Growth and reproduction of the limpet Patella rustica linnaeus, 1758 and heat stress physiology of the mediterranean patellid limpets

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GROWTH AND REPRODUCTION OF THE LIMPET *PATELLA RUSTICA* LINNAEUS, 1758 AND HEAT STRESS PHYSIOLOGY OF THE MEDITERRANEAN PATELLID LIMPETS

Doctoral thesis

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This doctoral thesis was performed at the University of Dubrovnik, Department of Aquaculture, under the guidance of Prof. Branko Glamuzina Ph.D., and at the University of Palermo, in the Laboratory of Experimental Ecology, under the guidance of Gianluca Sarà Ph.D., Associate Professor, as a part of the inter-university postgraduate studies of Applied Marine Sciences at the University of Split and University of Dubrovnik.

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CONTENTS

BASIC DOCUMENTATION CARD

University of Split and University of Dubrovnik Ph.D. thesis

GROWTH AND REPRODUCTION OF THE LIMPET *PATELLA RUSTICA* LINNAEUS, 1758 AND HEAT STRESS PHYSIOLOGY OF THE MEDITERRANEAN PATELLID LIMPETS

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Thesis performed at the University of Dubrovnik and University of Palermo

Abstract

First investigation of age, growth and reproduction cycle of the limpet *Patella rustica* on the south eastern Adriatic coast was performed. Marginal increment analysis showed annual periodicity of growth line formation, with annual growth line being deposited in May. Population structure was described and the von Bertalanffy growth curves were fitted for: asymptotic length L_∞=40.86 mm, asymptotic width W_∞=33.02 mm and asymptotic height H_∞=14.07 mm, with corresponding values of growth constant (K) of 0.23, 0.24 and 0.21 $year^{-1}$, respectively. Shells were found to grow allometrically (α=1.66). The maximum, mathematically defined longevity was 12.7 years, but only 2 individuals were observed to be more than 6 years old (6.75 and 7.75 years). Males and females were found to differ in size, with females becoming more prevalent from ~28 mm onwards, suggesting *P. rustica* is a protandrous hermaphrodite. *Patella rustica* has only one reproductive cycle per year with longer breeding period. The spawning occurred synchronously in November. The biggest oocytes were measured in ripe stage with the mean value of 115.6±44.1 µm for diameter and 329.0±125.4 µm for perimeter, while the smallest oocytes were recorded in early developmental stage, 21.3±9.7 µm for diameter and 60.2±30.5 µm for perimeter. Performed experimental research demonstrated that congeneric limpets *P. rustica*, *P. caerulea* and *P. ulyssiponensis* have different physiological responses to thermal stress related to their vertical zonation on the shore. Arrhenius breakpoint temperature for *P. rustica* was 37.9°C, for *P. caerulea* 35.9°C and for *P. ulyssiponensis* 32.2°C. Levels of *hsp70* increased at 34°C and kept increasing with temperature in *P. rustica*, while in *P. caerulea* reached a maximum at 36°C. This suggests that the high shore *P. rustica* is able to tolerate higher temperatures than the lower shore counterparts. Temperature influenced stability of haemocytes in both *P. rustica* and *P. caerulea*, with both species showing inability to recover damaged lysosomes after stress. The results showed that *Patella* congeners already live at the edges of their thermal window and further temperature changes may have large-scale consequences for these species.

(152 pages, 68 figures, 13 tables, 269 references, original in English)

Thesis deposited in National and University Library in Zagreb, Split University Library and Dubrovnik University Library.

Keywords: cardiac activity, growth, heat shock proteins expression (*hsp70*), lysosomal stability, *Patella rustica*, *P. caerulea, P. ulyssiponensis*, reproduction.

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RAST I RAZMNOŽAVANJE PRILJEPKA *PATELLA RUSTICA* LINNAEUS, 1758 I FIZIOLOGIJA TOPLINSKOG STRESA KOD SREDOZEMNIH VRSTA RODA *PATELLA*

Ivana Prusina

Rad je izrađen na Sveučilištu u Dubrovniku i Sveučilištu u Palermu

Sažetak

Provedeno je prvo istraživanje starosti, rasta i reproduktivnog ciklusa priljepka *Patella rustica* na jugoistočnoj obali Jadrana. Analiza rubnog prirasta pokazala je periodičnost formiranja linije rasta koja nastaje tijekom svibnja. Prikupljeni su podatci o sastavu populacije te su izračunati parametri von Bertalanffy krivulje rasta za asimptotsku dužinu ljušture L_∞=40,86 mm, širinu W_∞=33,02 mm i visinu H_∞=14,07 mm, dok su vrijednosti konstante rasta (K) iznosile 0,23 godina^{-1} za dužinu, 0,24 godina^{-1} za širinu i 0,21 godina^{-1} za visinu. Ljušture pokazuju alometrijski rast (α=1,66). Maksimalni životni vijek matematički je procijenjen na 12,7 godina, dok su samo dvije analizirane jedinke bile starije od 6 godina (6,75 i 7,75 godina). Mužjaci i ženke razlikuju se u veličini. Mužjaci dominiraju u manjim veličinskim kategorijama, dok ženke postaju brojem dominantnije pri dužinama većim od 28 mm što upućuje da je *P. rustica* protandrični hermafrodit. Utvrđen je jedan reproduktivni ciklus godišnje s produženim razdobljem sazrijevanja gonada. Mriješćenje se odvija sinkrono u studenom. Najmanje oocite su izmjerene u stadiju ranog sazrijevanja (srednjak za promjer 21,3±9,7 μm i za opseg 60,2±30,5 μm) a najveće u zrelim gonadama (srednjak za promjer 115,6±44,1 μm i za opseg 329,0±125,4 μm). Provedeni pokusi dokazali su da tri vrste priljepaka *P. rustica*, *P. caerulea* i *P. ulyssiponensis* imaju različite fiziološke odgovore na toplinski stres kao rezultat njihove prilagodbe na različito zonirana mikrostaništa. Arrheniusova prijelomna temperatura za vrstu *P. rustica* iznosila je 37,9°C, za vrstu *P. caerulea* 35,9°C, a za vrstu *P. ulyssiponensis* 32,2°C. Kod priljepka *P. rustica* proizvodnja proteina *hsp70* povećava se na 34°C te raste i nakon 38°C, dok se kod vrste *P. caerulea* smanjuje nakon 36°C, dokazujući da vrsta *P. rustica* može podnijeti više temperature od ostale dvije vrste. Temperatura značajno utječe na lizosomalnu stabilnost hemocita kod obje vrste, a u konačnici uzrokuje promjene u funkcioniranju metabolizma. Rezultati pokusa dokazuju da ove vrste već žive na rubu svojih temperaturnih niša, a daljnje promjene temperature mogu imati negativne posljedice.

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Ključne riječi: ekspresija proteina termalnog šoka (*hsp70*), lizosomalna stabilnost, *Patella rustica*, *P. caerulea, P. ulyssiponensis*, rast, reprodukcija, srčana aktivnost.

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1. INTRODUCTION

Limpets are undoubtedly one of the best known and most studied marine herbivores found on rocky shores worldwide (Southward, 1964; Powel, 1973; Underwood, 1979; Branch, 1981; Jenkins et al. 2005). Intertidal communities are fundamentally structured by limpet grazing (Hawkins et al. 1992; Underwood, 2000; Paine, 2002; Jenkins et al. 2005; Coleman et al. 2006), hence they are rightfully considered to be keystone species (sensu Power et al. 1996). In addition, they successfully inhabit varying levels of rocky shores in climatically different coastal regions, making them an excellent model of adaptational biology (Koufopanou et al. 1999; Nakano & Ozawa 2004, 2007; González-Wevar et al. 2010).

In the Mediterranean Sea the genus *Patella* is comprised of four species: *Patella rustica* Linnaeus, 1758, *P. caerulea* Linnaeus, 1758, *P. ulyssiponensis* Gmelin, 1791 and *P. ferruginea* Gmelin, 1971. Except the last one, these congeners were the focus of this study. *Patella ferruginea* is considered to be one of the most endangered marine invertebrates in the Mediterranean, its present geographical distribution is isolated to the western basin and it is considered to be at risk of extinction (Ramos, 1998; Espinosa & Ozawa 2006; García-Gómez et al. 2011). Limpets *P. rustica*, *P. caerulea* and *P. ulyssiponensis* are co-occurring on rocky shores but have different vertical zonation: *P. rustica* occurs in the upper intertidal, *P. caerulea* is dominant in the lower mid-littoral, while *P. ulyssiponensis* inhabits only low intertidal shore (Davies, 1969; Šimunović, 1995; Mauro et al. 2003).

Different aspects of biology of the Mediterranean *Patella* limpets have been investigated in the past decades, however, little is known about their fundamental life-history traits such as age, growth patterns or reproduction cycles, especially for *P. rustica*. These data are necessary in order to understand population dynamics of these species. For example, patterns of growth rate can give us insight into the ecological factors influencing growth, while mode of reproduction can have distributional implications for the species. Furthermore, there is a lack of information about thermal adaptation of these congeners over the narrow vertical tidal gradient that is characteristic of the Mediterranean. There, physical proximity of different species is extremely close and the variation in environmental conditions experienced over this gradient is expected to be compressed. Since habitat conditions mediate larger-scale climate effects, adjacent congeners with differing habitats may show different responses to thermal stress. Knowing how Mediterranean *Patella* limpets are physiologically adapted to their microhabitat is necessary for making synergetic conclusions on their ecology.

This thesis, although not strictly divided, is comprised of three parts. The first part investigates age and growth pattern of *P. rustica*. This was done by sectioning the shell and determining growth from annual lines in the shell cross sections (see Richardson, 2001). The second part of the thesis investigates reproductive biology of *P. rustica*. Histology was used as the primary method of staging gonad development during the reproductive cycle, since according to McCarthy et al. (2008) this technique has proven to be more accurate than macroscopical staging system developed by Orton et al. (1956). The third part of this thesis concerns experimental investigation performed in order to test physiological responses of *P. rustica*, *P. caerulea* and *P. ulyssiponensis* to thermal stress. To investigate the influence of temperature variation on these congeneric limpets, the Arrhenius breakpoint temperatures (ABT), which can represent the metabolic functioning of animals (see Stillman & Somero 1996), chaperone production (heat shock proteins, *hsp*s) and lysosomal stability of the haemocytes were measured.

1.1 Rationale and objectives

Despite their ecological significance, there is a paucity of information about the fundamental population processes of *Patella* spp. in the eastern Adriatic. In this study the main focus was on the high shore limpet *P. rustica*. This species lives in the harsh environment where food supplies are limited and desiccation stress occurs daily. How *P. rustica* channel its energy into growth and/or reproduction was one of the main questions addressed. In addition, the three Mediterranean limpet species, *P. rustica*, *P. caerulea* and *P. ulyssiponensis*, represent an excellent model to test the relationship between vertical zonation and physiological thermal tolerance. Despite the fact that the Mediterranean tidal range is very narrow (not more than 60 cm, see Sarà et al. 2013b), these congeners inhabit different tidal height and as a result will experience different microhabitats, including variation in the levels and duration of thermal stress (see Stillman & Somero 1996; Tomanek & Somero 1999). In the light of climate change, measuring an organism's thermal performance is crucial to an understanding how these species are adapted to their present day environments (Hochachka & Somero 2002). Gathered information from this study will contribute to the better understanding of biology and ecology of these Mediterranean limpets, required for their future management and conservation. In addition, knowing growth patterns and reproduction cycle of *P. rustica* in the eastern Adriatic and its physiological response to thermal stress, can help in understanding the distributional

patterns of this species whose shifts have already been recorded in Portugal and related to the recent warming (Lima et al. 2006; Sousa et al. 2012).

The aims of this thesis were to:

- validate growth line formation in the shells of *P. rustica*
- determine age and growth pattern of *P. rustica* living on the south-eastern Adriatic coast
- describe reproductive cycle of *P. rustica* using histology as the primary method of staging gonad development
- estimate the size at which *P. rustica* becomes sexually mature
- determine sex ratio of *P. rustica*
- estimate the size at which change of sex occurs for *P. rustica*
- experimentally test short-term physiological responses of *P. rustica*, *P. caerulea* and *P. ulyssiponensis* to thermal stress
- determine Arrhenius breakpoint temperatures of *Patella* congeners
- determine if heat shock protein expression will differ between *Patella* congeners
- determine cellular level of response to thermal stress in *Patella* congeners measuring lysosomal stability of haemocytes
- conclude if physiological responses between *Patella* congeners will be different related to their vertical position on the shore.

2. LITERATURE REVIEW

2.1 Order Patellogastropoda or true limpets – significance and main characteristics

Limpets belonging to the order Patellogastropoda (Lindberg, 1986), commonly named true limpets, are a group separated from gastropods early in molluscan evolution (Nakano & Ozawa 2004, 2007). They are quite distinct from other gastropods in their fundamental features such as secondarily uncoiled shell, two pairs of lateral radular teeth, shell microstructure including foliated and conical crossed-lamellar layers, pallial gills and rotation of the pericardium (Lindberg, 1998a; Brusca & Brusca 2003; Nakano & Ozawa 2007). Consequently, they are now considered to be the basal branch of the extant gastropods (Haszprunar, 1988; Ponder & Lindberg 1997; Ridgway et al. 1998), usually refer to as the most primitive group of living gastropods. Nonetheless, primitive does not mean they are unsuccessful animals, quite opposite: the true limpets are inhabitants of both tropical and polar regions, reaching their greatest diversity in temperate climates (Harasewych & McArthur 2000; Nakano & Ozawa 2007). They are very abundant in the intertidal, but can also be found in the subtidal zone, in the deep sea at hydrothermal vents and sulphide seeps, and there are even some species which live on sunken wood at the bottom of the ocean (Lindberg & Hedegaard 1996; Harasewych & McArthur 2000; Nakano & Ozawa 2007).

Being so universal and adapted to many habitats, limpets have been used as models in evolutionary studies (Lindberg & Wright 1985; Hocky et al. 1987; Byers, 1989; Ridgway et al. 1998) and also served as examples of adaptive radiation and historical biogeography (Koufopanou et al. 1999; Nakano & Ozawa 2004, 2007; González-Wevar et al. 2010). Patellogastropods have also great ecological significance since they are one of the most abundant molluscs on rocky intertidal shores. This wave-swept zone is extremely harsh environment and among most physically stressful on earth (Denny & Harley 2006). Intertidal organisms have to endure both terrestrial and marine conditions, altering on daily basis (Denny & Harley 2006; Helmuth et al. 2006). Nonetheless, limpets thrive there: they have adopted a variety of strategies to tolerate fluctuating thermal regimes, including morphological, behavioural and physiological adaptations (Garrity, 1984; Santini et al. 2001; Somero, 2002; Williams et al. 2005; Harley et al. 2009; Williams et al. 2011). Justifiably limpets are called keystone species (sensu Power et al. 1996): they are generalist grazers and can thus indirectly enhance or inhibit the establishment of other organisms (Ribeiro, 2008). Grazing on biofilms, they remove macroalgal propagules and invertebrate larvae and in that way influence

recruitment and play an important role in structuring intertidal rocky shore communities (Southward, 1964; Hawkins & Hartnoll 1983; Jernakoff, 1985; Hawkins et al. 1992; Jenkins et al. 2005; Coleman et al. 2006).

All living limpets have flattened cap-shaped shells with the apex situated at the centre of the shell or moved slightly towards the anterior (Denny, 2000). The coiling of the shell has been greatly reduced in limpets, resulting in a conical shell with a large aperture. Still, the shell shape can differ substantially and an array of functional and evolutionary interpretations has been assigned to explain these differences (see Branch, 1981; Vermeij, 1993; Denny, 2000). Shell morphology is usually related with the surrounding environment (Fretter & Graham 1962; Bannister, 1975) but can be highly variable, often leading to taxonomic confusions (Ridgway et al. 1998; Gonzáles-Wevar et al. 2010). In addition to calcite, proteins and aragonite, different concentrations of iron, potassium, sodium and strontium can be present as shell components (Cabral, 2005; Cabral & Jorge 2007). Furthermore, between species different layers in the shell can be recognized, as well as the orientation of the crystals in each of those layers (MacClintock, 1967). Characters of shell microstructure are of particular importance for identification of fossil limpets (Lindberg, 1988).

Limpets have one pair of cephalic tentacles anteriorly on the head, in addition with pallial tentacles distributed around the mantle edge (Figure 2.1.1) (Matoničkin et al. 1998). The broad foot is attached to the shell with a horseshoe - like muscle, called columellar muscle (Figure 2.1.2) (Thompson et al. 1998). The mouth (Figure 2.1.2, Figure 2.1.3) opens ventrally for feeding on the substrate. Inside the mouth is the primitive radula (Figure 2.1.3) called docoglossan, which consists of 3 lateral and 3 marginal robust teeth, brown in colour from the iron compounds (Matoničkin et al. 1998; Brusca & Brusca 2003). Patellogastropods are also specific for two gill configurations: the gill can be located around the edge of the foot (Figure 2.1.2) and extends around the aperture (*Patella* sp.), or the gill can be located over the head (*Acmaea* sp.), as it is in other gastropod species (Matoničkin et al. 1998).

Limpets have comparatively simple reproductive system. They have one, large gonad under a visceral mass (Figure 2.1.4), that grows in size during maturation and eventually can constitute up to half of body weight (Orton et al. 1956). True limpets are broadcast spawners and in their life cycle they go through a planktonic larval stage (Matoničkin et al. 1998; Ribeiro, 2008): from a free swimming trochophore stage to a veliger stage.

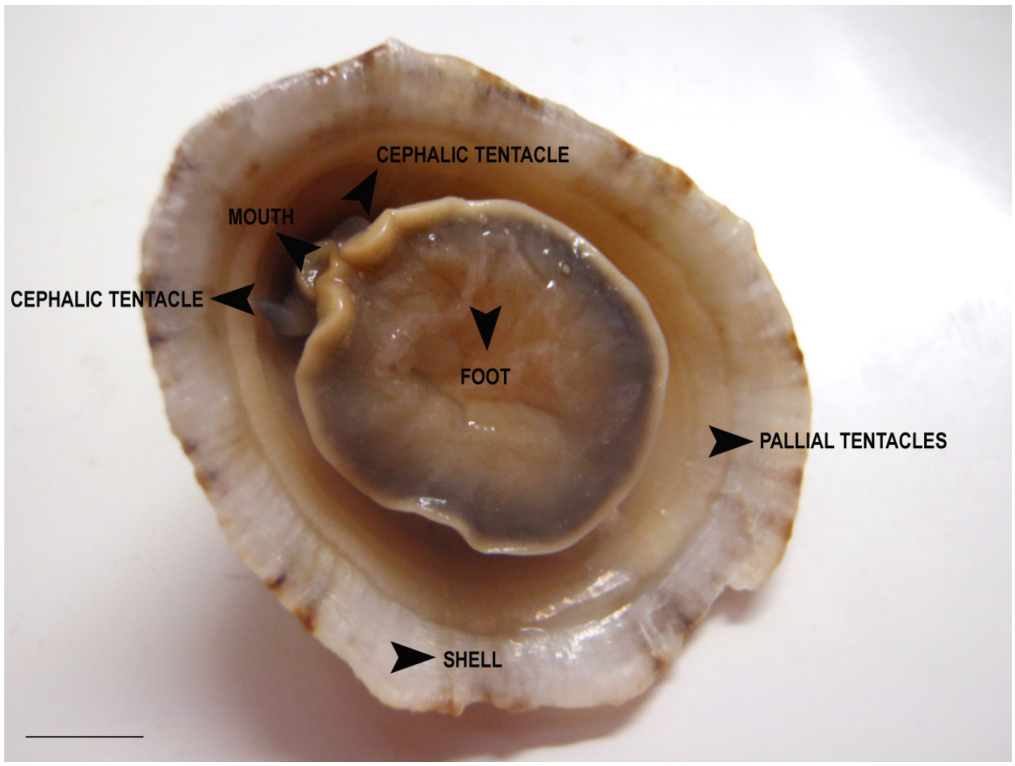

Figure 2.1.1 Underside of the limpet *Patella rustica* showing major body characteristics, scale bar 0.5 mm.

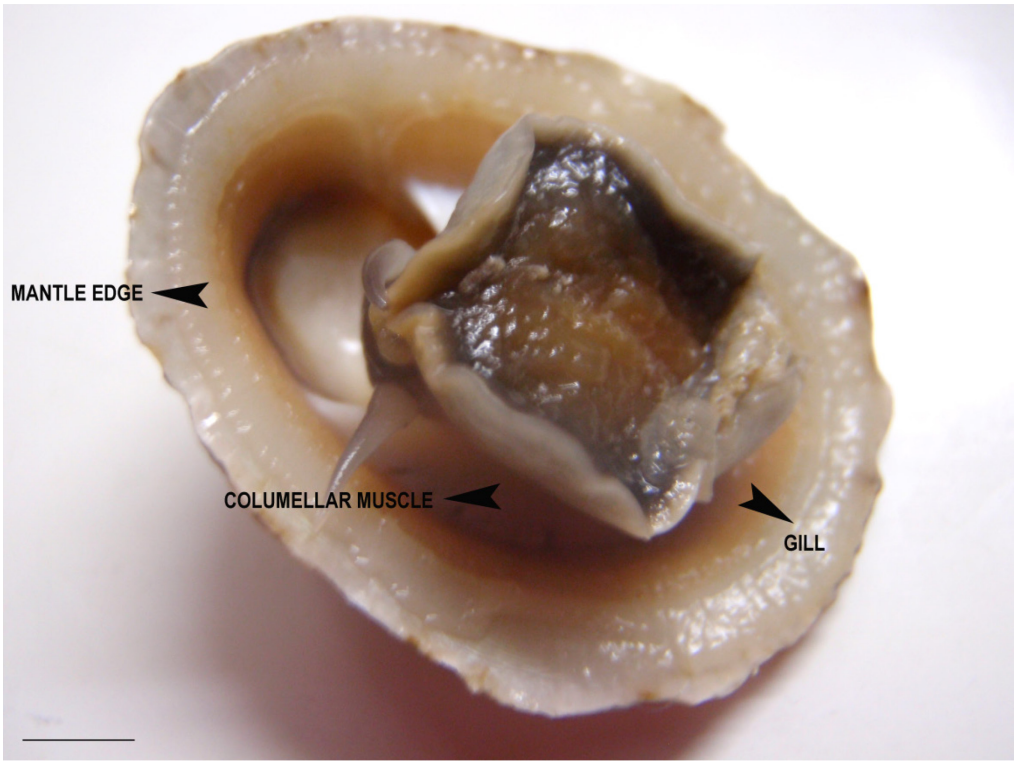

Figure 2.1.2 Underside of the limpet *Patella rustica* showing columellar muscle and pallial gill between the foot and the mantle edge, scale bar 0.5 mm.

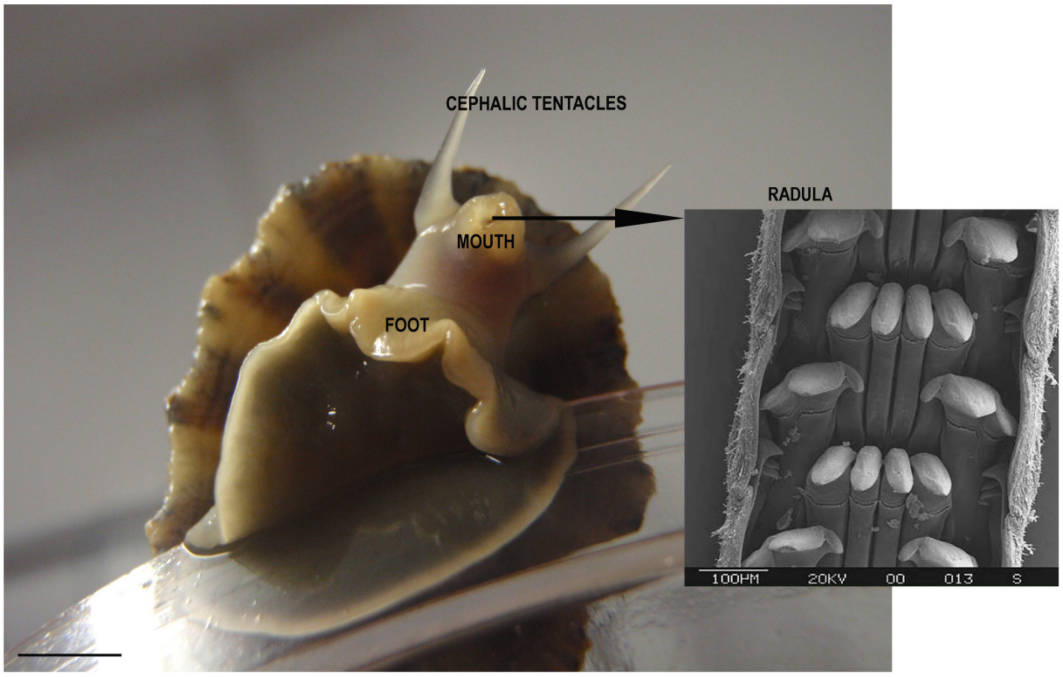

Figure 2.1.3 Underside of the limpet *Patella rustica*, showing head with cephalic tentacles and mouth, scale bar 0.5 mm; photo of docoglossan radula taken with electron microscope (Source: Salzburg University, http://www.uni-salzburg.at).

