



Elemental microchemistry, fatty acid profile and geometric morphometrics signatures of goose barnacles (*Pollicipes pollicipes*) reveal their place of origin

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Resumo

Os produtos provenientes do mar têm um importante papel a nível socioeconómico, gastronómico e no legado cultural das comunidades piscatórias e costeiras de Portugal. O Percebe *Pollicipes pollicipes* é na Península Ibérica o recurso biológico do intertidal mais explorado pelo ser humano, devido à sua enorme popularidade e conseqüente procura, fazendo em algumas ocasiões disparar o preço de mercado até 150€/kg, gerando na sobreexploração dos stocks existentes. No entanto, na área marinha protegida da Reserva Natural da Berlenga, a apanha do percebe é fortemente regulada, tendo-se tornado em Portugal num bom exemplo da gestão de recursos marinhos.

Com o intuito de prevenir fraudes, adulteração alimentar ou quaisquer outras práticas que possam induzir o consumidor em erro a Comissão Europeia declara que, o consumidor tem o direito de receber informação correcta acerca dos produtos que adquire, para além de definir regras para a correcta aplicação destas regras.

Métodos analíticos que possibilitem identificar a origem do percebe, tornam-se deste modo importantes ferramentas no desenvolvimento de um selo de Denominação de Origem Protegida (DOP) e na gestão comercial do produto.

Deste modo, investigou-se se o Percebe possui diferenças específicas de cada local de captura, através da forma da unha (CS), da composição microquímica da unha (EM) e do perfil de ácidos gordos (FA). A análise foi efectuada em indivíduos recolhidos em 3 locais na Reserva Natural das Berlengas e 7 ao longo de 300 km da costa Portuguesa.

Em cada indivíduo analisou-se a forma da unha (CS) através da morfometria geométrica, a composição microquímica da unha (EM) através de ICP-MS e o perfil de ácidos gordos do músculo através de GC-FID.

A análise das funções discriminantes (DFA) quer para a EM quer para a FA em separado obteve um elevado sucesso de reclassificação (77,6 % e 99% respectivamente, através de validação cruzada), enquanto que EM combinado com FA permitiu um sucesso de reclassificação de 100 %. A análise discriminante baseada apenas na CS, demonstrou um baixo sucesso (29,6 %).

Estes resultados demonstram que a composição microquímica da unha e o perfil de ácidos gordos do músculo de Percebe, poderá ser uma ferramenta de elevada importância, na determinação da origem do Percebe. Esta abordagem poderá ser utilizada para identificar a origem dos percebes comercializados, bem como ajudar no desenvolvimento de um selo DOP, aumentando ao mesmo tempo o valor potencial dos recursos biológicos provenientes de áreas marinhas protegidas em Portugal.

Palavras-chave: Percebe, ICP-MS, Perfil de ácidos gordos; Morfometria geométrica; Origem, Análise de função discriminante

Abstract

Seafood plays an important role in the socioeconomic, gastronomy and cultural heritage of Portuguese coastal communities. In the Iberian Peninsula, the goose barnacle *Pollicipes pollicipes* is the intertidal biological resource most heavily exploited by man, due to the great commercial demand and the high prices in the market which can reach 150€/kg resulting on the overexploitation of stocks. In the MPA of BNR *P.pollicipes* harvesting is however strictly regulated, making it a good example of marine resources management.

Aiming at the prevention of fraudulent practices, adulteration of food and any other practices which may mislead the consumer, the European Commission states that, every consumer have the right to be truthfully informed about fishery and aquaculture products, also laying down detailed rules for the application of this rules

Analytical methods able to identify the origin of goose barnacle would be an important tool to help in the establishment of a Protected Designation of Origin (PDO) and the management of the trade.

For such purpose, it was investigated whether *P. pollicipes* have site-specific differences based on its elemental microchemistry (EM), fatty acid profile (FA) and capitulum shape (CS). The analysis was performed on specimens collected from 3 sites in the BNR and 7 along a 300 km stretch of the Portuguese coast.

Each individual had analysed the largest lateral shell for EM using ICP-MS, the FA content of the muscle using GC-FID, and the CS using geometric morphometrics.

Discriminant function analyses (DFA) for both EM and FA separately provided a high reclassification success (77.6% and 99% respectively, of cross-validated cases correctly classified), while for EM combined with FA allowed for a 100% reclassification success. DFA analysis based only on CS, revealed a low classification success (29.6%).

These results show that EM and FA signatures can be a powerful tool to infer goose barnacles origin. Such “fingerprinting” approach can be used to track and identify goose barnacles origin, helping in establishing an origin certificate and increasing the potential value of biological resources from Portuguese MPAs.

Key words: Goose barnacle; ICP-MS; Fatty acid profile; Geometric morphometrics; Source of origin; Discriminant function analysis

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Abbreviations

Throughout this work the following abbreviations will be used.

DHA – *Docosahexaenoic Acid*

EPA – *Eicosapentaenoic Acid*

MUFA – *Monounsaturated Fatty Acid*

PUFA – *Polysaturated Fatty Acids*

ICP-MS – Inductively Coupled Plasma - Mass Spectrometry

GC-FID - Gas Chromatography- Flame Ionization Detector

1. Introduction

1.1 General background

Seafood plays an important role in the socioeconomic and culinary heritage and traditions of Portugal. According to Girin (1989), Portuguese people are one of the most avid consumers of fish and seafood in the entire world, being the only country where the Mediterranean diet has a bigger fish consumption than meat, most likely due to having a long coastline in contact with the Atlantic Ocean, with an average of 57.1 kg/year per capita, while the average for the rest of the world was only 16.4 kg/year (Laurenti, 2010).

With such an importance, it is common to find seafood festivals all over the country, from the northern inland city of Bragança almost 250 km away from the sea, to the southern coast of Olhão in Algarve, with many seafood festivals taking place especially during summertime in most of the coastal cities.

The goose barnacle *Pollicipes pollicipes* is in the Iberian Peninsula the most heavily exploited intertidal resource by man, due to the great commercial demand and the high prices in the market (Bernard, 1988; Jacinto, Cruz, Silva, & Castro, 2009) which can reach up to 150€/kg in some restaurants. The annual estimated consumption of 2000 tons in Spain (Proverbs, 1979), and a market value of 45 million Euros in this country results in the overexploitation of the stocks (Cunha & Weber, 2001).

The goose barnacles harvest technique is quite simple despite the involved risks. The task is carried out at low tides, when goose barnacles are emerged and harvest is possible. In order to reach the difficult and tricky areas where *P.pollicipes* are found, the “percebeiros” in the Berlengas Nature Reserve (BNR) use inflatable boats to access areas otherwise inaccessible, equipped only with a diving suit and a scraper (arrilhada), where it is not only a matter of harvest the biggest quantity possible but harvest the best specimens available, often in small crevices and near or below the sea level where the barnacles are usually large and considered of best quality (Novo Loureiro, 2000; Pérez, 1996). Since they are filter-feeding animals, the more beaten, colder and productive the area, the better quality the barnacles will be (H. Barnes & Reese, 1960; Cardoso & Yule, 1995; Molares, 1998; Rainbow, 1984), this is the case of the barnacles harvested in the

Berlengas Nature Reserve, which quality is highly appreciated and reflected in their market prices.

It is known consumers are not regularly able or trained to identify fish or other fisheries products species, when independently of origin morphological characteristics, such as shape, color, or size are either removed or seem identical, owing to its potential market value, and the lack of morphological distinctive characters, some suggest *P.pollicipes* sometimes being sold as harvested in the Berlengas Nature Reserve, are in fact provenant from harvest zones as far away from Berlengas Nature Reserve as Senegal. This practicum are potentiated by the increase in technology and road infrastructures which allow fisheries products to reach farther populations and opens the possibility of fraudulent adulteration, with the risk of falsification due to the profit that can be made (Mackie et al., 1999). An example of a common fraud is the substitution of one ingredient by a similar cheaper one which is difficult to recognize by the consumer and to detect by current analytical techniques, according to a European Regulation (Comission, 2001, 2002) consumers have to be informed about fishery and aquaculture products, also laying down detailed rules for the application of this rules.

Analytical methods, such as geometric morphometrics (Hard, Berejikian, Tezak, Schroder, & Knudsen, 2000), elemental microchemistry (Cabrera-Vique, Bouzas, & Oliveras-López, 2012; Geana et al., 2013; M.-M., Hansawek, Shiowatana, & Siripinyanond, 2011) and the fatty acid content (Richoux, Vermeulen, & Froneman, 2014) able to identify fisheries products species or real provenance are therefore important in preventing fraudulent or even unintentional substitution (Woolfe & Primrose, 2004) of high-value *P.pollicipes* from RNB with lower priced barnacles.

The rationale for this study is that since there are many distinct harvest locations for the goose barnacle, each origin location should present site-specific differences, allowing for a “fingerprint” approach, where each location has its own signature.

1.2 *Pollicipes pollicipes* – On its biology and ecology

Pollicipes pollicipes (Gmelin, 1790), commonly known as goose barnacle (percebe or perceve in Portuguese), is a marine crustacean, sessile for the most part of his life, inhabiting the rocky substrate of the intertidal and subtidal. Cirripedes are widely

accepted to be the most modified group of animals of all crustaceans, as the only resemblance of a crustacean being their larval phase. Taxonomically they belong to the Cirripedia Subclass and Thoracica Superorder easily distinguishably from all others for the presence of six cirrus pairs in the thorax:

- Phylum Arthropoda
 - Subphylum Crustacea
 - Class Maxillopoda
 - Subclass Thecostraca
 - Infraclass Cirripedia
 - Superorder Thoracica
 - Order Pedunculata
 - Infraorder Scalpelliformes
 - Family Pollicipedidae
 - Genus *Pollicipes*
 - Specie *P.pollicipes*

The Superorder Thoracica was described by Darwin in the XIX century, and then modified in 1994 by Anderson, describing the Pedunculata Order on which the pedunculated cirripedes like the *P.pollicipes* are included, this Superorder contains five more Orders characterized by the absence of the peduncle and therefore they directly hold to the carapace. Phylogenetically, the pedunculated cirripedes are the most primitive of all, being ancestors of the remaining cirripedes who evolved into cirripedes without peduncle, as the giant barnacle *Megabalanus azoricus* (Newman, 1987).

P.pollicipes is widely distributed (Fig.), it spreads for part of the continental European and African coasts, there are also reports of its occurrence in the South coast of the United Kingdom (Hayward & Ryland, 1990). Nowadays its northern range is well defined by the French region of Brittany where it continually spreads through the French coast (M. Barnes, 1996) Atlantic coast of the Iberia Peninsula (Borja, Bald, Muxika, & Liria, 2004; Teresa Cruz & Hawkins, 1998), Gibraltar strait (H. Barnes & Barnes, 1977) some regions of the Mediterranean (M. Barnes, 1996) to the Atlantic African coast from Morocco to Senegal where it reaches the species southern range (~15°N) (M. Barnes, 1996; Stubbings, 1967). *P.pollicipes* is found almost exclusively in areas heavily exposed

to the waves, with less predominance within quiet zones. Preferentially occupies holes, cracks and crevices of variable dimensions of the low and mid intertidal and subtidal where it forms dense aggregates in combination with other cirripeds of the *Balanus* e *Chthamalus* Genus and mussels (Molares, 1993), can also be found in the form of isolated individuals. Specialists distinguish between two types of barnacles: those in the sun and



Figure 1 – *Pollicipes pollicipes* geographical distribution (red line)

those in the shade or watery ones. The former grow in sunny areas and tend to have a short thick stalk, whereas the latter, which are less highly valued, possess a longer, thinner body and larger water content.

It has a soft malleable peduncle composed of chitin and covered with small calcareous scales through which it is attached to the substrate, it is the edible (muscle) part of the barnacle, at the other end lies the capitulum, which consists of two symmetrical sides with 8 main plates, and a variable number of small plates in the intersection area of the capitulum with the peduncle, the faces are separated from the center by a small aperture. The capitulum contains all thoracic appendices and every organ except the

gonads that can be found in the peduncle (Molares, 1993), it is a simultaneous hermaphrodite (Teresa Cruz & Hawkins, 1998), with internal cross-fertilization, where one of the individuals acts as a functional male and the other one as functional female. Gregariousness facilitates fertilization, although records of self-fecundation when isolated do exist, (M. Barnes, 1992), although more recent studies suggest self-fecundity is impossible (Teresa Cruz & Hawkins, 1998; Teresa Cruz, 2000), the male deposits sperm in the female's mantle cavity. The female stimulated by the sperm deposition carries the oocytes by forming two ovissacs to the oviduct where they are fertilized, the ovissacs subsequently acquire a laminar shape and are located on both sides of the prosoma, where they will be placed throughout embryonic development (Anderson, 1994).

The embryonic development of these individuals takes place in the mantle cavity (Walker, 1983), which benefits from oxygenation promoted by movements of the prosome, embryonic development of barnacles is dependent on temperature (20-30 days for temperatures of 13-23 ° C respectively for *P. polymerus*; (Hilgard 1960), the duration of the embryonic phase of *P. pollicipes* is not fully known in the wild, however, Molares (1993) refers to 25 days for embryonic development carried out in the laboratory at 20 ° C, after completion of embryological development a planktotrophic larvae is released into the water, larval release mechanisms in cirripedes appears to be hormonally regulated by a pheromone liberated in the mantle cavity (Clare, 1987).

The larval development is characterized by six nauplii stages and a late cypris stage, the first larval stage (nauplii I) is the only lecithotrophic stage, lasting a few hours, can be characterized by its visible thoraco-abdomen process and swimming appendices. From this larval stage on, individuals become planktotrophic feeding on phytoplankton of the water column. Larvae progressively increase their size through development initiating the extension of frontolateral horns, the dorsal spine becomes elongated and the thoraco-abdominal process develops and becomes forked. By the Nauplius III stage the frontolateral horns become nearly perpendicular to the body, with two teeth and a variable number of small spines developing on the posteriolateral margins of the labrum. Nauplius IV is characterized by the separation of the caudal spine from the carapace and the presence of a pair of large spines close to the base of the furca, the two teeth on the margin of the labrum become separate. The thoraco-abdominal process thickens by Nauplius V, three pairs of short spines emerge on the abdominal process in addition to the pair on the

base of the furca. In stage VI the larva undergoes a quite profound structural and functional transformation, which turns the individual into a cypris larvae (Anderson, 1994), the carapace is convex in shape, six pairs of spines forming parallel lines are present along the thoraco-abdominal process, by the end of this development stage two compound eyes are visible on both sides of the nauplii eye.

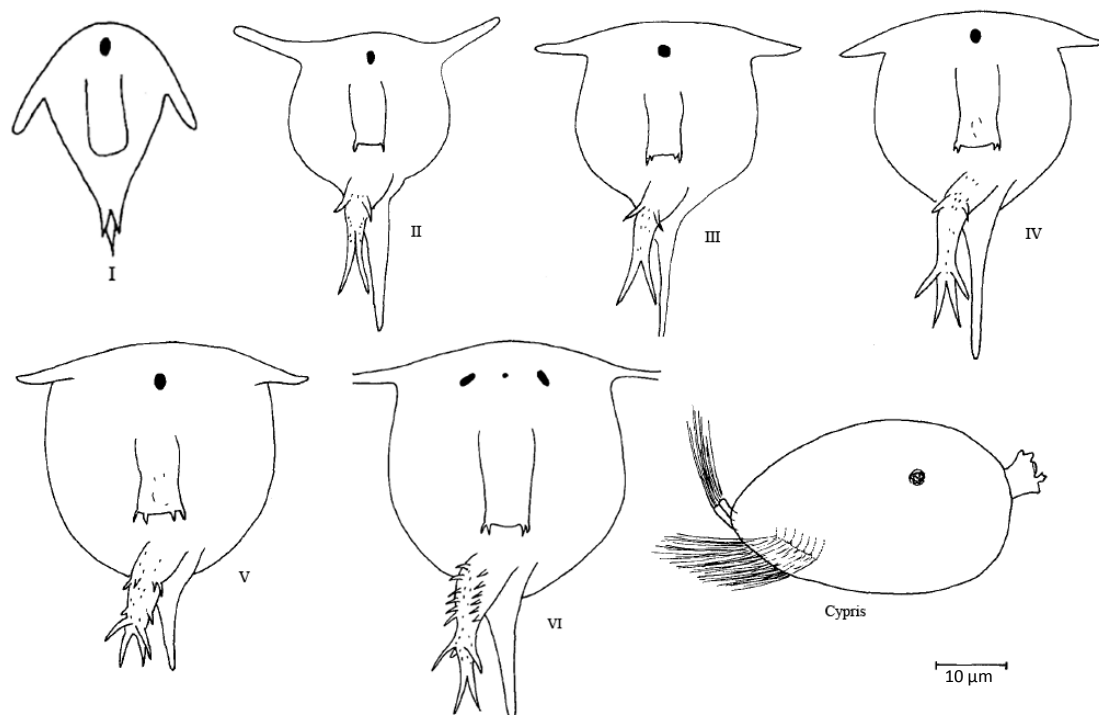


Figure 2 - Larval development of the goose barnacle *P.pollicipes* (adapted from Molaes et al. (1993))

Finally the larvae develops into a cypris, the edges of the carapace touch each other, giving the larvae an external appearance of a bivalve mollusc, with a rounded frontal margin and a ovoid like rear margin, a caudal furca, a pair of antennules, six pairs of natatory limbs and a big eye are visible.

The cypris larvae will at the end of its development find a substrate on which it secretes a fixing substance produced by the antennules, after this fixation takes place a complete metamorphosis that will origin to a juvenile (Anderson 1994). The post-metamorphosis juvenile is already similar to an adult individual, lacking only a variable number of moults to complete the functional differentiation, which is regulated by a hormonal system (Clare 1987). Studies carried out on *P.pollicipes* reproduction on the

coasts of France, Spain and Portugal describe breeding periods ranging from the beginning of spring and late summer, with up to four asynchronously spawns reported for each individual over this period (M. Barnes, 1992; T. Cruz & Araújo, 1999; Pavón, 2003).

Marine invertebrates spawns are usually influenced by biotic factors (age, size, density) and abiotic (water temperature, light, food), thus presenting exogenous spawning rhythms. The most important factors in spawning are temperature and food availability acting as dominant factors over others, and controlling the life cycle of individuals (H. Barnes & Barnes, 1977). These cues have been suggested as a strategy to match larval stages with favourable environmental conditions for larval development and offspring survival. In addition to its major importance in the geographical distribution of species, temperature plays an important role in spawning events, in the case of *P. pollicipes* that inhabits temperate waters, where environmental conditions have a less pronounced variation throughout the year, the breeding periods are often larger, with more spawns by individual/year. Although there are no records the temperature triggers the beginning of the spawning season (Cardoso & Yule, 1995), some authors have reported the influence of the temperature of either the sea or the air in reproductive activity (Teresa Cruz & Hawkins, 1998; Pavón, 2003). Photoperiod is another mechanism that may act in the regulation of the reproductive cycle, Cardoso and Yule (1995) consider the decrease in photoperiod as summer progresses can be a key signal for the end of the breeding season, while Macho et al. (2005) refers to regulation of lunar phases on larval emission timing.

Recruitment in older individuals acting as hosts is more pronounced in the basal half of the peduncle, though existing direct recruitment in the bedrock and other organisms, can be monitored with relative ease (M. Barnes, 1996; Hoffman, 1989). Size distributions of juvenile *P. polymerus* on adult peduncles were studied by Hoffman (1984) who found that juveniles were not randomly distributed. There was a gradient from the smallest and most abundant near the growth zone (the narrow zone between capitulum and peduncle) to the largest and fewest located near the base of the peduncle.

The goose barnacle is mainly made up of water (almost 90%), followed by protein (4%), fats (5%) and mineral salts (>3%) (Kameya & Zeballos, 1988). They supply large amounts of vitamins B1 and B2, potassium and other minerals, while possessing a low fat content, with about 66 calories per 100 g meat weight.

1.3 “Fingerprint” approach

1.3.1 Capitulum shape

Body shape is an important component of an individual’s phenotype, and it is directly linked on relevant traits as feeding efficiency, locomotor performance, vulnerability to predators, and of course reproductive success (Guill, Hood, & Heins, 2003), all of them with significant consequences for fitness.

Comparison of the anatomical characteristics of organisms has been for a long time a key element of biology. Taxonomic classification was itself historically based on the descriptions of morphological features. However in the early twentieth century a transition from a descriptive field to a more quantitative based science took place, which was also applied to the way morphology was analyzed (Bookstein, 1998). Morphological approaches were mainly based on measurable traits which were later summarized as mean values and compared between groups, but, the development of statistical methods such as the correlation coefficient (Pearson, 1985), analysis of variance (Fisher, 1935), and principal components analysis (Pearson, 1901) allowed for further advances of the rigor in morphological studies.

Referred to as geometric morphometrics (GM) techniques, it is used as an approach in the study of biological forms (Adams, Rohlf, & Slice, 2004; Bookstein, 1998), this approach describes individuals geometrically, composed of x;y (and even z) coordinates obtained from a set of landmarks. The principal advantages of geometric morphometrics is that it allows a way of visualizing the changes in shape and produces a new set of shape related variables which can afterwards be analysed using traditional multivariate statistic methods (F. Rohlf, 1993), these tools have been implemented to a wide range of questions regarding shape, including feeding ecology (Carpenter, 1996), geographic variation (Lezcano, González-José, Spivak, & Dellatorre, 2012), sexual dimorphism (Hood & Heins, 2000), conspecific cues (Duarte, Ré, Flores, & Queiroga, 2013) and systematics (Fink & Zelditch, 1997).

If traits, such as the aforementioned can induce changes in the shape of some organisms, then it is possible that distinct goose barnacle populations subjected to different environmental conditions (e.g. shade or sun, wave exposure, North-South

exposure, food availability and quality, etc.), would induce changes in the shape of the individuals, probably producing more than one distinct shape.

1.3.2 Elemental microchemistry

Trace elements in near shore water vary due to natural and anthropogenic reasons, and this variation may possibly cause distinct elemental signatures to individuals of the same species within different locations, these elemental signatures are called natural tags, since they act upon the different physical and chemical environments experienced by the organism.

The use of elemental signatures in calcified structures, is based on the analysis of trace elements imprinted in the structure, in this case the goose barnacle's capitulum, which grows continually for the duration of the individuals life, and are formed by the deposition of mainly calcium carbonated crystals, as the capitulum constituents accrete it imprints trace elements from the environment that have been absorbed per respiratory or feeding processes and entered the bloodstream (Campana, 1999).

