *de novo* biosynthesis of these fatty acids may also explain those high concentrations in limpet gonads. Without further studies, it is not possible to conclude whether or not both 18:1(n-7) and 18:1(n-9) fatty acids are of exogenous origin in these limpets.

According to Dalsgaard *et al.* (2003), an 22:6(n-3)/20:5(n-3) ratio lower than 1 and an 18:1(n-7)/18:1(n-9) ratio higher than 1 along with a high content of the fatty acid 20:5(n-3) is consistent with a more generalist herbivorous diet. All of these observations support the hypothesis that, diatom, bacterial and algae FATM may be transferred to limpets directly from grazing on microbial films and are in agreement with gut contents studies performed on limpets (Santelices & Correa 1985; Hill & Hawkins 1991; Della Santina *et al.* 1993). However, additional studies are required to confirm whether the occurrence of these fatty acids is a consequence of intake from the marine food chain and/or *de novo* synthesis.

A major difference between this study and previous studies on *P. depressa* gonads was the presence of very low amounts of 20:4(n-6) (ARA, arachidonic acid) in

both female and male limpets (1.76 to 1.99% and 0.95 to 1.07%, respectively). This macroalgal marker (Graeve *et al.* 2002) is quite abundant in prosobranch gastropods (Johns *et al.* 1980; Kawashima *et al.* 2002, 2008) and has been detected in higher amounts in previous studies of the gonad fatty acid composition of *P. depressa* (Brazão *et al.* 2003b; Morais *et al.* 2003). The significance of the relatively low amounts of this fatty acid in this study is unclear, but it may be related to its availability within the benthic food web, showing that limpets might be able to adjust their feeding behaviour in response to food availability.

On the other hand, the low 22:6(n-3) (DHA) level observed in *P. depressa* gonads (ranging between 0.04 and 1.39%) is unusual for marine molluscs, being normally one of the most abundant and essential fatty acids in marine lipids (Gabbott 1983). Nevertheless, trace amounts of DHA contents have also been reported in previous studies on *P. depressa* (Brazão *et al.* 2003b; Morais *et al.* 2003), as well as in other molluscs and prosobranch gastropods (Gardner & Riley 1972; Phleger *et al.* 2005; Kawashima *et al.* 2008). Given that zooplankton is the usual source of this marker, small amounts may also be indicative of a more herbivorous limpet diet.

Interestingly, significant differences related with gender were found in the analyses of individual fatty acids, as well as in the main fatty acid groups. SIMPER analysis revealed that female gonads were predominantly composed of SFA and MUFA groups (two important energetic reserves for the developing embryo), including the fatty acids 14:0, 16:0 and 18:1(n-9) (Morais *et al.* 2003). On the other hand, male gonads are richer in n-3, HUFA and PUFA fatty acids, structural fatty acids typically found in high amounts in spermatozoa of marine invertebrates, with 20:5(n-3) as major component (Morais *et al.* 2003). A curious result was also found with the ratio 20:5(n-3)/20:4(n-6), which had values lower than 10 for females (6.05±2.57) but higher than 10

for males (31.01±1.88), thus indicating a red calcareous algae input to males diet. The reason why only 20:5(n-3) was shown to have higher contribution on males and not in females may be related to a selective retention from diet of this fatty acid over others, although this cannot be proven with these data alone. The composition of PUFA can only be related to diet, given that marine molluscs are generally unable to synthesise PUFA *de novo* (Abad *et al.* 1995; Dalsgaard *et al.* 2003). However, recent studies have shown that some bacterial species of marine origin are capable of producing long chain PUFA, such as EPA (Nichols *et al.* 1997; Nichols *et al.* 2002). Marked differences between the fatty acid composition of male and females gonads have been also reported on the two dominant limpet species (*Cellana grata* Gould and *Collisella dorsuosa* Gould) from the northern coast of Japan (Kawashima *et al.* 2002), and in previous studies on gonad fatty acid composition of the limpet *P. depressa* in the central coast of Portugal (Brazão *et al.* 2003b; Morais *et al.* 2003). These differences may be related to diverse nutritional strategies, feeding performance and/or differences in lipid storage from diet associated with gender. However, additional studies (combining limpet gut contents, FA compositions and stable isotopes analyses) are required to support these hypotheses, since it was shown that some fatty acids might be biosynthesized by some of the limpet symbiotic organisms rather than by the limpet itself (Kawashima & Ohnishi 2006).