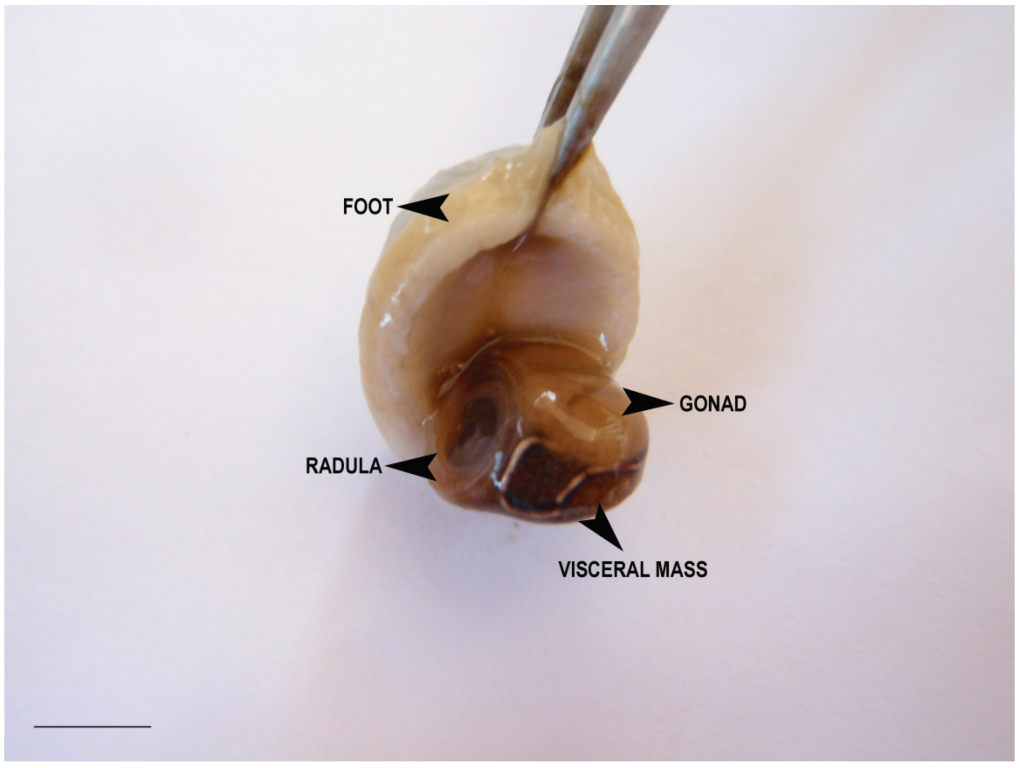

Figure 2.1.4 View of the limpet's body after the shell has been removed showing visceral mass (shell side), underlying gonad (foot side) and long radula (sideward), scale bar 0.5 mm.

A veliger larvae has an embryonic shell on dorsal side and beginning of the foot on ventral side. It will spend from 2 to 4 weeks in plankton, feeding and accumulating energy for upcoming metamorphosis (Matoničkin et al. 1998). The sexes are normally separated. Protandry, the most common form of sequential hermaphroditism in molluscs (Heller, 1993), has long been suggested for many species of the genus *Patella* (Ribeiro, 2008). This phenomenon has usually been correlated with territorial species - beginning life as males before becoming females, often upon the acquisition of a feeding territory (Branch, 1981). In the protandric species that are being harvested (e.g. *Patella ulyssiponensis* or *P. ferruginea*), protandry can increase species vulnerability, since larger individuals have greater probability of being collected resulting in decrease of reproductive output (Ribeiro, 2008).

Almost all limpet species exhibit homing behaviour, i.e. they have permanent place to live attached to the substratum on the rocks called home scar (Hyman, 1967; Fretter & Graham 1994). They move during feeding and return to the same place after. Migratory movements of patellogastropods are limited to a general up-shore pattern, and the upper limits are assumed to be set by tolerance to abiotic, environmental conditions (Davies, 1969, 1970; Branch, 1981).

Due to phenotypic variability among characters used for identification, such as shell morphology or the coloration of the foot, the taxonomy of limpets has always been considered as debatable subject (Ridgway et al. 1998; Hawkins et al. 2000; Sá Pinto et al. 2010). The true limpets were previously divided into three families based on the gill type: 1) Acmaeidae, with single ctenidium in the mantle cavity over the head, 2) Patellidae, where the ctenidium has been lost and replaced with a secondary gill surrounding the foot in the pallial groove, and 3) Lepetidae, with no gills at all, respiring through the body surface (Keen, 1960; Powell, 1973). Within then defined Patellidae, two sub-families were generally recognized: a) Patellinae (three pairs of lateral teeth) and b) Nacellinae (two pairs of lateral teeth). But further research demonstrated that shell microstructure and alimentary canal, such as radula, jaws and gut, can show the evolutionary history of the group (Sasaki & Okutani 1993; Lindberg, 1998a, b; Sasaki, 1998). Lindberg (1988) in his review of relationships in the Patellogastropoda, proposed a new division into two sub-orders: Patellina and Nacellina. According to Lindberg (1988), the sub-order Nacellina comprises four families: Nacellidae, Lepetidae, Acmaeidae and Lotiidae, distinguished by reductions of shell layers and simplifications of gills and radula. Recent molecular studies recognized at least seven families in the order Patellogastropoda: Lottiidae, Acmaeidae, Pectinodontidae, Patellidae, Lepetidae, Eoacmaeidae, and Nacellidae (Nakano & Ozawa 2007; González-Wevar et al. 2010).

2.2 Overview of the family Patellidae

While Patellogastropods are abundant worldwide (Branch, 1985a, b; Lindberg, 1988), patellid limpets i.e. members of family Patellidae, show two centres of diversity: one in the north-eastern Atlantic and the Mediterranean (Fretter & Graham 1976; Hawkins et al. 1992; Fretter & Graham 1994; Ridgway et al. 1998), and another one on the shores of southern Africa, where they reach their greatest diversity with 18 endemic species (see Ridgway et al. 1998). The taxonomy of family Patellidae has been reviewed by Ridgway et al. (1998) based on shell shape and shell microstructure, headfoot, pallial complex and gut loops, radula and alimentary system and sperm morphology. The proposed classification identified 38 patellid limpets divided in four genus: *Helcion* (four species distributed in southern Africa), *Cymbula* (eight species distributed in southern Africa, eastern Atlantic, southern Indian Ocean), *Scutellastra* (seventeen species distributed in southern and western Africa, Australia and Pacific) and *Patella* (nine species distributed in north-eastern Atlantic and Mediterranean). Koufopanou et al. (1999) performed a molecular phylogeny of patellid limpets (Figure 2.2.1), confirming that the genus *Patella* is indeed monophyletic and the sister taxon of all other patellids. In addition, same authors estimated that the radiation of modern species have occurred between 5 and 20 million years before present and most likely could have been influenced by the opening and closing of the Mediterranean.

Nine species (Ridgway et al. 1998) within the genus *Patella* are:

i. *Patella rustica*
ii. *Patella caerulea*
iii. *Patella ulyssiponensis*
iv. *Patella ferruginea*
v. *Patella candei* D'Orbigny, 1839
vi. *Patella depressa* Pennant, 1777
vii. *Patella vulgata* Linnaeus, 1758
viii. *Patella lugubris* Gmelin, 1791
ix. *Patella pellucida* Linnaeus, 1758

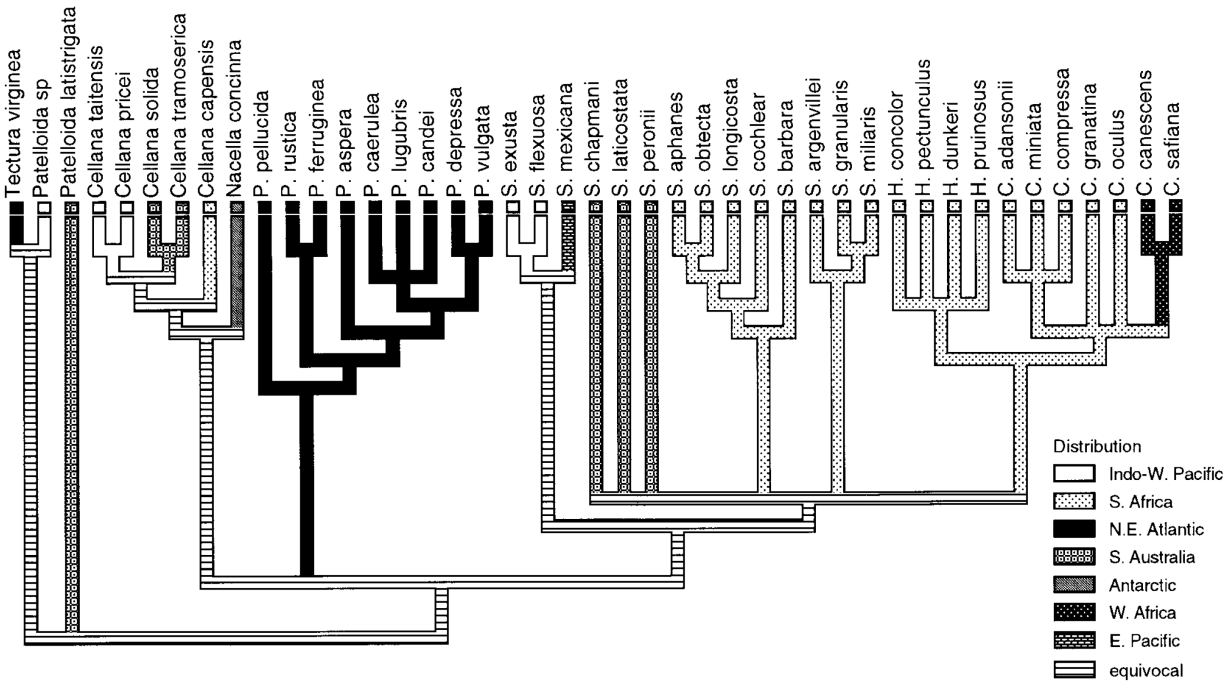

Figure 2.2.1 Molecular phylogeny of patellid species with geographical distributions (taken from Koufopanou et al. 1999).

2.3 Biological and ecological features of the investigated species

In this study, three congeneric limpet species were investigated: *Patella rustica*, *P. caerulea* and *P. ulyssiponensis.* All three species occur sympatrically along Mediterranean but inhabit different vertical heights on rocky shores (Davies, 1969; Sella et al. 1993; Šimunović, 1995; Mauro et al. 2003).

The limpet *P. rustica* (Figure 2.3.1), commonly named lusitanian limpet denoting its southern distribution, ranges from the Mediterranean to the Atlantic coasts of the Iberian Peninsula and northern Africa, including Macaronesian Islands (Christiaens, 1973; Ridgway et al. 1998; Lima et al. 2006). Its southern limit is Mauritania and northern is located in French Basque Country (Crisp & Fischer-Piètte 1959). Within this range there was a gap, placed between Portugal in the south and Spain in the north (Lima et al. 2006). However, Lima et al. (2006) reported about recent changes in the distribution of lusitanian limpet and its sudden expansion to northern Portuguese shores, bridging this historical distribution gap.

Figure 2.3.1 *Patella rustica* shell: top, bottom and side view (source: Malacologist's corner, http://www.elrincondelmalacologo.com).

The limpet *P. caerulea* (Figure 2.3.2), called blue limpet due to blue inner shell reflection, is endemic to the Mediterranean and is common in both eastern and western Basins (Christiaens, 1973; Ribeiro, 2008). *Patella caerulea* is a sedentary species, it colonises new isolated habitats and it is numerically abundant both on artificial structures (e.g. seawalls and harbour jetties) and natural rocky shores (Fauvelot et al. 2009).

Figure 2.3.2 *Patella caerulea* shell: top, bottom and side view (source: Malacologist's corner, http://www.elrincondelmalacologo.com).

Patella ulyssiponensis (Figure 2.3.3) is commonly named china limpet alluding to porcellanous white inner part of the shell, or also rough limpet due to the ridges on the outer surface. It has a widespread distribution and is present throughout the Mediterranean, the Macaronesian Islands, the northern African coasts and in Europe as far north as southern Norway and British Isles (Ribeiro, 2008). This species has been heavily exploited in the Azores for human consumption and consequently populations have been severely reduced in many of those islands (Hawkins et al. 2000; Ribeiro, 2008).

Figure 2.3.3 *Patella ulyssiponensis* shell: top, bottom and side view (source: Malacologist's corner, http://www.elrincondelmalacologo.com).

Patella rustica is easily distinguished by brown spots near the high shell apex, while *P. caerulea* and *P. ulyssiponensis* are morphologically variable and can even overlap in shell colouration and morphology in some Mediterranean localities (Cretella et al. 1990; Sella et al. 1993; Mauro et al. 2003). According to Cretella et al. (1990), the only character to distinguish *P. ulyssiponensis* in the field is the radial ribs on the outer shell and the yellow coloration of the foot. The Mediterranean intertidal region is very heterogeneous environment over a short vertical distance and this could explain the morphological and genetic variability of the mentioned limpet species (Mauro et al. 2003).

2.4 Previous studies

Limpets have often captivated biologists' attention, but the past couple of decades have aggregated a wealth of information in diverse aspects of research. Their small size allows easy manipulation and handling both *in situ* and in the laboratory. Considering their important role as key stone species in intertidal communities worldwide, it is not surprising that limpets have been widely used in biological and monitoring studies of the health of rocky shore communities (e.g. Jones & Baxter 1985; Marchán et al. 1999; De Pirro et al. 2001; Chelazzi et al. 2004; De Pirro & Marshall 2005). Reviewing the great extent of available information from limpets' studies, two major areas of interest are noticeable:

1. energy flow, including food acquisition and energy expenditure via growth and reproduction,

2. limpets' adaptation to physical factors and their role in determining vertical zonation and distribution.

Most of the true limpets are herbivorous grazers, scraping the food from the substratum by radula (Branch, 1981). Grazing is the most important link between primary producers and consumers in food chains and can consequently control biomass and productivity of ecosystems (Paine, 2002; Coleman et al. 2006). A number of extensive experimental manipulations of patellid grazers have been performed in order to test their key role in controlling macroalgal development and community complexity (Southward & Southward 1978; Jenkins et al. 1999; Benedetti-Cecchi et al. 2001; Williams et al. 2000; Benedetti-Cecchi et al. 2001; Boaventura et al. 2002; Jenkins et al. 2005; Coleman et al. 2006; Range et al. 2008). Food availability and diet composition were studied in three Mediterranean congeners, *P. rustica*, *P. caerulea* and *P. ulyssiponensis* and it was suggested that their zonation patterns are successful mechanism of diet segregation (Della Santina et al. 1993). The same study demonstrated that the diet differences were greater between *P. rustica* and *P. caerulea* than between *P. caerulea* and *P. ulyssiponensis*, probably due to the upward foraging of *P. rustica*, allowing it to exploit mainly supralittoral epilithic algae. Santini & Chelazzi (1995) demonstrated that the high shore *P. rustica*, being naturally exposed to prolonged periods of desiccation and starvation, is able to store more glycogen and has more efficient mechanism for energy conservation during these unfavourable conditions. Contrary, the lower shore *P. caerulea* foraging more regularly is able to store glycogen at lower concentrations and therefore has a lower capacity to reduce energy losses in periods of starvation (Santini & Chelazzi 1995).

A number of studies have been conducted in order to investigate the reproductive cycles of *Patella* congeners, e.g. *P. vulgata* (Orton et al. 1956; Thompson, 1980; Bowman & Lewis 1986; Delaney et al. 2002; McCarthy et al. 2008; Ribeiro et al. 2009), *P. depressa* (Orton & Southward 1961; Bowman & Lewis 1986; Brazão et al. 2003; Moore et al. 2007; Ribeiro et al. 2009), *P. ulyssiponensis* (Evans, 1953; Thompson, 1979; Bowman & Lewis 1986; Guerra & Gaudêncio 1986; McCarthy et al. 2008; Ribeiro et al. 2009), and *P. caerulea* (Frenkiel, 1975; Belkhodja et al. 2011). Reproduction cycle of *P. rustica* was previously described on Algerian (Frenkiel, 1975), Basque (Othaiz, 1994) and Portuguese coast (Ribeiro et al. 2009). None of the studies were conducted on the Adriatic shores, and only few of performed studies used histology as the primary method of staging gonad development (see McCarthy et al. 2008; Belkhodja et al. 2011).

Due to weaker growth patterns in gastropod shells, investigations of their age and growth have mostly been understudied (Richardson, 2001). Majority of the available studies used mark-recapture method to determine limpets' growth and longevity (Kenny, 1977; Bretos, 1978, 1980; Kido & Murray 2003; Gray & Hodgson 2003; Clark et al. 2004; Espinosa et al. 2008) or length frequency distributions method (Guerra & Gaudêncio 1986; Brethes et al. 1994; Khow, 2007). However, in intertidal molluscs like limpets, microgrowth lines are forming daily or tidally, and determining the periodicity of their deposition is important for the exact age determination (Richardson, 1989, 1990; Richardson & Liu, 1994; Richardson, 2001). Microgrowth patterns have been described in different limpet species including *P. vulgata* (Ekaratne & Crisp 1982), *Fissurella crassa* Lamarck, 1822 (Bretos, 1978), *Siphonaria gigas* G. B. Sowerby I, 1825 (Crisp et al. 1990), *Cellana toreuma* Reeve, 1854 (Richardson & Liu 1994), *Scutellastra granularis* Linnaeus, 1758 (Vat, 2000) or *Helcion pectunculus* Gmelin, 1791 (Gray & Hodgson 2003). Hitherto, no research was performed to describe growth patterns in *P. rustica* or its other congeners.

Research into limpets' adaptation to physical factors was always a synergy of physical environment, physiology and intertidal ecology and has often focused on the role of thermal stress (Newell, 1979; Somero, 2002; Helmuth et al. 2005; Denny et al. 2006; Harley et al. 2009). Stress is known to be affected by a variety of climatic factors (Helmuth, 2002; Harley & Helmuth 2003; Harley & Paine 2009) and under these conditions physical factors, such as desiccation, temperature and salinity, may be linked and can augment one another. Desiccation for limpets is usually considered the most important physical factor and the patterns of distribution of intertidal organisms have often been explained by responses to desiccation

(Chelazzi, 1990; Coleman, 2010). Osmolality of the haemolymph is considered to be a good measure of desiccation stress (Wolcott, 1973; Branch, 1981; Marshall & McQuaid 1992; Williams & Morritt 1995; Morritt et al. 2007). Aggregation behaviour of limpets was proposed to reduce water loss and desiccation stress (Gallien, 1985), since more water will be retained on the rock surface between them. However, Coleman (2010) demonstrated in his study with *Cellana tramoserica* Holten, 1802 that aggregation behaviour was not connected with desiccation reduction but more likely with predation defence.

Cardiac activity in invertebrates received an increasing interest in the last two decades due to development of the non invasive technique (Depledge & Anderson 1990). Measuring cardiac activity in limpets is of particular interest since there is a positive correlation between heart rate and oxygen consumption and therefore can be considered as a metabolic measurement (Marshall & McQuiad 1992; Santini et al. 1999). In their study of porcelain crabs, Stillman & Somero (1996) used Arrhenius break temperatures (ABTs) of cardiac performance (the temperature above which cardiac activity drops off dramatically) as an indicator of thermal limitation. The same measurement was successfully employed by Dong & Williams (2011) to test temperature - adaptive differences in cardiac thermal tolerance in two tropical limpet species *C. toreuma* and *C. grata* Gould, 1859. Furthermore, through cardiac activity many studies investigated direct physiological response of different limpet species to thermal stress, e.g. *S. granularis*, *Siphonaria oculus* Krauss, 1848 (Marshall & McQuaid 1992), *C. toreuma* and *C. grata* (Chelazzi et al. 1999, 2001; Dong & Williams 2011); variation in salinity, *P. caerulea, P. ulyssiponensis* (De Pirro et al. 1999) or oxygen tension, *S. granularis*, *S. oculus* (Marshall & McQuaid 1993). The most applicable measurement of cardiac activity is the one used as a prerequisite to identify limpets as possible bioindicators of chemical pollution (Depledge et al. 1995; Marchàn et al. 1999; De Pirro et al. 2001; Chelazzi et al. 2004; De Pirro & Marshall 2005).

The production of heat shock proteins (*hsp*) is another indicator of a species thermal sensitivity (reviewed by Feder & Hofmann 1999; Pörtner 2002). It is known that intertidal animals, when facing thermal stress, will show variable expression patterns of *hsp*s (Tomanek & Somero 2000; Dong et al. 2008; Dong & Williams 2011), reflecting thermal adaptation to their local habitats. This chaperone production was investigated in a number of limpet species, such as Antarctic limpet *Nacella polaris* Hombron & Jacquinot, 1841 (Clark et al. 2008), four Californian congeners *Lottia scabra* Gould, 1846, *L. austrodigitalis* Murphy, 1978, *L. pelta* Rathke, 1833 and *L. scutum* Rathke, 1833 (Dong et al. 2008), and tropical limpets *L. gigantea*

Gray in G. B. Sowerby I, 1834 (Miller et al. 2009), *C. toreuma* and *C. grata* (Dong & Williams 2011).

The lysosomes are a key mediator in major metabolic functions and have been used primarily as a reliable indicator of toxic injury at the cellular level and a good diagnostic biomarker of individual health status (Russo et al. 2009). Vital dye, such as neutral red (NR), has been used to test lysosomal permeability since it accumulates passively in lysosomes as long as the lysosomal membrane remains undamaged (Russo et al. 2009). Most of the work performed on different gastropod species used neutral red retention (NRR) assay or neutral red uptake (NRU) assay in order to test their sensitivity to different pollutants (Brown et al. 2004; Gopalakrishnan et al. 2009; Russo et al. 2009; Deschaseaux et al. 2011). Only a few studies, mainly on bivalves such as *Ostrea edulis* Linnaeus, 1758, *Crassostrea gigas* Thunberg, 1793 or *Mytilus edulis* Linnaeus, 1758, used NRR assay to test haemocyte lysosomal stability as a response to temperature changes (Hauton et al. 1998; Camus et al. 2000; Zhang & Li 2006; Munari et al. 2011). No research of lysosomal stability was performed on Mediterranean *Patella* congeners.

2.5 Limpets as a proxy for climate change

Warming of the world climate is evident and the recent increase in temperature has been regarded as a major driver of change in natural systems (Rosenzweig et al. 2007). Intertidal organisms are ectotherms and their growth, reproduction and general fitness are influenced with environmental temperature (Hochachka & Somero 2002). Therefore, since their survival and performance are strongly tied to their physical environment, these organisms can potentially serve as a sensitive bellwether of global climate change (Denny & Harley 2006). How species can adapt and response to unsteady environment is becoming an area of increasing interest (Walther et al. 2002; Harley et al. 2006; Helmuth et al. 2006; Parmesan, 2006; Williams et al. 2011). Furthermore, since intertidal is considered to be physically most stressful environment, intertidal species are often believed to live very close to their thermal tolerance limits and consequently are considered highly sensitive to climate change (Helmuth et al. 2002). Limpets have emerged as one of the best proxies for climate change studies since they are easy to monitor and manipulate in the field and in the laboratory, they are ubiquitous on rocky shores worldwide and usually have different zonation patterns depending on their specific thermal niches, making them even more suitable to test their adaptivity. Limpets are

also considered to be potential controlling species or keystone species, since their removal, or alterations of behaviour may have cascade effect on intertidal communities and fundamental ecosystem consequences.

A numerous studies have been conducted in order to test acclimation capacities of limpets to temperature stress, and most of them have been performed on tropical Hong Kong species *Cellana grata* and *C. toreuma* (Williams & Moritt 1995; Chelazzi et al. 1999, 2001; Williams et al. 2005; Moritt et al. 2007; Firth & Williams 2009; Harley et al. 2009; Dong & Williams 2011; Williams et al. 2011), but also on Australian *C. tramoserica* (Coleman, 2010), Patagonian limpet *Nacella magellanica* Gmelin, 1791 and *N. deaurata* Gmelin, 1791 (Pöhlman et al. 2011), Antarctic limpet *Nacella concinna* (Clark et al. 2008), North American limpets from the genus *Lottia* (Dong et al. 2008; Dong & Somero 2009; Harley et al. 2009; Miller et al. 2009), *P. vulgata* (Harley et al. 2009) and pulmonate limpet *S. gigas* (Harley et al. 2009). It is suggested that impacts of climate warming could be more severe in tropics than in temperate regions (Tewksbury et al. 2008). The reason for this is little temperature variation in tropics throughout the year, for which tropical ectotherms are believed to live near or above their optimal performance temperature (Deutsch et al. 2008).

Limpets have also emerged as model organisms for studies of biogeographic processes because of their near - linear geographic distributions (Sagarin & Gaines 2002) and climate-driven colonisation (Sousa et al. 2012). There are documented cases of recent changes of *P. rustica* distributional range, which is thought to be a direct consequence of global warming (Lima et al. 2006, 2007; Sousa et al. 2012).

3. MATERIAL AND METHODS

3.1 Area and dynamics of sampling

Limpets were collected on two locations in the Mediterranean, one in the Adriatic and the other in the Tyrrhenian Sea (Figure 3.1.1 A). For the research of growth and reproduction of the limpet *Patella rustica*, sampling was performed in Zaton Bay (Figure 3.1.1 B), in the south-eastern Adriatic Sea. Zaton is enclosed rocky shore bay situated 8 km northwest of Dubrovnik. Limpets were hand collected monthly from July 2011 to June 2012. For the experimental investigation of heat stress physiology, limpets *P. rustica*, *P. caerulea* and *P. ulyssiponensis* were hand collected in Addaura and Altavila (Figure 3.1.1 C) in the Tyrrhenian Sea of the western coast of Italy. Addaura and Altavilla are rocky shores situated northeast and southeast from Palermo Bay (Sicily), respectively. Dynamics of limpet sampling for each analysis is shown in Table 3.1.1 and Table 3.1.2.