Studies regarding calcified structures elemental signatures have turned to be in recent years increasingly popular for differentiating between fish stocks, assuming that environmental differences can induce changes enough to tell them apart (Fowler et al., 1995; Farrell and Campana, 1996; Tzeng, 1996; Dove, 1997), however little is known about the reason of these site-specific differences (S. E. Swearer, Forrester, Steele, Brooks, & Lea, 2003).

Some elements (Sr, Ba, Hg, Pb) seem to be accrete in calcified structures in relation to their own concentration the water (Bath et al., 2000; Geffen, Pearce, & Perkins, 1998; Milton & Chenery, 2001; Mugiya, Hakomori, & Hatsutori, 1991), while others do not appear to have a concentration-dependence, and some authors (Bath et al., 2000; Fowler, Campana, Jones, & Thorrold, 1995a,b) suggest other environmental conditions such as the water temperature, salinity, pH and dissolved oxygen to be of influence in how trace elements are imprinted.

Since the use of elemental signatures in calcified structures requires the water chemistry to have enough differences, to allow for a positive discrimination amongst sites, it is natural why the majority of the initial work has concentrate on both estuarine

and coastal systems, which are known to present more spatial variation in the water chemistry than the usually detected in the open ocean (Gillanders and Kingsford, 1996; Thorrold et al., 1998).

Inductively coupled plasma – mass spectrometry (ICP-MS) is known to be a powerful multi-element analytical technique, with low detection limits and multi-element capability, turning it into an appealing option in large range of applications (Linge & Jarvis, 2009). The basic principle of ICP-MS is elemental differentiation based on atomic mass, and since the isotopic composition of each element is well known, thus, easily predicted (Bohlke et al. 2005), nevertheless mass spectrometry (MS) alone is not able to distinguish amongst neutral atoms, making it necessary to ionize the atoms, in order to form positively charged particles, the inductively coupled plasma (ICP) comprising a strongly ionized phase at high temperatures.

It has been demonstrated by some authors (**insert citation**) the success in identifying populations less than 20 km apart from each other, suggesting this could also be the case for populations of goose barnacles of the Portuguese coast.

1.3.3 Fatty acids profile

The definition of these compounds is not consensual, may be considered as any biological molecule soluble in an organic solvent (Halim et al . 2012) or as fatty acids, derivatives and molecules with the biosynthesis / function similar to these (Woertz 2007; Xiao 2010). They are composed of triacylglycerides, phospholipids, fatty acids, waxes and pigments, having as main functions the intracellular signaling, source of metabolic energy as well as membrane and tissue formation. Lipids and fatty acids in marine organisms reflect the environmental and biochemical conditions of their habitat, making them suitable to be used as biomarkers (Bergé & Barnathan 2005; Simões 2011).

Photosynthetic cells are the only ones that can biosynthesize the n-3 and n-6 fatty acids, commonly known as omega (ω) 3 and 6, which are essential to heterotrophic organisms, leading them to have an important position in the food chain (Bergé & Barnathan 2005).

Fatty acids are carboxylic acids, usually with a long aliphatic chain, usually designated accordingly with the IUPAC nomenclature, or described in a shorter version C:D, where C is the number of carbon atoms in the fatty acid and D is the number of double bonds in the fatty acid. The position of the double bonds on PUFAs is represented as (*n*-Z) where *n* indicates the number of double bonds and Z the position of the first double bond after the methyl group. The figure below (Fig. 3) represents the standard structure of two polyunsaturated fatty acids – EPA and DHA.

The majority of fatty acids found in nature are composed by a pair number of carbon atoms, with carbon chains ranging from 4 to 25 atoms. Regarding the presence of double bonds within the carbon chain, fatty acids are classified as saturated fatty acids (SFA), if they do not present double bonds, in monounsaturated fatty acids (MUFA), when a single double bond is present and finally in polyunsaturated fatty acids (PUFA) when at least two double bonds are formed.

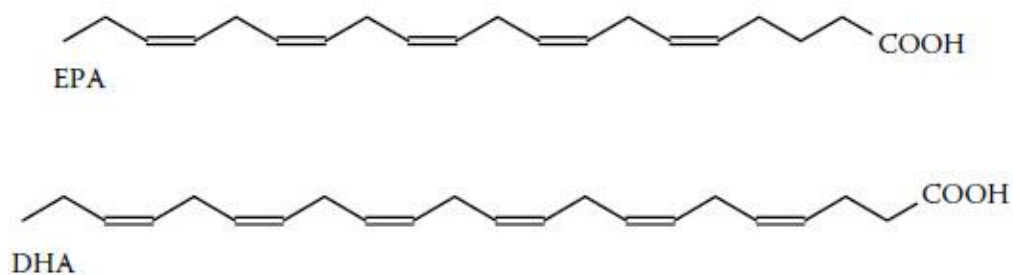


Figure 3 - Structure of EPA and DHA fatty acids (adapted from: Gunstone et al. (2007))

Saturated FA can be found in a wide variety of food, such as butter, coconut and palm oil. The most common MUFA is the oleic acid (C₁₈:1), found in high concentrations in olive and sunflower oil as well as in animal fat. PUFA are mainly present in fish and vegetable oils. The presence of palmitic acid (C₁₆:0) is related with energy storage, high amounts of this fatty acid are associated with moments of low metabolic rate, were individuals start to accumulate energy reserves (Stryer et al.. 2002), stearic acid (C₁₈: 0) is in crustaceans larvae associated with low mortality rates (Calado et al .. (2009). Linoleic (C₁₈:2 n-6) and linolenic (C₁₈:3 n-3) acids are essential fatty acids (EFA), meaning they cannot be synthesized by most animals, and have to be provided through diet. Both are

precursors of the also essential fatty acids arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA), which are constituents of membrane lipids.

PUFA are important structural components of the cellular membranes, reducing its inelasticity, especially in tissues such as the retina and human brain cells (Koletzko et al, 2001).

Knowing these molecules can depict the environmental conditions of each habit, there is the chance that different populations present site-specific fatty acids profile allowing for a positive discrimination among them.

1.4 The project “Percebe da Berlenga”

The goose barnacle fishery in Berlenga is of vital socio-economic relevance for Peniche and nearby cities due to high demand, especially in the summer, which naturally increases the market value of the fishery, despite being a regulated activity it does not currently have a dynamic management system, possibly leading to a scenario of overexploitation of the stocks.

In order to guarantee a sustainable fishery in the Berlenga Nature Reserve, this project aims at the evaluation of the stock and fishing effort, assess the recruitment, growth and population size structure, identification of specific natural markers, development of a shared dynamic management, and the economic valorization of marine resources from marine protected areas.

“Percebe da Berlenga – contributions to a sustainable fishery” is financed by the Polytechnic Institute of Leiria (IPL) and GAC-Oeste (Grupo de Ação Costeira do Oeste), led by the Research Group on Marine Resources (GIRM-IPL) with the collaboration of ICNF (Institute for the Conservation of Nature and Forests), University of Évora, University of Aveiro and the Association of Berlenga Harvesters.

2. Objectives

The present study aimed to evaluate and identify site-specific differences of the goose barnacle *Pollicipes pollicipes* collected from distinct sources, in order to infer the discriminate power of the analytical approaches.

The specific aims of the study were:

- Evaluate the discriminant power of:
 - Capitulum shape
 - Elemental microchemistry
 - Fatty acid profile

- Characterize:
 - Elemental micro chemical composition of the capitulum
 - Fatty acid profile of the muscle

3. Materials and Methods

3.1 Sampling site and collection

The goose barnacle samples were collected from 10 distinct sites on the Portuguese shore (Fig. 4; Table I)

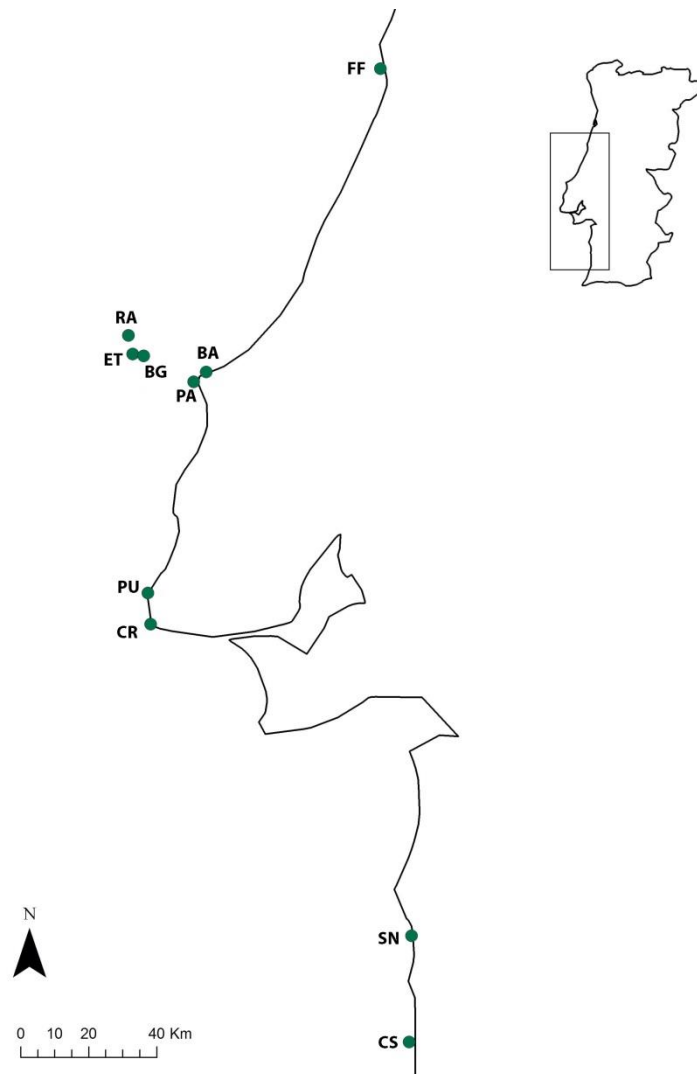


Figure 4 – Map of Portugal with the locations of sampling sites.

Sampling locations were selected based on the Portuguese regions with highest *P.pollicipes* harvest, and in the Central region also due to their proximity to Berlengas Nature Reserve (RNB). Exact sampling sites were selected based on accessibility to the rocky shore, except for the 3 sites part of the RNB – BG, ET, FA – which were accessed

by boat. On each site at least 5 clusters were collected by hand using gloves to avoid any potential trace metal contamination caused by the usually employed steel made scraper. Samples were transported to the laboratory in an icebox, individuals were manually separated and stored in labeled ziploc bags and stored at -80°C within 2-3 hours of collection.

Table I – Description of the sampling locations

| Location | Code name | Latitude N | Longitude W |
|---------------|-----------|------------|-------------|
| Figueira Foz | FF | 40.178598° | -8.906513° |
| Farilhões | FA | 39.473214° | -9.545214° |
| Estelas | ET | 39.424187° | -9.533622° |
| Berlengas | BG | 39.419675° | -9.504889° |
| Baleal | BA | 39.376588° | -9.339759° |
| Peniche | PA | 39.369378° | -9.381259° |
| Praia da Ursa | PU | 38.792253° | -9.493550° |
| Cabo Raso | CR | 38.710455° | -9.486362° |
| Sines | SN | 37.886998° | -8.796691° |
| Cabo Sardão | CS | 37.606126° | -8.816595° |

3.2 Sample preparation

In order to minimize contamination a strict procedure was followed. For all steps during all analysis Milli-Q 18MΩ water and all reagents were of certified trace metal purity. Solutions were stored in previously acid leached bottles (48 hours in a 25:75% solution of HCl (Hydrochloric acid fuming 37% Merck Emsure®) and Milli-Q 18MΩ HDPE). All plastic material employed during the samples processing was leached in acid solution (1N HCl) for 48 hours, rinsed in Milli-Q water, allowed to dry under a laminar flow chamber and stored in ziploc plastic bags. All other material such as petri dishes,

ceramic tipped forceps, etc, coming in contact with samples were also acid leached in a 0.01 N HCl solution and well rinsed in Milli-Q water.

Individuals from each site were sorted accordingly to their general appearance (i.e. complete capitulum and healthy peduncle coloration), ten individuals from each site were then randomly assigned for all of the 3 analyses.

Capitulum was manually removed from the peduncle, reserved for geometric morphology analyses and trace elemental fingerprinting, while the peduncle was kept for fatty acid profile analyses.

3.2.1 Capitulum shape

Morphogeometric analyses were restricted to Capitulum, because it is the rigid structural area of each individual, with the well-defined calcareous plates allowing for homologous landmarking.

Digital images of the capitulum were captured using a Nikon D90 digital camera coupled to a 105mm F2.8 EX DG OS HSM Macro Sigma lens. The software Nikon Camera Control Pro 2 was used for image acquisition, since it allows for a complete configuration of the shooting and live view visualization. Images were treated with Photoshop CC v14.0 (Adobe) for color balance and contrast levels.

A total of seven landmarks were defined along the capitulum as shown in Figure 5:

1. Tip of tergum
2. Intersection between tip of scutum and tergum
3. Tip of rostrum
4. Anterior capitulum-peduncle margin
5. Intersection between tip of carina and tergum
6. Tip of the first lateral plate
7. Posterior capitulum-peduncle margin

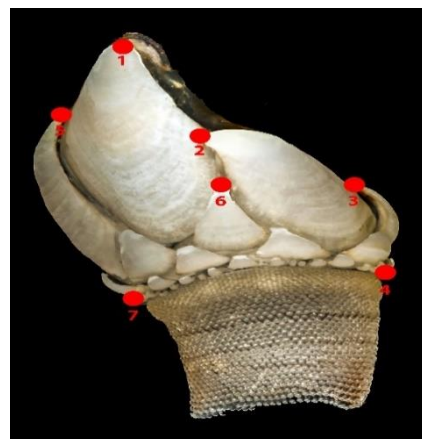


Figure 5 – Defined landmarks in the capitulum of goose barnacle

Landmarks were defined using the software tpsDig 2.14 (Rohlf, 2009), following standardized criteria (Adams et al., 2004; Zelditch et al., 2004).

After image digitizing, landmarks were aligned by a generalized least-squares superimposition procedure – Procrustes analysis – in order to remove shape-unrelated information, such as position, orientation and scale (Rohlf & Slice, 1990).

Change in morphology was evaluated using the thin plate spline – TPS – deformation procedure, fitting an interpolate function to a consensus configuration (Bookstein, 1991), producing shape variation in two components, an uniform component (UC) which refers to global change, and a non-uniform component describing local variation in particular regions (Zelditch et al., 2004).

At last, a relative warp (RW) analysis, equivalent to a principal components analysis, was carried out for the non-uniform component generating new shape variables (RWs) (Rohlf, 1993a, b). These procedures were done using software tpsRelw 1.46 (Rohlf, 2008).

Centroid size (CS), defined as the square-root of the summed squared distances between all landmarks, and the rostro-carinal (RC) length, was used to access confirm homologous growth.

3.2.2 Elemental microchemistry

In a clean room, using acid-dipped, porcelain-tipped forceps, Capitulum plates were manually scraped of debris and transferred to acid-washed plastic vials. Samples were then treated twice with 30 mL of 15% H₂O₂ (Suprapur; Merck) buffered with 0.05 M NaOH (Suprapur; Merck) in a microwave (400 W, 90 seconds) in order to obtain a complete removal of the organic matter and of the gelatinous matrix that keeps the Capitulum plates together, rinsed three times in 30 mL of ultra-pure water, sonicated 3 times for 5 minutes, finally the water was removed and samples transferred to acid-washed vials and allowed to dry under a laminar-flow hood placed in a petri dish.

The largest Lateral plate was removed from Capitulum with porcelain-tipped forced and digested in 15 mL acid-washed vials with 1 mL 60% HNO₃. (Ultrapur; Merck).

Acid digested samples were then diluted to reach a HNO₃ concentration of 5% for ICP-MS analyses.

After digestion, samples were analyzed by an inductively coupled plasma – mass spectrometer (ICP-MS) Thermo X Series equipped with a Burgener Myramist nebulizer, set up with a conical chamber Peltier cooled, quartz torch and Silver shielded Nickel cones, coupled to an autosampler Cetac ASX-510, Indium (In) and Terbium (Tb) were used as internal standards and CeO/Ce ratio <2%.

A total of 12 elements were quantified, Li; B; Mg; P; Ca; Cr; Mn; Zn; Sr; Cd; Ba and Pb, subsequently all data were converted to molar concentrations and expressed as ratios to Ca.

3.2.3 Fatty acid profile

After the pretreatment described above in 3.2, 150 mg of *P. pollicipes* muscle were weighed and the total lipids of the muscle were extracted according to the method proposed by Bligh and Dyer (1959) with minor changes. 5 mL of a methanol:chloroform solution (2:1) [Panreac; Scharlau] were added and the sample homogenized for 60s at 20 000 rpm, 1mL of chloroform were added and the sample homogenized for 30s at the same speed.

Vials containing the sample were centrifuged ($v=7000\text{rpm}$, $t=10\text{ min}$), the now clearly formed organic phase was collected to a new vial, another mL of chloroform was added and gently homogenized, vials were finally subjected to a N₂ current until complete evaporation of the solvents. Mass were weighed and total lipids determined gravimetrically after equation

$$\text{Total Lipids} = \frac{(M_f - M_i) \times V_t \text{ CHCl}_3}{\frac{V_e \text{ CHCl}_3}{\text{Biomass}}}$$

Equation A – Determination of total lipids (mg/100mg of muscle tissue). Initial weight (Mi) corresponds to the weight of the vials without sample, final weight (Mf) corresponds to the weighted mass of vials after the evaporation of the solvents (g). Total volume of Chloroform (Vt CHCl₃) stands to the volume used in the extraction (mL), while the evaporated volume (Ve CHCl₃) represents the amount of Chloroform evaporated (mL). Biomass refers to the amount of sample that was used (g)

For the fatty acid profile analysis, Lepage and Roy's method (1986) was used for the direct transesterification of samples. 1,5 mL of methanol:acetyl chloride (20:1) and 1 mL of n-hexane were added to the aforementioned sample. Vials containing the samples were homogenized and incubated for 1 hour at 80°C. Tubes were let to cool in ice until room temperature was attained, centrifuged to recover the upper phase which was collected and filtered through an anhydrous sodium sulfate column to remove any water present in the samples. Filtrate was stored in new vials, and subsequently used for the fatty acid profile analysis.

A Finnigan TRACE GC Ultra gas chromatograph (Thermo Electron Corporation), equipped with a Thermo TR-FAME capillary column (30m * 0.25 mm ID * 0.25µm film thickness), an AS 3000 auto sampler from Thermo Electron Corporation and a flame ionization detector were used for the FAME analysis, according to Tecelão *et al* (2010). The injector was set to operate in splitless mode and the detector temperatures were set at 250 and 260°C, respectively. Helium was used as the carrier gas, at a flow rate of 1.5 mL^{min}⁻¹. Air and Hydrogen were supplied to the detector at a flow rate of 350 mL^{min}⁻¹ and 35 mL^{min}⁻¹, respectively. The column temperature was briefly set at 60°C for a minute, then slowly raised 15°C^{min} to 150°C for a minute followed by an increase of 5°C^{min} to 180°C for 3 minutes, and finally increased at a rate of 10°C^{min} to 220°C for a minute.

Retention time for each fatty acid were identified comparing to the retention time of commercial standards [PUFA-1 Marine Source and PUFA-3 Menhaden oil (Supelco Analytical)], are described in Attachments – Table 8.1. Fatty acid profile were calculated in proportion to the total lipidic content.

3.3 Statistical analysis

Prior to statistical analyses, all data were transformed in order to meet the normality, homogeneity of variance assumptions of ANOVA and to place more emphasis on compositional differences among samples rather than on quantitative differences (Anderson, 2008). For this purpose either natural logarithm or fourth-root transformations were used.

Differences between sampling locations in Relative Warps, concentration of elements analysed and fatty acid profile was assessed using Analyses of Variance.

Three independent discriminant analyses for each set of variables analysed (capitulum shape, elemental microchemistry and fatty acid content) were performed to classify harvest locations. A jackknifed procedure was followed to calculate classification success, this ensured that the classification function used to determine group assignment was unbiased as the goose barnacle being classified was not used to construct the function.

Classification success was compared with the results of an additional discriminant analysis on the combination of the elemental microchemistry and fatty acid content. Variables introduced in each model were selected following a forward stepwise analysis with an F to enter of 1,8 and an F to remove of 0.

4. Results

4.1 Capitulum Shape

As previously described in section 3.2.1, in order to have an insight regarding on how goose barnacle's capitulum growth can be classified – analogous or homologous – and how reliable the results are a basic linear regression was performed using the rostro-carinal size and the Centroid size. According to the obtained linear relation $R^2 = 0.887$ (Fig. X), there is a strong relation between the two variables, meaning the growth of the sampled specimen is homologous, in other words, the capitulum grows proportionally.

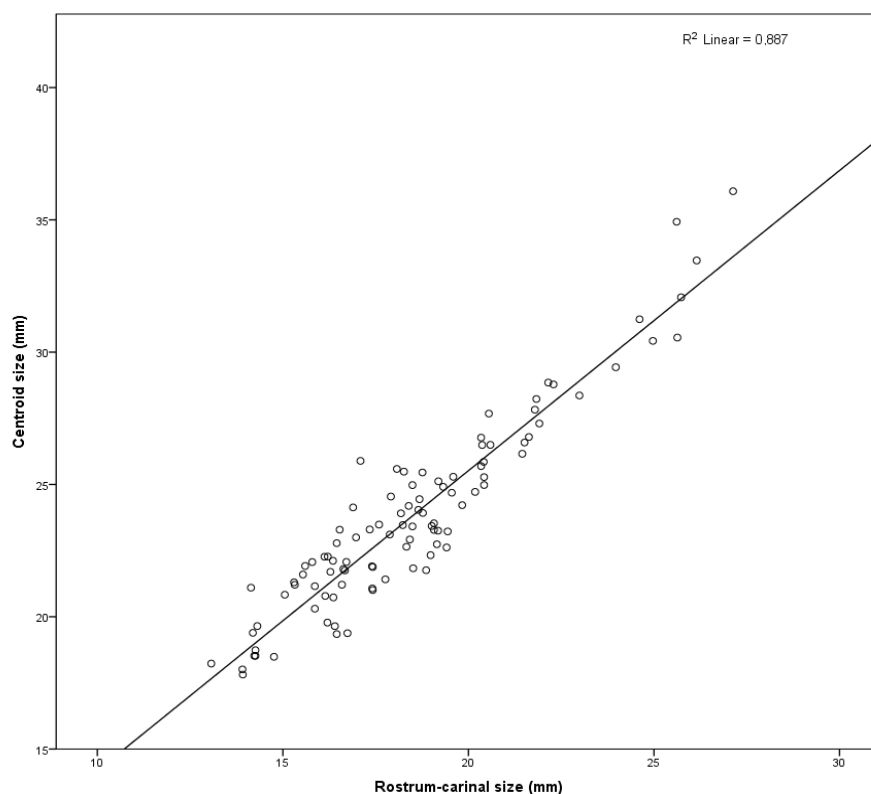


Figure 6 - Linear relation of Rostro-carinal size (mm) and Centroid size (mm)

$$y = 1,13x + 2,83$$

Equation B – Linear relation between Rostro-carinal size (mm) and Centroid size (mm), where **y** is defined as the Centroid size, and **x** as the Rostro-carinal size of the goose barnacle.