It would be expected that differing environmental conditions (natural and artificial habitats and even anthropogenic inputs) might had an effect in the fatty acid composition of the limpet gonads. However, such habitat effect was only detected in males. The patterns revealed by SIMPER showed that marina male populations have higher levels of 20:5(n-3), 20:1(n-9) and 16:1(n-9) than rocky shores male populations, most likely because red algae and diatoms may represent an important component of the

male diet in marinas. The occurrence of 20:1(n-9) and 16:1(n-9) isomers is consistent with the hypothesis that chain elongation is occurring, and the presence of these two isomers is of metabolic origin rather than a food-chain variable (Johns *et al.* 1980). Males in rocky shores have higher levels of 18:1(n-9), 16:0, 14:0, 18:1(n-7), 16:1(n-7), 20:4(n-3) and 17:0. Of these fatty acids, 20:4(n-3) is usually associated with the use of protozoa as food source, while the 14:0, 16:0 and 17:0 are common to bacteria (Johns *et al.* 1980; Fleurence *et al.* 1994; Dalsgaard *et al.* 2003). The presence of the MUFA 18:1n-9, 18:1n-7 and 16:1n-7 is probably consistent with brown, red and green algae, settling stage of invertebrates and protozoa, but also diatom and bacterial biomass intake from the biofilm that coats rocky shores (Fleurence *et al.* 1994; Dalsgaard *et al.* 2003). These results are in agreement with previous visual observations of the most common species at both habitats. Rocky shores showed a higher variability of algae species in contrast with marinas in which the most common algae were red algae (e.g. *C. elongata*, *Gelydium* sp., *Caulacanthus* sp.). The presence of high levels of 20:5n-3 in males from marinas are also in agreement with observations made by Della Santina *et al.* (1993) in Mediterranean artificial rocky substrata, where the red algae *C. elongata* was found to be more tolerant to pollutants and well developed on settled artificial rocky substrata. All these observations suggest that limpets might be able to adjust their feeding behaviour in response to food availability. However, additional studies are required to understand the relationship between the available food in the biofilm and that ingested by limpets on natural and artificial substrata.

These findings highlight the need for further research combining functional aspects of limpets gut, FA compositions (of both the biofilm ingested by limpets and limpet tissues) and stable isotopes analysis to elucidate the importance of diet in the fatty acid composition of limpets. To fully understand the ecology of limpets in natural

and artificial habitats, it is also important to understand the processes of uptake, incorporation and modification of dietary FA, *de novo* biosynthesis, mobilization of FA during starvation and reproduction and how all of these processes might interfere with the interpretation of FATM.

CHAPTER 4

General Discussion and Conclusions

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

4.1. Overall summary

The main questions raised in this work focus on the influence of gender and spatial variation in biochemical parameters of the limpets. Biochemical analyses were performed on the limpet *P. depressa* from artificial (i.e. marinas) and natural rocky shores of the Portuguese Coast using two marker approaches (pollution biomarkers and fatty acid trophic markers). The importance of taking into account gender and maturation stage in the measurement of biochemical parameters in limpets whenever spatial differences are to be interpreted was demonstrated. Additionally, the comparison of limpet biochemical responses between artificial (i.e. marinas) and natural habitats was a fundamental step to understand and assess the role of artificial structures in sustaining natural biochemical processes. In this final chapter, the major findings of the work were briefly summarised (section 4.1), before discussing the fundamental causes of the patterns described (section 4.2). Finally, some concluding comments and questions raised by the present study were outlined for further research (section 4.3).

4.2. Biochemical analyses

The pollution of the marine environment by a vast number of contaminants has increased during the last decade as a direct consequence of a wide variety of anthropogenic activities (Gray 1997; Cajaraville *et al.* 2000; Picado *et al.* 2007). In similarity with most coastal areas in the world, the Portuguese coastal environment has been altered by the development of tourism related infrastructures such as marinas, and by the leisure and intense use of rocky shores, especially during the summer. Since a further increase in these artificial coastal structures can be predicted in the next few

decades (Reilly *et al.* 1996; Thompson *et al.* 2002; Alves *et al.* 2007), it is essential to gather a comprehensive understanding of the potential impacts of these kind of human disturbances on organisms, in order to make ecologically sensitive future decisions about managing the development of man made structures in coastal environments and the maintenance of biodiversity.