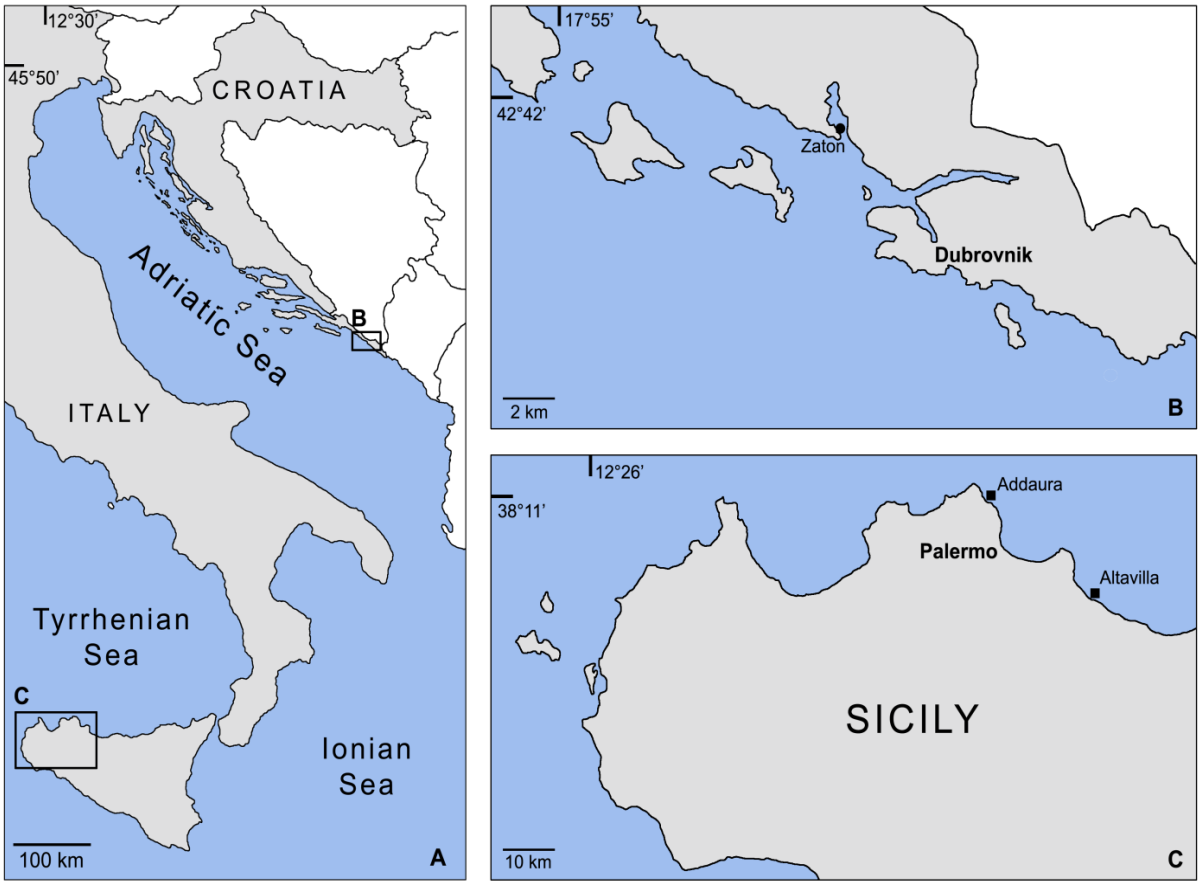

Figure 3.1.1 A: The map of Croatia and Italy with denoted sampling sites in black squares; **B**: Zaton Bay in Croatia (sampling site for growth and reproduction of the limpet *Patella rustica*); **C**: Addaura and Altavilla in Italy (sampling sites for the experimental investigation of heat stress physiology of the three Mediterranean limpet species).

Table 3.1.1 Sampling size and dynamic of limpet collection with respect to analysis performed; N - number of individuals per sampling, N_{tot} - total number of sampled individuals for each analysis, X - mean shell length in mm, SD - standard deviation.

Growth and reproduction of *Patella rustica*				
Analysis	N	Sampling dynamics	N_{tot}	X±SD
Marginal increment analysis	5	monthly (07/2011-06/2012)	60	14.4±1.6
Inner growth lines	120	once (09/2011)	120	20.2±6.2
Endoliths	5	once (06/2012)	5	32.6±2.3
Histology (medium sized)	30	monthly (07/2011-06/2012)	355*	24.2±2.9
Histology (smaller sized)	**	**	95	16.2±3.0
Sex change	***	once (09/2011)	70	23.6±5.3
1st sexual maturity	****	3-monthly (09/2011-11/2011)	65	15.5±3.8
Condition index (CI)	30	monthly (07/2011-06/2012)	348*	24.0±2.3

* in December only 25 limpets were collected for histology and 18 for CI
** N=10 in September, N=30 in October, N=25 in November, N=15 in February, N=10 in March, N=5 in May
*** N=30 medium sized, N=10 smaller sized and N=30 wider range of shell length
**** N=10 in September, N=30 in October, N=25 in November

Table 3.1.2 Sampling size of limpets collected during December 2011, with respect to analysis performed; N - number of individuals per sampling, N_{tot} - total number of sampled individuals for each analysis, X - mean shell length in mm, SD - standard deviation.

Heat stress physiology of Mediterranean patellid limpets					
Analysis	N	X±SD			N_{tot}
		P. rustica	***P. caerulea***	***P. ulyssiponensis***	
ABT	5	26.9±3.0	27.7±2.9	25.9±3.2	15
Hsp70	25	25.0±1.8	24.9±2.3	*	50
NRU	12	24.4±2.3	25.2±1.8	*	24

ABT=Arrhenius break temperature, *Hsp70*=heat shock proteins, NRU=Neutral red uptake assay
*due to inclement weather conditions, this species was not sampled for *hsp70* and NRU

3.2 Growth and reproduction study of the limpet *Patella rustica*

3.2.1 Hydrographic parameters

Average daily sea surface and air temperatures (°C) for Dubrovnik area for the period of July 2011 to June 2012 were obtained from Croatian Meteorological and Hydrological Service (http://meteo.hr). Salinity and dissolved oxygen (mg/L) were recorded in the Zaton Bay monthly at the sea surface (0 m) with YSI probe (YSI Model 85 Handheld Oxygen, Conductivity, Salinity and Temperature System) from August 2011 to June 2012.

3.2.2 Analysis of chlorophyll *a* concentration

Sea surface water was collected with bottle, monthly from July 2011 to June 2012 in the Zaton Bay. Chlorophyll *a* concentration (chl *a*) was determined from 500 mL sub-samples filtered through Whatman GF/F glass-fibre filters stored at −20°C. These were homogenized and extracted in 90% acetone for 24 hours at room temperature (Holm-Hansen et al. 1965). Samples were analyzed fluorometrically with a Turner TD-700 Laboratory Fluorometer (Sunnyvale, CA) calibrated with pure chl *a* (Sigma). Concentration of chl *a* was calculated according to Jeffrey & Welschmeyer (1997):

$$\text{Chlorophyll } a\ (\mu\text{g L}^{-1}) = \frac{\text{K}\text{F}_\text{m}\text{v}\ (\text{F}_0 - \text{F}_\text{a})}{\text{V}_\text{f}(\text{F}_\text{m} - 1)}$$

where:

K = relationship factor between concentration of standard and fluorescence reading of the standard (concentration of standard/reading of the standard),

F_m = relationship of fluorescence of the chl *a* standard before and after acidification (F_o /F_a),

F_o = fluorescence of the sample before acidification,

F_a = fluorescence of the sample after acidification,

v = volume of the extract (mL),

V_f = volume of the sample filtered (L).

3.2.3 Age and growth analysis

Limpets were sampled from July 2011 to June 2012 with sampling dynamics described in Table 3.1.1 for each analysis. In the laboratory, the meat of each limpet was carefully removed from the shell and shell length (L) - greatest distance between the anterior and posterior ends of the shell, width (W) - greatest distance between margins perpendicular to the anterior/posterior axis and height (H) - greatest vertical distance from the apex to the base of the shell were measured with Vernier calliper to the nearest 0.1 mm and weighted with analytical scale to the nearest 0.001 g. Measured dimensions are shown in Figure 3.2.3.1.

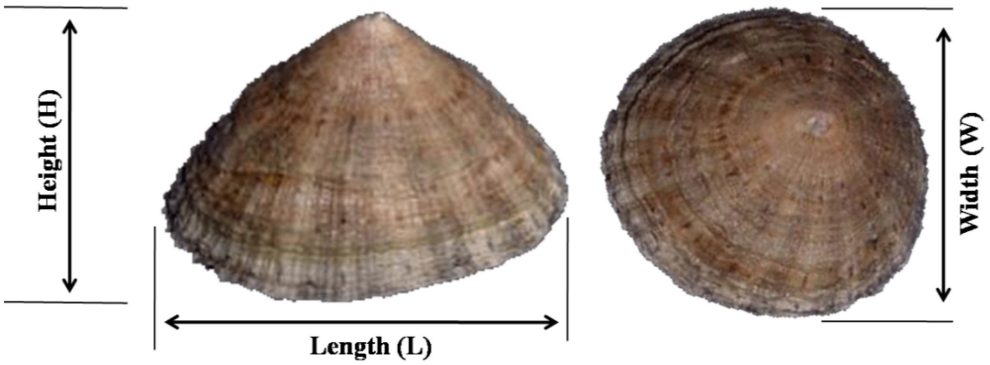

Figure 3.2.3.1 Measured dimensions for each sampled individual: shell length (L), shell width (W) and shell height (H).

The age was determined from acetate peel replicas of shell sections. Shells were embedded in epofix resin (Struers, Figure 3.2.3.2 A-B) and sectioned through the apex along the axis of maximal growth using Struers Labotom cut-off saw (Figure 3.2.3.2 C-D). The cut surface were ground on trimite (80, 120, 220, 500 and 1200) and polished (Figure 3.2.3.2 C, E) with 3 µm diamond paste. The polished cut sections were washed in detergent, air-dried and etched in 0.1 M HCl for 1 minute after which they were again washed in water and air-dried (Figure 3.2.3.2 F). Acetate peel replicas of the polished and etched sections were prepared according to Richardson et al. (2001). Acetone was pipetted onto the etched cut section abundantly to avoid any bubbles after which an acetate sheet was carefully placed over the cut section and air-dried for 15 minutes. The acetate was peeled off from the cut section and mounted on microscopic slides. Photographs of acetate peel replicas were made with a Zeiss AxioCam ERc 5s at 2.5× magnification and the number of growth lines was counted.

The age and growth analysis requires validation of growth line formation. In this study the marginal increment analysis was applied: the analysis of smaller individuals with higher growth rate over a one year period. Smaller individuals (14.4±1.6 mm, mean length±SD) were collected monthly, their shells were embedded in raisin, sectioned from the apex to the margin and acetate peels were made as described above.

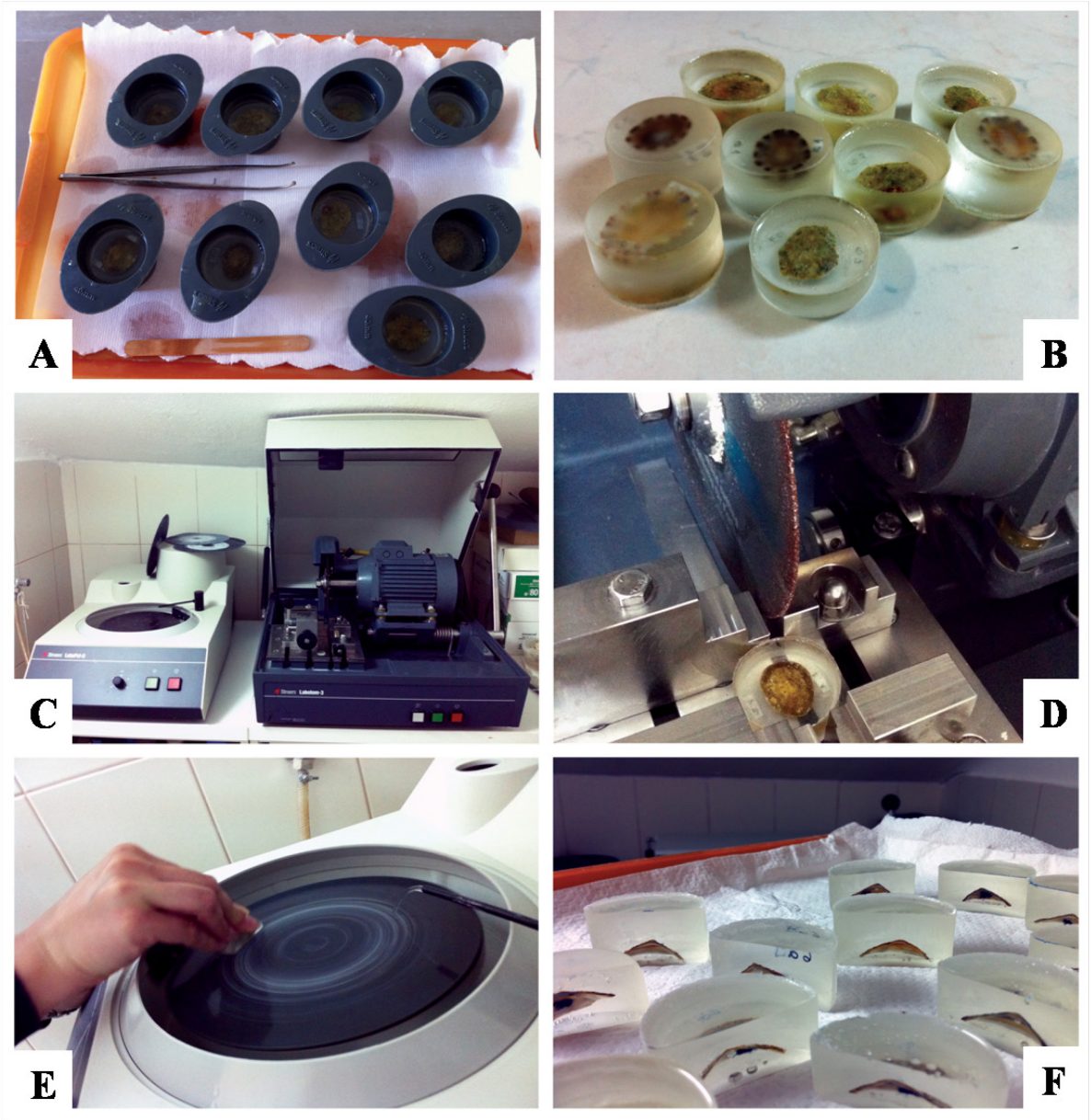

Figure 3.2.3.2 Preparation of acetate peel replicas from shell sections **A**: moulds for embedding the shells in epofix resin; **B**: shells embedded in resin; **C**: Struers Labotom cut-off saw and Labopol machine for grounding and polishing; **D**: sectioning of embedded shell from the apex to the margin using Struers Labotom cut-off saw; **E**: polishing cut sections; **F**: polished and washed cut sections prior to preparation of acetate peels replicas.

To determine the timing of growth line formation, the distances from the last visible growth line and the shell margin were measured using Axio Vision Rel 4.8 software. Following validation of an annual periodicity of growth line formation, each growth line in acetate peel replicas from collected individuals was identified and their age determined. Obtained age, length, width and height data for each of the 119 individuals, ranging in shell length from 8.1 to 33.6 mm (20.2±6.2 mm) were fitted to the von Bertalanffy growth function (Sparre & Venema, 1998):

$$L_t = L_\infty(1 - e^{-K(t-t_0)})$$

where L_t is the shell length at the time t; L_∞ is the asymptotic maximum length i.e. the length at which further growth is stopped; K is growth constant and t_0 is the length at time zero (Gulland, 1983), usually called initial condition parameter and it represents the hypothetical time (age) at which the length of the organism is zero. The same was applied for width and height changing L_t for W_t or H_t and obtaining asymptotic maximum width (W_∞) and height (H_∞). The longevity was calculated according to Taylor (1958):

$$A_{95} = t_0 - \frac{\ln(1 - 0.95)}{K}$$

where A_{95} is a life span to attain 95% of L_∞, t_0 is the length at time zero, 0.95 is 95% and K is growth constant.

3.2.4 Analysis of endoliths in the limpet *Patella rustica*

For identification of the endolithic species involved in penetrating *Patella rustica* shells, modified method from Golubić et al. (1970) was used. After collection, the meat of each limpet was removed from the shell and shell length, width and height were measured with Vernier calliper to the nearest 0.1 mm. The shells were decalcified in 8% hydrochloric acid solution (HCl). Decalcification was done two times per 3 hours, after which the soft organic parts were mounted on microscopic slides. Slides were analyzed with Zeiss Axio Lab.A1 microscope at 100×, 200× and 400× magnification and photographs were made with a Zeiss AxioCam ERc. The species were identified according to Golubić et al. (2005), Royer et al. (2006) and Riascos et al. (2008).

3.2.5 Histological analysis

For the qualitative and quantitative histological analysis, 30 medium-sized individuals were sampled monthly (N=355, 24.2±2.9 mm), except in December 2011 when only 25 individuals were collected due to inclement weather conditions (Table 3.1.1). In addition, during the study period, different size classes of limpets were also sampled randomly and processed histologically for different analysis (Table 3.1.1). Total of 30 individuals with wider range of shell length, from 14.6 to 33.6 mm (22.4±5.3 mm) were sampled in September 2011 to obtain an estimate of size at which change of sex occurs. Furthermore, smaller limpets (N=95), ranging in length from 10.1 to 22.4 mm (16.2±3.0 mm) were sampled in September 2011 (N=10), October 2011 (N=30), November 2011 (N=25), February 2012 (N=15), March 2012 (N=10) and May 2012 (N=5) and used for qualitative histological analysis. Out of these, 65 individuals collected during the 3-month period when late gametogenesis and maximal gonad activity was observed (September, October and November 2011) were used to obtain an estimate of minimal size at first sexual maturity. To estimate size at first sexual maturity, the data were fitted in equation:

$$P = \frac{1}{1 + e^{a - b \times L}}$$

where P is probability that individuals are sexually matured and L is their length. The length when 50% of analyzed individuals were mature was calculated according to Sparre & Venema (1998):

$$L_{50\%} = \frac{a}{b}$$

For histological analysis, length, width and height of each limpet was measured to the nearest 0.1 mm using Vernier caliper. In the laboratory, limpets were dissected and since it was not possible to precisely remove gonad tissue from digestive gland, both tissues were fixed in 10% buffered formalin. The purpose of fixation was to preserve tissues permanently in as life-like a state as possible. Fixation was followed by tissue processing that included dehydration in increasingly concentrated ethanol (70%, 80%, 95% and 100%) and tissue clearing or removal of the dehydrant with chloroform. Finally, tissue was embedded in paraffin (Histowax, Leica) and sectioned on microtome at 5 μm. Sections were adhered to the slides over a warm water

bath that helped remove wrinkles and later stained by haematoxylin and eosin dyes. Haematoxylin, being a basic dye, has an affinity for the nucleic acids of the cell nucleus and dye them blue to purple, while eosin is an acidic dye with an affinity for cytoplasmic components of the cell and dye them pink (Kozarić, 1997).

For qualitative analysis, histological slides were examined using Zeiss Axio Lab.A1 microscope at 50×, 100× and 400× magnification, sexed and assigned to a development stage adopted from McCarthy et al. (2008) and Belkhodja et al. (2011) and modified for this species. For males, five stages were determined (Table 3.2.5.1): early active (3), late active (4), ripe (5), spawning (2) and spent (1). For qualitative analysis of female gonads seven stages were applied (Table 3.2.5.2): inactive (0), early active (3), late active (4), ripe (5), atresic (1.5), spawning (2) and spent (1). Individuals where sex was not possible to determine were marked as undetermined.

A mean gonad index (MGI) was calculated for each month, separately for males and females, to estimate the proportion of individuals in different reproductive stages. Values of MGI were obtained by multiplying the number of individuals from each developmental stage by the numerical ranking of that stage, and dividing the result by the number of individuals for each sex (Gosling, 2003).

For quantitative analysis, one photograph of random visual field was made with a Zeiss AxioCam ERc 5s at 100× magnification for each histological slide. Each visual field had an area of 0.585 mm^2, inside of which all oocytes were measured. Oocyte size, expressed as oocyte perimeter and diameter, were measured using Axio Vision Rel 4.8 software.

Table 3.2.5.1 Description of gonad stages in *Patella rustica* males, adopted from McCarthy et al. (2008) and Belkhodja et al. (2011) and modified for this species.

Stage No	Description	♂ Male gonad development
3	**EARLY**	Islet of small compact acini. One or two layers of spermatogonia line the edges of testes lobes. Connective tissue still abundant.
4	**LATE**	Layers of reproductive cells completely fill the lumen. These cells are in different stages of maturation forming a regular pattern from the tubular wall to the lumen - spermatogonia, spermatocytes and spermatides. Tails of first spermatozoa are in the tubular lumen resulting in pink color of lumen.
5	**RIPE**	Tubules are enlarged with only one layer of spermatogonia attached to the tubular wall. Free spermatozoa fill the lumen. Light pink connective tissue very thin and stretched between large acini.
2	**SPAWNING**	The tubules are smaller in size surrounded with loose and abundant connective tissue. Large germinal cells abundant around the edges. The number of spermatozoa inside the tubules decreased.
1	**SPENT**	Thick connective tissue present in the gonad. Haemocytes can be observed in the interstitial and connective tissue surrounding residual spermatozoa.

Table 3.2.5.2 Description of gonad stages in *Patella rustica* females, adopted from McCarthy et al. (2008) and Belkhodja et al. (2011) and modified for this species.

Stage No	Description	♀ **Female gonad development**
0	**INACTIVE**	Abundant connective tissue in the gonad. Only a few oogonia are present.
3	**EARLY**	Acini lumen large and mostly empty. Small oogonia in proliferation adhering to the acini wall. Previtellogenic oocytes of various size can also be found. Connective tissue still abundant.
4	**LATE**	Gonadal acini are enlarged. Reduction of connective tissue. Reproductive cells fill the tubule and are in different stages of maturation. Fewer oogonia attached to the acini wall. Large previtellogenic oocytes and few oocytes in the final stages of vitelogenesis are present. Oocytes undergoing different stages of atresia can be present also.
5	**RIPE**	Gonadal acini large with dominance of completely developed free vitellogenic oocytes. Mature oocytes undergoing atresia or complete lysis can be found. Acini with oocytes in advanced vitellogenesis showing signs of atresia can also be present in this stage.
2	**ATRESIC**	Gonadal tissue is still abundant but majority of oocytes are undergoing high degree of atresia. A few previtellogenic and vitellogenic oocytes can be present also.
2	**SPAWNING**	Decrease in free vitellogenic oocytes in the lumen, some of which showing signs of atresia. Symptoms of total voidance of gametes characterized by small acini with no generative activity in their walls. Pedunculated oocytes attached to acini wall can be observed.
1	**SPENT**	Ovaries are slack showing signs of tissue destruction. Abundant connective tissue present around empty acini. Only few degenerative oocytes can be present. Haemocytes can be observed in the interstitial and connective tissue.

3.2.6 Condition index analysis

For the condition index (CI) analysis, 30 *Patella rustica* (N=348, 24.0±2.3 mm) were collected monthly, except in December 2011 when only 18 individuals were collected and frozen for later laboratory analysis. Prior to the analysis, *P. rustica* individuals were defrosted and placed on filter paper to remove excess of water (Figure 3.2.6.1 A-B). Length, width and height of each limpet were measured to the nearest 0.1 mm using Vernier caliper. Soft tissue was carefully removed from the shell and meat and shell of each individual were weighted to the nearest 0.001 g using analytical scale after which they were dried at 105°C for 24 h to constant weight. Samples were then re-weighted to the nearest 0.001 g to obtain dry weight of meat and shell (Figure 3.2.6.1 C-D). The CI was determined according to Mann (1978):

$$\mathrm{CI} = \frac{\text{dry meat weight (g)}}{\text{dry shell weight (g)}} \times 1000$$

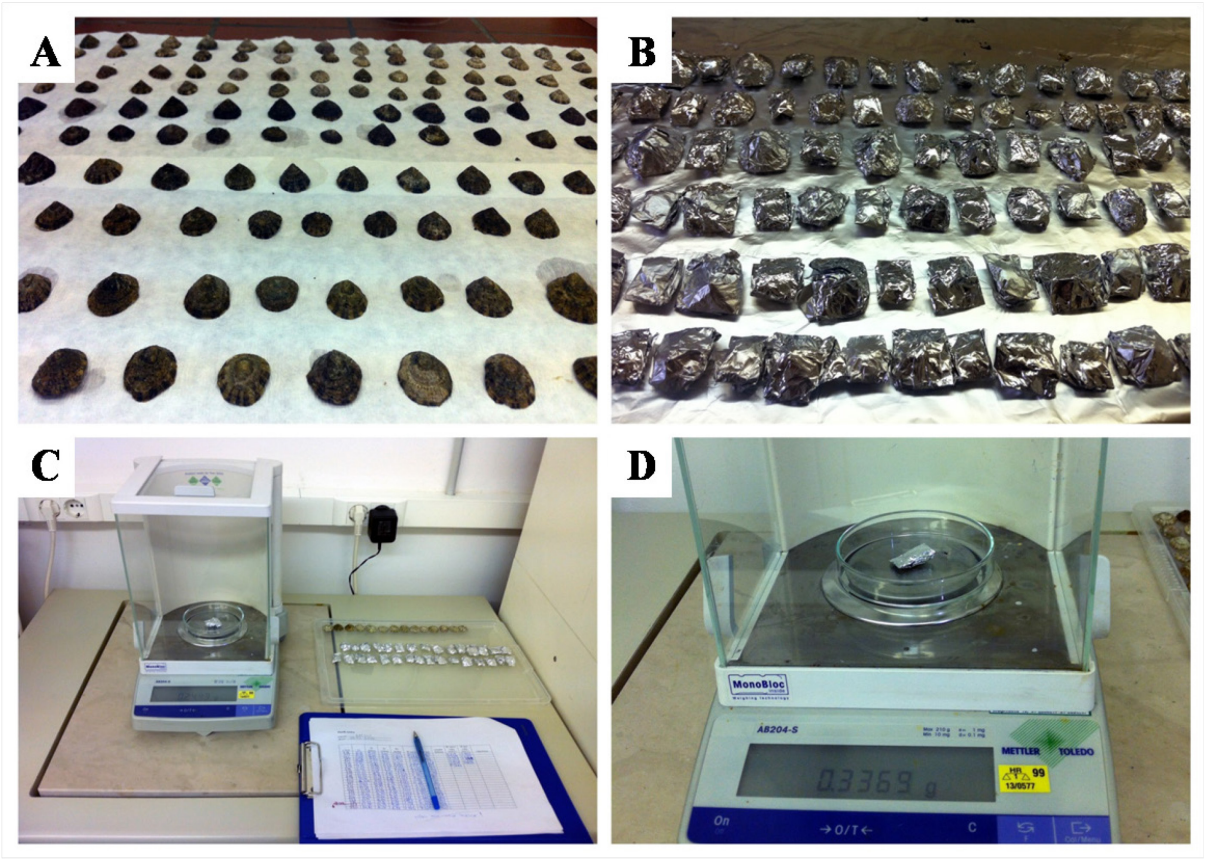

Figure 3.2.6.1 Laboratory processing of limpets for condition index calculation **A**: limpets on filter paper; **B**: limpets prepared for drying in the oven; **C, D**: weighing limpets on the analytical scale (the weight of the aluminium foil was later detracted from the total weight).