The obtained Relative Warps were analysed for differences between Sites (Table II). Of the 10 variables, 6 were found to show statistical differences. RW 1 and RW 4

respectively present the strongest statistical differences ($p < 0,0001$), followed by RW 5, RW 3 and RW 8 with ($p < 0,01$) and finally RW 7 and RW 8 exhibit statistical significant differences of ($p < 0,05$).

Table II - One-way ANOVA testing for differences in the Capitulum shape based Relative Warps of *Pollicipes pollicipes* between sites

| | Source | SS | df | MS | F | p |
|--------------|--------|----------|----|----------|----------|----------|
| RW 1 | Site | 1098,829 | 9 | 122,0921 | 8,018113 | 0,000000 |
| | Error | 1339,980 | 88 | 15,2270 | | |
| RW 2 | Site | 266,405 | 9 | 29,60060 | 1,673933 | 0,107301 |
| | Error | 1556,127 | 88 | 17,68326 | | |
| RW 3 | Site | 430,826 | 9 | 47,86954 | 2,721990 | 0,007568 |
| | Error | 1547,588 | 88 | 17,58623 | | |
| RW 4 | Site | 609,750 | 9 | 67,75003 | 5,189470 | 0,000012 |
| | Error | 1148,866 | 88 | 13,05529 | | |
| RW 5 | Site | 289,2489 | 9 | 32,13877 | 3,338662 | 0,001485 |
| | Error | 847,1091 | 88 | 9,62624 | | |
| RW 6 | Site | 102,441 | 9 | 11,38235 | 0,670154 | 0,733688 |
| | Error | 1494,652 | 88 | 16,98468 | | |
| RW 7 | Site | 341,259 | 9 | 37,91763 | 2,193353 | 0,029804 |
| | Error | 1521,301 | 88 | 17,28752 | | |
| RW 8 | Site | 423,240 | 9 | 47,02663 | 2,625279 | 0,009752 |
| | Error | 1576,344 | 88 | 17,91300 | | |
| RW 9 | Site | 108,004 | 9 | 12,00050 | 0,571824 | 0,816760 |
| | Error | 1846,800 | 88 | 20,98636 | | |
| RW 10 | Site | 264,802 | 9 | 29,42245 | 1,206203 | 0,301503 |
| | Error | 2146,550 | 88 | 24,39261 | | |

The performed discriminant function analysis of the capitulum shape, entered all the variables presenting statistical differences, the first variable to enter analysis was RW1 followed by RW4; RW 5; RW 3; RW 7 and finally RW 8, as shown in Table III. However, despite being statistical significant, variables display low Lambdas scores, with the highest belonging to the first variable to enter analysis (0,549).

Table III - Order the variables were entered in the stepwise analysis, and standardized canonical coefficients (DF weights) for the first two discriminant functions.

| Step | Entered | Lambda | DF1 | DF2 | DF3 | p |
|------|---------|--------|--------|--------|--------|---------|
| 1 | RW1 | 0,549 | 0,533 | 0,837 | -0,032 | <0,0001 |
| 2 | RW4 | 0,361 | 0,688 | -0,479 | -0,250 | <0,0001 |
| 3 | RW5 | 0,267 | -0,525 | 0,007 | -0,152 | <0,01 |
| 4 | RW3 | 0,202 | -0,141 | 0,310 | 0,774 | <0,01 |
| 5 | RW7 | 0,157 | 0,459 | -0,243 | 0,469 | <0,05 |
| 6 | RW8 | 0,121 | -0,285 | 0,489 | -0,332 | <0,05 |

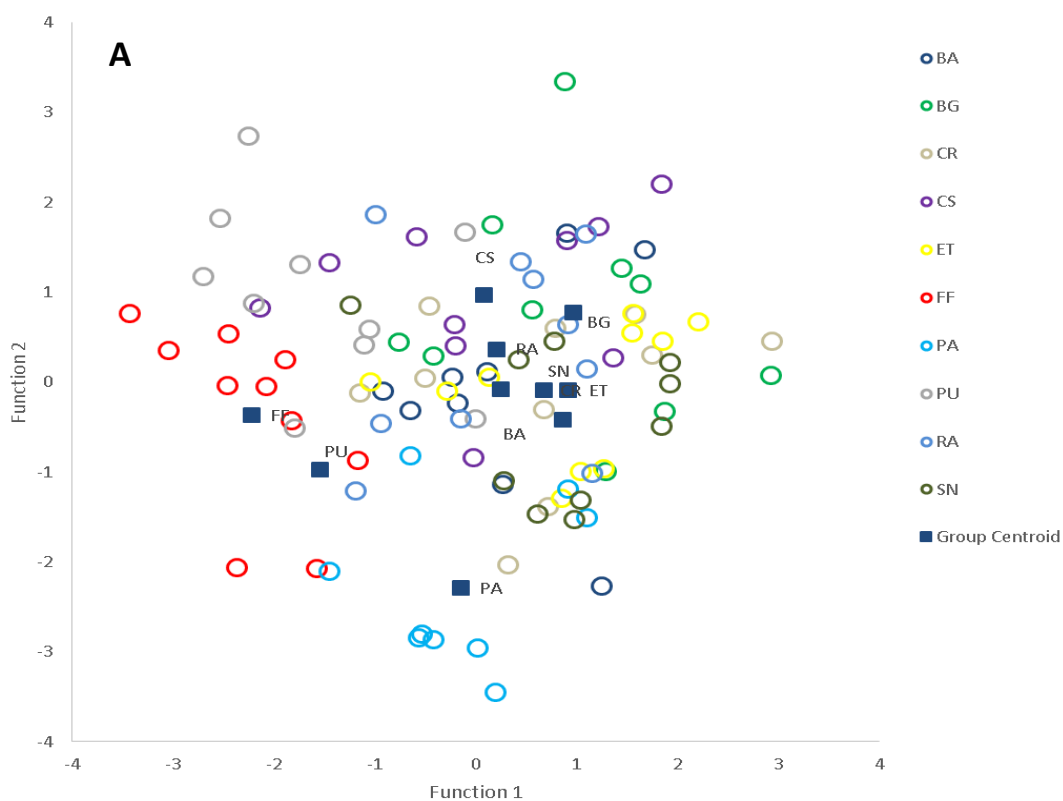
The linear discriminant function analysis yielded 6 canonical discriminant functions which were used in the analysis to classify samples according to their group of origin, the first three canonical discriminant functions are able to explain 85,4% of the shape variation in *P.pollicipes* capitulum as present below in Table IV.

Table IV - Eigenvalues, % of Variance, Cumulative % and Canonical correlation of the canonical functions used to in the analysis

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|----------|------------|---------------|--------------|-----------------------|
| 1 | 1,182 | 41,4 | 41,4 | 0,736 |
| 2 | 0,868 | 30,4 | 71,9 | 0,682 |
| 3 | 0,387 | 13,6 | 85,4 | 0,528 |
| 4 | 0,240 | 8,4 | 93,9 | 0,440 |
| 5 | 0,104 | 3,6 | 97,5 | 0,307 |
| 6 | 0,072 | 2,5 | 100 | 0,259 |

In order to obtain a two dimensional image of the results, the canonical functions which better explain the variation were plotted (Fig 7). Function 1 (F1) was responsible for 41,4%, Function 2 (F2) for 30,4% and Function 3 (F3) for 13,6% of the total variance in this analysis, thus generates 3 plots (F1*F2; F1*F3; F2*F3) as shown below in Fig.7. Examining the plot, it becomes evident a general dispersion of all samples, without any clear pattern, absence of clouds within their own group of origin and most important, the samples from the Berleugas Nature Reserve do not appear to be in any way distinguishable from the remaining set.

It is safe to say that although the first 3 canonical functions explain 85,4% of all observed variance, they do not seem suffice to discriminate samples amongst sites.



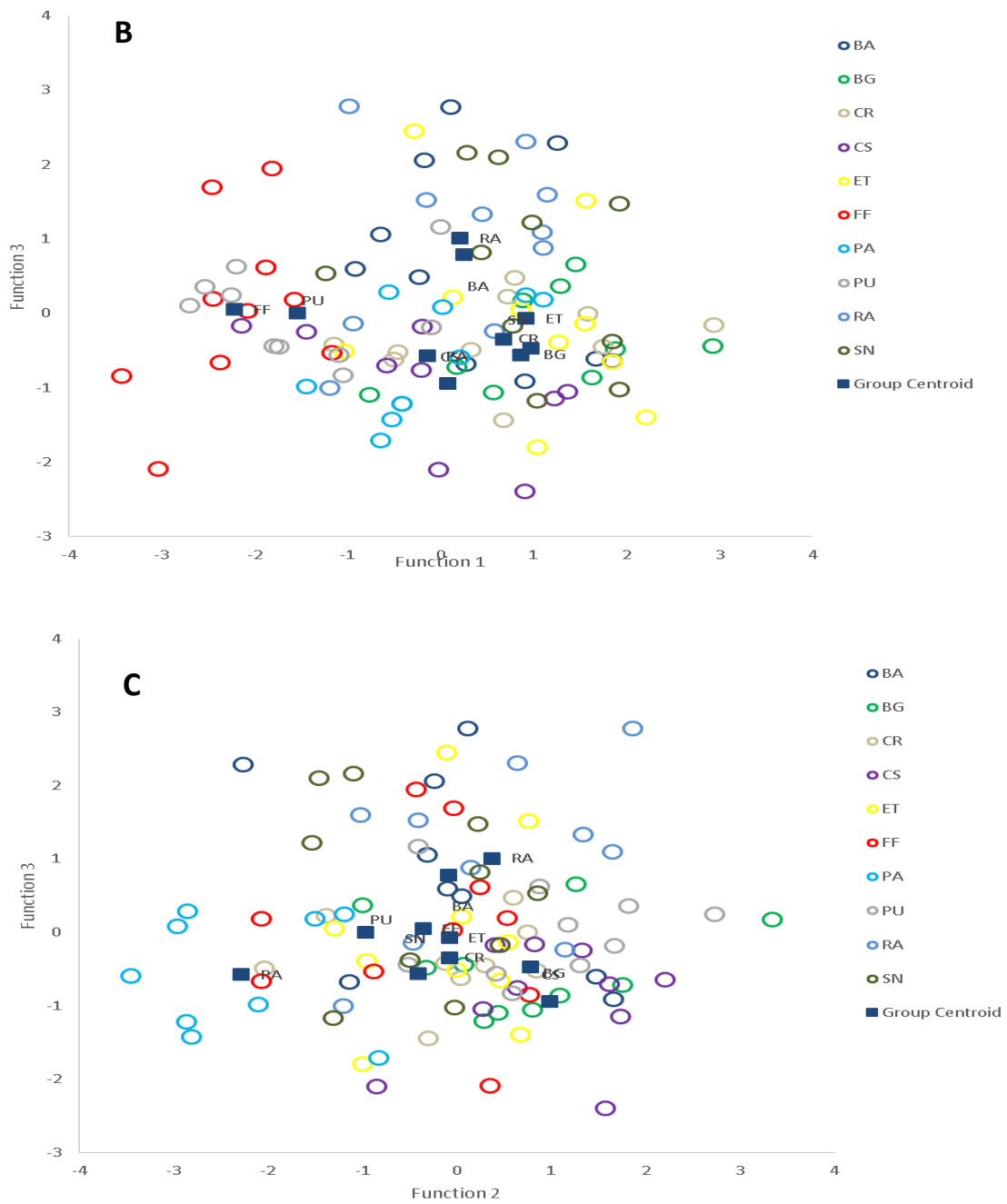


Figure 7 - Results of the Canonical Discriminant Functions. A Function 1*Function2, B Function 1*Function3, C Funtion2*Function3. Colored circles represent Sites, each circle being a sample

This is once again clearly shown in Table V, the reclassification success is extremely low in all sites, with results as low as 11,1% in Baleal – BA, and in the opposite side Peniche – PA with the highest reclassification success (66,7%), the sites from the BNR present a success of 30%;10% and 10% for Berlengas (BG), Estelas (ET) and Farilhões (RA) respectively. Such results unveil an average success of 29,6% of cross-validated cases correctly classified regarding the Capitulum shape of goose barnacles.

Table V - Percentage of goose barnacles correctly classified to each origin in the capitulum shape. Columns are the predicted group for each individual, rows are the origin group of each individual. Data are cross-validated

| Sites | Predicted Group Membership | | | | | | | | | | Total |
|-----------------|----------------------------|-----------|-----------|-----------|-----------|-----------|-------------|-----------|-----------|-----------|-------|
| | BA | BG | CR | CS | ET | FF | PA | PU | RA | SN | |
| Cross validated | | | | | | | | | | | |
| BA | 11,1 | 11,1 | 0 | 11,1 | 11,1 | 0 | 0 | 11,1 | 33,3 | 11,1 | 100 |
| BG | 0 | 30 | 10 | 40 | 20 | 0 | 0 | 0 | 0 | 0 | 100 |
| CR | 0 | 10 | 20 | 10 | 10 | 0 | 10 | 20 | 0 | 20 | 100 |
| CS | 0 | 20 | 0 | 30 | 10 | 0 | 10 | 30 | 0 | 0 | 100 |
| ET | 20 | 20 | 20 | 0 | 10 | 0 | 10 | 10 | 10 | 0 | 100 |
| FF | 0 | 0 | 0 | 0 | 0 | 40 | 20 | 30 | 10 | 0 | 100 |
| PA | 0 | 0 | 11,1 | 0 | 11,1 | 0 | 66,7 | 0 | 0 | 11,1 | 100 |
| PU | 10 | 10 | 0 | 10 | 0 | 20 | 0 | 50 | 0 | 0 | 100 |
| RA | 20 | 20 | 0 | 0 | 0 | 10 | 10 | 0 | 10 | 30 | 100 |
| SN | 20 | 0 | 20 | 0 | 10 | 0 | 10 | 10 | 0 | 30 | 100 |

4.2 Elemental Microchemistry

The elemental microchemistry analysis of the largest lateral shell of *P.pollicipes* capitulum, as Table VI shows reveal Mg and Sr to have the highest concentrations (27041,88 mmol/mol Ca⁻¹; 3946,54 mmol/mol Ca⁻¹ respectively) of the 11 analysed elements, while Cd and Pb are the elements showing the lowest concentration (0,0349 μmol/mol Ca⁻¹; 0,0122 μmol/mol Ca⁻¹ respectively).

The univariate results of the performed ANOVA unveal statistical significant differences (p<0,0001) in all of the 11 elements analysed for microchemistry (Table VII) between sites.

The stepwise discriminant analysis shows Cr, Zn, Mn and Cd to be the first entered variables in the analysis, establishing them as most discriminant elements amongst sites as observed in Table VIII, with FF having a higher concentration in Cr (3,856 μmol/mol Ca⁻¹) while CS and ET show concentrations in this element near zero, Zn was found in higher concentrations in CR (27,982 μmol/mol Ca⁻¹) with PA and SN exhibiting the lowest concentrations amongst sites (2,486 μmol/mol Ca⁻¹ and 2,786 μmol/mol Ca⁻¹ respectively), as for Mn it was found to have the pattern of Cr, higher concentrations in FF (4,394 μmol/mol Ca⁻¹) and the lowest in ET (0,959 μmol/mol Ca⁻¹), Cd presented low concentrations in all sites with CR to show the highest value (0,130 μmol/mol Ca⁻¹).

The aforementioned elements are shown in Fig. 8 to a better perception of the variability between sites.

Table VI – Mean concentration \pm SD of analysed elements. Element concentration is shown in $\mu\text{mol/mol Ca}^{-1}$ for Li; Mn; Cr; Zn; Ba; Cd; PB and mmol/mol Ca^{-1} for B; Mg; P; Sr. Average concentration on top, Standard deviation on bottom

| | BA | BG | CR | CS | ET | FF | PA | PU | RA | SN |
|-----------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Li | 15,336
3,708 | 14,699
1,502 | 14,967
4,103 | 12,996
1,768 | 13,548
1,448 | 20,575
2,590 | 18,532
1,792 | 15,597
0,746 | 15,637
1,680 | 16,121
1,494 |
| B | 69,820
11,756 | 54,059
6,389 | 70,142
16,914 | 64,418
10,120 | 64,851
12,470 | 40,704
6,545 | 43,483
5,789 | 59,857
7,644 | 62,968
10,256 | 58,850
4,845 |
| Mg | 24073,1
3377,5 | 24210,8
2371,5 | 27446,3
4643,7 | 24236,9
1775,3 | 27521,4
2328,9 | 23915,3
1485,8 | 25024,2
2561,1 | 28969,4
4407,4 | 30326,6
2358,1 | 34694,7
4204,6 |
| P | 386,45
24,25 | 408,91
51,45 | 539,09
136,11 | 469,59
81,88 | 498,72
40,13 | 615,80
49,12 | 505,96
24,734 | 514,46
68,348 | 543,99
82,063 | 562,86
43,29 |
| Cr | 0,196
0,099 | 0,447
0,101 | 0,684
0,349 | -0,020
0,009 | -0,016
0,017 | 3,856
1,535 | 1,515
0,630 | 1,273
0,557 | 0,711
0,513 | 1,442
0,607 |
| Mn | 2,413
1,108 | 1,112
0,268 | 2,629
1,286 | 1,740
0,509 | 0,959
0,464 | 4,394
1,60 | 2,279
0,632 | 1,783
0,486 | 1,258
0,618 | 1,666
0,460 |
| Zn | 4,412
3,782 | 5,329
3,007 | 27,982
16,481 | 16,803
7,646 | 13,562
11,048 | 8,493
3,855 | 2,486
1,392 | 6,387
1,634 | 13,371
8,735 | 2,786
0,662 |
| Sr | 3528,9
1218,4 | 3837,5
277,7 | 3737,6
161,7 | 3791,7
208,8 | 3966,6
196,1 | 4384,1
123,2 | 4396,2
252,9 | 3958,9
205,8 | 3849,9
247,6 | 4013,9
228,2 |
| Cd | 0,018
0,016 | 0,043
0,038 | 0,130
0,077 | 0,035
0,030 | 0,022
0,029 | 0,012
0,013 | 0,003
0,005 | 0,017
0,014 | 0,041
0,023 | 0,027
0,019 |
| Ba | 5,591
0,632 | 5,608
0,623 | 6,651
0,572 | 5,342
0,591 | 5,563
0,420 | 5,794
0,365 | 4,895
0,410 | 5,337
0,478 | 5,291
0,248 | 6,411
0,456 |
| Pb | 0,022
0,018 | 0,005
0,006 | 0,010
0,016 | 0,021
0,011 | 0
0,001 | 0,003
0,006 | 0,004
0,009 | 0,020
0,027 | 0,008
0,016 | 0,030
0,015 |