Marine limpets such as *P. depressa* are dominant grazers along the Portuguese coast on rocky shores and other hard substrates (such as marinas) having an important role in the functioning of Portuguese rocky shore communities (Guerra & Gaudêncio 1986; Boaventura *et al.* 2002a,b). In the present study, all the biochemical analyses were carried out on organisms representative of natural populations, with smaller individuals naturally inhabiting natural rocky shores by comparison with those from artificial rocky shores (i.e. marinas). These existing size differences were suggested in this study to be related to variations in type and food availability, predation pressure, wave action and/or due to genotype. Actually, a few studies from different geographical areas have already suggested that populations of limpets may differ in abundances, size, movement patterns, homing behaviour and reproductive output between artificial and natural rocky shores (Chapman 2003; Bulleri & Chapman 2004; Bulleri *et al.* 2004; Guerra-Garcia *et al.* 2004; Bulleri *et al.* 2005; Moreira *et al.* 2006). All these studies strongly suggested a potential problem in the dynamics and sustainability of populations of limpets living on artificial habitats, highlighting the importance of the present study to investigate the biochemical responses of limpets on artificial structures.

In the present study, an interesting and important result was the effect that gender had on biochemical responses of limpets. AChE and MT levels were found respectively to be higher in female foot muscle and soft body. Curiously, strong gender differences were also found on the gonad fatty acid composition. Higher amounts of

total lipid contents were observed in female gonads, confirming previous observations where higher degree of energetic storage fatty acids (SFA and MUFA) were found in female gonads, while more structural storage fatty acids (PUFA) were observed in male gonads (Blackmore 1969; Simpson 1982; Kawashima *et al.* 2002; Brazão *et al.* 2003b; Morais *et al.* 2003). Such gender differences were suggested in the present study to be related to sex reproductive strategies in lipid storage from diet. According to Dalsgaard *et al.* (2003), the FA composition of an organism resembles the diet closely, being the similarities more obvious in animals ingesting more algae. Gonad fatty acid composition analyses were indicative of a diatom-based diet, but also suggested bacteria as the most likely potential food sources available to the limpets at both habitats, and thus being consistent with a more generalist herbivorous diet. In addition, an interesting result was found with the ratio 20:5(n-3)/20:4(n-6), which showed values lower than 10 for females but higher than 10 for males, thus indicating a red calcareous algae input to males diet. This observation suggests a selective retention of this fatty acid over others from diet in males but not in females.

Simultaneously, food supply and dietary ingestion are thought to be major routes of metals uptake and accumulation in invertebrates (Bryan 1984; Depledge & Rainbow 1990). Qiu *et al.* (2001), working on the slipper limpet *Crepidula onyx* Sowerby highlighted bacteria as a potential importance source of metal accumulation in this gastropod. All these observations suggest that differences: (1) in the storage and selective retention of specific fatty acids; (2) in metal uptakes from diet and (3) in food types and availabilities amongst locations were likely the main causes responsible for the limpet biochemical responses and gender differences found in the present study.

Despite the lack of literature explaining gender variation in gastropod molluscs, these differences may also be related to variation in hormonal control, metabolic and

intake rates, diverse nutritional and reproductive strategies, feeding performance and/or differences in animal movement, which could be indirectly related to contamination through diet. However, additional laboratorial and field experimental studies are required to investigate all these hypotheses. Understanding the processes controlling dietary uptake and assimilation efficiency of contaminants by limpets, the investigation of contaminants concentrations in their tissues and in the ingested biofilm, and the study of the effects of contaminants on gametogenesis may provide useful information on the exposure and transfer of potentially toxic elements through food.