3.3 Heat stress physiology of the Mediterranean patellid limpets

Cardiac activity (expressed as Arrhenius breakpoint temperatures), heat shock response *(hsp70* production) and lysosomal stability (neutral red uptake assay) were measured in congeneric limpets *Patella rustica*, *P. caerulea* and *P. ulyssiponensis* in order to analyze the abrupt exposure and short-term responses of limpets to temperature changes. Limpets, *P. rustica*, *P. caerulea* and *P. ulyssiponensis* were collected in December 2011 on Sicilian rocky shores (Table 3.1.2). The procedure of animal handling after collection was the same for every experiment and was as follows. Limpets were collected on the ebbing tide and so were assumed to have been actively feeding before collection and were about to become inactive prior to emersion (see Williams et al. 2005). Individuals were immediately placed in Petri dishes and kept moist with seawater during transportation (<1 hour) to the Laboratory of Experimental Ecology at the University of Palermo. In the laboratory, limpets in their Petri dishes were placed into a tank with seawater spray at room temperature (20°C) for 1 hour to allow them to regain mantle water, discard metabolic wastes and recover from stress due to transportation (see Williams et al. 2005). During this period limpets did not feed and settled onto the Petri dishes.

3.3.1 Arrhenius breakpoint temperature

Heart rates were measured using the non-invasive technique developed by Depledge & Anderson (1990), modified by Chelazzi et al. (1999) and improved by Burnett et al. (2013). The shell of each limpet was cleaned and individuals were placed on a Petri dish with the side wall removed to allow drainage. An infrared sensor was glued onto the limpet shell (Loctite Super, Italy) in a position directly over the heart (Figure 3.3.1.1)

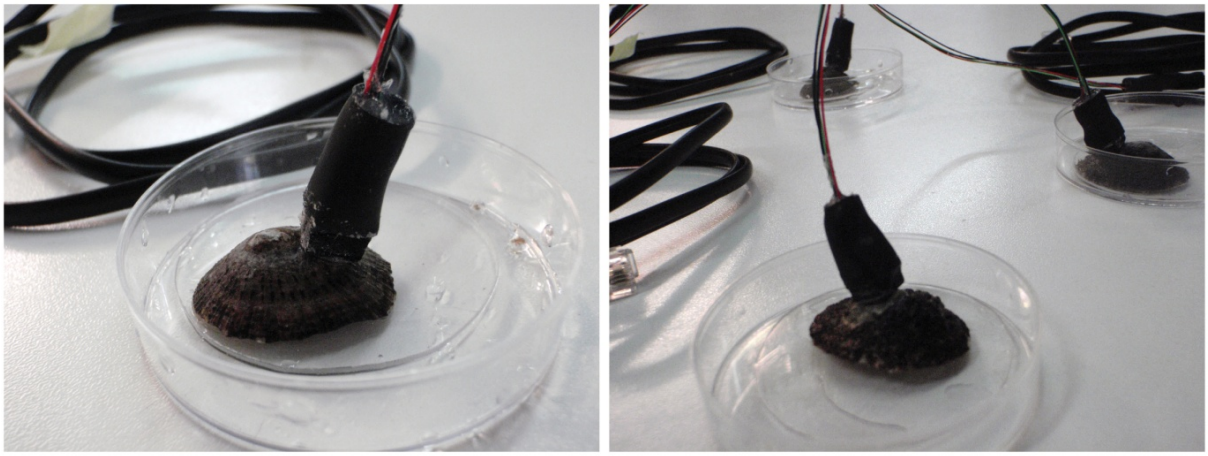

Figure 3.3.1.1 The placement of an infrared sensor onto the limpet shell.

The infrared sensor (Figure 3.3.1.2 A-B) consisted of an infrared-light-emitting diode from which the signal was filtered, amplified (AMP-3, Newshift Lda, Portugal, Figure 3.3.1.2 A-B) and recorded using a portable Oscilloscope (PicoScope 2203, Figure 3.3.1.2 A-B). Heart rate traces could subsequently be viewed and analyzed using PicoScope (version 6.6.13.15, Figure 3.3.1.2 A). Animals were then returned, in their Petri dishes, back to the seawater spray for 1 hour to recover from handling.

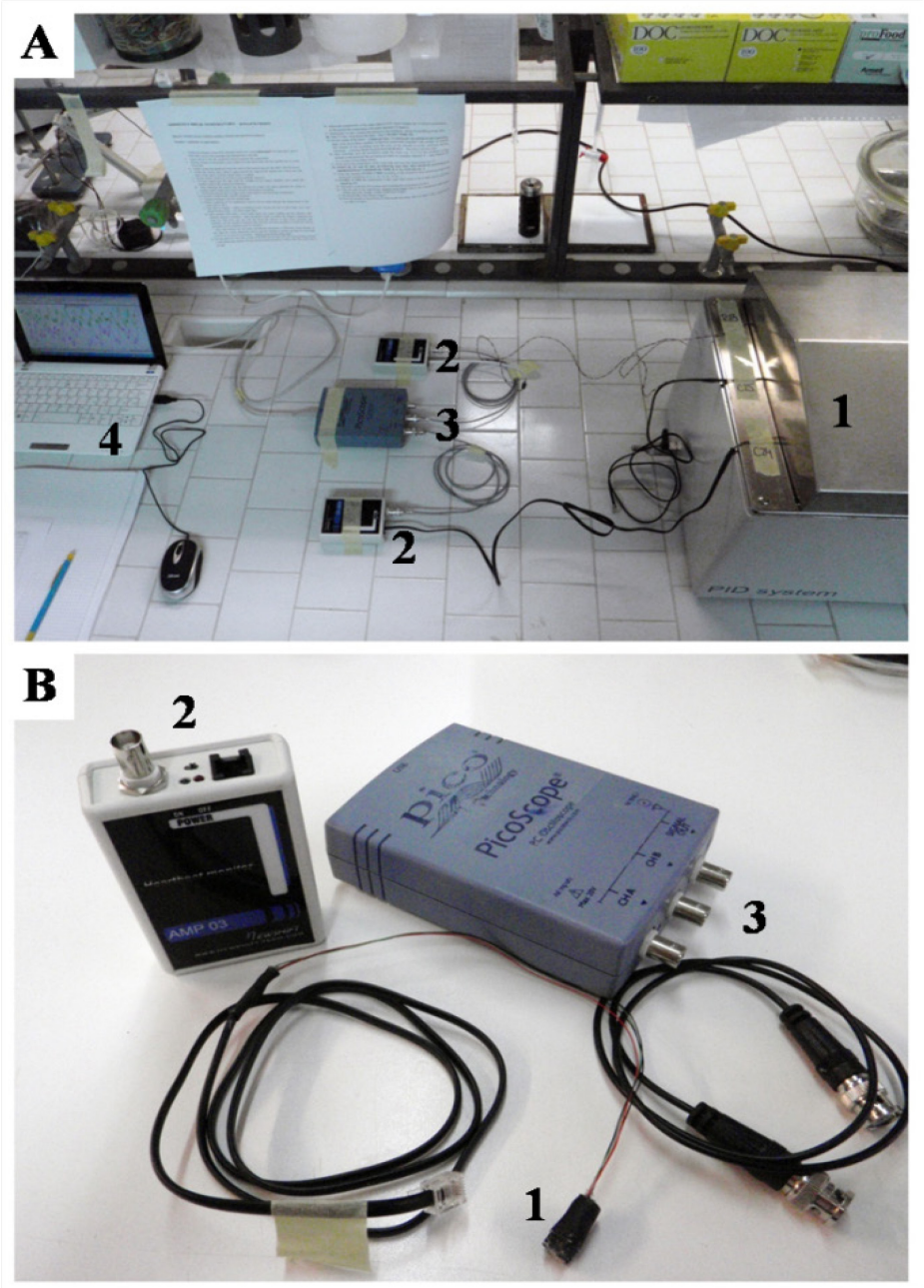

Figure 3.3.1.2 Recording of cardiac activity **A**: two limpets with attached sensors inside the water bath (1), each sensor connected to amplifier (2), two amplifiers connected to Oscilloscope (3), recorded signal viewed on personal computer using Picoscope software (4); **B**: infrared sensor (1), signal amplifier (2), portable Oscilloscope for signal recording (3).

Five individuals of each of the three species were used to determine Arrhenius breakpoint or Arrhenius Break Temperatures (ABT, the temperature above which cardiac activity drops off dramatically; Stillman & Somero 1996). Each limpet in their Petri dish was placed into a beaker (diameter=45 mm, height=70 mm) in air, which was then partly immersed in a programmable water bath (PID system, MPM Instruments s.r.l., type M428-BM) at 20°C (Figure 3.3.1.3).

Figure 3.3.1.3 Programmable water bath with beakers placed inside.

Animals were allowed 15 minutes to acclimatize, during which the temperature inside the water bath, and subsequently of the air in the beaker, was raised to 23°C. The temperature was then continuously increased at a rate of ~3°C per 15 minutes, mimicking an emersion period in the natural environment (Sarà et al. 2013b) and monitored until heart beat was lost (see De Pirro et al. 2001). To ensure temperatures were stable, air temperature inside the beaker and bath water temperature were recorded every minute using data loggers (iButton Inc, ±0.5°C). In general, limpet body temperatures are within a degree of air and/or substrate temperature (Denny & Harley 2006; Denny et al. 2006). Using a similar methodology, limpet body temperatures were found to typically be within ±0.2°C of the vial surface temperatures (KA Villarta, unpublished data), so it is assumed that there is a strong relationship between measured temperature and limpet body temperature. At the end of the experiment, each limpet was measured (±0.1 mm) and weighed (±0.001 g).

Real time heart rates (beats per seconds) of five individuals of each species were recorded every 5 minutes. To estimate heart rate, at least five heart beats were counted during three continuous oscilloscope frames of 10 seconds each, within each 5 minutes recording. Individual average heart rates from the three counts were computed and transformed to the natural logarithm of beats per seconds for Arrhenius plots. Arrhenius break temperatures were determined for each individual by generating the two intersecting linear regressions that best fitted the data, and calculated from the intersection of these two lines (see Dahlhoff & Somero 1993; Stillman & Somero 1996). To test potential differences in temperature sensitivity, Q10 relationships (rate of metabolism change as a consequence of increasing the temperature by 10°C, Van't Hoff, 1884) were calculated in the range of 23°C to 33°C for all three species.

3.3.2 Heat shock proteins

To determine the expression of heat shock proteins (*hsp70*) under different thermal regimes 25 individuals of *Patella rustica* and 25 of *P. caerulea* were exposed to different temperatures and different durations of specific temperatures. *Hsp70* levels could not be measured for *P. ulyssiponensis* due to inclement weather conditions preventing collection of animals. A possible exposure scenario (Denny et al. 2006) was mimicked, where limpet's body temperature rises during emersion to a maximum level, after which the animal would be immersed by the rising tide or splashed by waves. Limpets were randomly taken from the holding tank in seawater spray (20°C), measured (±0.1 mm), wet weighed (±0.001 g), placed on individual Petri dishes and left again in seawater spray at 20°C to recover from handling. After 1 hour, each limpet was put into a beaker, in air, which was immersed in the programmable water bath with initial temperature of 20°C (as described in previous experiment). Five non-heated limpets, held only in seawater spray at 20°C, of each species were used as controls. The temperature in the water bath was increased at a rate of 2°C per 15 minutes to reach 38°C and maintained at 38°C for 2 hours (8°C per 1 hour, see Denny et al. 2006; Dong et al. 2008). Temperatures in the beaker and in the water bath were recorded every minute (as described in previous experiment). Five individuals of each species were randomly collected at 20°C, 34°C, 36°C, 38°C after 60 minutes and 38°C after 120 minutes of heat duration, and placed under seawater spray (20°C) for 2 hours to allow expression of *hsp70* (as described in Dong et al. 2008; Dong & Williams 2011). After 2 hours, individuals were removed, rapidly dissected, and the foot muscle was macerated in an Eppendorf tube and stored in RNAlater (Sigma-Aldrich) prior to *hsp* estimation. Total RNA was isolated from ~20 mg of foot tissue by TrizolReagent (Invitrogen, USA).

A sample of 1 μg of total RNA was used as the template for synthesis of the first strand of cDNA using PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). Four degenerated primer pairs: SeaActinF and SeaActinR (Clark et al. 2008, Table 3.3.2.1), CAL5 and CAL6 (Jennings & Etter 2011), BtubF1 and ABtub4r (Einax & Voigt 2003), NS4 and AB1 (Lin et al. 2013) were used to amplify the *β-actin*, *calmodulin*, *beta-tubulin* and *18S ribosomal RNA* gene; and two degenerated primers, dAIHSP70F and dAIHSP70R, were used to amplify the *hsp70* gene (Song et al. 2006, Table 3.3.2.1). A 488 bp partial *β-actin* gene (GenBank accession No. KF494231, KF494235), a 258 bp *calmodulin* gene (GenBank accession No. KF494234), a 1212 bp *beta-tubulin* gene (GenBank accession No. KF494230, KF494234), a 879 bp *18S ribosomal RNA* gene (GenBank accession No. KF494227, KF494228) and a 626 bp partial *hsp70* gene (GenBank accession No. KF494229, KF494232) were amplified from the limpets. Comparison of the similarity of the sequences using a BLAST search in GenBank confirmed that the heat shock protein genes amplified from the two limpets were inducible isoforms of *hsp70*.

The expression of *hsp70* was determined by using real-time quantitative PCR with primers qrHSP70F, qrHSP70R, qcHSP70F and qcHSP70R, which were designed based on the partial *hsp70* gene. The reference genes were selected from *18S ribosomal RNA*, *β-actin*, *β-tubuli*n and *calmodulin* using GeNorm Algorithm (Primer Design, Ltd., Southampton University, UK) as described by Etschmann et al. (2006). GeNorm is a bioinformatics tool designed to rank candidate reference genes by using a normalization factor calculated on the basis of the geometric mean of the expression levels of the candidate reference genes in an array of representative samples. For *P. rustica* and *P. caerulea* the expression stability measures (M values) of *18S ribosomal RNA*, *β-actin*, *β-tubulin* and *calmodulin* were 2.449, 1.775, 1.775 and 2.553 and 1.416, 0.733, 0.733 and 1.046 respectively, when all genes were included in the calculation of M. Based on its low M values, partial sequences of the *β-actin* gene were selected as reference housekeeping genes to normalize the level of *hsp 70* expression. The partial *β-actin* gene, amplified using the primers qactionF and qactionR, was selected as an internal control.

Real-time PCR conditions were the same for all set of primers and was carried out on a ABI 7500 Real-Time PCR System (Applied Biosystems, USA, Figure 3.3.2.1) in a 20 μL reaction volume containing 10 μL of 2×FastStart Universal SYBR Green Master (Roche, Swiss), 0.8 μL of each primer (10 nmol μL^{-1}), 1 μL of cDNA template and 7.4 μL of RNAse-free water. PCR conditions were as follows: 50°C for 2 minutes; 95°C for 10 minutes; 40

cycles of 95°C for 20 seconds; 55°C for 20 seconds and 72°C for 40 seconds with a final dissociation curve step. All samples were measured in triplicate. The cycle threshold (Ct) values were analyzed using the ABI 7500 System Software (Applied Biosystems, USA). A Ct value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold, and it is inversely proportional to the original relative expression level of the gene of interest. The abundance of *hsp70* mRNA was expressed as the relative expression ratios (RU) of the *hsp70* compared to the *β-actin* between the control and the treated samples as described by Pfaffl (2001).

Figure 3.3.2.1 ABI 7500 Real-Time PCR System (Applied Biosystems, USA) used for PCR *hsp70* gene expression analysis (source http://www.sequences.crchul.ulaval.ca/eng/appareils.html).

Table 3.3.2.1 Sequences of primers used for gene clone and real-time PCR analysis in *Patella rustica* and *P. caerulea*

Primer name	Primer Sequences (5' - 3')	Amplicon size (bp)	PCR efficiency	Source
Gene clone				
SeaActinF	ACCGACTACYTSAKKAAGATCCT	488		Clark et al. 2008
SeaActinR	GAVGCVAGGATGGAGCCRCC			
dAIHSP70F	CAGGAATTCAARCGYAAACAC	626		Song et al. 2006
dAIHSP70R	TTGGTCATKGCTCGYTCTCC			
CAL5	TTYGACAAGGAYGGHGATGG	258		Jennings & Etter 2011
CAL6	TCGGCGGCACTGATGAANCCGTTNCCGTC			
BtubF1	CAGGCYGGNCAGTGYGGHAACCAGATTGG	1212		Einax & Voigt 2003
ABtub4r	GCYTCNGTGAARTCCATYTCGTCCAT			
NS4	CTTCCGTCAATTCCTTTAAG	879		Lin et al. 2013
AB1	GGAGGATTAGGGTCCGATTCC			
Real-time PCR analysis				
qactinF	ATATCAACATCGCACTTCAT	87	1.95, *P. rustica* 2.05, *P. caerulea*	Self-designed
qactinR	ACTCTTCCAACCTTCCTT			Self-designed
qrHSP70F	TTATTGGTGGATGTAGCC	123	1.86	Self-designed
qrHSP70R	AGCATAAGTTGTGAATATCTG			Self-designed
qcHSP70F	AACATCGCAGATATTCACAAC	75	1.86	Self-designed
qcHSP70R	GCTCGCTCTCCTTCATAG			Self-designed
qCALF	AACCATTACAACCAAGGA	178	1.972, *P. rustica* 2.001, *P. caerulea*	Self-designed
qCALR	TTCTTCTTCACTGTCTGT			Self-designed
q18SF	ATGGAATAATGGAATAGGA	180	1.955, *P. rustica* 1.950, *P. caerulea*	Self-designed
q18SR	TTCGTTCTTGACTAATGA			Self-designed
qBTUBF	TTCTGTTCTTGATGTTGT	138	2.050, *P. rustica*	Self-designed
qBTUBR	GGATATTCTTCACGGATT		1.953, *P. caerulea*	Self-designed

3.3.3 Neutral red uptake assay

To determine the responses of limpets to temperature changes on cellular level, the lysosomal stability of the haemocytes of the limpets *Patella rustica* and *P. caerulea* was assessed using the neutral red uptake assay (NRU). This assay (Test Method Protocol NHK, 2003) provides a quantitative estimation of the number of viable cells in a culture.

A possible exposure scenario (Denny et al. 2006) was mimicked, as described in previous experiment with heat shock proteins. Total of 24 limpets, 12 individuals of *P. rustica* and 12 of *P. caerulea*, were taken from the holding tank with spray seawater (20°C) and randomly placed into a plastic beaker, which was immersed in a programmable water bath with initial temperature of 20°C. Three non-heated limpets of each species, held only in seawater spray at 20°C, were used as controls. The temperature in the water bath was then increased at a rate that mimicked an emersion period in the natural environment (3°C per 15 minutes, Sarà et al. 2013b) to reach the maximum temperature of 38°C and maintained at 38°C for 30 minutes. Temperature in the bottom of the beaker and in the water bath was recorded every minute using thermo data loggers (iButton Inc, ±0.5°C). Three limpets of each species were randomly collected at 4 sampling periods: T_0=20°C, T_1=32°C, T_2=38°C after 30 minutes and T_3=38°C after 60 minutes of heating. Limpets collected at 38°C after 30 minutes of heating were immediately sampled and the ones collected after 60 minutes were placed under seawater spray (20°C) for 1 hour to allow recovery, after which they were processed.

Haemolymph samples were taken by direct puncture of the foot muscle using 0.1 mL plastic syringe (Figure 3.3.3.1 A) in which anticoagulant (pH=7.6) consisted of 0.05 M tris HCl, 2% glucose, 2% NaCl and 0.5% EDTA, was immediately added. After centrifugation, resuspended cells were washed in filtered seawater and cells were counted in Neubauer counting chambers under the light microscope to reach the appropriate number of cells (10^5 cells mL^{-1}). To 100 µL of cellular suspension (10^6 cells mL^{-1}) 20 µL of neutral red (0.2%) was added (Figure 3.3.3.1 B) and cells were allowed to incubate on microtiter 96-well plate for 1 hour at room temperature.

Supernatant was removed and cells were carefully washed two times with enough amount of filtered sea water after which they were incubated for another 30 minutes (Figure 3.3.3.1 C) with extraction buffer (1% acetic acid; 50% ethanol). The release of neutral red pinocytosed within the cells was measured using the ELISA microplate reader (Labsystem

Uniskan® I, type 362) at 490 nm (Figure 3.3.3.1 D). The optical density value (OD) of neutral red is proportional to the amount of dye taken from the lysosomes of viable cells.

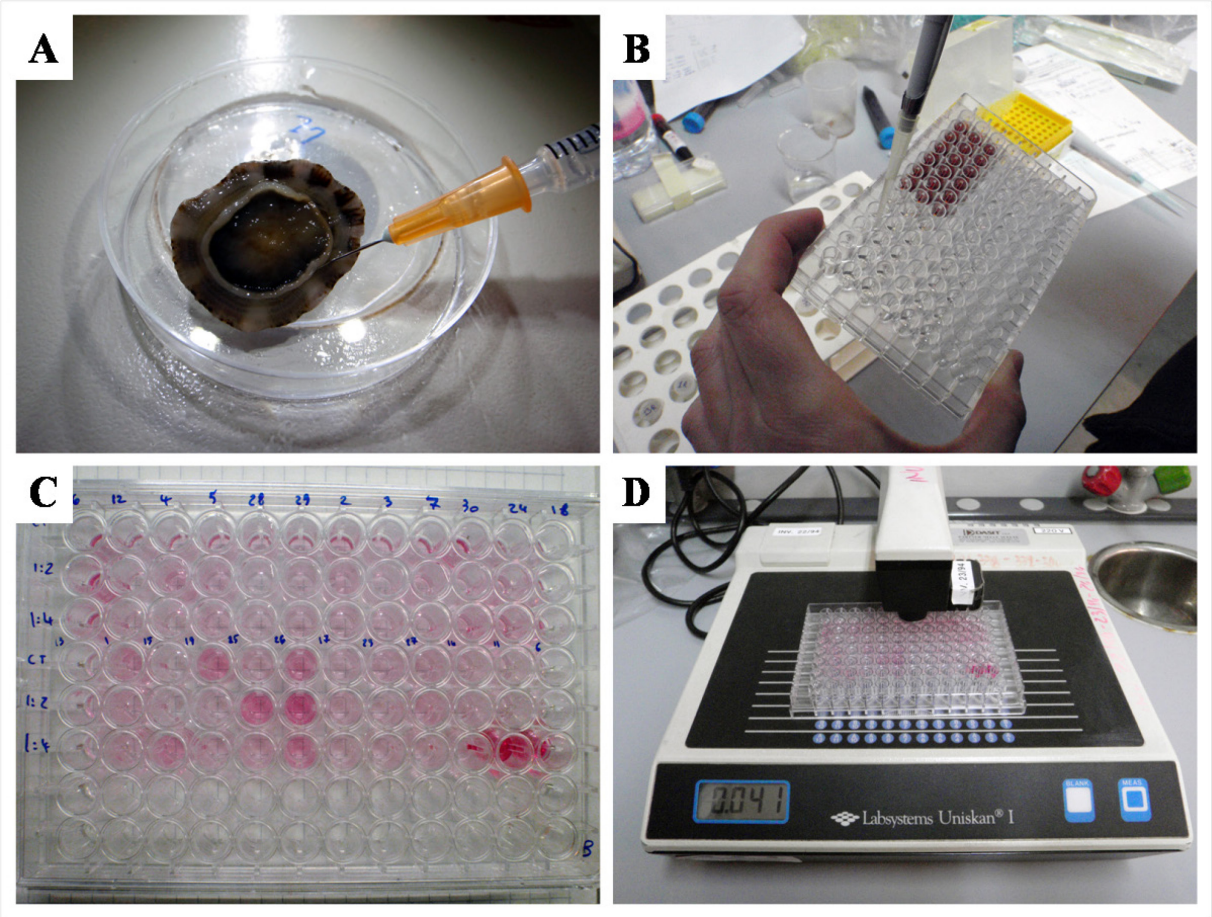

Figure 3.3.3.1 Neutral red uptake assay **A**: haemolymph extraction; **B**: pipetting neutral red to the cells; **C**: incubation of cells with extraction buffer on microtiter 96-well plate; **D**: ELISA microplate reader.

3.4 Statistical data processing

All collected data were entered into a database using Microsoft Excel 2007. Descriptive statistical analysis, including means, standard deviations and minimum and maximum values, were performed in the same program. Further statistical analyses were performed using statistical packages Minitab V.16, Statistica v.8 (StatSoft Ltd.) and PRIMER (PRIMER-E Ltd). Prior to analysis, when necessary, the assumption of homoscedasticity of variances was tested using Levene's test.

Sex ratios were tested using a chi-square goodness of fit test (χ^2). Non-parametric Kruskal-Wallis test was used to compare condition index values with respect to sampling months, since the data variances were not homogenous. Spearman's correlation analysis was

applied to the data to determine the degree of association between mean gonad index, hydrographic parameters and condition index.