Table VII - One-way ANOVA testing for differences in elemental concentration (relative to mean Ca concentration) in the lateral shell of *Pollicipes pollicipes* between sites

| | Source | SS | df | MS | F | p | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-----------|--------|----------|----|----------|----------|----------|-----------|------|----------|---|----------|----------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|----------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|----------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|----------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|----------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|----------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|---------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|---------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|---------|----------|-------|----------|----|----------|-----------|------|----------|---|----------|---------|----------|-------|
| Li | Site | 0,3135 | 9 | 0,0348 | 9,494 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,3229 | 88 | 0,0037 | | | B | Site | 0,6204 | 9 | 0,0689 | 12,825 | 0,000000 | Error | 0,4730 | 88 | 0,0054 | Mg | Site | 0,232 | 9 | 0,026 | 7,00 | 0,000000 | Error | 0,324 | 88 | 0,004 | P | Site | 0,2578 | 9 | 0,0286 | 6,58 | 0,000000 | Error | 0,3829 | 88 | 0,0044 | Cr | Site | 3,844991 | 9 | 0,427221 | 50,10215 | 0,000000 | Error | 0,750376 | 88 | 0,008527 | Mn | Site | 3,029981 | 9 | 0,336665 | 14,14660 | 0,000000 | Error | 2,094247 | 88 | 0,023798 | Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | Error | 4,71860 | 88 | 0,05362 | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error |
| B | Site | 0,6204 | 9 | 0,0689 | 12,825 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,4730 | 88 | 0,0054 | | | Mg | Site | 0,232 | 9 | 0,026 | 7,00 | 0,000000 | Error | 0,324 | 88 | 0,004 | P | Site | 0,2578 | 9 | 0,0286 | 6,58 | 0,000000 | Error | 0,3829 | 88 | 0,0044 | Cr | Site | 3,844991 | 9 | 0,427221 | 50,10215 | 0,000000 | Error | 0,750376 | 88 | 0,008527 | Mn | Site | 3,029981 | 9 | 0,336665 | 14,14660 | 0,000000 | Error | 2,094247 | 88 | 0,023798 | Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | Error | 4,71860 | 88 | 0,05362 | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | |
| Mg | Site | 0,232 | 9 | 0,026 | 7,00 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,324 | 88 | 0,004 | | | P | Site | 0,2578 | 9 | 0,0286 | 6,58 | 0,000000 | Error | 0,3829 | 88 | 0,0044 | Cr | Site | 3,844991 | 9 | 0,427221 | 50,10215 | 0,000000 | Error | 0,750376 | 88 | 0,008527 | Mn | Site | 3,029981 | 9 | 0,336665 | 14,14660 | 0,000000 | Error | 2,094247 | 88 | 0,023798 | Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | Error | 4,71860 | 88 | 0,05362 | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | |
| P | Site | 0,2578 | 9 | 0,0286 | 6,58 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,3829 | 88 | 0,0044 | | | Cr | Site | 3,844991 | 9 | 0,427221 | 50,10215 | 0,000000 | Error | 0,750376 | 88 | 0,008527 | Mn | Site | 3,029981 | 9 | 0,336665 | 14,14660 | 0,000000 | Error | 2,094247 | 88 | 0,023798 | Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | Error | 4,71860 | 88 | 0,05362 | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cr | Site | 3,844991 | 9 | 0,427221 | 50,10215 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,750376 | 88 | 0,008527 | | | Mn | Site | 3,029981 | 9 | 0,336665 | 14,14660 | 0,000000 | Error | 2,094247 | 88 | 0,023798 | Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | Error | 4,71860 | 88 | 0,05362 | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Mn | Site | 3,029981 | 9 | 0,336665 | 14,14660 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 2,094247 | 88 | 0,023798 | | | Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | Error | 4,71860 | 88 | 0,05362 | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Zn | Site | 7,96434 | 9 | 0,88493 | 16,50353 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 4,71860 | 88 | 0,05362 | | | Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | Error | 0,046 | 88 | 0,001 | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Sr | Site | 0,052 | 9 | 0,006 | 11,0 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,046 | 88 | 0,001 | | | Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | Error | 5,6533 | 88 | 0,0642 | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cd | Site | 6,4893 | 9 | 0,7210 | 11,2237 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 5,6533 | 88 | 0,0642 | | | Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | Error | 0,18487 | 88 | 0,00210 | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Ba | Site | 0,15019 | 9 | 0,01669 | 7,944 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 0,18487 | 88 | 0,00210 | | | Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Pb | Site | 1,523912 | 9 | 0,169324 | 7,35993 | 0,000000 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | Error | 2,024540 | 88 | 0,023006 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Table VIII - Order the variables were entered in the stepwise analysis, and standardized canonical coefficients (DF weights) for the first two discriminant functions

| Step | Entered | Lambda | DF1 | DF2 | DF3 | <i>p</i> |
|------|---------|--------|--------|--------|--------|----------|
| 1 | Cr | 0,163 | 1,275 | -0,194 | -0,308 | <0,001 |
| 2 | Zn | 0,061 | 0,191 | -0,550 | -0,756 | <0,001 |
| 3 | Mn | 0,021 | -0,589 | 0,175 | 1,106 | <0,001 |
| 4 | Cd | 0,010 | -0,207 | -0,533 | 0,204 | <0,001 |
| 5 | Mg | 0,005 | -0,115 | 1,220 | -0,677 | <0,001 |
| 6 | Ba | 0,003 | -0,230 | -0,752 | 0,319 | <0,001 |
| 7 | Pb | 0,002 | -0,231 | 0,308 | 0,227 | <0,001 |
| 8 | B | 0,001 | -0,119 | 0,267 | 0,049 | <0,001 |
| 9 | Sr | 0,001 | -0,002 | 0,646 | -0,057 | <0,001 |
| 10 | Li | 0,001 | 0,399 | -0,446 | -0,011 | <0,001 |

The DFA yielded the canonical functions observed in Table IX, the first 3 functions are responsible for explaining 82,8% of the observed variance, function 1 (F1) accounts for almost half of the total, with 49,9%, function 2 (F2) with 19,4% and function 3 (F3) justifying 13,5%.

Table IX - Eigenvalues, % of Variance, Cumulative % and Canonical correlation of the canonical functions used to in the analysis

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|----------|------------|---------------|--------------|-----------------------|
| 1 | 8,988 | 49,9 | 49,9 | 0,949 |
| 2 | 3,497 | 19,4 | 69,3 | 0,882 |
| 3 | 2,435 | 13,5 | 82,8 | 0,842 |
| 4 | 1,655 | 9,2 | 92,0 | 0,790 |
| 5 | 0,694 | 3,9 | 95,9 | 0,640 |
| 6 | 0,369 | 2,0 | 97,9 | 0,519 |
| 7 | 0,269 | 1,5 | 99,4 | 0,461 |
| 8 | 0,093 | 0,5 | 99,9 | 0,292 |
| 9 | 0,015 | 0,1 | 100 | 0,122 |

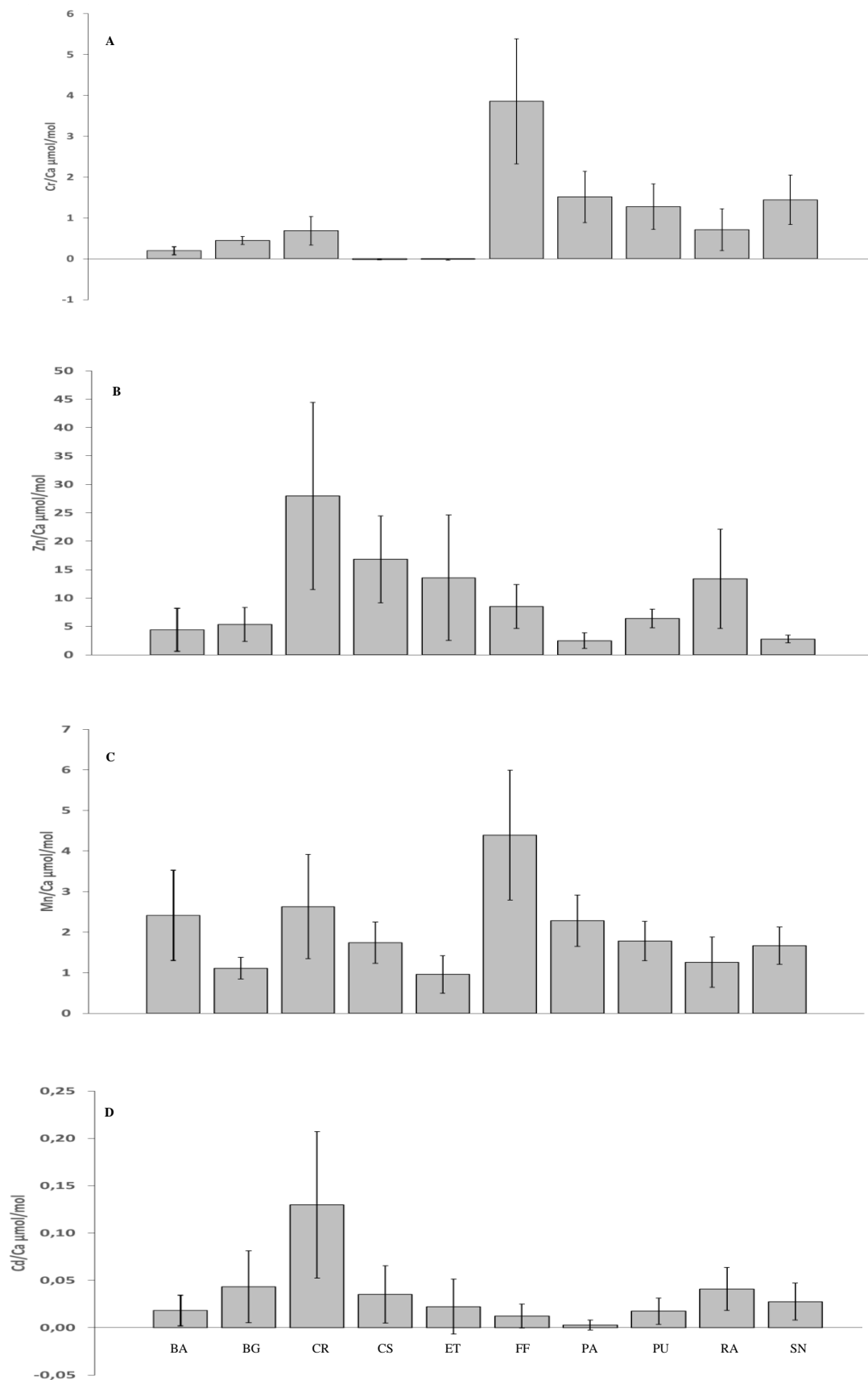
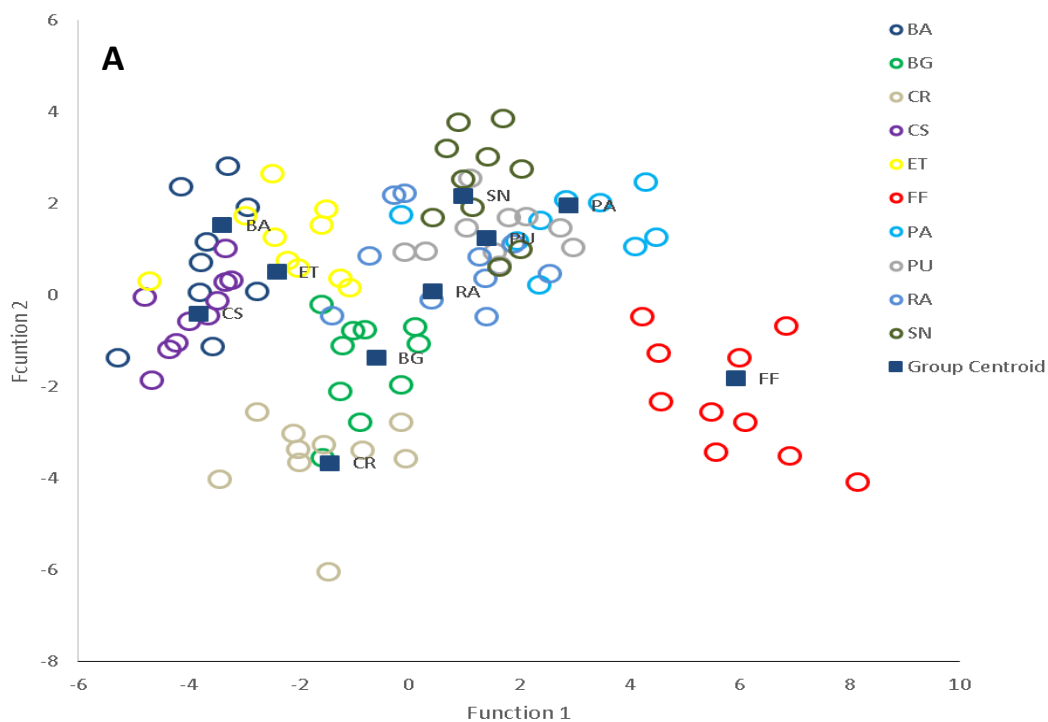


Figure 8 - Element concentrations (relative to mean Ca concentration) mean (\pm SD) of the 4 most discriminant elements in the largest lateral plate of *Pollicipes pollicipes* capitulum. A Cr $\mu\text{mol/mol Ca}^{-1}$, B Zn $\mu\text{mol/mol Ca}^{-1}$, C Mn $\mu\text{mol/mol Ca}^{-1}$ and D Cd $\mu\text{mol/mol Ca}^{-1}$

These canonical functions were plotted against each other to help graphically comprehend the differences. (Fig. 9). It is clear the grouping amongst sites, with individuals of each site closer to their own group centroid, FF is in the X axis completely separated from the other groups due to a higher concentration in Cr, as shown in Table VIII DF values, function 1 distinguishes primarily groups based on Cr concentrations, since FF has the highest values it appears leaning to the positive side of the Axis, while BA leans to the negative half of the Axis due to low concentration in Mn. Function 2 shows CR leaning towards the negative side due high amounts in Zn, unlike SN, PA and BA owing to low concentrations in Zn and Cd their positive trend. Function 3 is influenced by concentration in Mn and Zn in the positive and negative half respectively.

The patterns shown below in (Fig. 9) indicate an ability to differentiate and classify samples to their own group of origin, which is confirmed in Table X, the number of cross-validated samples correctly reclassified is between 60% and 100% (RA and FF respectively). Sites from the BNR scores range from the mentioned 60% for RA to 70% in BG and 80% in ET, both of them close to the average of grouped cases correctly classified of 77,6%.



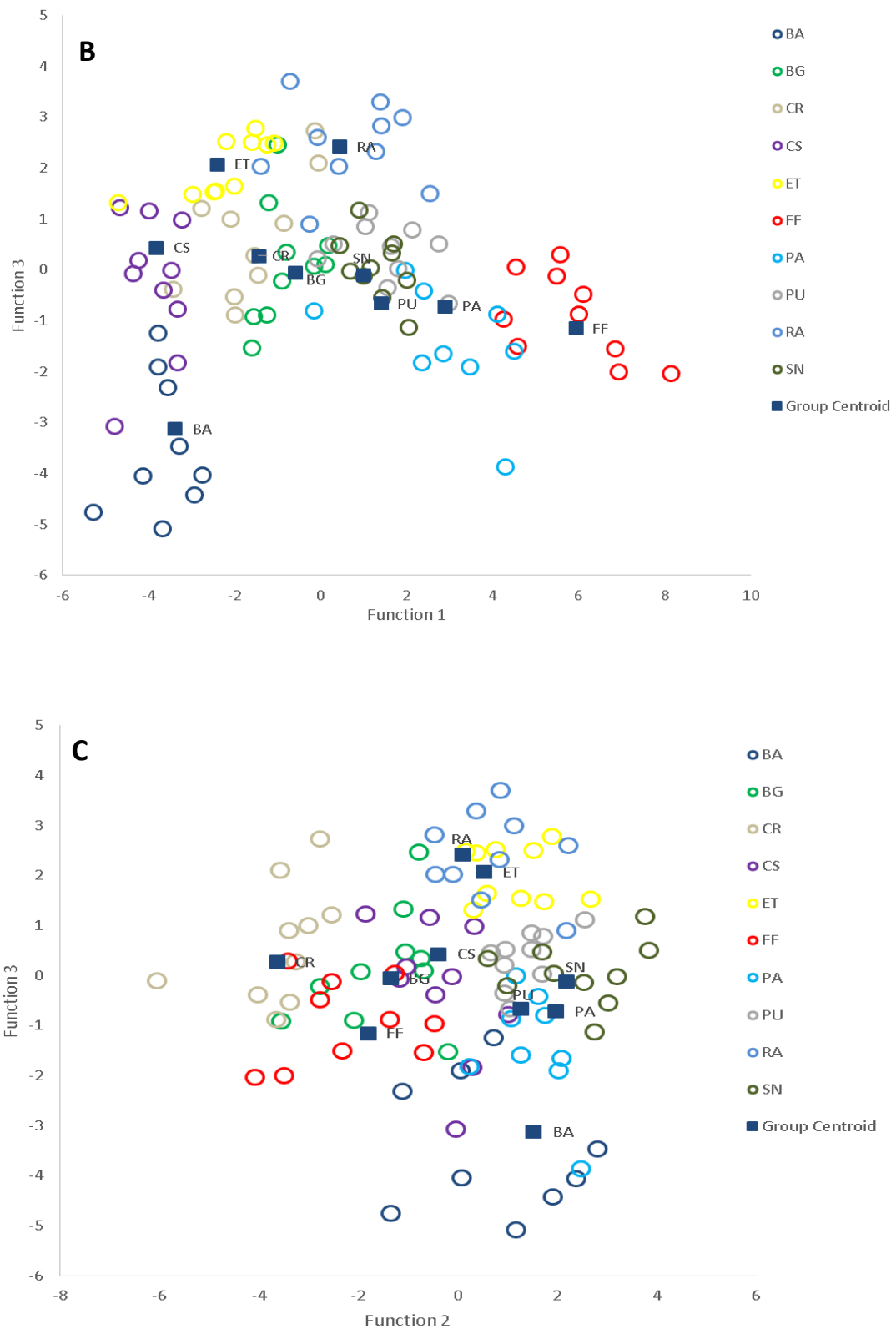


Figure 9 - Results of the Canonical Discriminant Functions of the elemental microchemistry. A Function 1*Function2, B Function 1*Function3, C Function 2*Function3. Colored circles represent Sites, each circle being a sample

Table X - Percentage of goose barnacles correctly classified to each origin in the elemental microchemistry. Columns are the predicted group for each individual, rows are the origin group of each individual. Data are cross-validated

| Sites | Predicted Group Membership | | | | | | | | | | |
|-----------------|----------------------------|-----------|-----------|-----------|-----------|------------|-------------|-----------|-----------|-----------|-------|
| | BA | BG | CR | CS | ET | FF | PA | PU | RA | SN | Total |
| Cross validated | | | | | | | | | | | |
| BA | 77,8 | 0 | 0 | 11,1 | 0 | 0 | 0 | 0 | 0 | 11,1 | 100 |
| BG | 0 | 70 | 10 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 100 |
| CR | 0 | 30 | 70 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| CS | 10 | 0 | 0 | 80 | 10 | 0 | 0 | 0 | 0 | 0 | 100 |
| ET | 0 | 10 | 0 | 10 | 80 | 0 | 0 | 0 | 0 | 0 | 100 |
| FF | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 |
| PA | 0 | 0 | 0 | 0 | 0 | 0 | 88,9 | 11,1 | 0 | 0 | 100 |
| PU | 0 | 0 | 0 | 0 | 10 | 0 | 0 | 70 | 10 | 10 | 100 |
| RA | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 10 | 60 | 10 | 100 |
| SN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 80 | 100 |

4.3 Fatty acid profile

The total lipids found to be present in the sampled goose barnacles ranged from 3,249 mg/100 mg in SN to 6,954 mg/100 mg in FF.

In the fatty acid profile analysis chromatograms similar to Fig. 10 were acquired.

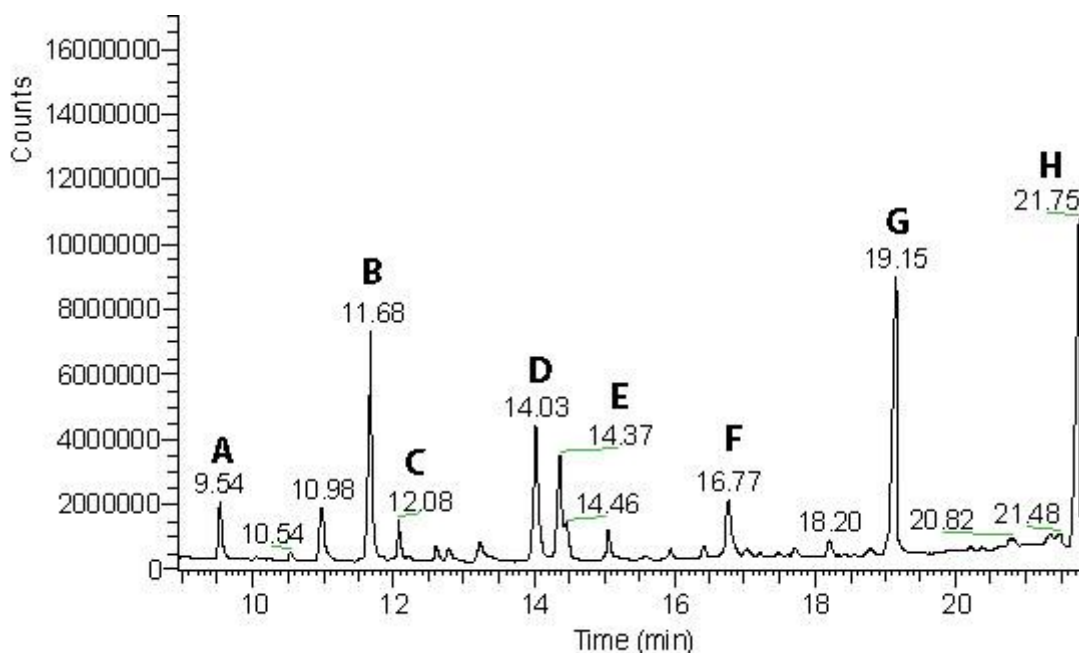


Figure 10 - Illustrative chromatogram of the fatty acid profile of *P. pollicipes*. Letters identify the most represented fatty acids, where A Myristic acid (C14:0), B Palmitic acid (C16:0), C Palmitoleic acid (C16:1 n7), D Stearic acid (C18:0), E Oleic acid (C18:1 n9), F Eicosenoic acid (C20:1 n9), G Eicosapentaenoic acid (C20:5 n3) and H Docosahexaenoic acid (C22:6 n3)

Regarding the fatty acid mean (\pm SD) content, a summary is shown in Table XI, 14 fatty acids were successfully identified while only 4 were not possible to identify and therefore named FA 1 to FA 4 according to their retention time in the chromatogram. When grouping FAs in classes, saturated FAs (SFA) represented 0,976 mg/100mg, monounsaturated FAs (MUFA) represented 0,6759 mg/100mg, as for the polyunsaturated FAs (PUFA) were the most important class of FAs recorded in the sampled goose barnacles, with an average content of 1,945 mg/100mg accounting for more than 50% of all FAs.

Regarding only the identified FA's, stearic acid (C18:0) was the most abundant fatty acid with an average of 0,6516 mg/100mg closely followed by Docosahexaenoic

acid - DHA (C22:6 n3) with 0,6298 mg/100mg and Eicosapentaenoic acid (EPA) (C20:5 n3) with 0,5761 mg/100mg, in the opposite side the least abundant were Palmitic acid (C16:0) and Arachidonic acid - ARA (C20:4 n6) with 0,0102 mg/100mg and 0,0157 mg/100mg correspondingly. Only 3 fatty acids – C14:0; C16:1 and C18:0 - were accounted for in all 10 sites.