Currently it is interesting to examine the mechanism in which the fatty acid profile in an animal is influenced by its diet and/or by the conditions under which it lives. It would be expected that different environmental conditions (natural and artificial habitats and even anthropogenic inputs) had an effect in the fatty acid composition of the limpet gonads. However, such habitat effect was only detected in males. Males from marinas showed higher concentrations of 20:5(n-3) than males from rocky shores most likely indicating that red algae and diatoms could be an important component of the male diet in marinas, and showing that limpets might be able to adjust their feeding behaviour in response to food availability. These results were in agreement with visual observations of the most common species at both habitats. Rocky shores showed a higher variability of algae species in contrast with marinas in which the most common algae were red algae (e.g. *C. elongata*, *Gelydium* sp., *Caulacanthus* sp.). Della Santina *et al.* (1993) also observed in Mediterranean artificial rocky substrata that the red algae *C. elongata* was more tolerant to pollutants and well developed on settled artificial rocky substrata.

The organisms that inhabit and successfully reproduce in metal contaminated environments, such as marinas, are likely to have developed specific biochemical

mechanisms to confer tolerance to elevated environmental metal levels. The influence of different locations on pollution biomarkers (AChE and MT levels) were also tested in this study. Organisms collected at marinas were expected to be more exposed to hydrocarbons and metals from the maritime traffic and marina associated boating activities (e.g. increasing concentration of Cu in antifouling paints) (An & Kampbell 2003).

Despite the influence of seawater temperature and body size, respectively, in AChE and MT levels, both biomarker results showed a general artificial/natural substrata trend. Lower AChE activities were found in limpets at artificial rocky shores (i.e. marinas) than on natural rocky shores, and it was suggested that these differences could be in part neurotoxin-mediated resulting from several classes of contaminants such as PAHs from maritime traffic and sewage and ships' antifouling compounds (Tributyltin and Cu). Similar lowest AChE activities were found in mussels collected close to harbours in the Baltic and Mediterranean Sea (Schiedek *et al.* 2006; Damiens *et al.* 2007). Unexpectedly, limpets from artificial rocky shores showed lower MT concentrations than those from natural rocky shores, despite the fact that the organisms collected at artificial rocky shores were probably more exposed to hydrocarbons and metals from the maritime traffic and marina associated boating activities (e.g. increasing concentration of Cu in antifouling paints).

Despite several authors have noted that single species that live in a medium polluted by metals have higher concentrations of MT (e.g. Noël-Lambot *et al.* 1980; Bebianno & Machado 1997; Leung *et al.* 2001; Amiard *et al.* 2004), especially within or close to harbours/marinas (Pérez *et al.* 2004; Schiedek *et al.* 2006; Damiens *et al.* 2007), some authors detected no change or even reduction of MT concentrations in invertebrates from sites where metals (especially Cu) were present and bioavailable at

high concentrations (Bebianno *et al.*, 2003; Tanguy *et al.*, 2003). This observation is probably related to two well-documented metal detoxification mechanisms common in invertebrates that have been related to increase metal tolerance and even genetic resistance of organisms in polluted environments: (1) metal-binding to MT and (2) insolubilisation of metals in the form of granules, which are stored for life or regularly eliminated (Amiard *et al.* 2004, 2006). It was suggested in the present study that insolubilisation of metals (mainly Cu) in the form of granules might be the most important detoxification strategy adopted by limpets and responsible for the lower MT concentrations found in limpets from marinas.

When considered all together and despite the lack of detailed analyses of contaminants in limpet tissues, the data reported here suggest that artificial rocky shores may be submitted to integrated effects of several classes of contaminants such as PAHs from maritime traffic and sewage and antifouling compounds (TBT and Cu), which affected both biomarker responses in this species. Additionally, Cu is still used in antifouling paints and has been found in increasing concentrations in limpets from Mediterranean harbours (Campanella et al, 2001; Bebianno *et al.*, 2003). Also, several studies have already dealt with limpets sensitivity and ability to concentrate Cu in their tissues (Noël-Lambot *et al.* 1980; Campanella *et al.* 2001; Cubadda *et al.* 2001; Hung *et al.* 2001; Bebianno *et al.* 2003; Conti & Ceccheti 2003; Brown *et al.* 2004; Cravo *et al.* 2004; Cravo & Bebianno 2005; Hamed & Emara 2006; Nakhlé *et al.* 2006) but also PCBs and PAHs (Tena & Montelongo 1999; Peña-Méndez *et al.* 2001). This fact was suggested to be responsible for the tolerance of this genus to contaminated environments (Howard & Nickless 1977; Alyakrinskaya 2002).