To compare differences in Arrhenius breakpoint temperature and Q10 relationships between the three species, one-way ANOVA was performed followed by Tukey *post hoc* comparisons. For the analysis of *hsp70* mRNA expression, data were log transformed and differences in *hsp70* mRNA between different temperatures and species analyzed using two factor PERMANOVA (fixed factors: species and temperature) using an unbalanced design, as *hsp70* could not be measured in some replicates due to technical difficulties. After PERMANOVA, *post hoc* pairwise tests were used to determine differences for each temperature and species. Because of the small permutation number in pairwise tests, p-values were calculated using Monte-Carlo simulation. To investigate interspecific differences in neutral red uptake, non-parametric Kruskal-Wallis test was used since the data variances were not homogenous. In order to investigate intraspecific differences with different thermal regimes, one-way ANOVA was performed followed by Tukey *post hoc* comparisons where data were homogenous, and Kruskal-Wallis test was used on non homogenous data. Critical probability value for each test was set at 0.05.

4. RESULTS

4.1 Growth and reproduction study of the limpet *Patella rustica*

4.1.1 Hydrographic parameters

Sea surface temperature (Figure 4.1.1.1) ranged from 12.5°C in February 2012 to 25.8°C in September 2011, with yearly average of 19.3±4.8°C. The mean daily air temperatures ranged from 7.5°C in February 2012 to 27.1°C in August 2011, while yearly air temperature mean was 17.5±6.9°C. Salinity ranged from unusually low value in April of 18 to 38 measured in October (Figure 4.1.1.2). Sea surface dissolved oxygen value (Figure 4.1.1.2) was lowest in August (5.7 mg/L) and highest in March (8.5 mg/L) while mean dissolved oxygen value for the surface layer was 7.2±1.0 mg/L.

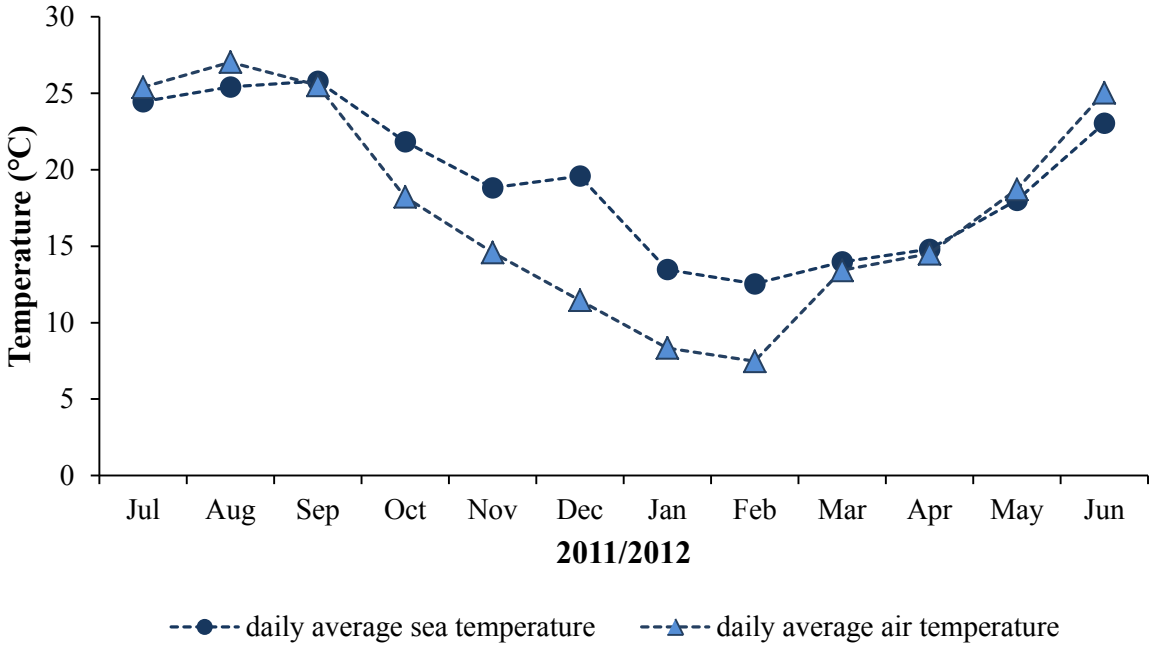

Figure 4.1.1.1 Mean daily sea surface and air temperatures for Dubrovnik area, data obtained from Croatian Meteorological and Hydrological Service.

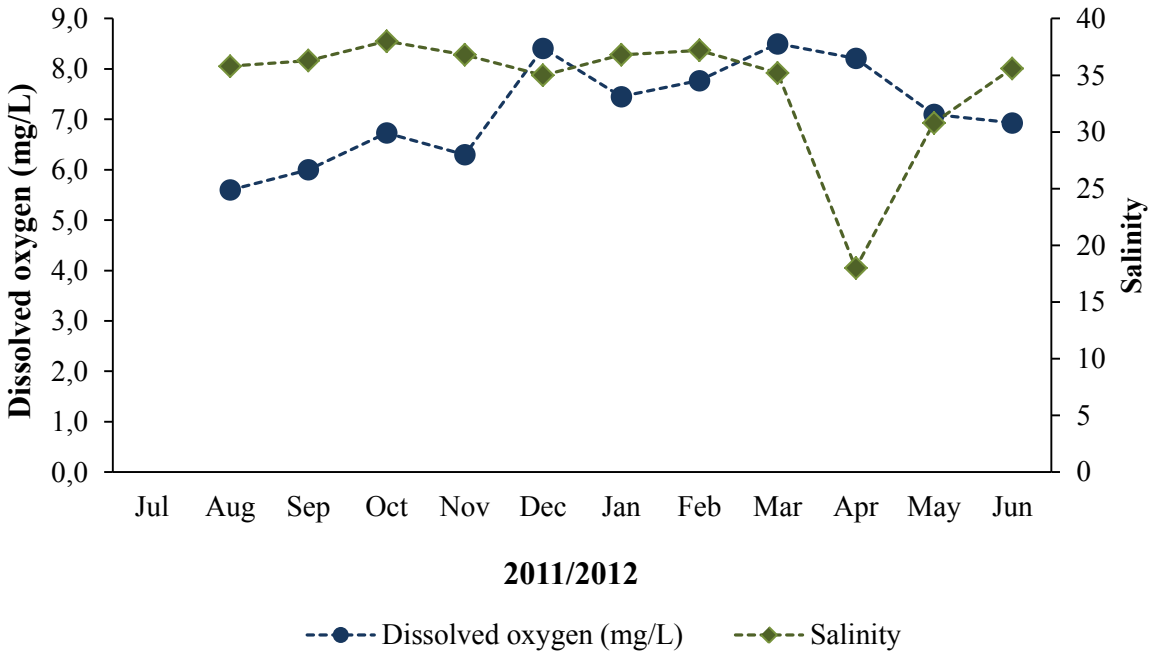

Figure 4.1.1.2 Dissolved oxygen and salinity in surface layer, measured with YSI probe in the Zaton Bay during the study period.

4.1.2 Analysis of chlorophyll *a* concentration

Chlorophyll *a* concentration values were measured from the sea surface water samples collected in the Zaton Bay on each sampling month from July 2011 to June 2012 (Figure 4.1.2.1). Chl *a* values ranged from 0.03 µg/L in September to 1.68 µg/L in April. Mean value and corresponding standard deviation was 0.38±0.45 µg/L.

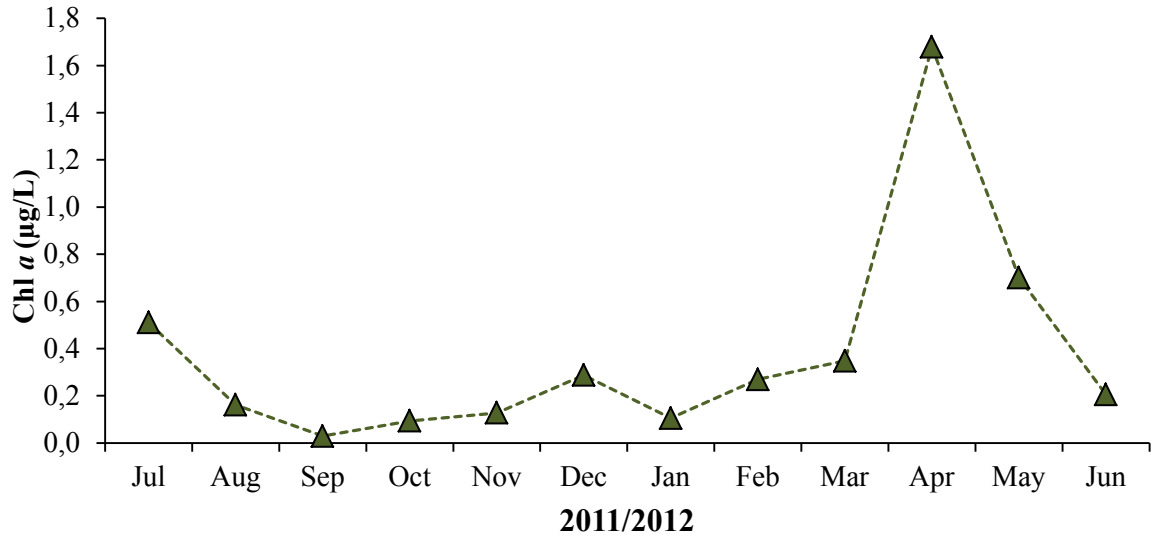

Figure 4.1.2.1 Chlorophyll *a* concentration values measured from the sea surface water samples collected in the Zaton Bay on each sampling month from July 2011 to June 2012.

4.1.3 Age and growth analysis

Population structure. For age and growth analysis, 120 individuals of *Patella rustica* were sampled randomly in September 2011. Limpets' lengths ranged from 8.1 to 33.6 mm, widths from 6.2 to 27.8 mm and heights from 2.8 to 11.8 mm. Mean shell length of all analyzed individuals was 20.2±6.2 mm, mean width was 16.2±5.2 mm, while mean height was 6.6±2.1 mm. Length, width and height frequency distribution are shown in Figure 4.1.3.1 - Figure 4.1.3.3. Total of 15% of collected individuals had shell length equal or greater than 25 mm, 12.5% limpets had shell width equal or greater of 21 mm while 21.7 % had shell height equal or greater of 8 mm.

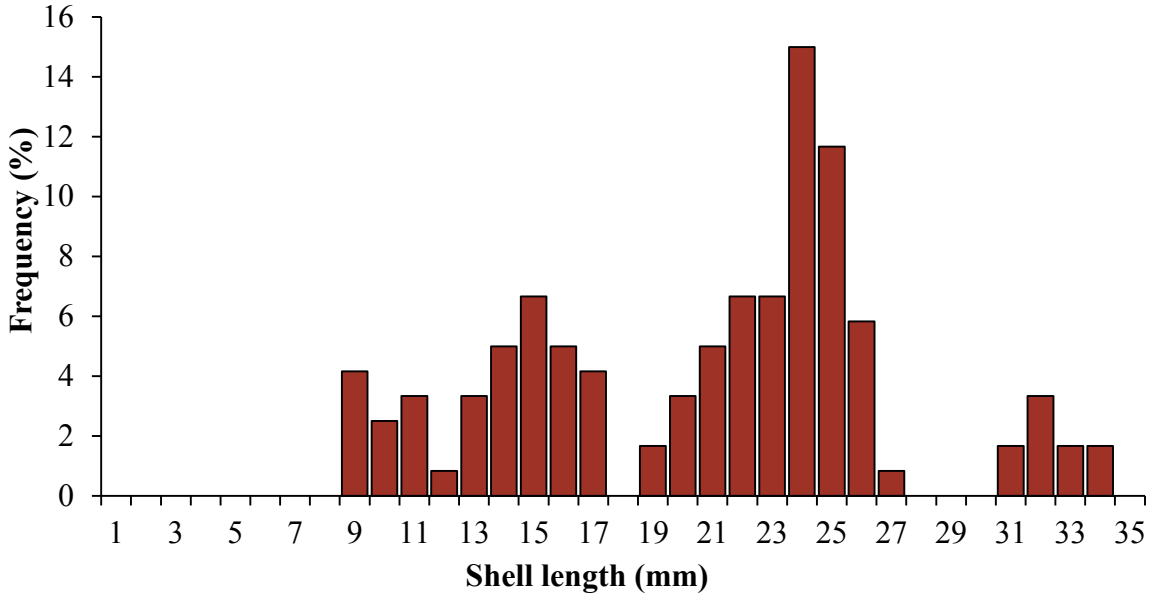

Figure 4.1.3.1 The length frequency histogram of 120 *Patella rustica* individuals randomly collected in September 2011 for age analysis.

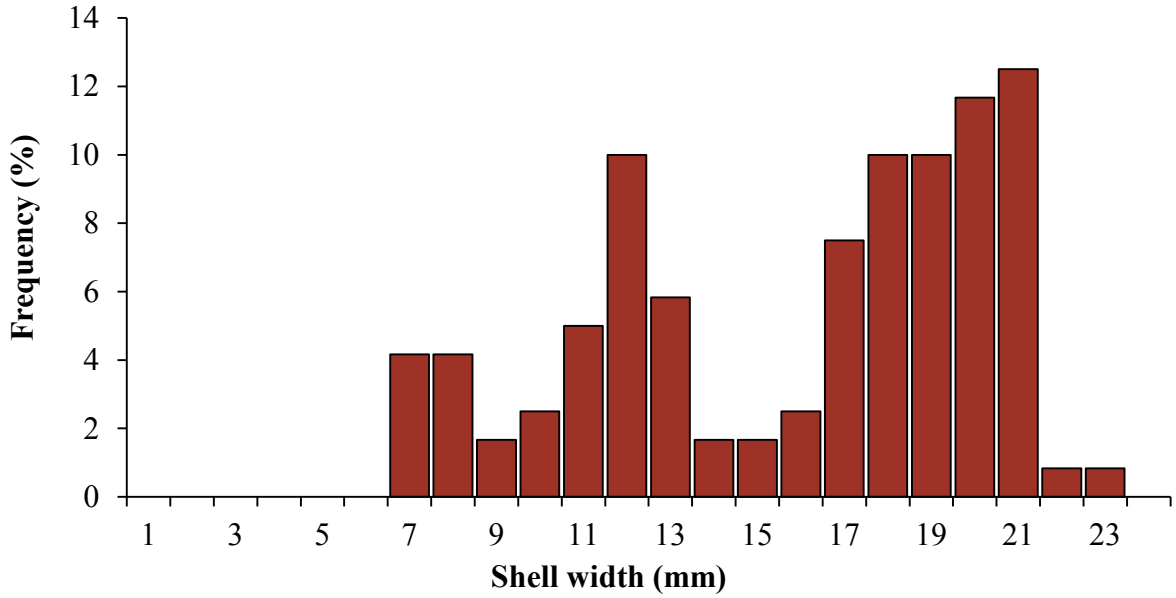

Figure 4.1.3.2 The width frequency histogram of 120 *Patella rustica* individuals randomly collected in September 2011 for age analysis.

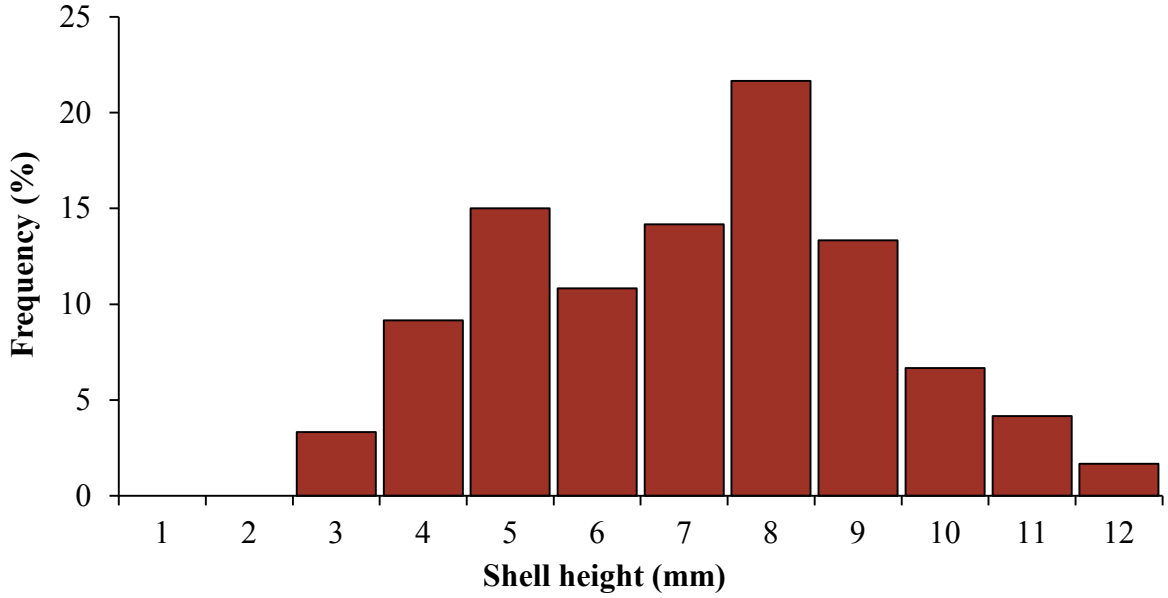

Figure 4.1.3.3 The height frequency histogram of 120 *Patella rustica* individuals randomly collected in September 2011 for age analysis.

For all 120 *Patella rustica* relationships between shell length (L) and shell weight (w), as well as shell length (L) and shell height (H) were calculated. Relationship between length (L) and weight (w) was calculated using functional regression: $w=a\times L^{b}$ (Figure 4.1.3.4). The ratio of limpet height (H) and length (L) were related by the function: $H=c\times L^{\alpha}$, where α is the

constant of allometry. A plot of log shell length against log shell height (Figure 4.1.3.5) provides an indication that the growth is allometric (α=1.66).

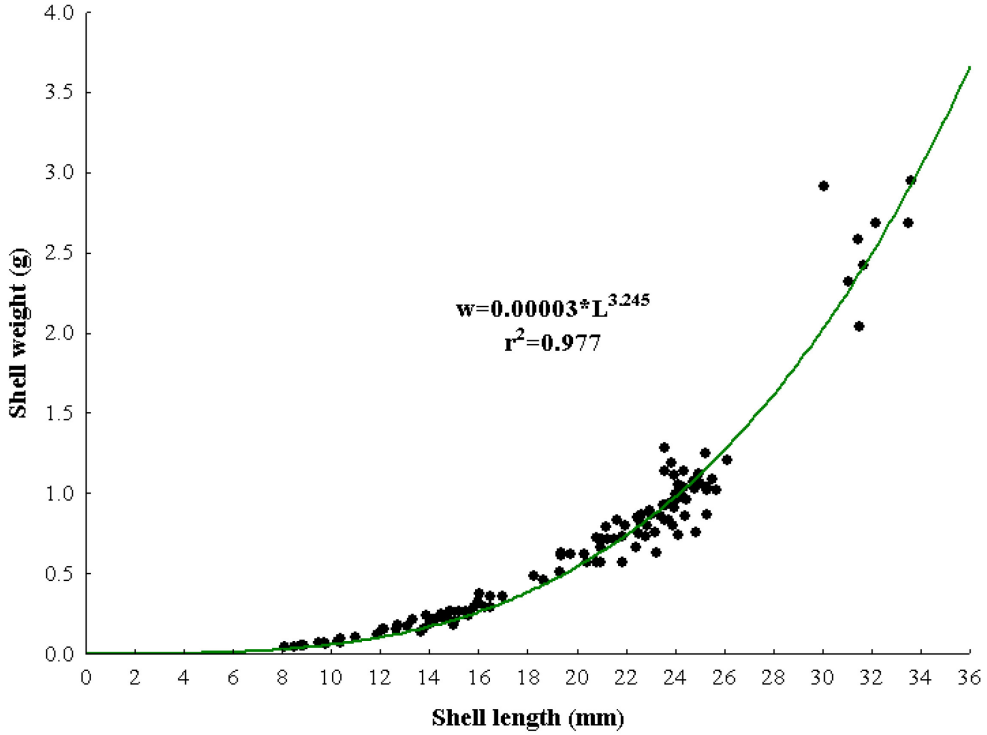

Figure 4.1.3.4 Shell length to weight relationship of *Patella rustica* individuals collected in September 2011.

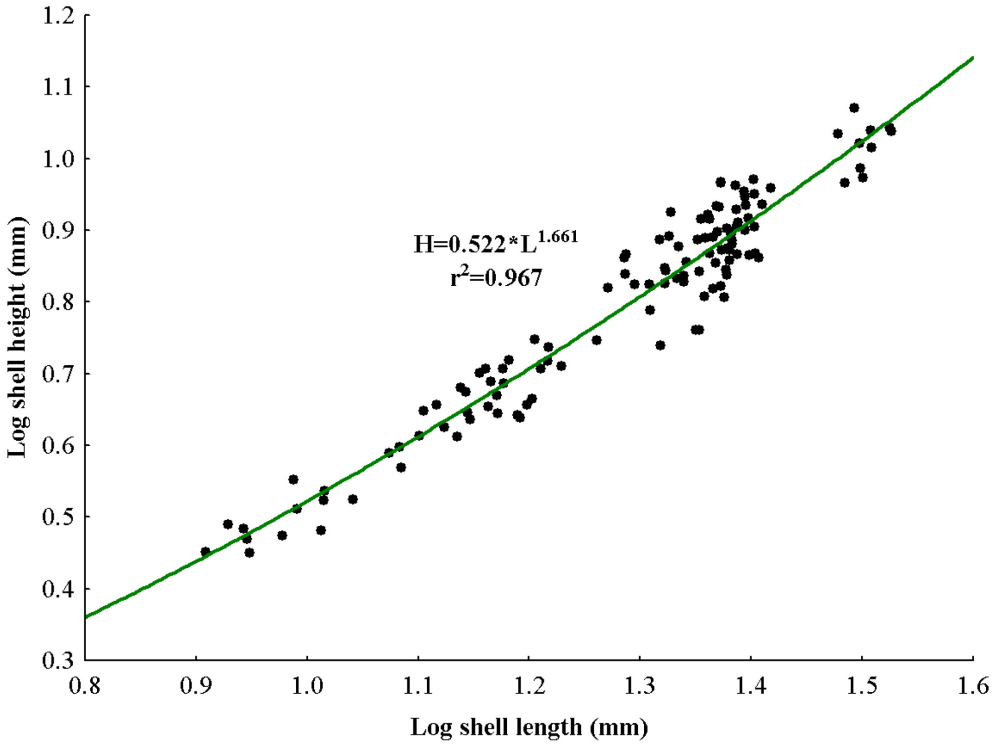

Figure 4.1.3.5 Shell length to height relationship of *Patella rustica* individuals collected in September 2011.

Validation of growth line. An annual periodicity of line formation was validated using marginal increment analysis of smaller individuals collected monthly in a period from July 2011 to June 2012. Acetate peels replicas of a total 60 individuals ranging in length from 11.4 to 16.9 mm (14.4±1.6 mm) were examined, out of which 6 individuals (10%) were omitted from the analysis due to poor growth line visibility. The results showed that one dark growth line is formed annually. A dark growth line representing slow shell deposition was visible at or near the margin of shells collected during the period from May to October. Based on these data, growth line formation was set in May. The variations in the distance from the last growth line to the shell margin are visible on the Figure 4.1.3.6. An example of acetate peel replica of shell section of an individual (15.2 mm length) sampled in November 2011 and used in validation of growth line is illustrated in Figure 4.1.3.7.

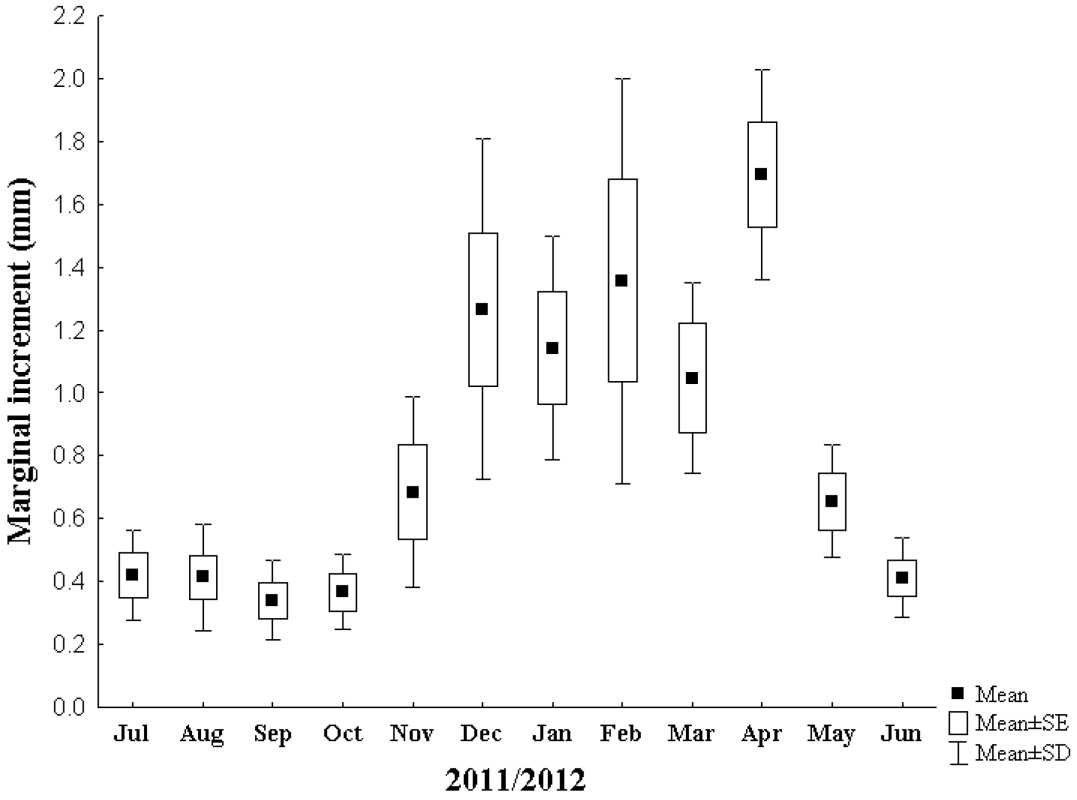

Figure 4.1.3.6 Seasonal variations in the distance from the last growth line to the shell margin (marginal increment width) of *Patella rustica* collected from July 2011 to June 2012; SE–standard error, SD–standard deviation.