Table XI - Mean content \pm SD of Total Lipids (TL) and fatty acid profile of *Pollicipes pollicipes* per site. Total lipids and fatty acid concentration is shown in mg/100mg. Average content on top, standard deviation on bottom

| | BA | BG | CR | CS | ET | FF | PA | PU | RA | SN |
|-----------------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|
| TL | 3,435 | 4,129 | 6,365 | 5,318 | 3,670 | 6,954 | 4,472 | 5,582 | 3,898 | 3,249 |
| | 1,365 | 2,107 | 1,105 | 1,664 | 1,313 | 1,636 | 1,636 | 2,184 | 1,183 | 0,809 |
| C14:0 | 0,165 | 0,278 | 0,3938 | 0,238 | 0,376 | 0,578 | 0,294 | 0,317 | 0,286 | 0,217 |
| | 0,071 | 0,223 | 0,110 | 0,171 | 0,145 | 0,144 | 0,182 | 0,152 | 0,098 | 0,116 |
| C16:0 | 0,009 | 0,008 | 0,015 | 0,011 | - | 0,017 | 0,011 | 0,013 | 0,010 | 0,007 |
| | 0,004 | 0,004 | 0,005 | 0,003 | - | 0,004 | 0,003 | 0,005 | 0,003 | 0,002 |
| C16:1 n7 | 0,070 | 0,169 | 0,187 | 0,145 | 0,941 | 0,504 | 0,163 | 0,249 | 0,016 | 0,065 |
| | 0,058 | 0,103 | 0,076 | 0,112 | 0,313 | 0,137 | 0,077 | 0,137 | 0,050 | 0,091 |
| C16:2 n4 | 0,081 | - | - | 0,014 | 0,039 | - | 0,018 | 0,110 | - | - |
| | 0,062 | - | - | 0,033 | 0,065 | - | 0,029 | 0,051 | - | - |
| C18:0 | 0,756 | 1,078 | 0,880 | 1,247 | 0,417 | 0,470 | 0,433 | 0,509 | 0,674 | 0,039 |
| | 0,340 | 0,377 | 0,401 | 0,297 | 0,153 | 0,176 | 0,127 | 0,237 | 0,203 | 0,064 |
| C18:1 n7 | 0,422 | - | - | 0,161 | 0,268 | 0,772 | 0,425 | - | 0,361 | 0,236 |
| | 0,245 | - | - | 0,217 | 0,111 | 0,261 | 0,242 | - | 0,163 | 0,068 |
| C18:1 n9 | - | 0,239 | 0,556 | - | - | - | - | 0,475 | - | - |
| | - | 0,116 | 0,209 | - | - | - | - | 0,208 | - | - |
| C18:2 n6 | 0,186 | - | 0,256 | 0,102 | 0,008 | 0,806 | - | 0,173 | - | 0,011 |
| | 0,156 | - | 0,04 | 0,095 | 0,026 | 0,677 | - | 0,088 | - | 0,036 |
| FA 1 | - | - | - | 0,315 | - | - | - | - | - | - |
| | - | - | - | 0,861 | - | - | - | - | - | - |

Results

| | | | | | | | | | | |
|-----------------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|
| C18:4 n3 | 0,010 | - | 0,044 | 0,004 | - | - | - | 0,102 | - | - |
| | 0,030 | - | 0,075 | 0,014 | - | - | - | 0,060 | - | - |
| C20:1 n9 | 0,116 | 0,057 | 0,008 | 0,005 | - | 0,038 | 0,019 | 0,079 | - | - |
| | 0,072 | 0,064 | 0,025 | 0,012 | - | 0,044 | 0,030 | 0,068 | - | - |
| FA 2 | - | - | - | 0,100 | - | - | 0,041 | - | 0,010 | - |
| | - | - | - | 0,081 | - | - | 0,044 | - | 0,032 | - |
| FA 3 | 0,045 | - | - | 0,002 | 0,018 | 0,121 | 0,067 | - | - | - |
| | 0,090 | - | - | 0,008 | 0,057 | 0,055 | 0,047 | - | - | - |
| C20:4 n6 | 0,064 | - | 0,010 | - | - | - | 0,005 | 0,077 | - | - |
| | 0,044 | - | 0,030 | - | - | - | 0,015 | 0,046 | - | - |
| C20:5 n3 | 0,519 | 0,944 | 1,068 | 1,030 | 0,900 | 1,038 | - | - | 0,127 | 0,132 |
| | 0,286 | 0,306 | 0,458 | 0,223 | 0,380 | 0,403 | - | - | 0,088 | 0,060 |
| C22:5 n3 | 0,114 | 0,061 | 0,012 | 0,0306 | - | - | 1,046 | 0,961 | 1,140 | 1,902 |
| | 0,060 | 0,059 | 0,037 | 0,049 | - | - | 0,325 | 0,239 | 0,495 | 0,448 |
| C22:6 n3 | 0,395 | 1,099 | 0,803 | 0,613 | 0,687 | - | 0,569 | 1,049 | 0,667 | 0,413 |
| | 0,209 | 0,477 | 0,251 | 0,237 | 0,378 | - | 0,461 | 0,427 | 0,416 | 0,231 |
| FA 4 | - | - | - | - | 0,014 | 0,005 | - | - | - | - |
| | - | - | - | - | 0,031 | 0,016 | - | - | - | - |
| Σ-3 | 1,039 | 1,999 | 1,909 | 1,624 | 1,725 | 2,034 | 1,864 | 2,231 | 1,630 | 1,455 |
| | 0,567 | 0,852 | 0,496 | 0,572 | 0,628 | 0,468 | 0,957 | 0,927 | 0,564 | 0,300 |
| Σ-6 | 0,251 | - | 0,266 | 0,102 | 0,008 | 0,806 | 0,005 | 0,250 | - | 0,011 |
| | 0,189 | - | 0,044 | 0,095 | 0,026 | 0,677 | 0,015 | 0,119 | - | 0,036 |
| SFA | 0,930 | 1,365 | 1,29 | 1,496 | 0,793 | 1,066 | 0,748 | 0,840 | 0,971 | 0,264 |
| | 0,393 | 0,556 | 0,434 | 0,658 | 0,229 | 0,298 | 0,219 | 0,250 | 0,333 | 0,113 |
| MUFA | 0,609 | 0,466 | 0,752 | 0,312 | 1,209 | 1,316 | 0,608 | 0,804 | 0,377 | 0,302 |
| | 0,186 | 0,108 | 0,260 | 0,087 | 0,444 | 0,374 | 0,196 | 0,210 | 0,178 | 0,111 |
| PUFA | 1,372 | 2,105 | 2,195 | 1,795 | 1,635 | 1,844 | 1,640 | 2,473 | 1,935 | 2,460 |
| | 0,189 | 0,495 | 0,441 | 0,406 | 0,387 | 0,455 | 0,415 | 0,448 | 0,452 | 0,700 |

Other than FA 4, all of the analysed FAs, when tested for differences between sites showed statistical significant differences ($p < 0,01$) for Σ -3 and ($p < 0,0001$) for the remaining fatty acids (Table XII).

Table XII - One-way ANOVA testing for differences in the fatty acid content of *Pollicipes pollicipes* muscle between sites

| | Source | SS | df | MS | F | p |
|-----------------|--------|----------|----|----------|----------|----------|
| C14:0 | Site | 1,175326 | 9 | 0,130592 | 5,96944 | 0,000002 |
| | Error | 1,925151 | 88 | 0,021877 | | |
| C16:0 | Site | 0,002064 | 9 | 0,000229 | 15,214 | 0,000000 |
| | Error | 0,001327 | 88 | 0,000015 | | |
| C16:1 n7 | Site | 7,20370 | 9 | 0,80041 | 30,63339 | 0,000000 |
| | Error | 2,29933 | 88 | 0,02613 | | |
| C16:2 n4 | Site | 1,09456 | 9 | 0,12162 | 14,4212 | 0,000000 |
| | Error | 0,74213 | 88 | 0,00843 | | |
| C18:0 | Site | 7,026396 | 9 | 0,780711 | 38,55709 | 0,000000 |
| | Error | 1,781839 | 88 | 0,020248 | | |
| C18:1 n7 | Site | 0,568807 | 9 | 0,063201 | 25,10880 | 0,000000 |
| | Error | 0,221503 | 88 | 0,002517 | | |
| C18:1 n9 | Site | 10,14385 | 9 | 1,12709 | 190,6816 | 0,000000 |
| | Error | 0,52016 | 88 | 0,00591 | | |
| C18:2 n6 | Site | 8,18308 | 9 | 0,90923 | 52,25969 | 0,000000 |
| | Error | 1,53105 | 88 | 0,01740 | | |
| FA 1 | Site | 3,1040 | 9 | 0,3449 | 4,705 | 0,000041 |
| | Error | 6,4504 | 88 | 0,0733 | | |
| C18:4 n3 | Site | 8,2698 | 9 | 0,9189 | 14,2736 | 0,000000 |
| | Error | 5,6650 | 88 | 0,0644 | | |
| C20:1 n9 | Site | 0,137837 | 9 | 0,015315 | 8,670047 | 0,000000 |
| | Error | 0,155448 | 88 | 0,001766 | | |
| FA 2 | Site | 7,0759 | 9 | 0,7862 | 11,7047 | 0,000000 |
| | Error | 5,9111 | 88 | 0,0672 | | |
| FA 3 | Site | 1,13482 | 9 | 0,12609 | 12,8369 | 0,000000 |
| | Error | 0,86439 | 88 | 0,00982 | | |
| C20:4 n6 | Site | 9,8457 | 9 | 1,0940 | 27,4238 | 0,000000 |
| | Error | 3,5104 | 88 | 0,0399 | | |
| C20:5 n3 | Site | 1,599212 | 9 | 0,177690 | 47,70786 | 0,000000 |
| | Error | 0,327760 | 88 | 0,003725 | | |
| C22:5 n3 | Site | 24,80772 | 9 | 2,75641 | 190,9146 | 0,000000 |
| | Error | 1,27054 | 88 | 0,01444 | | |
| C22:6 n3 | Site | 0,729632 | 9 | 0,081070 | 11,62877 | 0,000000 |
| | Error | 0,613493 | 88 | 0,006972 | | |

| | | | | | | |
|-------------|-------|---------|----|---------|----------|----------|
| FA 4 | Site | 0,3166 | 9 | 0,0352 | 1,65 | 0,114793 |
| | Error | 1,8817 | 88 | 0,0214 | | |
| Σ -3 | Site | 0,3115 | 9 | 0,0346 | 3,125 | 0,002612 |
| | Error | 0,9746 | 88 | 0,0111 | | |
| Σ -6 | Site | 4,31434 | 9 | 0,47937 | 59,27139 | 0,000000 |
| | Error | 0,71172 | 88 | 0,00809 | | |
| SFA | Site | 0,2780 | 9 | 0,0309 | 18,49 | 0,000000 |
| | Error | 0,1470 | 88 | 0,0017 | | |
| MUFA | Site | 3,5012 | 9 | 0,3890 | 16,3247 | 0,000000 |
| | Error | 2,0971 | 88 | 0,0238 | | |
| PUFA | Site | 0,1418 | 9 | 0,0158 | 3,87 | 0,000366 |
| | Error | 0,3584 | 88 | 0,0041 | | |

Despite the unidentified fatty acids recording low concentrations, they were kept in the analysis for discriminant function analysis since only FA 4 did not show statistical significant differences. The DFA entered 21 variables, excluding C18:2 n6 and C18:4 n3, (**Table XV**), the first variables to enter the stepwise analysis were C22:5 n3 (DPA), C18:1 n9, Σ -6 followed by the most abundant C18:0, variability among sites between these FAs can be effortlessly observed in **Fig. 11**.

Table XIII - Order the variables were entered in the stepwise analysis, and standardized canonical coefficients (DF weights) for the first two discriminant functions

| Step | Entered | Lambda | DF1 | DF2 | DF3 | <i>p</i> |
|------|-----------|--------|--------|--------|--------|----------|
| 1 | C22:5 n-3 | 0,049 | 1,152 | -0,090 | -0,231 | <0,001 |
| 2 | C18:1 n-9 | 0,020 | 0,002 | 0,994 | -0,283 | <0,001 |
| 3 | Σ-6 | 0,000 | -0,162 | -0,161 | -0,177 | <0,001 |
| 4 | C18:0 | 0,000 | -0,521 | 0,515 | 0,482 | <0,001 |
| 5 | C16:1 n-7 | 0,000 | -0,403 | -0,183 | 0,058 | <0,001 |
| 6 | C20:4 n-6 | 0,000 | 0,029 | 0,058 | 0,061 | <0,001 |
| 7 | C20:5 n-3 | 0,000 | -0,335 | -0,391 | -0,069 | <0,001 |
| 8 | FA 2 | 0,000 | 0,125 | -0,228 | 0,160 | <0,001 |
| 9 | C14:0 | 0,000 | 0,149 | -0,091 | 0,034 | <0,001 |
| 10 | PUFA | 0,000 | -0,172 | -0,286 | -1,166 | <0,001 |
| 11 | FA 1 | 0,000 | 0,254 | -0,205 | -0,096 | <0,001 |
| 12 | C20:1 n-9 | 0,000 | 0,514 | 0,154 | 0,596 | <0,001 |
| 13 | MUFA | 0,000 | -0,177 | 0,145 | 0,345 | <0,001 |
| 14 | C18:1 n-7 | 0,000 | 0,008 | 0,021 | 0,007 | <0,001 |
| 15 | SFA | 0,000 | 0,079 | -0,002 | 0,188 | <0,001 |
| 16 | C16:2 n-4 | 0,000 | 0,051 | -0,244 | -0,197 | <0,001 |
| 17 | C22:6 n-3 | 0,000 | -0,096 | 0,030 | 0,179 | <0,001 |
| 18 | C16:0 | 0,000 | 0,203 | 0,089 | 0,489 | <0,001 |
| 19 | FA 4 | 0,000 | 0,205 | 0,010 | -0,485 | <0,001 |
| 20 | Σ-3 | 0,000 | 0,036 | 0,049 | 0,001 | <0,001 |
| 21 | FA 3 | 0,000 | -0,094 | -0,241 | -0,489 | <0,001 |

Due to a sample comprised of 10 sites, DFA returns 9 canonical functions, as seen below in Table XIV, Function 1 accounts for 46% of variance, Function 2 to 24,9% and Function 3 to 11,4 summing up to 82,2% of the total variance explained by the first 3 functions, exhibiting strong canonical correlations (<0,96).

Table XIV - Eigenvalues, % of Variance, Cumulative % and Canonical correlation of the canonical functions used to in the analysis

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|----------|------------|---------------|--------------|-----------------------|
| 1 | 49,333 | 46,0 | 46,0 | 0,990 |
| 2 | 26,677 | 24,9 | 70,8 | 0,982 |
| 3 | 12,229 | 11,4 | 82,2 | 0,961 |
| 4 | 8,350 | 7,8 | 90,0 | 0,945 |
| 5 | 5,335 | 5,0 | 95,0 | 0,918 |
| 6 | 2,693 | 2,5 | 97,5 | 0,854 |
| 7 | 1,799 | 1,7 | 99,2 | 0,802 |
| 8 | 0,593 | 0,6 | 99,7 | 0,610 |
| 9 | 0,290 | 0,3 | 100 | 0,474 |

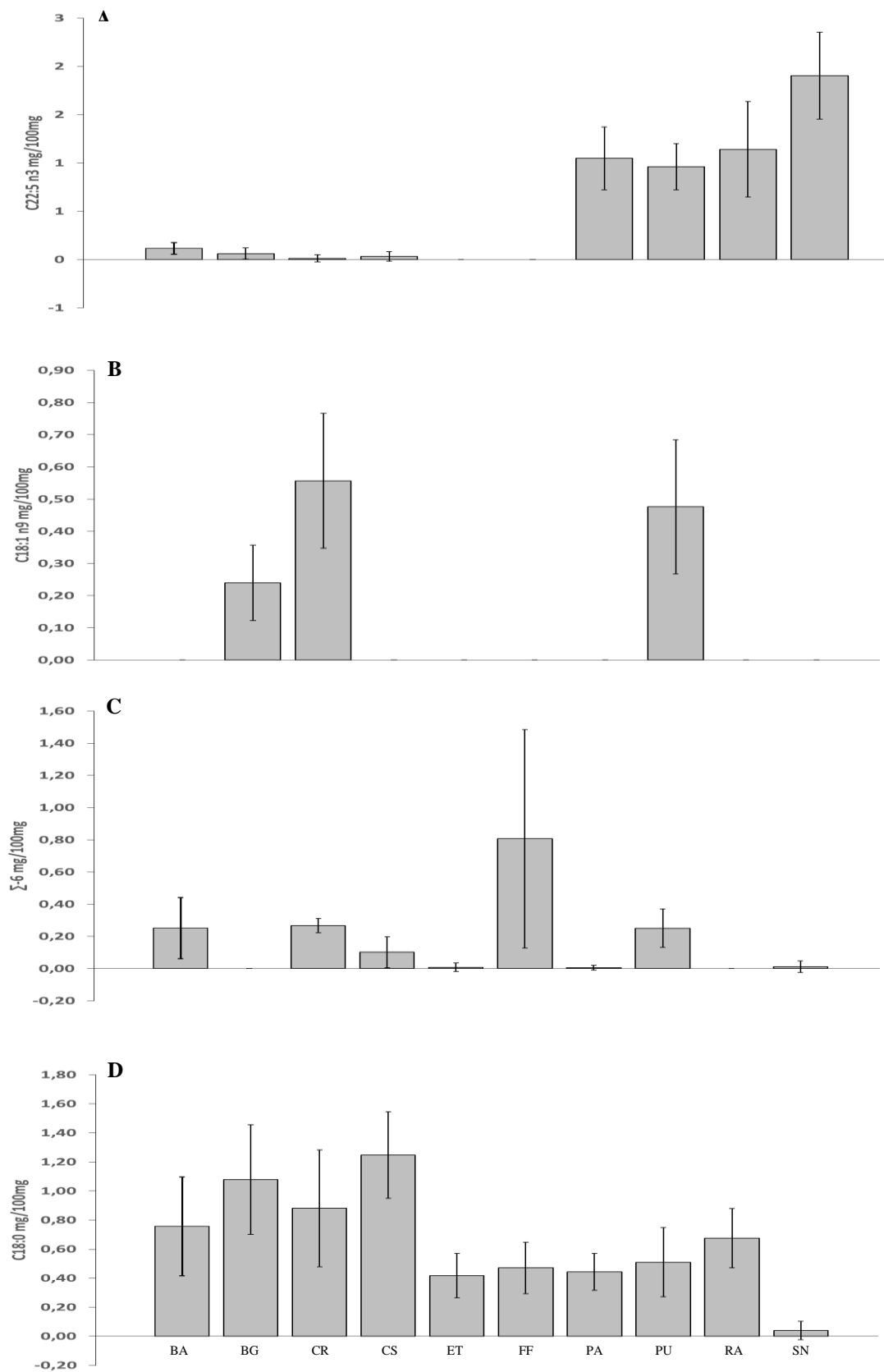
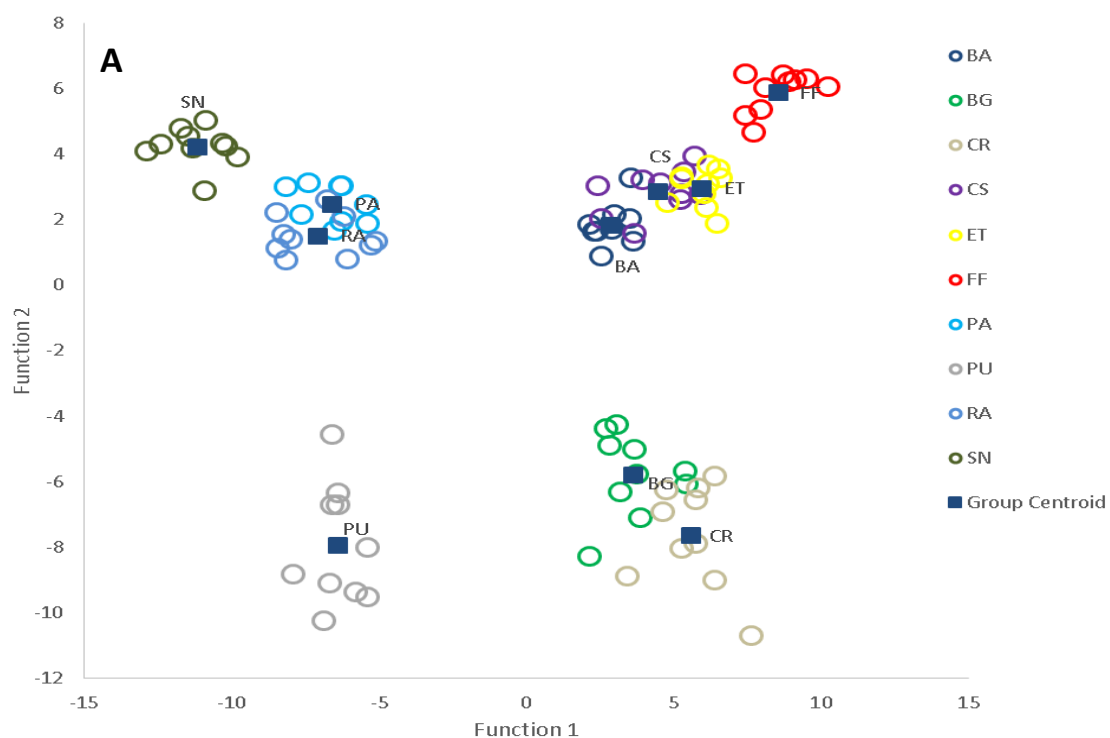


Figure 11 - Fatty acid content (relative to 100mg), mean (\pm SD) of the 4 most discriminant fatty acids in *Pollicipes pollicipes* muscle. A C22:5 n3 mg/100mg, B C18:1 n9 mg/100mg, C Σ -6 mg/100mg and D C18:0 mg/100mg

With the plot of the first 3 canonical functions (Fig. 12), most of the sites are separated and clearly clustered on their group centroid, indicating the entered variables have sufficient differences to distinguish individuals and to classify them correctly to the group of origin. Function 1 is distinctly separating SN, PA, RA and PU to one side and the remaining sites to the opposite side based on the content in DPA – docosapentaenoic acid, function 2 is mainly able to distinguish sites based on content in Oleic acid (C18:1 n9), with sites PU, BG and CR leaning to the negative side of the axis while all the remaining sites, without the presence of Oleic acid remain in the positive side of the axis. Finally function 3 differentiates sites due to content in Docosahexaenoic acid – DHA, as demonstrated by the negative side position of site FF and the positive half of the axis position of BG.