Biomarker results of the present study stressed out the importance of taking into account potential confounding abiotic and biotic factors (e.g. gender, maturation stage,

shell length, tissue weight and water temperature) when interpreting AChE and MT levels in *P. depressa*, and clearly demonstrated the need to better understand if and how limpet biochemical processes are affected by such factors. As such abiotic and biotic factors are susceptible to interact with contaminants on biomarker responses, it is important to know and understand their effects to avoid misinterpretation of results in future environmental studies.

In order to apply biomarkers for assessing pollution effects in such variable ecosystems, it is essential to estimate the natural variability of biomarkers and separate the variations due to pollution from the natural variability. Although more research is needed to identify biomarkers variability due to abiotic and biotic parameters, evidence from the present study confirms that the measurement of AChE and MT levels in limpets are valuable tools that should be incorporated to a battery of biomarkers (indicating effects at different biological levels). This should be combined with chemical analysis, in order to measure an integrated *in situ* response index, and thus provide an overall physiological status for the organism. A multibiomarker approach using multivariate statistical data analyses could be effective in future studies in estimating the toxicity and impact of complex mixtures of contaminants present in coastal marine ecosystems. The use of control specimens collected at a pristine site of the studied ecosystem, the use of depurated organisms at laboratory conditions and/or the use of recovery techniques (when a suitable control site is not available), could be valuable tools in the interpretation of future results. Considering the limited knowledge about *P. depressa* AChE and MT levels, it is also recommended that field and laboratory experiments should be run concurrently and complementary to each other, to evaluate the effects of specific classes of environmental contaminants and mixtures on these species responses under controlled conditions and thus validate the field results.

4.3. Concluding remarks and future research

Despite the limitations and logistic constraints outlined, this work stresses the importance of taking into account gender whenever differences between field locations in biochemical levels are to be interpreted. Furthermore, the measurement of biomarkers in adult limpets, which were not subjected to seasonal and sexual variations and having gender in consideration, proved to be a useful tool when comparing biomarker responses in different locations. The results obtained here also underline the importance of identifying the potential interfering abiotic and biotic factors (e.g. water temperature, body size and weight), as well as spatial variability and natural ranges in biomarker responses, in order to distinguish effects induced by pollutants from those induced by the natural intrinsic characteristics of animals (Chapter 2). A Fatty Acid Trophic Marker (FATM) approach combined with multivariate statistical techniques to interpret a large number of fatty acid data proved to be a powerful tool for exploring and demonstrating gender and habitat differences in limpet diets, but also useful to identify the fatty acids, and therefore the dietary items, responsible for those differences (Chapter 3). This study allowed a better understanding of biochemical responses of limpets from marinas and rocky shores of the Portuguese coast. Clearly, additional studies investigating the ecological and biochemical consequences of the introduction of artificial structures within natural coastal habitats are needed.

Thus, in order to provide useful information on the exposure and transfer of potentially toxic elements through food it is important in future studies to: (1) understand the processes controlling the dietary uptake and detoxification of metals in limpets; (2) investigate trace metal concentrations in limpet tissues and in the biofilm ingested; (3) study the effects of contaminants on gametogenesis; (4) study the processes of uptake, incorporation and modification of dietary FA and, (5) investigate

de novo biosynthesis and mobilization of FA during starvation and reproduction. Accordingly, the most suitable approach for future biochemical studies on limpets could be combining functional aspects of limpets gut, FA compositions, stable isotopes, pollution biomarkers and contaminant analyses in a multivariate statistical data investigation, in order to understand the relationship between contaminants uptake, the available food in the biofilm and that ingested by limpets on natural and artificial substrata.

The use of *P. depressa* as a sentinel species for biomonitoring potential toxic effects *in situ* appears feasible using a multi-biomarker approach, where the relationships between biomarkers, contaminants and physiological status indicators of limpets can be examined. Knowledge of all these factors could not only greatly reduce erroneous results improving data interpretation, but also evaluate the robustness of these organisms for routine use in marine monitoring aiming to fully clarify their actual accumulation patterns.

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