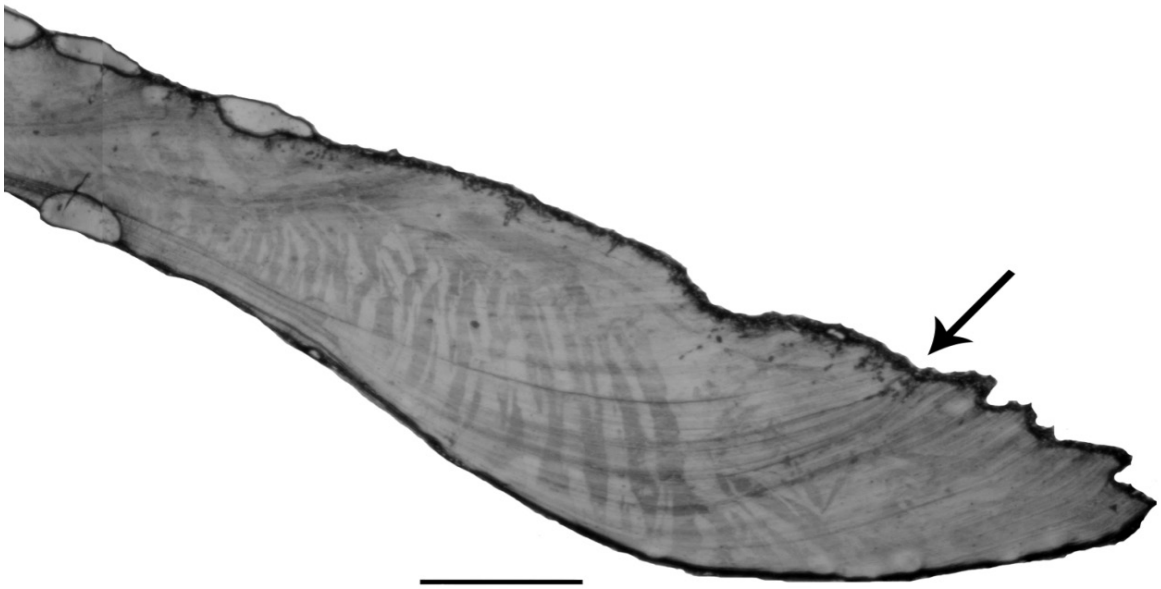

Figure 4.1.3.7 Acetate peel replica of an individual *Patella rustica* collected in November; growth line near the shell margin is marked with black arrow, scale bar 0.5 mm.

Age analysis. The analysis of inner growth lines from acetate peel replicas of shell sections were performed on 120 individuals, ranging in length from 8.1 to 33.6 mm, randomly collected in September 2011. Since the peak of *Patella rustica* spawning season is in November (see further down results from reproduction), settlement date or birth date was set as December 1st. Based on these data it was concluded that a first growth line actually represents nine months instead of one full year. This was taken into account when estimating the age and growth of *P. rustica*. The estimated age of 120 individuals ranged from 0.75 to 7.75 years, with a mean age of 2.9±1.4 years (Figure 4.1.3.8). An example of inner growth lines, visible in acetate peel replica of shell section from an individual (32.3 mm length) is shown on Figure 4.1.3.9.

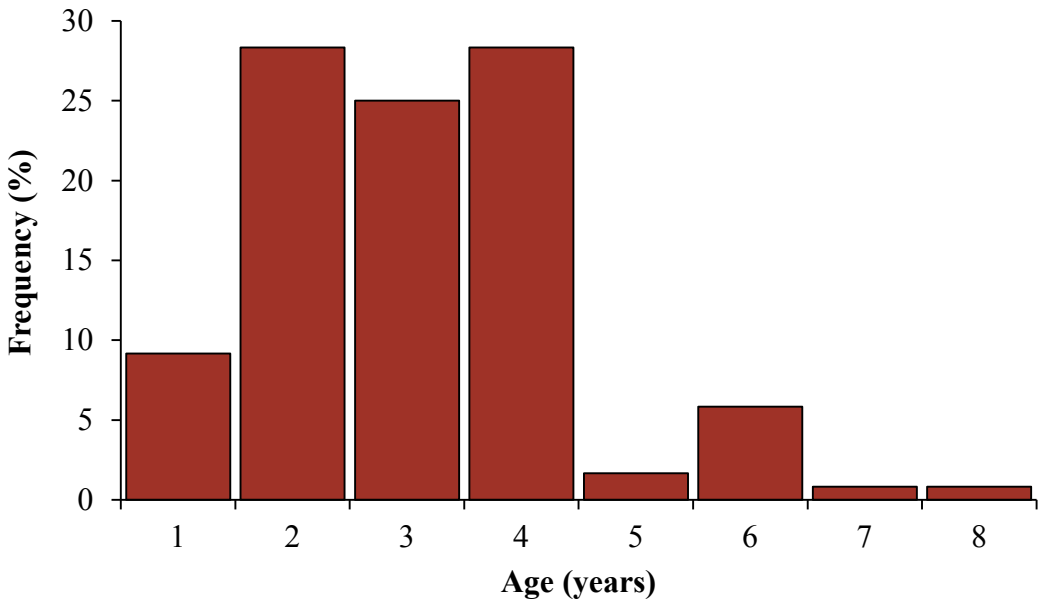

Figure 4.1.3.8 Population structure of *Patella rustica* based on estimated age data.

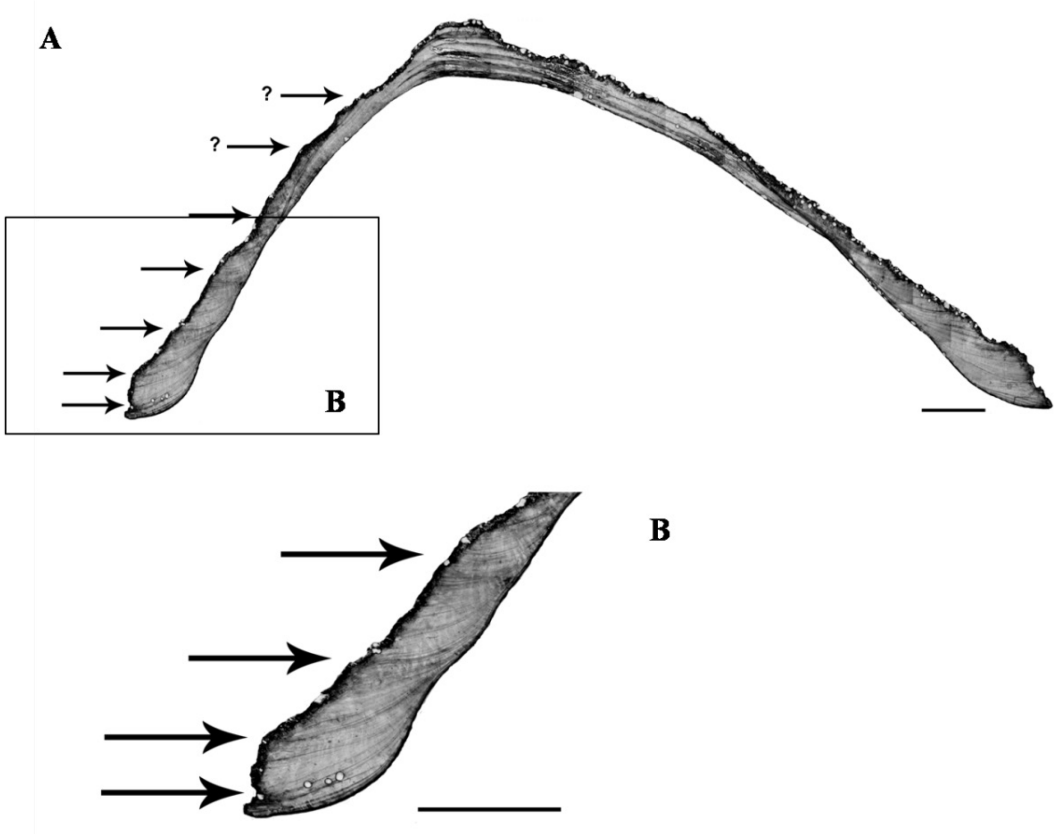

Figure 4.1.3.9 **A**: Acetate peel replica of *Patella rustica* individual with shell length of 32.3 mm; **B**: close up of one shell side; inner growth lines are marked with black arrow, scale bar 2 mm.

From the age, length, width and height data of each individual, von Bertalanffy growth (VBG) functions were calculated and the estimated VBG coefficients (L_∞, W_∞ and H_∞) and values of growth constant (K) are shown in Table 4.1.3.1. Von Bertalanffy growth curves for each measured shell dimension are showed in Figure 4.1.3.10. Mean length, width and height data for each estimated year are shown in Table 4.1.3.2. Overall, 90.8% of collected individuals were <4 years old with 34 individuals (28.3%) belonging to second (1.75 year) and fourth (3.75 years) age class. Only two (1.6%) individuals were more than 6 years old (6.75 and 7.75 years). The maximum longevity was estimated to 12.7 years.

Table 4.1.3.1 Von Bertalanffy growth parameters for each measured dimension (L_∞ - asymptotic maximum length, W_∞ - asymptotic maximum width, H_∞ - asymptotic maximum height), K (growth constant), t_0 (initial condition parameter) and r^2 (correlation coefficient).

	L_∞ / W_∞ / H_∞ (mm)	K (year^{-1})	t_0 (year)	r^2
Length	40.86	0.23	-0.33	0.958
Width	33.02	0.24	-0.22	0.954
Height	14.07	0.21	-0.32	0.934

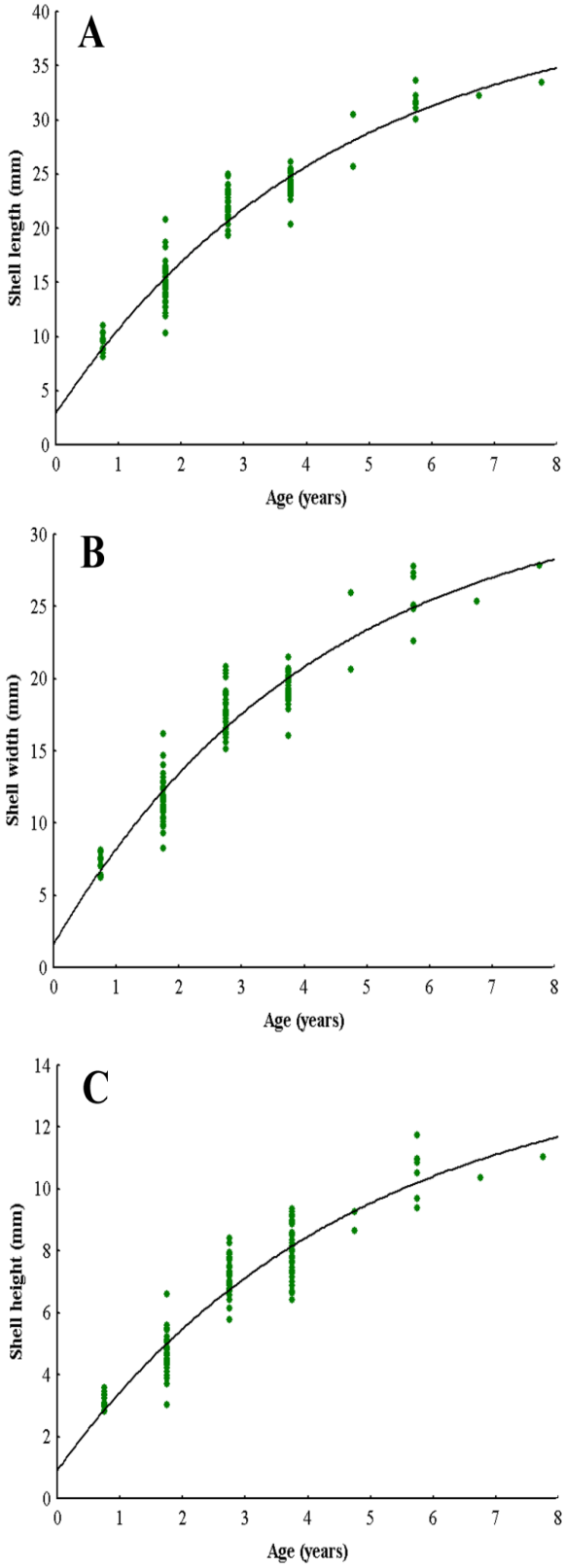

Figure 4.1.3.10 Von Bertalanffy growth functions fitted to the data obtained from the annually resolved growth lines of 120 analyzed *Patella rustica* individuals **A**: age at length data; **B**: age at width data; **C**: age at height data.

Table 4.1.3.2 Age-length key for 120 analyzed individuals of *Patella rustica*.

Length class (mm)	Age (years)								Total
	0.75	1.75	2.75	3.75	4.75	5.75	6.75	7.75	
9	5								5
10	3								3
11	3	1							4
12		1							1
13		4							4
14		6							6
15		8							8
16		6							6
17		5							5
18									0
19		2							2
20			4						4
21			6						6
22			8						8
23			6	2					8
24			6	12					18
25			2	12					14
26				6	1				7
27				1					1
28									0
29									0
30									0
31					1	1			2
32						4			4
33						1	1		2
34						1		1	2
Total (N)	11	33	32	33	2	7	1	1	120
%	9.2	27.5	26.7	27.5	1.7	5.8	0.8	0.8	100
mean length (mm)	9.4	14.8	22.0	24.1	28.1	31.7			
SD (mm)	0.9	2.1	1.5	1.1	3.4	1.1			
Max (mm)	10.9	20.8	25.0	26.2	30.5	33.6			
Min (mm)	8.1	10.3	19.3	20.3	25.7	30.1			

4.1.4 Analysis of endoliths in the limpet *Patella rustica*

During the age and growth analysis, it was determined that limpets' shells were in different degree of degradation (Figure 4.1.4.1). Shell infestation resulted in difficulties to define the exact position of the first growth line in majority of examined limpets (Figure 4.1.4.2). This analysis was performed as an identification snapshot of endolithic algae present in *Patella rustica* shell, and not as determination of the degree of shell infestation.

Figure 4.1.4.1 Limpet *Patella rustica* with different degree of shell degradation, scale bar 0.5 mm.

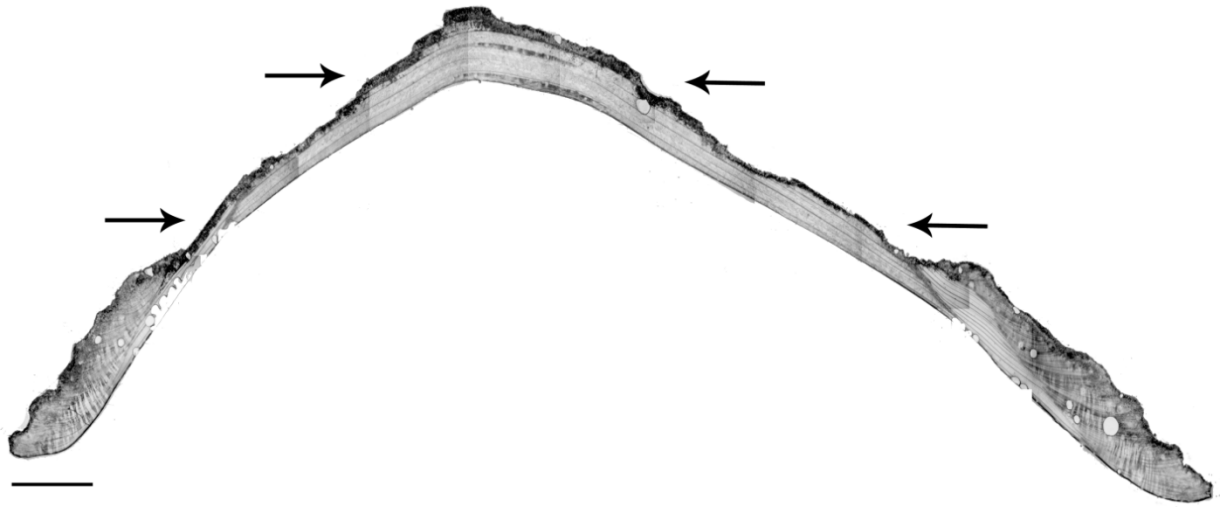

Figure 4.1.4.2 An example of acetate peel replica of a degraded *Patella rustica* shell; black arrows indicate areas of shell loss; scale bar 2 mm.

All identified species were filamentous cyanobacteria that colonize as epiliths or penetrate inside the limpet shell (and shells in general) as endoliths in between periostracum and prismatic layer. Five taxa were identified: *Mastigocoelus testarum* Lagerheim, 1886, *Hormathonema paulocellulare* Ercegović, 1929, *Hyella caespitosa* Bornet & Flahault, 1888, *Leptolyngbya* sp. Anagnostidis & Komárek, 1988 and *Calothrix* sp. Agardh, 1886.

Mastigocoleus testarum (Figure 4.1.4.3 A) was identified by its characteristic branching pattern and the presence of terminal heterocysts. *Hormathonema paulocellulare* (Figure 4.1.4.3 A) is characterised with blue-grey gelatinous envelope forming parallel tunnels perpendicular to the surface with few lateral branchings. *Hyella caespitosa* (Figure 4.1.4.3 B-D) is characterised by larger, scarcely branched pseudofilaments that penetrate perpendicular to the shell surface. Pseudofilaments are with one or more rows of cells while mucilaginous sheaths are firm, thick, usually layered, rarely gelatinized and commonly visible also between cells. *Leptolyngbya* sp. (Figure 4.1.4.3 E) has long filaments, solitary or coiled into clusters and fine mats with usually colourless facultative sheaths opened at the apical end. *Calothrix* sp. (Figure 4.1.4.3 F) is an epilithic cyanobacteria that has basal heterocysts and long filaments, surrounded by thin, colourless mucilaginous envelope (trichomes).

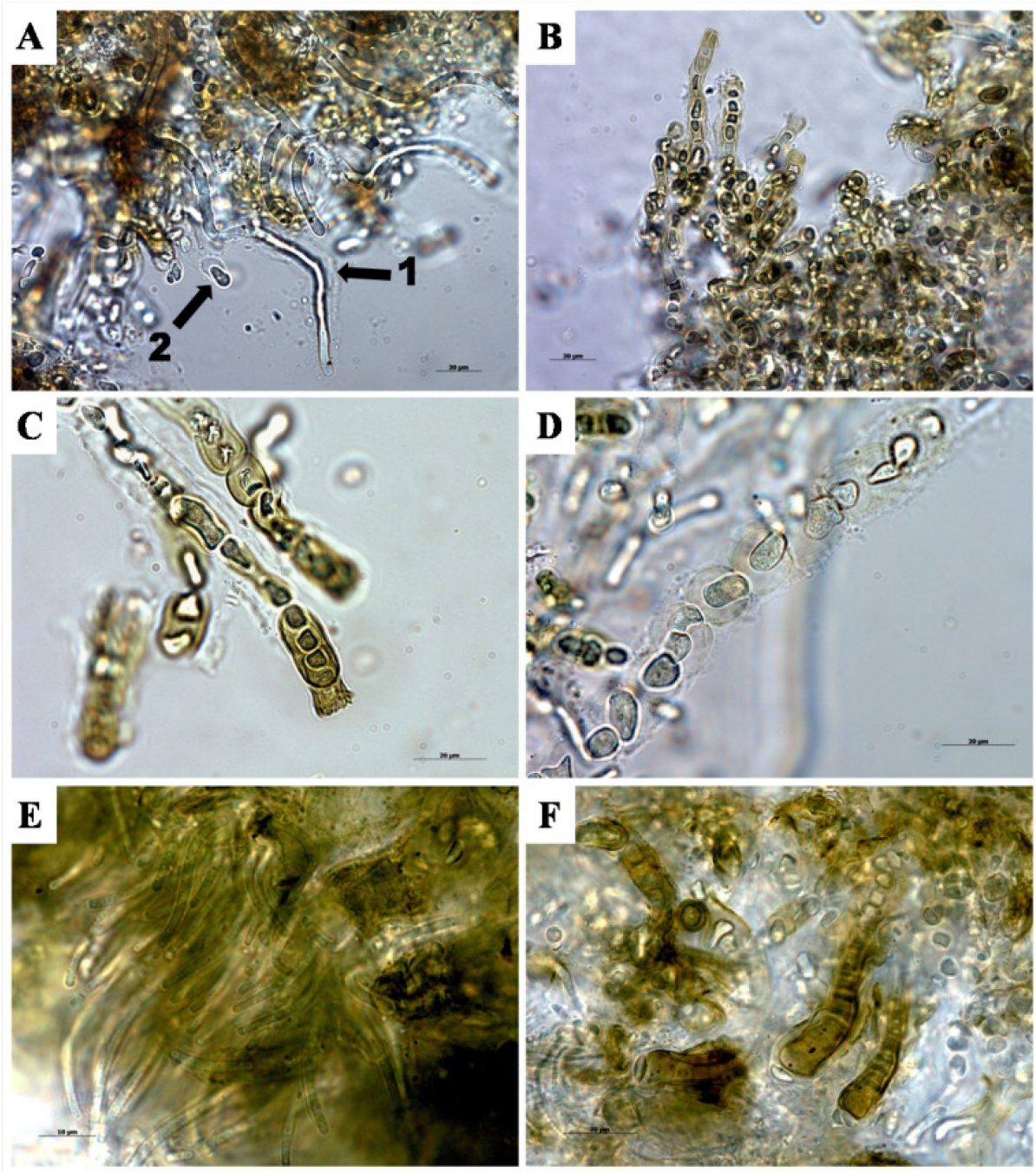

Figure 4.1.4.3 Cyanobacteria infesting *Patella rustica* shell **A**: 1 *Mastigocoelus testarum* and 2 *Hormathonema paulocellulare*; **B - D**: *Hyella caespitosa*; **E**: *Leptolyngbya* sp.; **F**: *Calothrix* sp.

4.1.5 Histological analysis

Patella rustica gonads were examined histologically using qualitative and quantitative methods in order to determine the exact period of spawning and to describe developmental stages of oogenesis and spermatogenesis. Gonad samples were collected from the total of 355 medium-sized *P. rustica* individuals (24.2±2.9 mm), out of which 1 individual (0.3%) was lost in processing, 142 individuals (40.0%) on which it was not possible to determine sex were marked as undetermined and 3 individuals (0.8%) were identified as hermaphrodite. The chi-square test showed statically significant differences between the number of males and females in medium sized class (χ^2=82.1, p<0.001, Table 4.1.5.1) with the female to male ratio of 4:1. In addition, gonad samples were collected from the total of 95 smaller individuals (16.2±3.0 mm), out of which 40 (42.1%) were males, 9 individuals (9.5%) were females and 46 were marked as undetermined (48.4%). The chi-square test also showed statically significant differences between the number of males and females in smaller sized class (χ^2=19.6, p<0.001, Table 4.1.5.1) and female: male ratio was 1:4.

Table 4.1.5.1 Analysis of the sex ratio of *Patella rustica* individuals according to shell length categories. 1: medium sized (N=355), 2: smaller sized (N=95); X- mean shell length in mm, SD - standard deviation, χ2 - chi-square goodness of fit test, p - p value (set at 0.05).

category	X length (mm) ± SD	length range (mm)	♀	♂	χ2	p
1	24.2±2.9	14.9-33.5	170	39	82.110	<0.001
2	16.2±3.0	10.6-22.4	9	40	19.612	<0.001
1+2			179	79	38.760	<0.001

Qualitative histological analysis. Qualitative histological analysis of *P. rustica* gonads showed only one reproductive cycle per year. Developmental patterns were uniform for both males and females, with only few noticeable differences. A descriptive staging for gametogenesis was made after examining all sampled months and is shown in Table 3.2.5.1 and Table 3.2.5.2.

The stages of gonad development for males were assigned based on the presence or absence of spermatogonia, spermatocytes and spermatides. Spermatogonia (male primordial germ cell) undergo meiosis and produce spermatozoa. The initial cells in this pathway are called primary spermatocytes. The primary spermatocyte divides into two secondary spermatocytes; each secondary spermatocyte then divides into two spermatids that develop into mature spermatozoa or sperm cells.

- **Early developmental stage** (Figure 4.1.5.1) for males is characterised with islets of small compact acini filled with spermatogonia. In June 2012, 100% of males had gonads in early developmental stage.
- **Late developmental stage** (Figure 4.1.5.2) is characterised with reproductive cells in different stages of maturation, forming regular layers from the tubular wall to the lumen: spermatogonia, spermatocytes and spermatids. In September 2011, 100% of male gonads were in late development.
- **Ripe developmental stage** (Figure 4.1.5.3) is defined with a band of spermatogonia attached to the wall and free spermatozoa filling the lumen. In November 2011, 60% of males had ripe gonads.
- **Spawning stage** (Figure 4.1.5.4) is characterised with large germinal cells around the edges and visible spermatozoa tails in the centre of the tubule. Spawning occurred during November 2011.
- **Spent stage** (Figure 4.1.5.5) is characterised with abundant connective tissue inside shrunk tubules and presence of haemocytes surrounding residual spermatozoa. In December 2011, 100% of males had spent gonads.