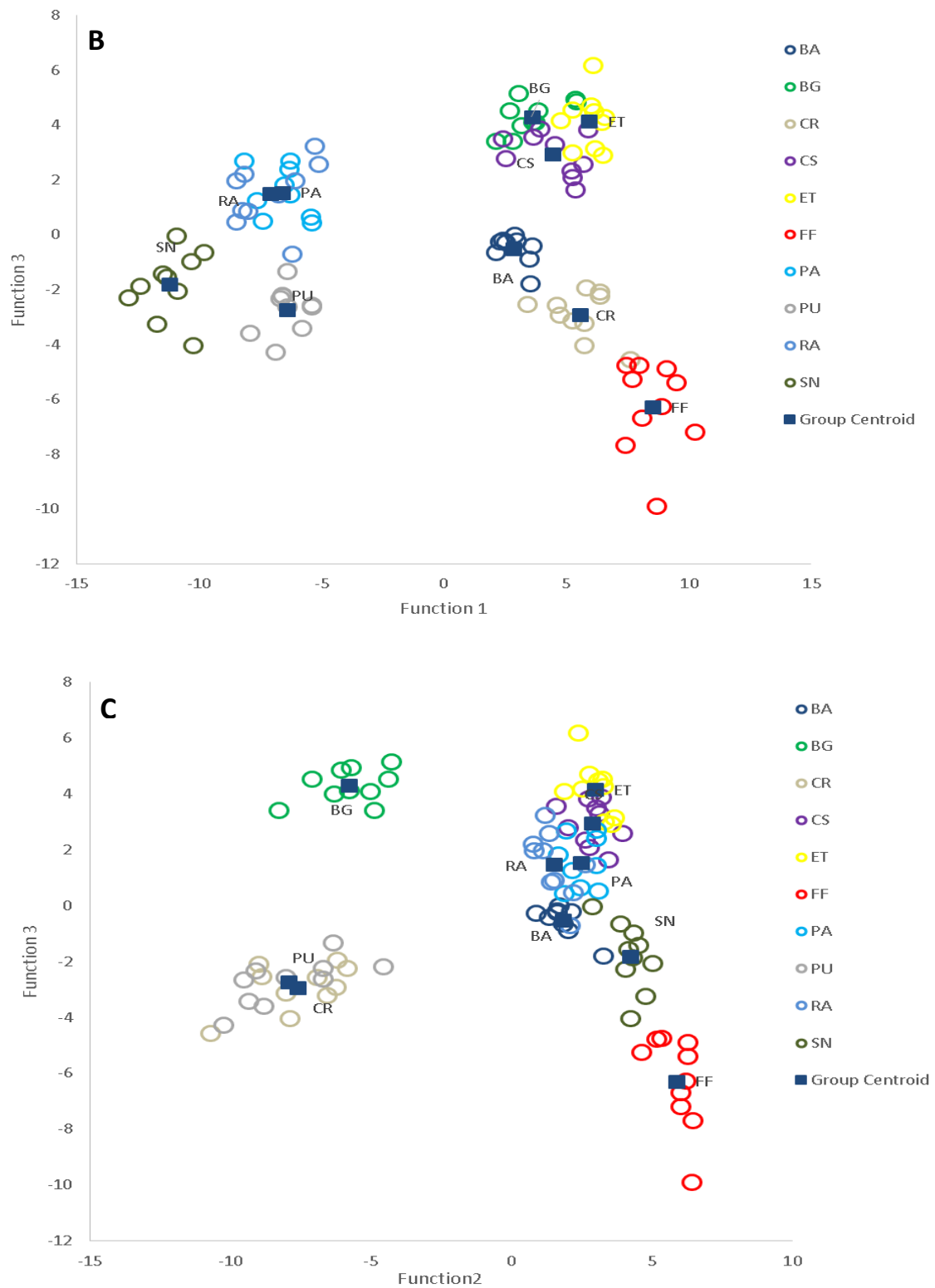


Figure 12 - Results of the Canonical Discriminant Functions of the fatty acid content. A Function 1*Function2, B Function 1*Function3, C Funtion2*Function3. Colored circles represent Sites, each circle being a sample

Table XV - Percentage of goose barnacles correctly classified to each origin in the fatty acid profile. Columns are the predicted group for each individual, rows are the origin group of each individual. Data are cross-validated

| Sites | Predicted Group Membership | | | | | | | | | | Total |
|-----------------|----------------------------|------------|------------|------------|------------|------------|------------|------------|-----------|------------|-------|
| | BA | BG | CR | CS | ET | FF | PA | PU | RA | SN | |
| Cross validated | | | | | | | | | | | |
| BA | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| BG | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| CR | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| CS | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| ET | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 100 |
| FF | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 |
| PA | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 100 |
| PU | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 100 |
| RA | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 | 90 | 0 | 100 |
| SN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 |

Inspecting Table XV, it confirms the Fig. 12 previously suggested good reclassification success, only RA had one misclassified individual to PA – Peniche, the remaining individuals were all successfully reclassified in relation to provenance location, generating an average reclassification success of 99%.

4.4 Combined approach – Elemental microchemistry and fatty acid profile

Subsequently to the previous analysis, due to the good results obtained with both Elemental microchemistry of the capitulum's largest lateral shell and the muscle Fatty acid content of *P. pollicipes*, a final stepwise discriminant function analysis consisting of all EM and FA variables was performed to determine the effect of variables from distinct analysis computed together.

To no surprise, owing to the fatty acid content reclassification performance, DFA entered the same 3 first variables as in the FA analysis alone (C22:5 n3; C18:1 n9 and Σ -6), nonetheless the fourth entered variable was Cr and not C18:0 as in the FA single analysis (Table XVI).

Table XVI - Order the variables were entered in the stepwise analysis, and standardized canonical coefficients (DF weights) for the first two discriminant functions

| Step | Entered | Lambda | DF1 | DF2 | DF3 | <i>p</i> |
|------|-------------|--------|--------|--------|--------|----------|
| 1 | C22:5 n3 | 0,049 | 1,190 | 0,085 | 0,104 | <0,001 |
| 2 | C18:1 n9 | 0,020 | -0,194 | 1,081 | 0,151 | <0,001 |
| 3 | Σ -6 | 0,000 | -0,085 | -0,035 | 1,040 | <0,001 |
| 4 | Cr | 0,000 | 0,332 | -0,027 | 0,738 | <0,001 |
| 5 | C18:0 | 0,000 | -0,607 | 0,444 | -0,216 | <0,001 |
| 6 | C16:1 n7 | 0,000 | -0,234 | -0,341 | 0,043 | <0,001 |
| 7 | C20:5 n3 | 0,000 | -0,297 | -0,414 | 0,063 | <0,001 |
| 8 | Zn | 0,000 | -0,177 | 0,110 | 0,030 | <0,001 |
| 9 | Mn | 0,000 | -0,477 | 0,348 | -0,143 | <0,001 |
| 10 | FA 2 | 0,000 | 0,124 | -0,248 | -0,256 | <0,001 |
| 11 | C14:0 | 0,000 | 0,088 | -0,022 | -0,032 | <0,001 |
| 12 | MUFA | 0,000 | 1,333 | -1,488 | -0,425 | <0,001 |
| 13 | Pb | 0,000 | 0,208 | 0,061 | 0,264 | <0,001 |
| 14 | C20:1 n-9 | 0,000 | 0,085 | 0,071 | 0,084 | <0,001 |
| 15 | PUFA | 0,000 | -0,855 | -0,013 | -0,484 | <0,001 |
| 16 | FA 1 | 0,000 | 0,236 | -0,150 | -0,019 | <0,001 |
| 17 | C18:1 n-7 | 0,000 | -0,955 | 0,898 | 0,689 | <0,001 |
| 18 | C18:2 n-6 | 0,000 | -0,140 | 0,063 | -0,150 | <0,001 |
| 19 | Cd | 0,000 | 0,090 | -0,106 | -0,299 | <0,001 |
| 20 | C16:2 n-4 | 0,000 | 0,510 | 0,189 | -0,447 | <0,001 |
| 21 | C16:0 | 0,000 | 0,076 | -0,019 | -0,095 | <0,001 |
| 22 | SFA | 0,000 | -0,021 | -0,286 | -0,131 | <0,001 |

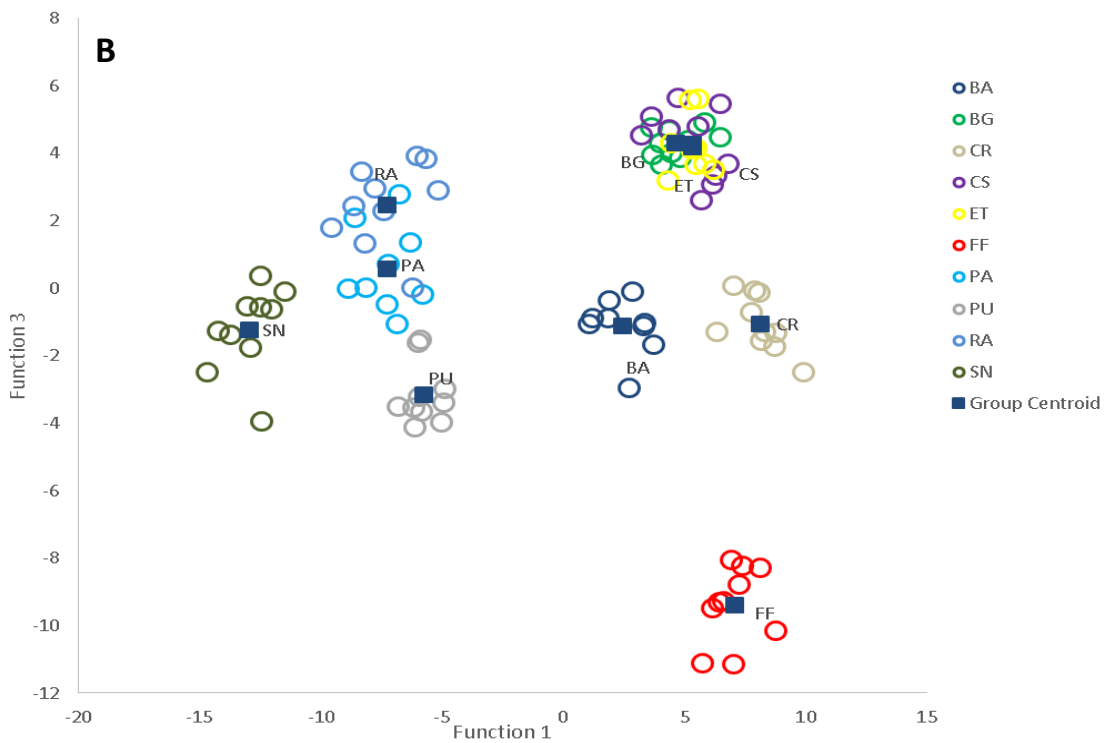
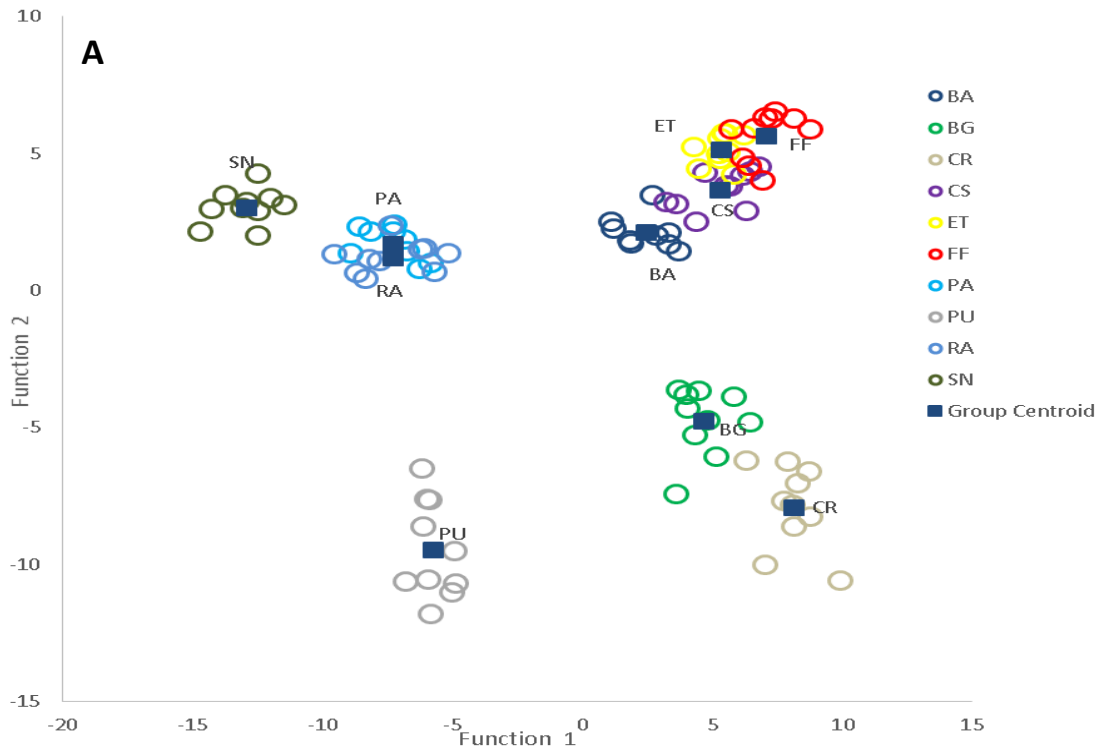
| | | | | | | |
|----|-----------|-------|-------|--------|--------|--------|
| 23 | Mg | 0,000 | 0,093 | -0,163 | 0,008 | <0,001 |
| 24 | C22:6 n-3 | 0,000 | 0,022 | -0,235 | 0,438 | <0,001 |
| 25 | FA 4 | 0,000 | 0,222 | -0,047 | -0,433 | <0,001 |

In regard to the canonical functions, as described in Table XVII, the first 3 variables slightly lowered their percentage of cumulative variance to 78,5% with 42,2% in Function 1, 22,3% in Function 2 and 13,8% in Function 3, however increasing the canonical correlation (<0,97)

Table XVII - Eigenvalues, % of Variance, Cumulative % and Canonical correlation of the canonical functions used to in the analysis

| Function | Eigenvalue | % of Variance | Cumulative % | Canonical Correlation |
|----------|------------|---------------|--------------|-----------------------|
| 1 | 56,930 | 42,4 | 42,4 | 0,991 |
| 2 | 29,978 | 22,3 | 64,7 | 0,984 |
| 3 | 18,498 | 13,8 | 78,5 | 0,974 |
| 4 | 10,977 | 8,2 | 86,7 | 0,957 |
| 5 | 6,548 | 4,9 | 91,6 | 0,931 |
| 6 | 5,343 | 4,0 | 95,5 | 0,918 |
| 7 | 3,573 | 2,7 | 98,2 | 0,884 |
| 8 | 1,782 | 1,3 | 99,5 | 0,800 |
| 9 | 0,621 | 0,5 | 100 | 0,619 |

Analysing the canonical functions plotted against each other (Fig. 13), and as expected they yield the same outcome as the stand alone analysis of FA, exhibiting groups closer to their centroid with some sites distanced from others. The only major difference in terms of the canonical discriminant functions is related with function 3, which contrasts with the function 3 of the Fatty acid analysis due to an increased influence of the Σ -6 over DHA as previously described.



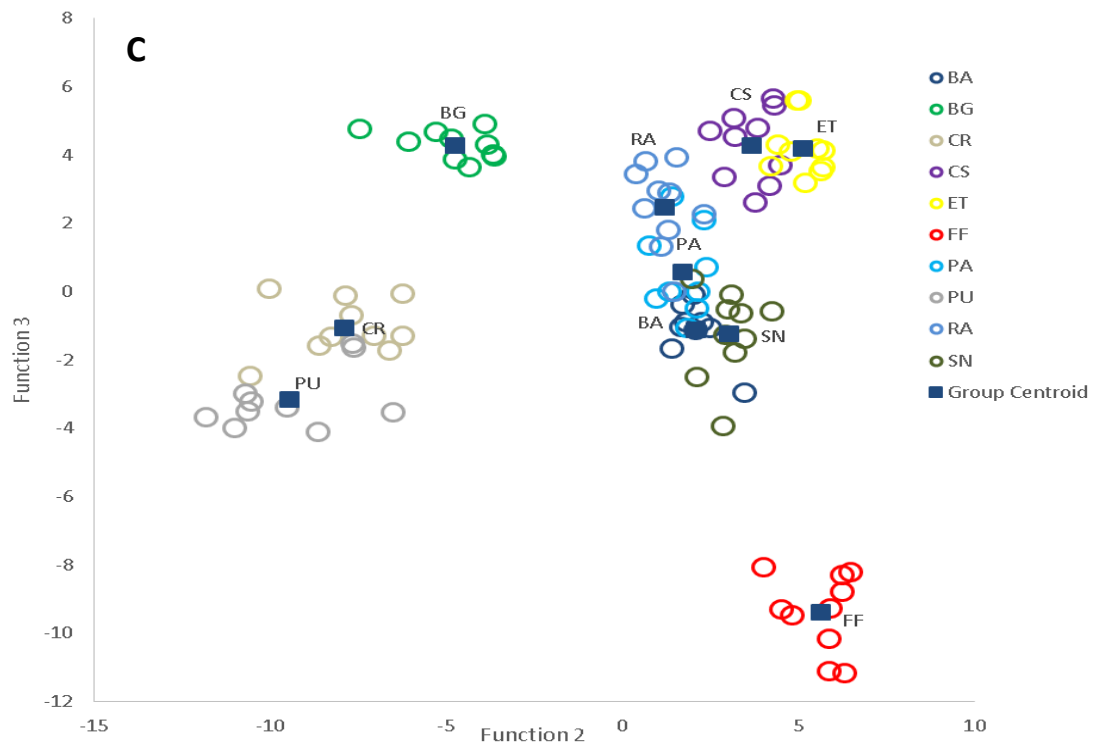


Figure 13 - Results of the Canonical Discriminant Functions of elemental microchemistry combined with fatty acid content. **A** Function 1*Function2, **B** Function 1*Function3, **C** Function 2*Function3. Colored circles represent Sites, each circle being a sample

Table XVIII - Percentage of goose barnacles correctly classified to each origin in the combined approach. Columns are the predicted group for each individual, rows are the origin group of each individual. Data are cross-validate

| Sites | Predicted Group Membership | | | | | | | | | | Total | |
|-----------------|----------------------------|------------|------------|------------|------------|------------|------------|------------|------------|----|------------|-----|
| | BA | BG | CR | CS | ET | FF | PA | PU | RA | SN | | |
| Cross validated | | | | | | | | | | | | |
| BA | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| BG | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| CR | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| CS | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| ET | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 100 |
| FF | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 100 |
| PA | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 |
| PU | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 100 |
| RA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 100 |
| SN | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 |

Examining Table XVIII, confirms the good reclassification success suggested in Fig. 13, with a reclassification success of cross-validated cases of 100%, confirming that all sampled individuals were successfully reclassified in relation to provenance location.

5. Discussion

The main objective of the present work is to develop a method able to determine distinguishable differences between populations of *P.pollicipes* that would allow the establishment of a Protected Designation of Origin for the goose barnacles harvested in the Berlengas Nature Reserve.

Despite its high commercial value, especially in the Iberian Peninsula, the majority of published studies have focused on the biology, ecology and management (Bald, Borja, & Muxika, 2006; Teresa Cruz & Hawkins, 1998; Jacinto et al., 2009; Sousa et al., 2013; A. T. M. Stewart, Fragoso, Clímaco, & Icely, 2014) , to the present date no work has been done regarding the shape and elemental composition of the capitulum, and only Fratini et al, (2012) reported the fatty acid profile of the goose barnacles in Galicia, Spain, nevertheless, the traceability of the species was never evaluated before.

5.1 Capitulum Shape

The relationship between centroid size and rostro-carinal ($R^2= 0.887$) imply a homologous growth of the capitulum, this goes in accordance with the length-weight relationship described by (Sestelo & Roca-Pardiñas, n.d.), suggesting a gradual and proportional increase in the rostro-carinal and the square-root of the summed squared distances between all 7 landmarks - centroid size.

Relative warps (RW) were selected to assess differences in shape between locations since they are the principal components of the variation amongst samples, analogous to a PCA analysis (Adams et al., 2004), and are independent variables, one would assume the greater the number of landmarks the best for inferring differences in the shape, however according to (F. J. Rohlf, 2000) landmarks should be, at least, less $(n-1)$ than the number of individuals per location, therefore only 7 landmarks were selected.

The geometric morphometric analysis did not demonstrate clear differences in the capitulum shape of *P.pollicipes* between the 10 distinct locations studied, it is known that individuals may go through alternative development footpaths in both favorable and unfavorable habitat traits (West-Eberhard 2003). Unlike many other marine or freshwater

crustaceans (see Naceur, Ben, Jenhani, & Romdhane, 2013; Lezcano, González-José, Spivak, & Dellatorre, 2012; Zuykova, Bochkarev, & Katokhin, 2012; Stewart, 1997), it appears that the sampled goose barnacle populations do not present phenotypic plasticity.

Knowing that barnacles attach themselves to highly exposed rocky shores, it is possibly they all suffer from the same environmental trait, therefore conducting to the same plasticity response. Also, the lack of phenotypic divergence between populations may indicate that a species is stable, or that an underlying process is concealing the expression of morphological traits, or even that the difference between populations is fine (Martin & Bermingham, 2000). Quinteiro et al. (2007) reported a high level of gene flow between populations ranging from France to the Canary Islands (47°N-28°N), similar to levels estimated for other marine invertebrates, which could equally explain the shape similarities.

5.2 Elemental Microchemistry

The relationships between an individual habitat and both biotic and abiotic factors influencing its calcified structures trace elemental composition are poorly understood, although, recent technological advances such as inductively coupled plasma – mass spectrometry show distinguished importance.

Despite the scarce knowledge about the drivers of site-specific differences in calcified structures, some authors have explored the value of using these structure such as fish otoliths or bivalve shells to identify the probable source populations of larvae (Becker, Levin, Fodrie, & McMillan, 2007; Swearer, Warner, Caselle, & Lea, 1999; Thorrold et al., 2002), the elemental deposition appears to be controlled by a complex set of factors, apart from the known influence of elements available from the surrounding environment becoming permanently incorporated into the structure (Campana, 1999), salinity, temperature, pH, dissolved oxygen have been shown to have an effect on elemental deposition in calcified structures (T. C. Barnes & Gillanders, 2013), in the opposite side, the incorporation of metabolic relevant and common elements, such as, P, K, S, Mg and Cu do not appear to be influenced by their relative concentration in the environment (Campana, 1999), The present study successfully apply for the first time this technique to identify harvest origin on *P. pollicipes* by finding local singularities in the geochemical composition of their capitulum.

In this work, the elemental microchemistry of the largest lateral plate of the capitulum was analysed in order to guarantee the same plate was analysed for every individual, since the species rostrum-carinal growth rate is reported to be in SW Portugal $15,7 \text{ mm year}^{-1}$ (Teresa Cruz, Castro, & Hawkins, 2010), capitulum plates deposition is associated with growth, and most of the sampled individuals were larger than 15 the whole plate was acid digested to obtain a balanced elemental composition of each individual for at a minimum period of 1 year.

All of the analysed elements showed statistical significant differences between locations (Table VII), Mg, Sr and P were the most abundant elements (Table VI). Similar results were reported for the striped shore crab *Pachygrapsus crassipes* (DiBacco & Levin, 2000) and for juvenile mussel shells *Mytilus californianus* and *M. galloprovincialis* (Becker et al., 2005). Nevertheless the 4 most discriminant elements were Cr, Zn, Mn and Cd, suggesting that the most common elements follow similar patterns of incorporation along the study area. The concentration of Cr was much higher in Figueira da Foz – FF, than in the remaining locations (Fig. 8), which might be related to the proximity of a cement factory and their waste and production emissions (Kumar et al., 2008), sitting only a few meters away from the shoreline and sampling location. Regarding to Zn, Mn and Cd, the higher concentration were found in Cabo Raso – CR located just outside of Lisbon metropolitan area and the Tagus estuary and again Figueira da Foz – FF next to the Mondego estuary respectively. These elements are generally associated with anthropogenic activity such as industrial, agricultural, domestic discharges and fishing building up the concentration of metals (Campana, 1999; R. S et al., 2012).

The results indicate that the lateral plate of *P. pollicipes* incorporate site-specific trace elements allowing a high discrimination success at geographical scales of 1s to 100s of km along the Portuguese coastline. An overall jackknifed reclassification success of 77.6% was obtained, suggesting that even subtle differences in physiochemical parameters of proximal water masses can lead to different elemental concentration. Small scale differences such as the ones found, could be generated by eddies, upwelling, temperature, waste water discharge, industrial pollution (Cutter, 1991; Thorrold, Zacherl, & Levin, 2006) or possibly even the substrate type where the individuals grow on.

5.3 Fatty acid content

The predominance of PUFAs, over SFAs and MUFAs in the fatty acids profile of the goose barnacles muscle has previously been observed in *P. pollicipes* with similar proportions in a study conducted in Galicia (Fratini et al., 2012), with the same pattern commonly observed in a variety of crustacean and molluscan species, such as the volcano barnacle *Tetraclita serrata* (Richoux et al., 2014), the Giant tiger prawn *Penaeus monodon* (Glencross, Smith, Thomas, & Williams, 2007), the Green tiger prawn *Penaeus semisulcatus*, the Kuruma prawn *Marsupenaeus japonicus*, the Caramote prawn *Melicertus kerathurus*, the Deepwater pink shrimp *Penaeus longirostris*, and the Speckled shrimp *Metapenaeus monoceros* (Ayas, Ozogul, & Yazgan, 2013), the giant river prawn *Macrobrachium rosenbergii* (Cavalli, Tamtin, Lavens, & Sorgeloos, 2001), and in other marine species such as the fan shell *Pinna nobilis* (Najdek, Blažina, Ezgeta-Balić, & Peharda, 2012), the Noah's ark shell *Arca noae*, the European flat oyster *Ostrea edulis*, and the Mediterranean mussel *Mytilus galloprovincialis* (Ezgeta-Balić, Najdek, Peharda, & Blažina, 2012), and *Astarte sulcata* (Olsen, Grahl-Nielsen, & Schander, 2009).

Except FA4, all the remaining FAs showed statistical significant differences amongst locations. Although for many other crustaceans (Cavalli et al., 2001; Fratini et al., 2012) the most abundant saturated fatty acid was palmitic acid (C16:0), the results of this study showed a reverse pattern (Table XI), where predominant SFAs were stearic acid (C18:0) followed by myristic acid (C14:0). Nevertheless, the results of the present study agree with many others (Fratini et al., 2012; Lim, Ako, Brown, & Hahn, 1997; Najdek et al., 2012) on the prevalence of DHA and EPA for the PUFAs group. In relation to MUFAs, vaccenic acid (C18:1 n7) was the most represented fatty acid, which had also been found as the most abundant in the volcano barnacle *Tetraclita serrata* (Richoux et al., 2014) and the bearded mussel *Modiolus barbatus* (Ezgeta-Balić et al., 2012).

The 4 most discriminant FAs in the analysis were DPA (C22:5 n-3), oleic acid (C18:1 n9), Σ -6 and stearic acid (C18:0) (Table XII). Regarding DPA its content was found to be much higher in 4 locations (Peniche – PA; Praia da Ursa – PU; Farilhões – RA; Sines – SN) than in the remaining ones. Since it is known that DPA can act as a source for both EPA and DHA (Arts, Ackman, & Holub, 2001; Kaur et al., 2010) this suggests that due to a metabolic event this individuals were storing this FA. Oleic acid

was only detected in 3 locations (Berlenga – BG; Cabo Raso – CR; Praia da Ursa – PU). Oleic acid is a biosynthetic precursor of a family of fatty acids with the (n-9) terminal structure and with chain-lengths of 20 to 24 or more (Gunstone et al., 2007), such as the eicosenoic acid (C20:1 n-9). The 3 previously mentioned locations show low content in eicosenoic acid, suggest a biosynthesis delay or a stop of production at the time.

Sines - SN is the location showing the lowest content in stearic acid, which might be related to the proximity of an important fishing harbor, and a large industrial complex with thermoelectric power plants and petrochemical refineries (Jones, Catarino, Sergio, & Bento - Pereira, 1981; Llop, Pinho, Matos, Pereira, & Branquinho, 2012) and its possible impact on food availability and quality.

The observed results of the fatty acid content of *P.pollicipes* muscle, imply that there are enough differences capable of discriminating harvest locations with a high success at small geographical scales. Although the overall jackknifed reclassification success of 99%, the well known seasonal variability in the FA profile of seafood (Ayas et al., 2013; Ezgeta-Balić et al., 2012), might prevent the use of fatty acid profiles as a reliable tool for origin traceability, out of the season studied in the present study.

5.4 Capitulum shape, elemental microchemistry and fatty acid profile as tools for detection of origin

In order to undoubtedly detect the real place of origin of a given product, in this study the goose barnacle *P.pollicipes*, there is the need to test different approaches, increasing the odds of being successful.

In this work, 3 different approaches were chosen - Capitulum shape, elemental microchemistry of the capitulum and the fatty acid content of the muscle – only 1 of the selected technics was unsuccessful with an extremely low reclassification success of just 29,6%, while the elemental microchemistry and fatty acid content exhibit higher success with 77,6% and 99% respectively. The combination of both elemental microchemistry and fatty acid profile provided a reclassification success of 100%.

This does not necessarily mean that a geometric morphometric approach is completely discarded for the purpose of origin discrimination in general, but might not

be useful for *P.pollcipes*. For instance Hard et al. (2000) was able to differentiate wild and captively reared adult salmon and Sara et al. (1999) successfully distinguished the sharpsnout seabream *Diplodus puntazzo* reared in different conditions.

Elemental microchemistry has been previously used with success in the geographical characterization of diverse food products, ranging from the wine industry (Geana et al., 2013), mining industry (Abduriyim & Kitawaki, 2006), olive oils characterization (Cabrera-Vique et al., 2012) to the classification of mussels cultivated in Galician rías with protected designation of origin (Costas-Rodríguez, Lavilla, & Bendicho, 2010). Nevertheless the present work is the first suggesting this methodology to detect a product origin at a small geographical scale in the marine environment.

Equally broadly used is, the fatty acid content to tell apart populations or geographical origin of seafood (Ezgeta-Balić et al., 2012; Richoux et al., 2014). Despite Fratini et al.(2012) work in the profile of goose barnacles fatty acids, this study is pioneer in characterizing the fatty acid profile with the objective of tracing the harvest location of the specimens, with extremely high success.

It is clear that both elemental microchemistry and fatty acid content combined have proved to be highly efficient, and have proved the existence, of site-specific signatures.

However, in practical terms it may not be required to apply both approaches. It is important to note the constraints of the methods. Sample preparation in both methods are time and resources consuming. The analysis should to be performed by a trained operator in certified laboratories, which would most likely increase the costs of operation, therefore the use of a single valid method would be advised.

Although reclassification success was higher when using FA, seasonal variability on its profile might increase the benefices of the elemental microchemistry analyses. Nevertheless, more analyses about the stability of the microchemistry signature might be performed to ensure its reliability.

6. Conclusion and future perspectives

The present work aimed to identify and evaluate different methodological approaches for the discrimination of goose barnacles from 10 distinct locations along the Portuguese coast.

The capitulum shape of the goose barnacle *P.pollicipes*, showed small differences amongst the sampled individuals, with a reclassification success 29,6 % the technique does not appear to be suitable for this purpose.

Considering the elemental microchemistry of largest lateral plate of the goose barnacle capitulum, all of the analysed elements showed statistical significant differences amongst locations, while Mg, Sr and P were the most abundant elements the most discriminant elements in the analyses were Cr, Zn, Mn and Cd, probably associated with anthropogenic activities. The results indicate that the goose barnacle capitulum does incorporate site-specific trace elements, allowing a high discrimination success at small geographical scales, with an overall reclassification success of 77,6 %.

Regarding the fatty acid profile of the muscle, there is a clear predominance of PUFAs over SFAs and MUFAs, stearic acid (C18:0) was the most abundant SFA while the vaccenic acid (C18:1 n-7) was the most representative MUFA, finally DHA and EPA were the most abundant PUFAs, however, DPA, oleic acid, Σ -6 and stearic acid proved to be more important in terms of site discrimination, enabling a reclassification success of 99 %.

Finally, in the combined approach using both elemental microchemistry and the fatty acid profile returned a reclassification success of 100 %, which does mean that every individual was in fact correctly reclassified regarding its origin.

The Elemental microchemistry, fatty acid profile and the combined signatures of the two exhibited strong discrimination power, proving beyond question to be valid methodological approaches in the detection of a product origin at a small geographical scale in the marine environment.

This results are one step forward in the desired establishment of a Protected Denomination of Origin for the goose barnacles harvested in the Berlengas Nature

Reserve, and in the valorization of marine resources from Portuguese Marine Protected Areas.

The present study focused in the spatial variability of the site-specific signatures, however in the future, it is of relevance to evaluate the stability of the spatial signatures and whether or not there is a temporal variability of the signatures, in order to determine the best approach for the detection of the harvest location.

7. References

- Abduriyim, A. & Kitawaki, H., 2006. APPLICATIONS OF LASER ABLEATION – INDUCTIVELY COUPLED PLASMA – MASS SPECTROMETRY (LA-ICP-MS) TO GEMOLOGY. *Gems & Gemology*.
- Adams, D.C., Rohlf, F.J. & Slice, D.E., 2004. Geometric morphometrics: Ten years of progress following the “revolution.” *Italian Journal of Zoology*, 71(1), pp.5–16. Available at: <http://www.tandfonline.com/doi/abs/10.1080/11250000409356545>.
- Anderson, D.T., 1994. *Barnacles — Structure, Function, Development and Evolution*. L. Chapman and Hall, ed., Cambridge University Press. Available at: http://journals.cambridge.org/abstract_S0016756800009195 [Accessed September 28, 2014].
- Anderson, M.J. & Gorley, R.N., 2008. *PERMANOVA for PRIMER: guide to software and statistical methods.*, PRIMER-E Ltd.,
- Arts, M.T., Ackman, R.G. & Holub, B.J., 2001. “Essential fatty acids” in aquatic ecosystems: a crucial link between diet and human health and evolution. *Canadian Journal of Fisheries and Aquatic Sciences*, 58(1), pp.122–137. Available at: <http://www.nrcresearchpress.com/doi/abs/10.1139/f00-224>.
- Ayas, D., Ozogul, Y. & Yazgan, H., 2013. The effects of season on fat and fatty acids contents of shrimp and prawn species. *European Journal of Lipid Science and Technology*, 115(3), pp.356–362. Available at: <http://doi.wiley.com/10.1002/ejlt.201200081> [Accessed September 27, 2014].
- Bald, J., Borja, A. & Muxika, I., 2006. A system dynamics model for the management of the gooseneck barnacle (*Pollicipes pollicipes*) in the marine reserve of Gaztelugatxe (Northern Spain). *Ecological Modelling*, 194(1-3), pp.306–315. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0304380005005284> [Accessed March 10, 2012].
- Barnes, H. & Barnes, M., 1977. Studies on the reproduction of cirripedes. I. Introduction: Copulation, release of oocytes, and formation of the egg lamellae. *Journal of Experimental Marine Biology and Ecology*, 27(3), pp.195–218. Available at: <http://www.sciencedirect.com/science/article/pii/0022098177901241> [Accessed September 28, 2014].
- Barnes, H. & Reese, E.S., 1960. THE BEHAVIOUR OF THE STALKED INTERTIDAL BARNACLE *POLLICIPES POLYMERUS* J. B. SOWERBY, WITH SPECIAL. *Journal of Animal Ecology*, 29(1), pp.169–185.
- Barnes, M., 1996. Pedunculate cirripedes of the genus *Pollicipes*. *Oceanography and Marine Biology: An Annual Review*. Available at: <http://www.emodnet-biology.eu/component/imis/?module=ref&refid=27033&basketaction=add> [Accessed September 28, 2014].

- Barnes, M., 1992. The reproductive periods and condition of the penis in several species of common cirripedes. *Oceanography and Marine Biology: An Annual Review*. Available at: <http://www.vliz.be/en/imis?refid=212228> [Accessed September 28, 2014].
- Barnes, T.C. & Gillanders, B.M., 2013. Combined effects of extrinsic and intrinsic factors on otolith chemistry : implications for environmental reconstructions. *Canadian Journal of Fisheries and Aquatic Sciences*, 70(October), pp.1159–1166.
- Becker, B.J. et al., 2007. Complex larval connectivity patterns among marine invertebrate populations. *Proceedings of the National Academy of Sciences of the United States of America*, 104(9), pp.3267–72. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1802003&tool=pmcentrez&rendertype=abstract> [Accessed March 6, 2012].
- Becker, B.J. et al., 2005. Spatial and temporal variation in trace elemental fingerprints of mytilid mussel shells : A precursor to invertebrate larval tracking. , 50(1), pp.48–61.
- Bergé, J.-P. & Barnathan, G., 2005. Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. *Advances in biochemical engineering/biotechnology*, 96, pp.49–125. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16566089> [Accessed September 30, 2014].
- Bernard, F.R., 1988. Potential Fishery for the Gooseneck Barnacle *Pollicipes polymerus* (Sowerby, 1833) in British Columbia. *Fisheries Research*, 6, pp.287–298.
- Bligh, E.G. & Dyer, W.J., 1959. A RAPID METHOD OF TOTAL LIPID EXTRACTION AND PURIFICATION. *Canadian Journal of Biochemistry and Physiology*, 37(8), pp.911–917. Available at: <http://www.nrcresearchpress.com/doi/abs/10.1139/o59-099> [Accessed July 14, 2014].
- Bookstein, F.L., 1998. A hundred years of morphometrics. *Acta Zoologica Academiae Scientiarum Hungaricae*, (44), pp.7–59. Available at: http://www.experts.scival.com/uwashington/pubDetail.asp?n=Fred+L.+Bookstein&u_id=267&oe_id=1&o_id=&id=32325057 [Accessed September 29, 2014].
- Bookstein, F.L., 1991. *Morphometric Tools for Landmark Data. Geometry and Biology.*, Cambridge: Cambridge University Press. Available at: <http://doi.wiley.com/10.1002/bimj.4710350416> [Accessed September 30, 2014].
- Borja, Á. et al., 2004. Borja A, Bald J, Muxika I, Liria P. 2004. El recurso marisquero del percebe (*Pollicipes pollicipes*) en el biotopo marino protegido de Gaztelugatxe y en áreas explotadas de Bizkaia.
- Cabrera-Vique, C., Bouzas, P.R. & Oliveras-López, M.J., 2012. Determination of trace elements in extra virgin olive oils: A pilot study on the geographical characterisation. *Food Chemistry*, 134(1), pp.434–439. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0308814612002762> [Accessed July 7, 2014].

- Calado, R. et al., 2009. Effect of different diets on larval production, quality and fatty acid profile of the marine ornamental shrimp *Lysmata amboinensis* (de Man, 1888), using wild larvae as a standard. *Aquaculture Nutrition*, 15(5), pp.484–491. Available at: <http://doi.wiley.com/10.1111/j.1365-2095.2008.00614.x> [Accessed September 30, 2014].
- Campana, S.E., 1999. Chemistry and composition of fish otoliths: pathways, mechanisms and applications.
- Cardoso, a. C. & Yule, a. B., 1995. Aspects of the reproductive biology of *Pollicipes pollicipes* (Cirripedia; Lepadomorpha) from the southwest coast of Portugal. *Netherlands Journal of Aquatic Ecology*, 29(3-4), pp.391–396. Available at: <http://link.springer.com/10.1007/BF02084238>.
- Carpenter, K.E., 1996. *Morphometric pattern and feeding mode in emperor fishes (Lethrinidae, Perciformes)* L. F. Marcus et al., eds., Boston, MA: Springer US. Available at: <http://link.springer.com/10.1007/978-1-4757-9083-2> [Accessed September 29, 2014].
- Cavalli, R.O. et al., 2001. Variations in lipid classes and fatty acid content in tissues of wild *Macrobrachium rosenbergii* de Man / females during maturation. , pp.311–324.
- Clare, A., 1987. *Endocrinology of cirripedes In: Crustaceans Issue 5* Southward A.J. A. A. Balkema, ed., Rotterdam. Available at: <http://www.google.pt/books?hl=pt-PT&lr=&id=rvkyMrgwdQcC&pgis=1> [Accessed September 28, 2014].
- Comission, E., 2001. Changes to lists of commercial designations and requirements governing consumer information. , 2001(2065), pp.2000–2002.
- Comission, E., 2002. REGULATION (EC) No 178/2002 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food saf.
- Costas-Rodríguez, M., Lavilla, I. & Bendicho, C., 2010. Classification of cultivated mussels from Galicia (northwest Spain) with European Protected Designation of Origin using trace element fingerprint and chemometric analysis. *Analytica chimica acta*, 664(2), pp.121–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20363393> [Accessed June 8, 2014].
- Cruz, T., 2000. *Biologia e ecologia do percebe Pollicipes pollicipes (Gmelin, 1790) no litoral sudoeste português*. Universidade de Évora.
- Cruz, T. & Araújo, J., 1999. Reproductive patterns of *Pollicipes pollicipes* (Cirripedia: Lepadomorpha) on the southwestern coast of Portugal. *Journal of Crustacean Biology*, (19(2)), pp.260–267.
- Cruz, T., Castro, J.J. & Hawkins, S.J., 2010. Recruitment, growth and population size structure of *Pollicipes pollicipes* in SW Portugal. *Journal of Experimental Marine*

- Biology and Ecology*, 392(1-2), pp.200–209. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0022098110001413> [Accessed June 3, 2012].
- Cruz, T. & Hawkins, S.J., 1998. Reproductive Cycle of Pollicipes Pollicipes at Cabo De Sines, South-West Coast of Portugal. *Journal of the Marine Biological Association of the United Kingdom*, 78(02), pp.483–496. Available at: http://www.journals.cambridge.org/abstract_S0025315400041576.
- Cunha, I. & Weber, M., 2001. La actividad reproductora del percebe Pollicipes pollicipes en el Norte de Portugal. *Dirección General de Pesca y Alimentación (Gobierno de Cantabria) (eds) Libro de resúmenes VII Congreso Nacional de Acuicultura*, pp.173–175. Available at: <http://www.fundacao-ela.pt/index.php/educacao/publicacoes-de-referencia> [Accessed September 28, 2014].
- Cutter, G.A., 1991. TRACE-ELEMENTS IN ESTUARINE AND COASTAL WATERS - UNITED-STATES STUDIES FROM 1986-1990. *REVIEWS OF GEOPHYSICS*, 29. Available at: http://apps.webofknowledge.com/full_record.do?product=UA&search_mode=GeneralSearch&qid=1&SID=R1P2RHDWd4I5VwvXKeh&page=1&doc=1 [Accessed September 26, 2014].
- DiBacco, C. & Levin, L. a., 2000. Development and application of elemental fingerprinting to track the dispersal of marine invertebrate larvae. *Limnology and Oceanography*, 45(4), pp.871–880. Available at: http://www.aslo.org/lo/toc/vol_45/issue_4/0871.html.
- Duarte, R.C. et al., 2013. Conspecific cues affect stage-specific molting frequency, survival, and claw morphology of early juvenile stages of the shore crab *Carcinus maenas*. *Hydrobiologia*, 724(1), pp.55–66. Available at: <http://link.springer.com/10.1007/s10750-013-1712-5> [Accessed March 11, 2014].
- Ezgeta-Balić, D. et al., 2012. Seasonal fatty acid profile analysis to trace origin of food sources of four commercially important bivalves. *Aquaculture*, 334-337, pp.89–100. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0044848611010234> [Accessed September 27, 2014].
- Fink, W.L. & Zelditch, M.L., 1997. Shape Analysis and Taxonomic Status of Pygocentrus Piranhas (Ostariophysi, Characiformes) from the Paraguay and Paraná River Basins of South America. *Copeia*, 1, pp.179–182. Available at: <http://www.jstor.org/discover/10.2307/1447854?uid=3738880&uid=2&uid=4&sid=21104703924217> [Accessed September 29, 2014].
- Fisher, R.A., 1935. The Logic of Inductive Inference. *Journal of the Royal Statistical Society*, 98(1), pp.39–82.
- Fratini, G. et al., 2012. Volatile profile of Atlantic shellfish species by HS-SPME GC/MS. *Food Research International*, 48(2), pp.856–865. Available at:

- <http://linkinghub.elsevier.com/retrieve/pii/S0963996912002372> [Accessed January 10, 2013].
- Geana, I. et al., 2013. Geographical origin identification of Romanian wines by ICP-MS elemental analysis. *Food chemistry*, 138(2-3), pp.1125–34. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23411223> [Accessed January 31, 2014].
- Girin, M. & FAO, 1989. *A regional survey of the aquaculture sector in the Mediterranean : including Albania, Algeria, Cyprus, Egypt, France, Greece, Israel, Italy, Libya, Lebanon, Malta, Morocco, Portugal, Spain, Syria.*, Rome: United Nations Development Programme ;Food and Agriculture Organization of the United Nations.
- Glencross, B.D. et al., 2007. Optimising the essential fatty acids in the diet for weight gain of the prawn , *Penaeus monodon*.
- Guill, J.M., Hood, C.S. & Heins, D.C., 2003. Body shape variation within and among three species of darters (Perciformes : Percidae). , (1952), pp.134–140.
- Gunstone, F.D. et al., 2007. *The Lipid Handbook*,
- Halim, R., Danquah, M.K. & Webley, P. a, 2012. Extraction of oil from microalgae for biodiesel production: A review. *Biotechnology advances*, 30(3), pp.709–32. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22266377> [Accessed March 5, 2012].
- Hard, J.J. et al., 2000. Evidence for morphometric differentiation of wild and captive reared adult coho salmon : a geometric analysis. , pp.61–73.
- Hayward, P.J. & Ryland, J.S., 1990. *The Marine Fauna of the British Isles and North-West Europe: Vols. 1+2*, Oxford, UK.: Clarendon Press. Available at: http://books.google.pt/books/about/The_Marine_Fauna_of_the_British_Isles_an.html?id=09UUAQAIAAJ&pgis=1 [Accessed September 28, 2014].
- HILGARD, G.H., 1960. A STUDY OF REPRODUCTION IN THE INTERTIDAL BARNACLE, MITELLA POLYMERUS, IN MONTEREY BAY, CALIFORNIA. *Biol. Bull.*, 119(2), pp.169–188. Available at: <http://www.biolbull.org/content/119/2/169.short> [Accessed September 28, 2014].
- Hoffman, D.L., 1989. Settlement and recruitment patterns of a pedunculate barnacle, *Pollicipes polymerus* Sowerby, off La Jolla, California. *Journal of Experimental Marine Biology and Ecology*, (125), pp.83–98.
- Hood, C.S. & Heins, D.C., 2000. Ontogeny and Allometry of Body Shape in the Blacktail Shiner,. *Copeia*, (1), pp.270–275.
- Jacinto, D. et al., 2009. Stalked barnacle (*Pollicipes pollicipes*) harvesting in the Berlengas Nature Reserve, Portugal: temporal variation and validation of logbook data. *ICES Journal of Marine Science*, 67(1), pp.19–25. Available at: <http://icesjms.oxfordjournals.org/cgi/doi/10.1093/icesjms/fsp226>.