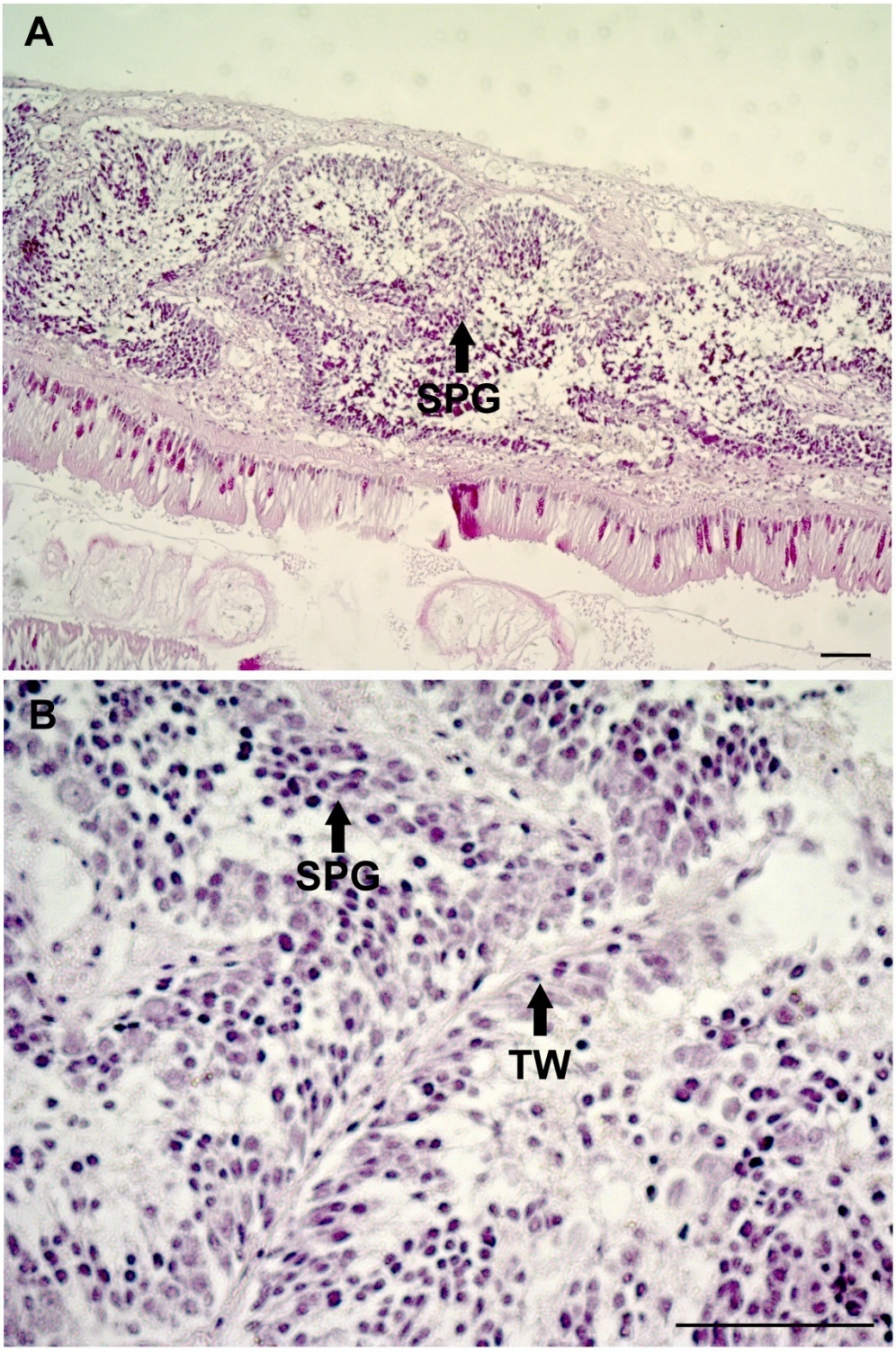

Figure 4.1.5.1 Light photomicrographs of early developmental stage of *Patella rustica* male, magnification: **A**: 100×; **B**: 400×. **SPG** spermatogonia, **TW** tubular wall. Scale bar 50 µm.

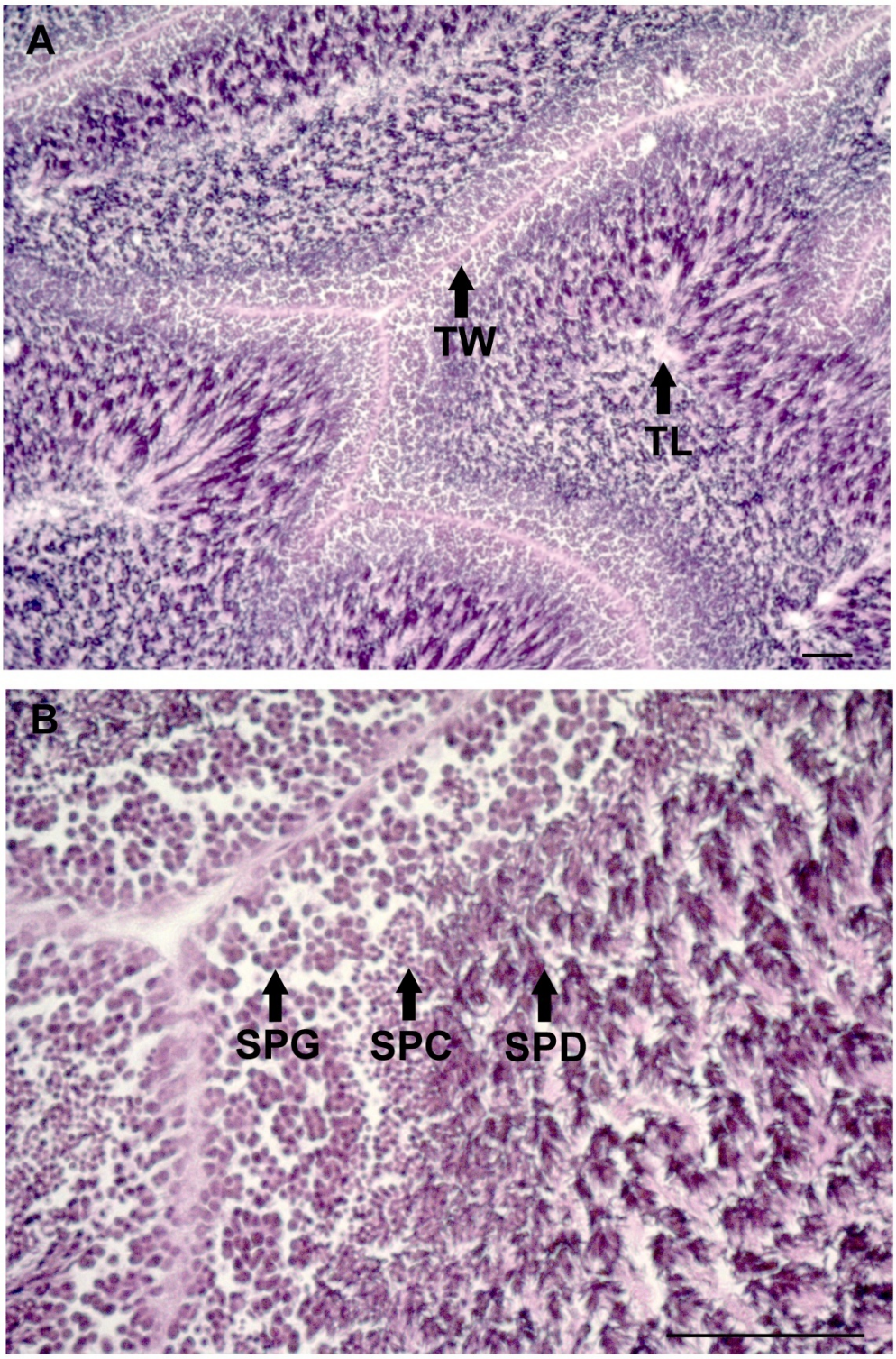

Figure 4.1.5.2 Light photomicrographs of late developmental stage of *Patella rustica* male, magnification: **A**: 100×; **B**: 400×. **TW** tubular wall, **TL** tubular lumen, **SPG** spermatogonia, **SPC** spermatocytes, **SPD** spermatids. Scale bar 50 µm.

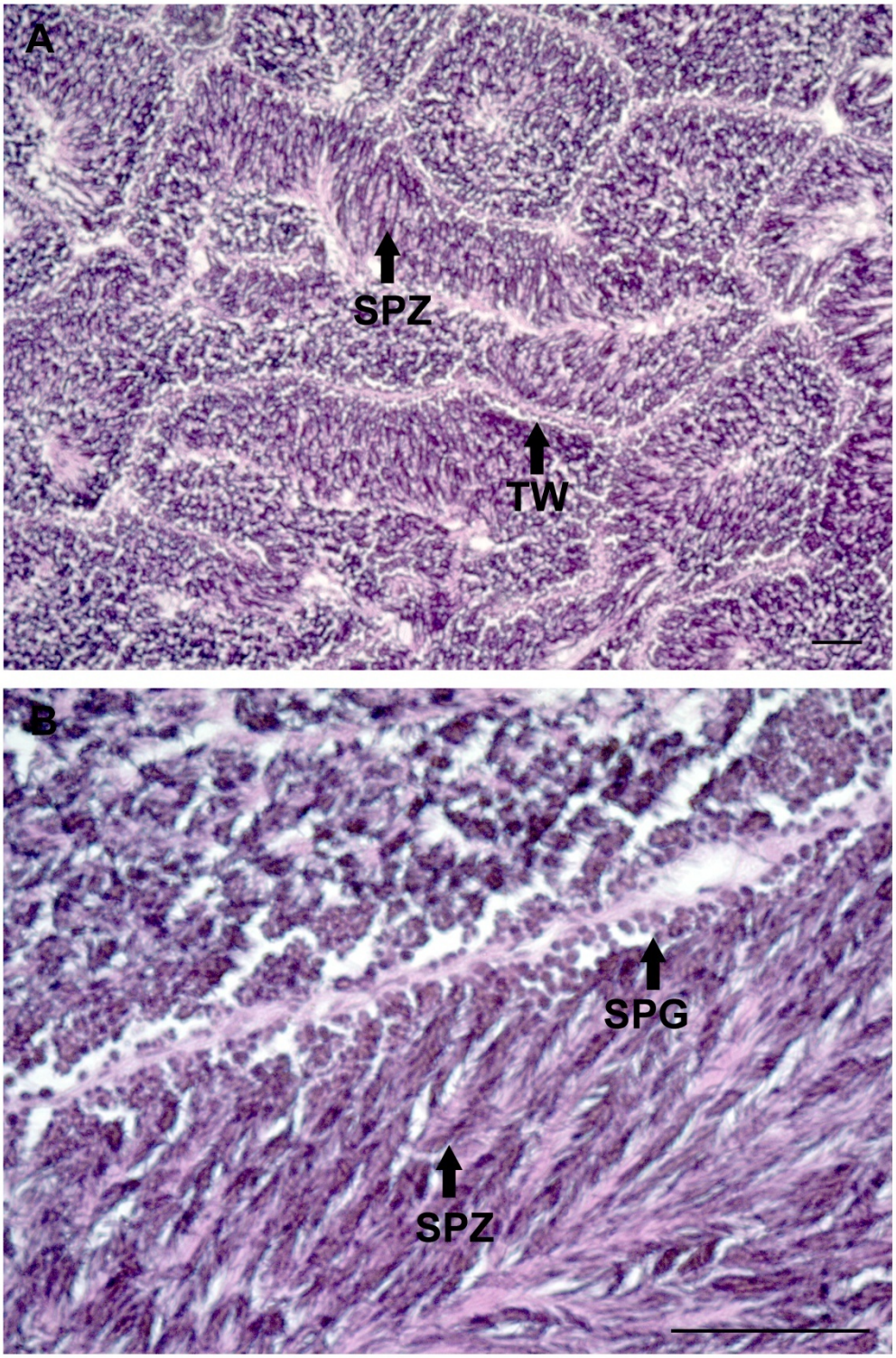

Figure 4.1.5.3 Light photomicrographs of ripe developmental stage of *Patella rustica* male, magnification: **A**: 100×; **B**: 400×. **SPZ** spermatozoa, **TW** tubular wall, **SPG** spermatogonia. Scale bar 50 μm.

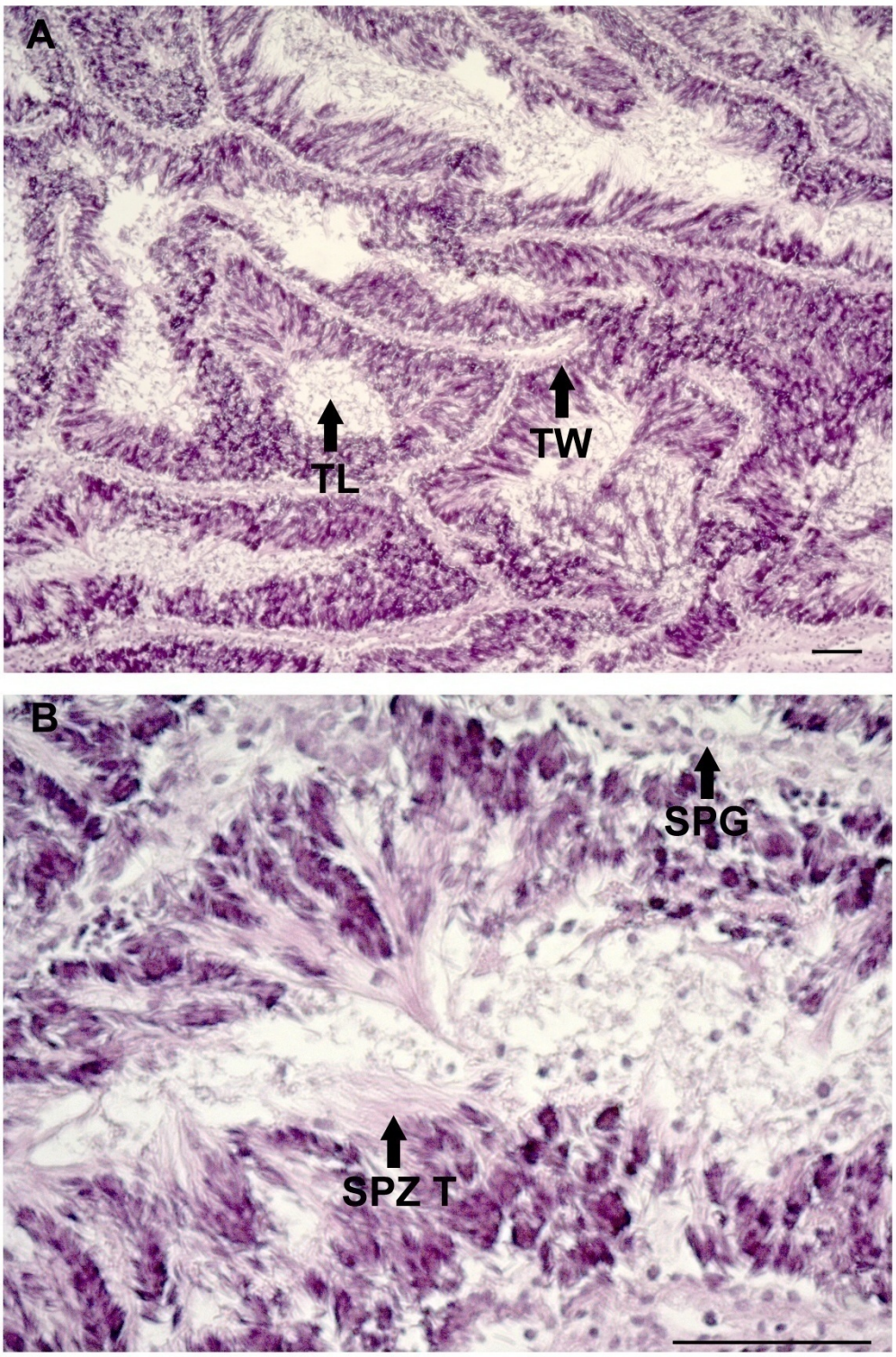

Figure 4.1.5.4 Light photomicrographs of spawning stage of *Patella rustica* male, magnification: **A**: 100×; **B**: 400×. **TL** tubular lumen, **TW** tubular wall, **SPG** spermatogonia, **SPZ T** spermatozoa tails. Scale bar 50 μm.

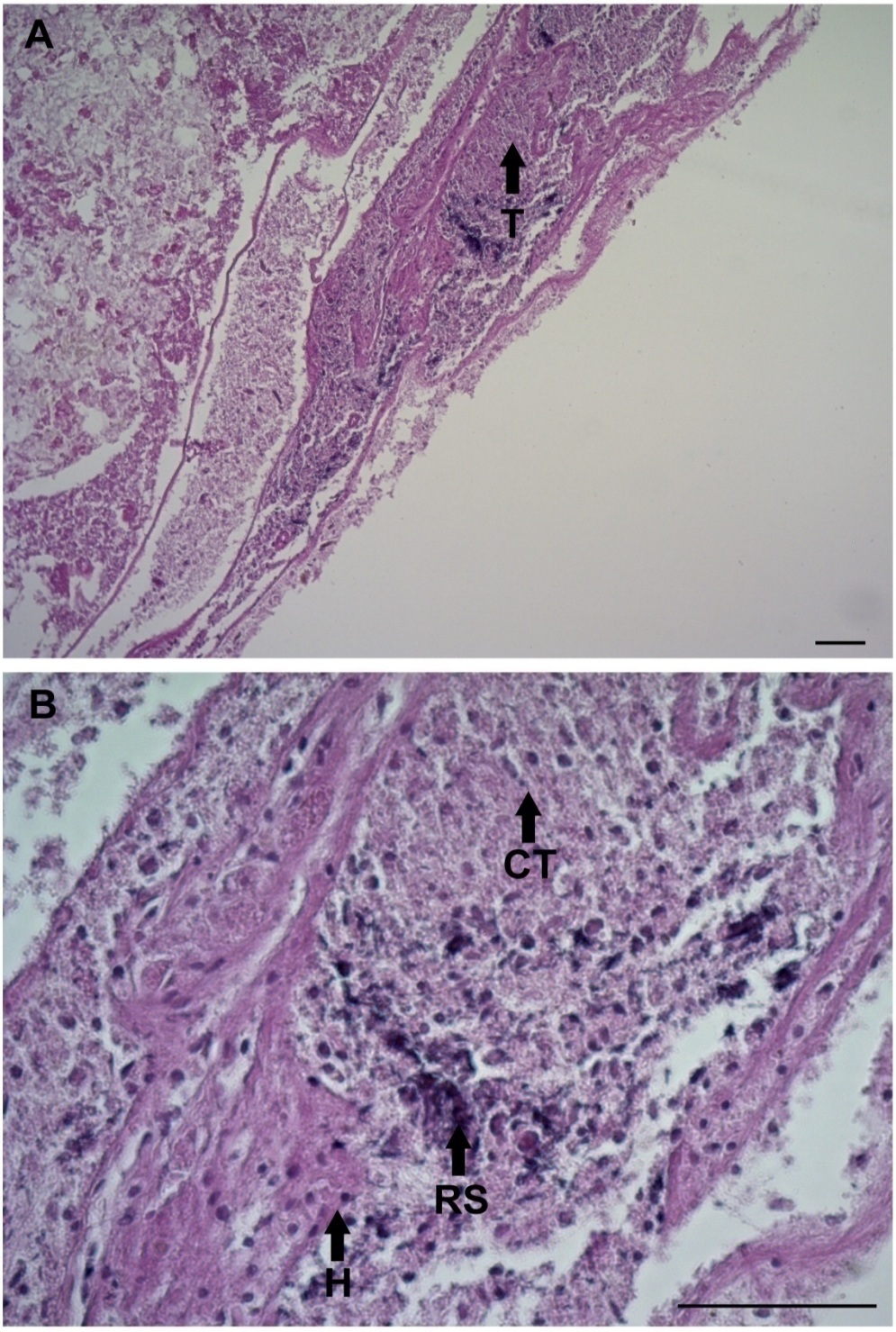

Figure 4.1.5.5 Light photomicrographs of spent stage of *Patella rustica* male, magnification: **A**: 100×; **B**: 400×. **T** tubule in degeneration, **CT** connective tissue, **H** haemocytes, **RS** residual spermatozoa. Scale bar 50 μm.

The stages of gonad development for females were determined based on the presence of oogonia, previtellogenic and vitellogenic oocytes. Primary oogonia undergo repeated mitotic division e.g. multiplying mitosis from which secondary oogonia develops followed by first meiotic prophase. The remaining meiotic stages are terminated after fertilization. Oocytes than undergo vitellogenesis and increase in size due to accumulation of lipid droplets and small amount of glycogen. Part of the oocytes undergoes lysis and different stages of atresia during the whole period of development, and particularly at the beginning of spawning season.

- **Inactive stage** (Figure 4.1.5.6) is characterised with abundant connective tissue and the presence of only few, very small, oogonia. Inactive stage was not included in quantitative analysis. This stage was present throughout the whole sampling year, with highest percentage in January 2012 (50%).
- **Early developmental stage** (Figure 4.1.5.7) is defined with clusters of oogonia and previtellogenic oocytes attached to the acini wall. Early development for females starts in February, while 96% of females in May 2012 had early developed gonads.
- **Late developmental stage** (Figure 4.1.5.8) is characterised with enlarged gonad acini and the presence of large previtellogenic oocytes and few oocytes in the final stages of vitelogenesis. In September, 70% of females had gonads in late developmental stage.
- **Ripe developmental stage** (Figure 4.1.5.9) is characterised with dominance of completely developed free vitellogenic oocytes. Oocytes with different degree of atresia can be present as well. In October 2011, 67% of females had ripe gonads.
- **Atresic stage** (Figure 4.1.5.10) is identified with abundant vitellogenic oocytes: some of them still with the ability to spawn, but majority undergoing different degree of atresia. This stage was found during ripening of gonads, in September and October 2011.
- **Spawning stage** (Figure 4.1.5.11) is characterised with the decrease in free vitellogenic oocytes in the lumen, the presence of pedunculated oocytes attached to the wall and with empty space inside the lumen. Spawning occurred in November 2011, where 33% of females had gonads in spawning stage.
- **Spent stage** (Figure 4.1.5.12) is characterised with slack ovaries and signs of tissue destruction. Only few degenerative oocytes can be present surrounded with haemocytes. In December 2011, 100% of females had spent gonads.

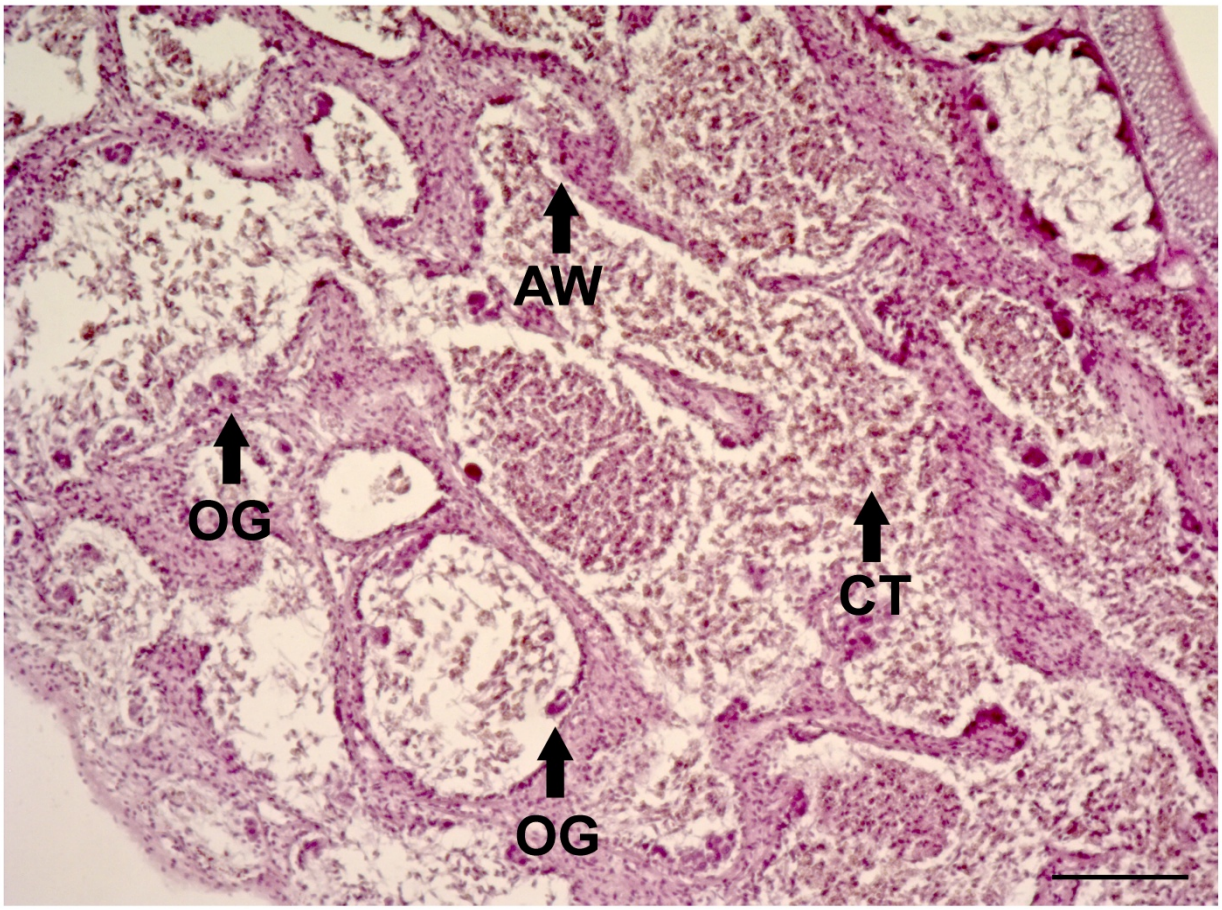

Figure 4.1.5.6 Light photomicrographs of inactive stage of *Patella rustica* female, magnification 100×. **AW** acinus wall, **OG** oogonia, **CT** connective tissue. Scale bar 100 µm.