- Jones, M.P. et al., 1981. THE SINES INDUSTRIAL COMPLEX MONITORING 2 . Description of the Site and Its Vegetation. *Environmental Monitoring and Assessment*, 1, pp.163–173.
- Kameya, A. & Zeballos, J., 1988. Distribucion y densidad de percebes. *Pollicipes elegans* (Crustacea: Cirripedia) en el Mediolitoral Peruano (Yasila, Paita, Chilca, Lima). *Boletín del Instituto del Mar del Perú*, (12 (1)), pp.5–22.
- Kaur, G. et al., 2010. Short-term docosapentaenoic acid (22:5 n-3) supplementation increases tissue docosapentaenoic acid, DHA and EPA concentrations in rats. *The British journal of nutrition*, 103(1), pp.32–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19650956> [Accessed September 28, 2014].
- Koletzko, B. et al., 2001. Long chain polyunsaturated fatty acids (LC-PUFA) and perinatal development. *Acta paediatrica (Oslo, Norway : 1992)*, 90(4), pp.460–4. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11332943> [Accessed September 30, 2014].
- Kumar, S.S. et al., 2008. IMPACT OF DUST EMISSION ON PLANT VEGETATION IN THE VICINITY OF CEMENT PLANT. , 7(1), pp.31–35.
- Laurenti, G., 2010. 1961–2007 fish and fishery products world apparent consumption statistics based on food balance sheets. *FAO*.
- Lepage, G. & Roy, C.C., 1986. Direct transesterification of all classes of lipids in a one-step reaction. *Journal of Lipid Research*, 27, pp.114–120.
- Lezcano, A.H. et al., 2012. Geographic differences in the carapace shape of the crab *Cyrtograpsus affinis* (Decapoda: Varunidae) and its taxonomic implications. *Scientia Marina*, 76(2), pp.329–337. Available at: <http://scientiamarina.revistas.csic.es/index.php/scientiamarina/article/view/1334/1423> [Accessed September 21, 2014].
- Linge, K.L. & Jarvis, K.E., 2009. Back to Basics Review Quadrupole ICP-MS : Introduction to Instrumentation , Measurement Techniques and Analytical Capabilities. , 33, pp.445–467.
- Llop, E. et al., 2012. The use of lichen functional groups as indicators of air quality in a Mediterranean urban environment. *Ecological Indicators*, 13(1), pp.215–221. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1470160X11001762> [Accessed September 10, 2014].
- M.-M., P. et al., 2011. Geographical origin classification of gem corundum using elemental fingerprint analysis by laser ablation inductively coupled plasma mass spectrometry. *International Journal of Mass Spectrometry*, 306(1), pp.57–62. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1387380611002260> [Accessed June 3, 2012].

- Macho, G., Molares, J. & Vázquez, E., 2005. Timing of larval release by three barnacles from the NW Iberian Peninsula. *Marine Ecology Progress Series*, 298(1), pp.251–260. Available at: <http://www.int-res.com/abstracts/meps/v298/p251-260/>.
- Mackie, I. et al., 1999. Challenges in the identification of species of canned fish. *Trends in Food Science & Technology*, 10(1), pp.9–14. Available at: <http://www.sciencedirect.com/science/article/pii/S0924224499000138> [Accessed September 28, 2014].
- Martin, a P. & Bermingham, E., 2000. Regional endemism and cryptic species revealed by molecular and morphological analysis of a widespread species of Neotropical catfish. *Proceedings. Biological sciences / The Royal Society*, 267(1448), pp.1135–41. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1690641&tool=pmcentrez&rendertype=abstract>.
- Molares, J., 1998. BIOLOGÍA Y EXPLOTACIÓN DEL PERCEBE (POLLICIPES CORNUCOPIA) EN LAS COSTAS DE GALICIA. En: *Penas Patiño, X. (ed.) Marisqueo en Galicia: 3ª Xornadas de Medio Mariño e Acuicultura. Edicios do Castro, Sada*, pp.135–158. Available at: <http://www.fundacionoesa.es/trabajos-cientificos/biologia-y-explotacion-del-percebe-pollicipes-cornucopia-en-las-costas-de-galicia> [Accessed September 28, 2014].
- Molares, J., 1993. Estudio del ciclo biológico del percebe (*Pollicipes cornucopia* Leach) de las costas de Galicia. *Alimentaria: Revista de tecnología e higiene de los alimentos*, (248), p.9. Available at: <http://dialnet.unirioja.es/servlet/articulo?codigo=132613>.
- Naceur, H. Ben et al., 2013. Morphometric characterization of adult *Artemia* (Crustacea : Branchiopoda) populations from costal and inland Tunisian salt lakes. , 54(December), pp.543–555.
- Najdek, M. et al., 2012. Diets of fan shells (*Pinna nobilis*) of different sizes: fatty acid profiling of digestive gland and adductor muscle. *Marine Biology*, 160(4), pp.921–930. Available at: <http://link.springer.com/10.1007/s00227-012-2144-x> [Accessed September 27, 2014].
- Newman, W.A., 1987. Evolution of cirripedes and their major groups. In: *Southward, A.J. (Ed.), Barnacle Biology. Balkema A.A. Rotterdam,* (Crustacean Issues 5), pp.3–42.
- Novo Loureiro, S., 2000. ¿ Un marisco?...: el percebe. *Terralia*, (16), pp.50–58. Available at: <http://dialnet.unirioja.es/servlet/articulo?codigo=792677>.
- Olsen, B.R., Grahl-Nielsen, O. & Schander, C., 2009. Population study of *Astarte sulcata*, da Costa, 1778, (Mollusca, Bivalvia) from two Norwegian fjords based on the fatty acid composition of the adductor muscle. *Biochemical Systematics and Ecology*, 37(5), pp.662–669. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S030519780900146X>.

- Pavón, C., 2003. *Biología y variables poblacionales del percebe, Pollicipes pollicipes (Gmelin, 1790) en Asturias*. Universidade de Oviedo.
- Pearson, K., 1985. Note on Regression and Inheritance in the Case of Two Parents. *Proceedings of the Royal Society*, (58), pp.240–242.
- Pearson, K., 1901. On lines and planes of closest fit to systems of points in space. *Philosophical Magazine*, (2), pp.559–572.
- Pérez, A.C., 1996. El percebe en Galicia. *Fundacion Caixa Galicia - Aula del mar*. Available at: [https://openlibrary.org/works/OL9126134W/El_percebe_en_Galicia_\(Aula_del_mar\)](https://openlibrary.org/works/OL9126134W/El_percebe_en_Galicia_(Aula_del_mar)).
- Proverbs, T., 1979. A status report on recent British Columbia gooseneck barnacle industry. *M.S. Interdepartmental Report, Department of Fisheries and Oceans*, p.10.
- Quinteiro, J., Rodríguez-Castro, J. & Rey-Méndez, M., 2007. Population genetic structure of the stalked barnacle *Pollicipes pollicipes* (Gmelin, 1789) in the northeastern Atlantic: influence of coastal currents and mesoscale hydrographic structures. *Marine Biology*, 153(1), pp.47–60. Available at: <http://www.springerlink.com/index/10.1007/s00227-007-0783-0> [Accessed November 14, 2012].
- R. S, R. et al., 2012. Heavy Metal Contamination and Risk Assessment in the Marine Environment of Arabian Sea, along the Southwest Coast of India. *American Journal of Chemistry*, 2(4), pp.191–208. Available at: <http://article.sapub.org/10.5923.j.chemistry.20120204.03.html> [Accessed September 26, 2014].
- Rainbow, P.S., 1984. An introduction to the biology of British littoral barnacles. *Field Studies*, (6), pp.1–51.
- Richoux, N., Vermeulen, I. & Froneman, P., 2014. Fatty acid profiles reveal temporal and spatial differentiation in diets within and among syntopic rocky shore suspension-feeders. *Marine Ecology Progress Series*, 495, pp.143–160. Available at: <http://www.int-res.com/abstracts/meps/v495/p143-160/> [Accessed September 27, 2014].
- Rohlf, F., 1993a. *Relative warp analysis and an example application to mosquito wings C. to M. C.* In: Marcus L.F., Bello E., García-Valdecasas A. (eds.), ed., Available at: <http://www.google.pt/books?hl=pt-PT&lr=&id=GquJJ-J2dqsC&pgis>.
- Rohlf, F. J., 2008. TpsRelw, relative warps analysis, version 1.46. Department of Ecology and Evolution, State University of New York at Stony Brook.
- Rohlf, F. J., 2009. TpsDig, digitize landmarks and outlines, version 2.05. Department of Ecology and Evolution, State University of New York at Stony Brook.

- Rohlf, F.J. & Slice, D., 1990. Extensions of the Procrustes Method for the Optimal Superimposition of Landmarks. *Systematic Zoology*, 39(1), p.40. Available at: <http://sysbio.oxfordjournals.org/content/39/1/40> [Accessed September 30, 2014].
- Sara, M., Favaloro, E. & Mazzola, A., 1999. Comparative morphometrics of sharpsnout seabream (*Diplodus puntazzo* Cetti , 1777), reared in different conditions. , 19, pp.195–209.
- Sestelo, M. & Roca-Pardiñas, J., 2011. A New Approach to Estimation of the Length—Weight Relationship of *Pollicipes pollicipes* (Gmelin, 1789) on the Atlantic Coast of Galicia (Northwest Spain): Some Aspects of Its Biology and Management. *Journal of Shellfish Research*, 30(3), pp.939–948. Available at: <http://www.bioone.org/doi/abs/10.2983/035.030.0336> [Accessed September 5, 2014].
- Simões, T. et al., 2013. Seasonal variation in proximate composition and fatty acid profile of grey triggerfish (*Balistes capriscus*) captured along the coast of Portugal. *Journal of food science*, 78(5), pp.C691–5. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23551246> [Accessed September 3, 2014].
- Sousa, A. et al., 2013. Patterns of distribution and abundance of the stalked barnacle (*Pollicipes pollicipes*) in the central and southwest coast of continental Portugal. *Journal of Sea Research*, 83, pp.187–194. Available at: <http://dx.doi.org/10.1016/j.seares.2013.04.005>.
- Stewart, A.T.M. et al., 2014. Evaluation of stakeholder perspectives on the management of the stalked barnacles (*Pollicipes pollicipes*) resource in the Parque Natural do Sudoeste Alentejano e Costa Vicentina, Portugal. *Marine Policy*, 43, pp.71–79. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0308597X13000924> [Accessed August 24, 2014].
- Stewart, B.A., 1997. Morphological and genetic differentiation between populations of river crabs (Decapoda : Potamonautidae) from the Western Cape , South Gecarcinautes brincki. , pp.1–21.
- Stryer, L., Berg, J.M. & Tymoczko, J.L., 2002. *Biochemistry, 5th edition*, New York: W H Freeman. Available at: <http://www.ncbi.nlm.nih.gov/books/NBK21154/> [Accessed September 30, 2014].
- Stubbings, H., 1967. *The Cirriped fauna of tropical West Africa*, London: British Museum (Natural History).
- Swearer, S.E. et al., 1999. Larval retention and recruitment in an island population of a coral-reef ® sh. *Nature*, 402(December), pp.799–802.
- Swearer, S.E. et al., 2003. Spatio-temporal and interspecific variation in otolith trace-elemental.pdf. *Estuarine, Coastal and Shelf Science*, 56, pp.1111–1123.
- Tecelão, C. et al., 2010. Production of human milk fat substitutes enriched in omega-3 polyunsaturated fatty acids using immobilized commercial lipases and Candida


- parapsilosis lipase/acyltransferase. *Journal of Molecular Catalysis B: Enzymatic*, 65, pp.122–127.
- Thorrold, S.R. et al., 2002. QUANTIFYING LARVAL RETENTION AND CONNECTIVITY IN MARINE POPULATIONS WITH ARTIFICIAL AND NATURAL MARKERS. , 70(1), pp.291–308.
- Thorrold, S.R., Zacherl, D.C. & Levin, L.A., 2006. Population Connectivity and Larval Dispersal Using Geochemical Signatures in Calcified Structures. , 20(3), pp.80–89.
- Walker, G., 1983. A study of the ovigerous frenae of barnacles. *Proceedings of the Royal Society*, (B218), pp.425–42.
- West-Eberhard, M.J., 2003. *Developmental Plasticity and Evolution*.
- Woolfe, M. & Primrose, S., 2004. Food forensics: using DNA technology to combat misdescription and fraud. *Trends in biotechnology*, 22(5), pp.222–6. Available at: <http://www.sciencedirect.com/science/article/pii/S0167779904000848>.
- Zelditch, M.L. et al., 2004. *Geometric Morphometrics for Biologists*, New York: Elsevier. Available at: <http://www.sciencedirect.com/science/article/pii/B9780127784601500004>.
- Zuykova, E.I., Bochkarev, N. a. & Katokhin, A. V., 2012. Identification of the Daphnia species (Crustacea: Cladocera) in the lakes of the Ob and Yenisei River basins: morphological and molecular phylogenetic approaches. *Hydrobiologia*, 715(1), pp.135–150. Available at: <http://link.springer.com/10.1007/s10750-012-1423-3>.

8. Attachments

Table 8.1 – List of the identified fatty acids.

| Nomenclature | Carbon:Double bonds designation |
|-----------------------------|---------------------------------|
| Myristic acid | C14:0 |
| Palmitic acid | C16:0 |
| Palmitoleic acid | C16:1 n-7 |
| Hexadecadienoic acid | C16:2 n-4 |
| Stearic acid | C18:0 |
| Vaccenic acid | C18:1 n-7 |
| Oleic acid | C18:1 n-9 |
| Linoleic acid | C18:2 n-6 |
| Octadecatetraenoic acid | C18:4 n-3 |
| Eicosenoic acid | C20:1 n-9 |
| Arachidonic acid (ARA) | C20:4 n-6 |
| Eicosapentaenoic acid (EPA) | C20:5 n-3 |
| Docosapentaenoic acid (DPA) | C22:5 n-3 |
| Docosahexaenoic acid (DHA) | C22:6 n-3 |

Elemental microchemistry, fatty acid profile and geometric morphometrics signatures of goose barnacles (*Pollicipes pollicipes*) reveal their place of origin



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July 2014




Introduction

“Consumers have to be informed about fishery and aquaculture products”
(European Commission Regulation Nº 2065/2001)

“Consumers have the right to receive truthful information about the food they buy.”
(European Commission Regulation Nº 178/2002)

- Aims at the prevention of **fraudulent** practices, **adulteration** of food and **any other** practices which may mislead the consumer.

Introduction Objectives Methods Results Discussion

Figure 8.1 – Oral conference presented at the International Meeting on Marine Resources (IMMR), Peniche (Portugal) - July 2014



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Using elemental microchemistry, fatty acids content and capitulum shape to identify goose barnacle populations from MPAs

Abstract

The Berlengas Natural Reserve (BNR) is a Portuguese archipelago and MPA where the goose barnacle *Pollicipes pollicipes* is exploited. The species is the most heavily exploited intertidal biological resource in the Iberian Peninsula, resulting on overexploitation of stocks. In the BNR goose barnacle's harvesting is strictly regulated however, making it a good example of marine resources management. Analytical methods able to identify the origin of goose barnacle would be an important tool to help the management of the trade. For such purpose, we investigated whether goose barnacles have site-specific differences based on its elemental microchemistry (EM), fatty acid content (FA) and capitulum shape (CS). The analysis was performed on specimens collected from 3 sites in the BNR and 7 along a 300 km stretch of the Portuguese coast. For each individual we analysed the largest lateral shell for EM using ICP-MS, the FA content of the muscle using GC-FID, and the CS using geometric morphometrics. Discriminant function analyses (DFA) for both EM and FA separately provided a high reclassification success (77.6% and 99% respectively, of cross-validated cases correctly classified), while for EM combined with FA allowed for a 100% reclassification success. DFA analysis based only on CS, had a low classification success (29.6%). These results show that EM and FA signatures can be a powerful tool to infer goose barnacles origin, helping in establishing an origin certificate and incrementing the value of biological resources from Portuguese MPAs.

Key words: Goose barnacle; ICP-MS; Fatty acid profile; Geometric morphometrics; Source of origin; Discriminant function analysis

Study area and sampling

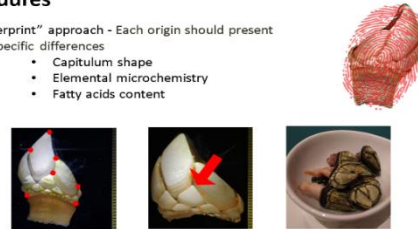
- Portuguese Atlantic coast from Figueira da Foz to Cabo Sardão (Fig. 1)
- 10 sites distributed over a 300 km stretch of the Portuguese Atlantic coast, including 3 sites in the BNR
- Specimens were hand collected at low tide between July-August 2012 and frozen at -80°C



Fig 1 - Goose barnacle collection sites (BNR - Berlengas Natural Reserve)

Procedures

- "Fingerprint" approach - Each origin should present site-specific differences
 - Capitulum shape
 - Elemental microchemistry
 - Fatty acids content



Results



Fig 2 - Results of the canonical discriminant analysis of goose barnacle signatures based on the Capitulum Shape

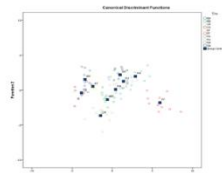


Fig 3 - Results of the canonical discriminant analysis of goose barnacle signatures based on the Elemental microchemistry

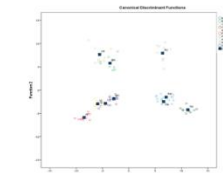


Fig 4 - Results of the canonical discriminant analysis of goose barnacle signatures based on the Fatty acids muscle content

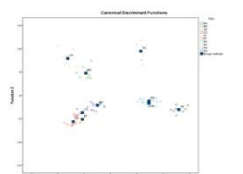


Fig 5 - Results of the canonical discriminant analysis of goose barnacle signatures based on the Elemental microchemistry combined with the Fatty acids muscle content

Discriminant function analysis (DFA) was used to classify the differences in the capitulum shape, elemental microchemistry, fatty acids content of goose barnacles amongst the 10 aforementioned sites. According to our results, we were able to clearly identify our sampling locations into all the 10 sampling sites based on the signatures.

Capitulum shape performed poorly with a low reclassification success (29,6 % of cross-validated grouped cases correctly classified), while elemental microchemistry (77,6 % of cross-validated grouped cases correctly classified) and fatty acids content (99 % of cross-validated grouped cases correctly classified) allow a high reclassification success.

Combining both elemental microchemistry and fatty acid content yielded the highest reclassification success (100% of cross-validated grouped cases correctly classified).

Conclusions

The sampled individuals of *Pollicipes pollicipes* showed differences in microchemistry and fatty acids content that allowed discrimination, while the capitulum shape was not a promising technique.

These results show that a "fingerprint" approach can be an effective instrument to infer goose barnacles source.

It can help in establishing an origin certificate and raise the value of biological resources from Portuguese MPAs.

What's next?

- Temporal stability of EM and FA signatures
- Promote a Geographical Indication scheme for goose barnacles from BNR
- Assess other tools to identify the source origin
- Evaluate the nutritional quality of BNR barnacles



References

Mull, J., Botta, A., & Munkittrick, J. (2006). A system dynamics model for the management of the goose-neck barnacle (*Pollicipes pollicipes*) in the marine reserve of Gacelago (Northern Spain). *Ecological Economics*, 59(1), 306-315.

Cruz, T., Castro, J. J., & Haskins, S. J. (2010). Recruitment, growth and population size structure of *Pollicipes pollicipes* in SW Portugal. *Journal of Experimental Marine Biology and Ecology*, 393(1-3), 200-209.

Macho, G., Viegas, L., Calado, R., & Moleiro, J. (2010). Spatial and temporal distribution of barnacle larvae in the partially mixed estuary of the Ria de Aveiro (Spain). *Journal of Experimental Marine Biology and Ecology*, 393(1-3), 123-130.

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Figure 8.2 – Poster presented at the International Council for the Exploration of the Sea – Annual Science Conference (ICES-ASC), A Coruña (Spain) - September 2014