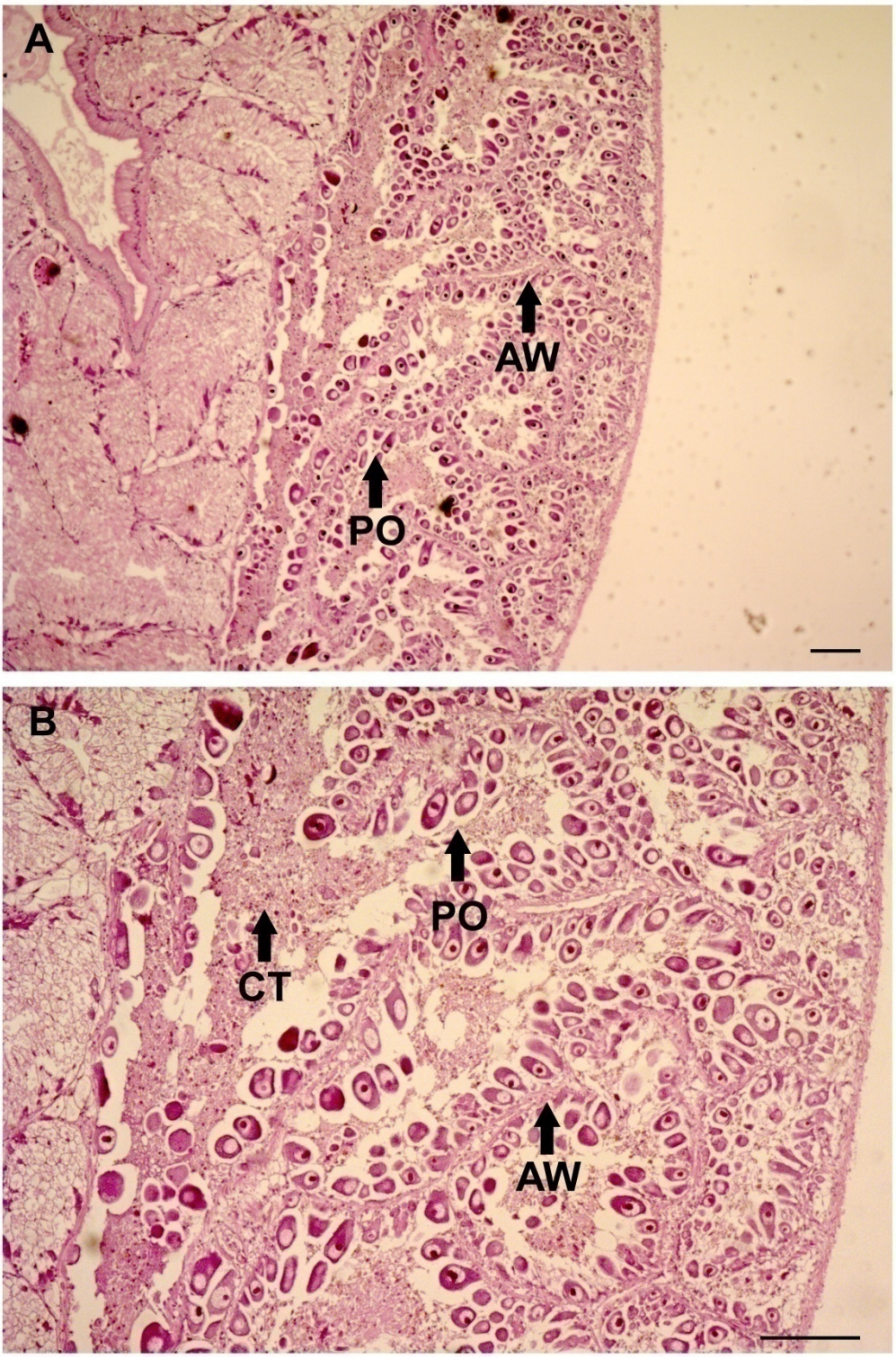

Figure 4.1.5.7 Light photomicrographs of early developmental stage of *Patella rustica* female, magnification **A**: 50×; **B**: 100×. **AW** acinus wall, **PO** previtellogenic oocyte, **CT** connective tissue. Scale bar 100 µm.

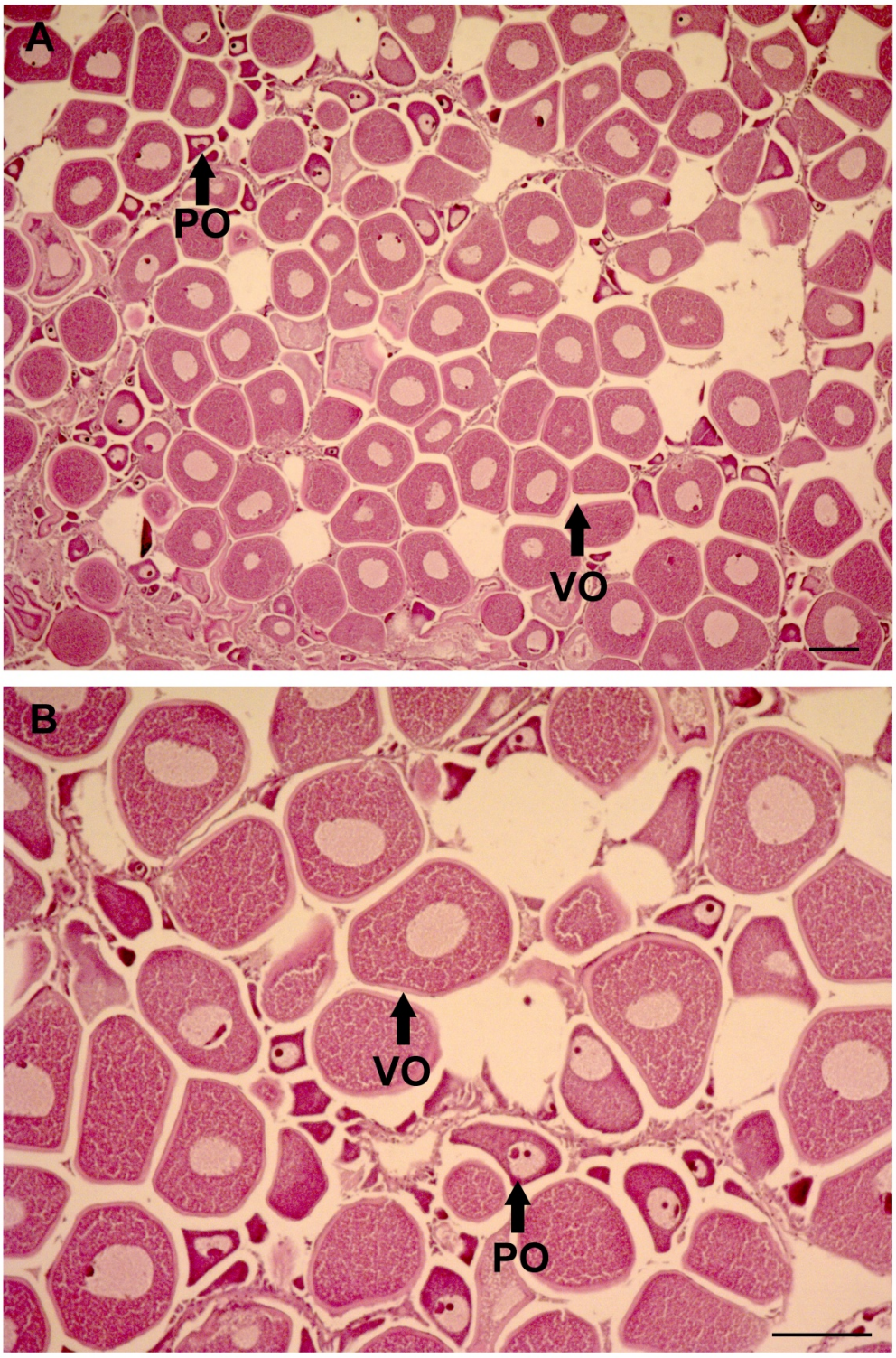

Figure 4.1.5.8 Light photomicrographs of late developmental stage of *Patella rustica* female, magnification **A**: 50×; **B**: 100×. **PO** previtellogenic oocyte, **VO** vitellogenic oocyte. Scale bar 100 µm.

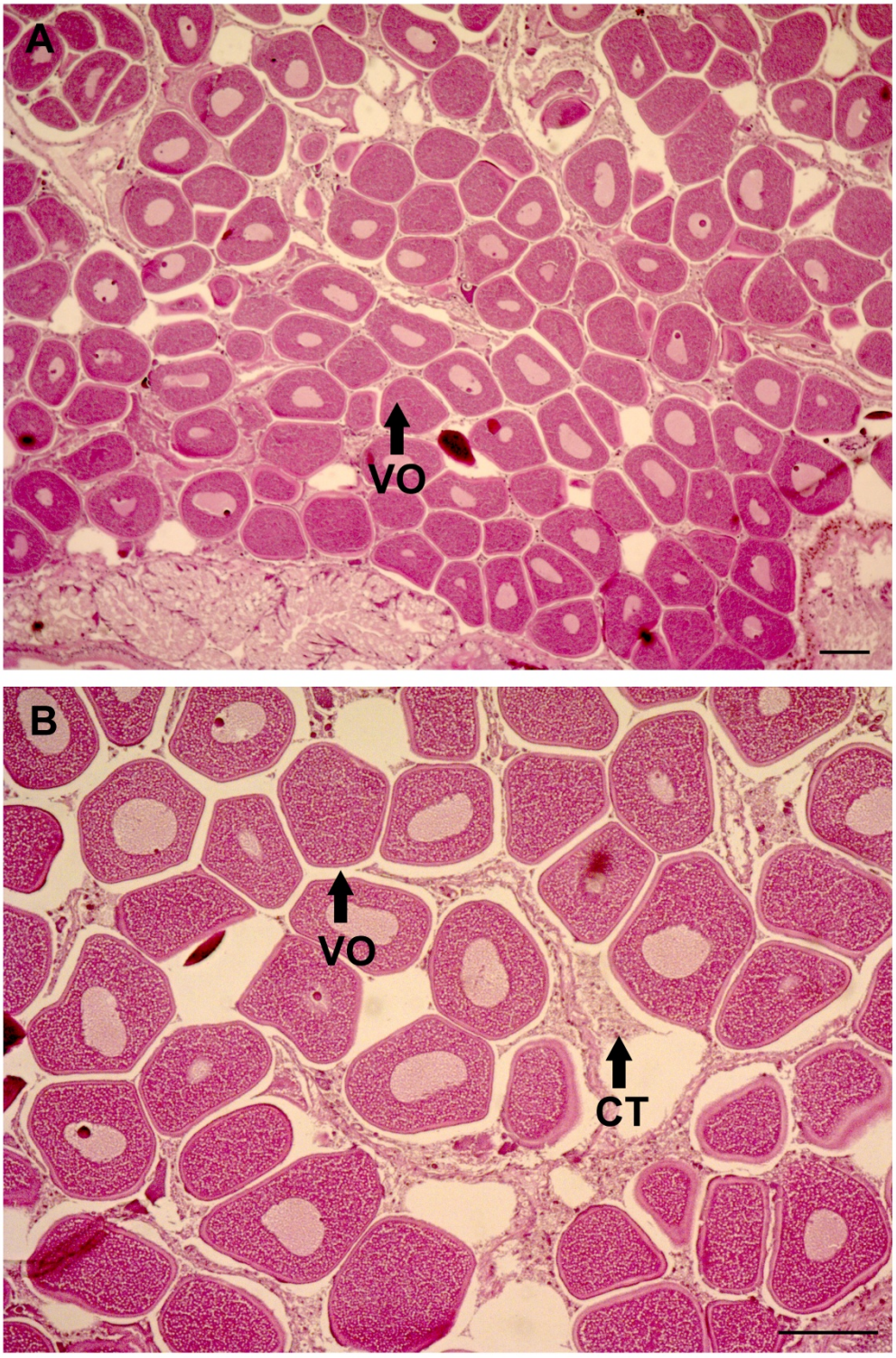

Figure 4.1.5.9 Light photomicrographs of ripe developmental stage of *Patella rustica* female, magnification **A**: 50×; **B**: 100×. **VO** vitellogenic oocyte, **CT** connective tissue. Scale bar 100 µm.

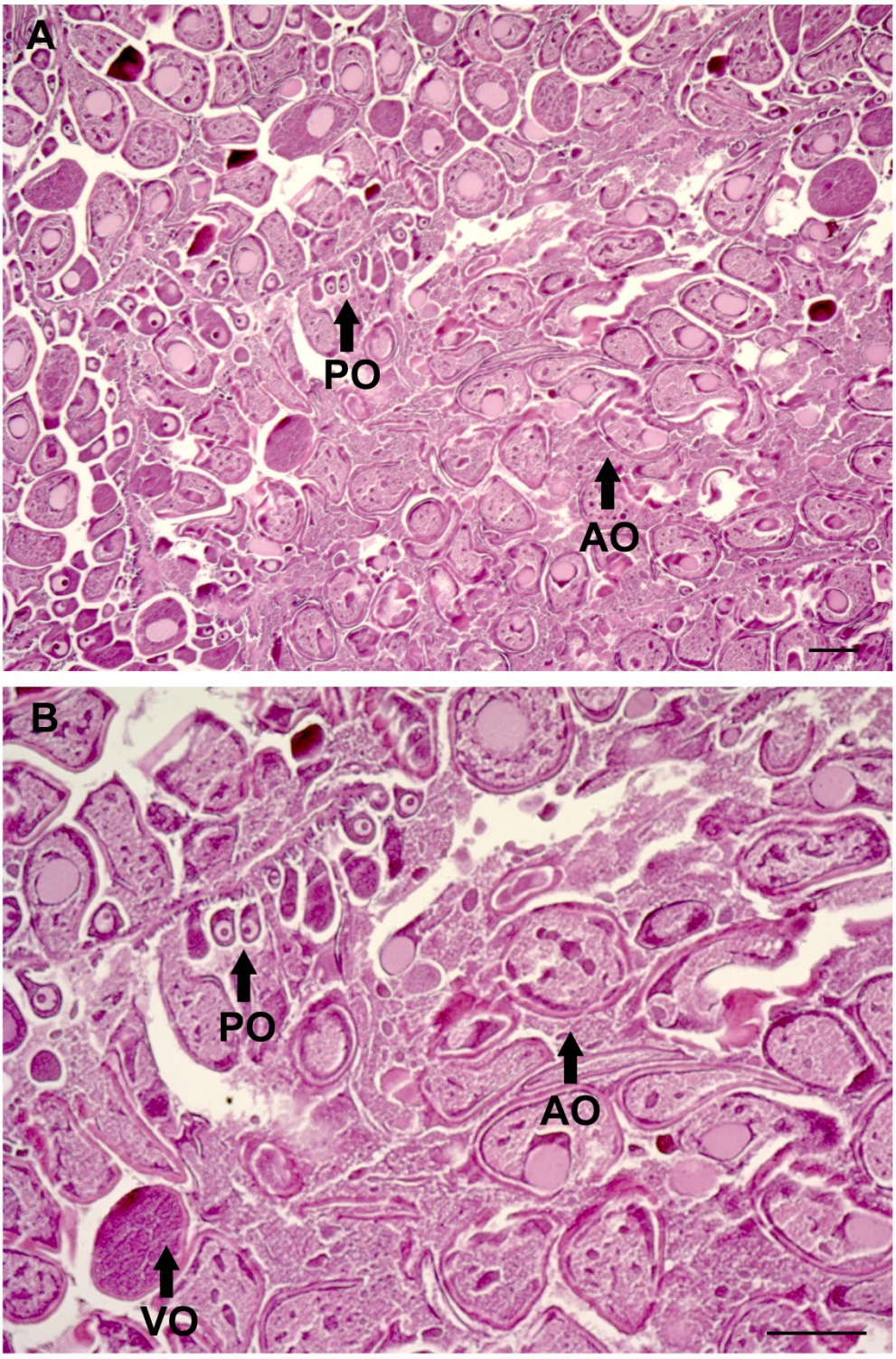

Figure 4.1.5.10 Light photomicrographs of atresic stage of *Patella rustica* female, magnification **A**: 50×; **B**: 100×. **PO** previtellogenic oocytes, **AO** atresic oocyte, **VO** vitellogenic oocyte. Scale bar 100 µm.

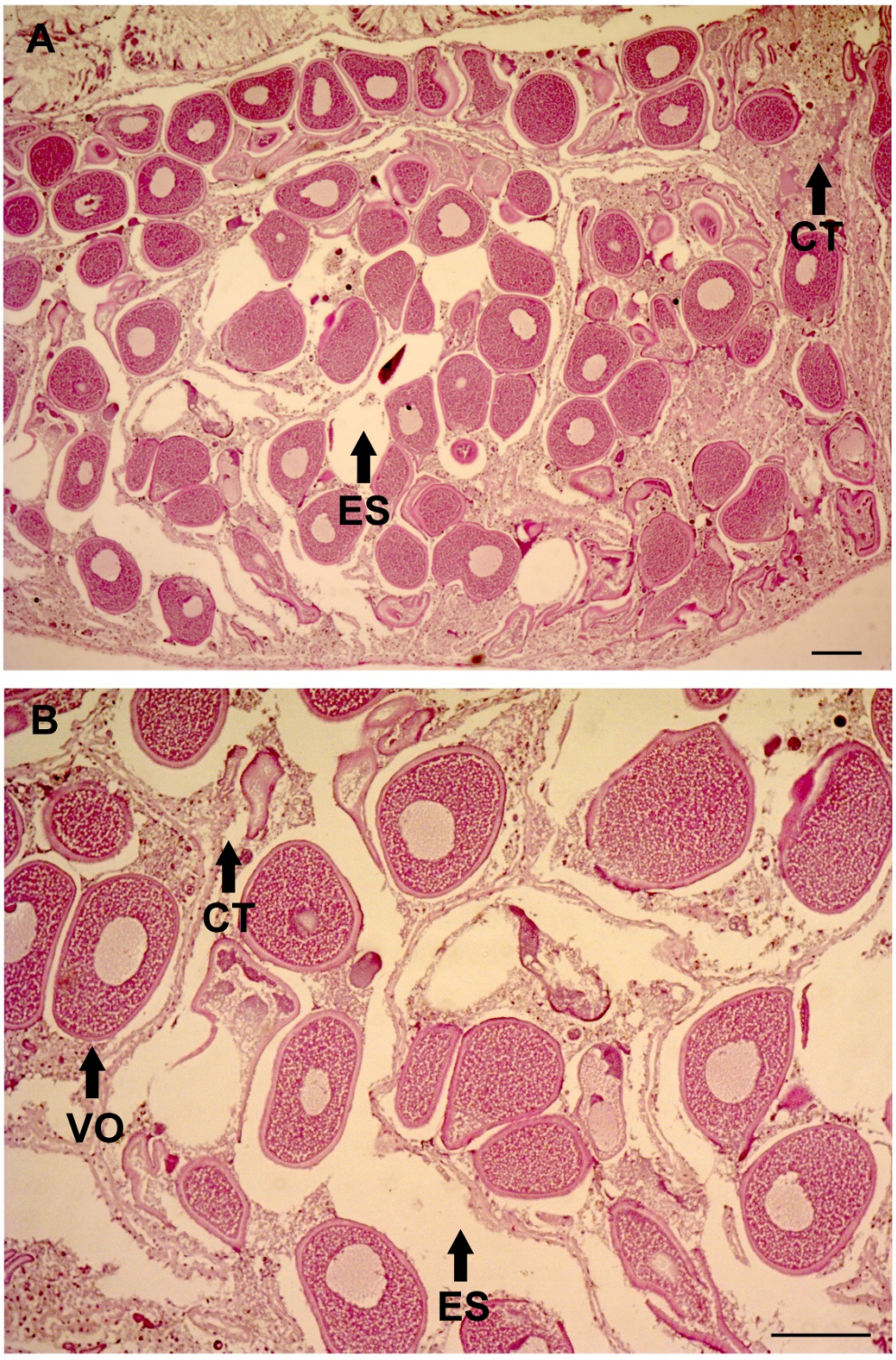

Figure 4.1.5.11 Light photomicrographs of spawning stage of *Patella rustica* female, magnification **A**: 50×; **B**: 100×. **CT** connective tissue, **ES** empty space, **VO** vitellogenic oocyte. Scale bar 100 µm.

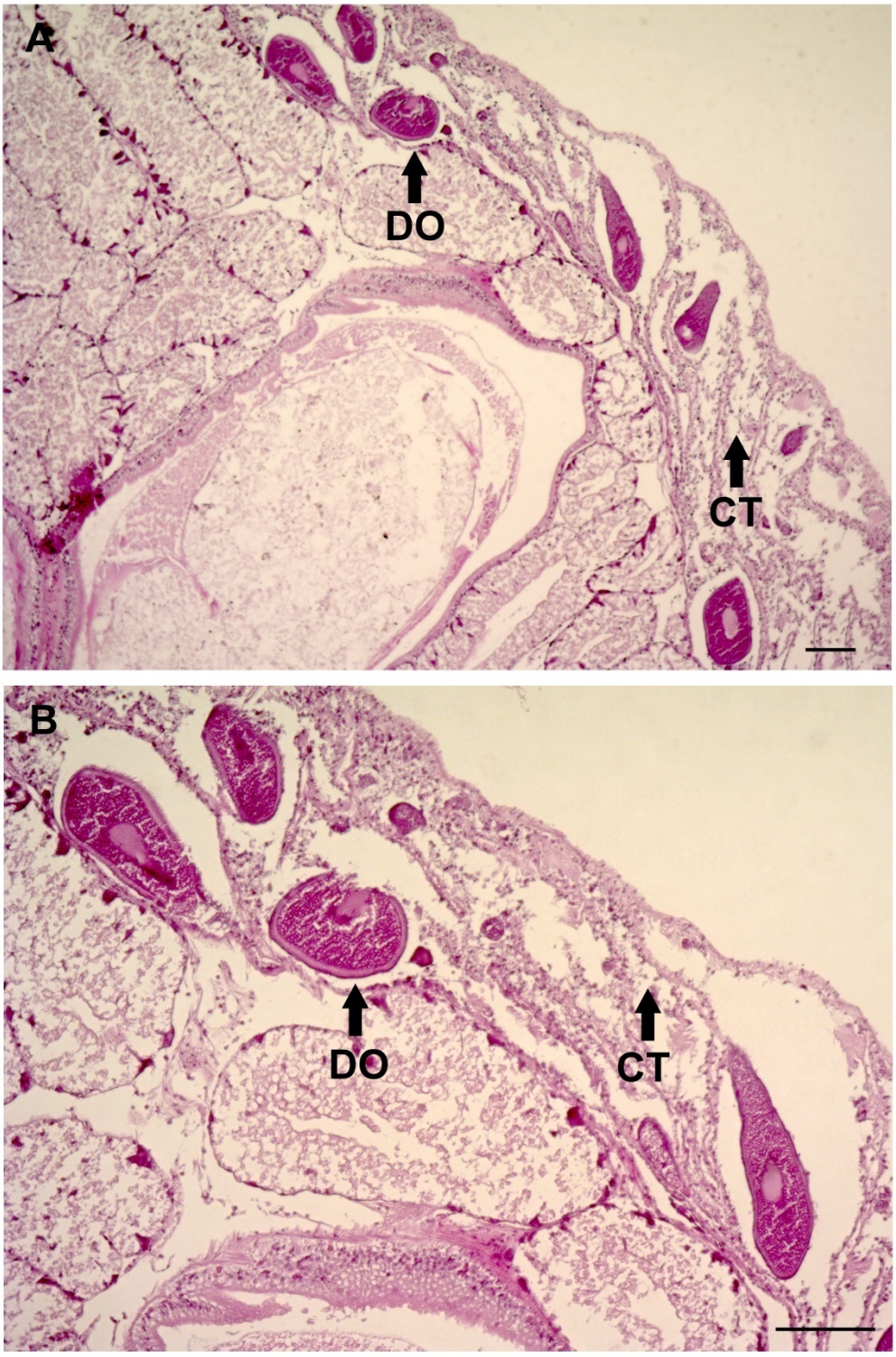

Figure 4.1.5.12 Light photomicrographs of spent stage of *Patella rustica* female, magnification **A**: 50×; **B**: 100×. **DO** degenerative oocyte, **CT** connective tissue. Scale bar 100 µm.

In *Patella rustica* sampled over the study period, the overall ratio of males to females to hermaphrodites to undetermined limpets was 0.48:0.11:0.01:0.40. Undetermined limpets were considered those whose sex could not be differentiated. Hermaphrodites were individuals with

both male and female characteristics (Figure 4.1.5.13). Total number of hermaphrodic individuals was three, two of them sampled in August and one in September. The relative frequencies of the entire sample are illustrated in Figure 4.1.5.14. The highest percentage of females was recorded in May (90%) while the highest percentage of males was present in November (50%). Undetermined individuals were recorded in each sampling month, except November where exactly 50% of analyzed individuals were females and 50% were males. January was the month with the most undetermined individuals (87%) and that trend, although descending, continued in latter months. However, the assumption is that among these undetermined individuals, great number were males and most likely in inactive stage that could not be determined with certainty. This would, however, explain the lack of male individuals in the samples from January to June.

Monthly variations of frequencies of different gametogenic stages for males are shown in Figure 4.1.5.15. In June and August 100% of male individuals were in early developmental stage. However, it is important to stress that in the total sample from February to May and July no males were recorded. Maturation of male gonads occurred from October to November. In November, 60% of males had ripe gonads, 27% were spawning and 13% of individuals were still in late gametogenic stage. The annual frequency of each gametogenic stage for males is shown in Figure 4.1.5.16.

Monthly variations of frequencies of different gametogenic stages for females are shown in Figure 4.1.5.17. Gametogenesis for females started in February while maturation of female gonads occurred from September to November. In November, 47% of females were with ripe gonads, 33% were classified as spawning while 13% were already spent. During September and October a certain percentage (5.0% and 4.8% respectively) of female gonads were in atresic stage characterised with previtellogenic oocytes and mature oocytes undergoing atresia or even complete lysis. The annual frequency of each gametogenic stage for females is shown in Figure 4.1.5.18.

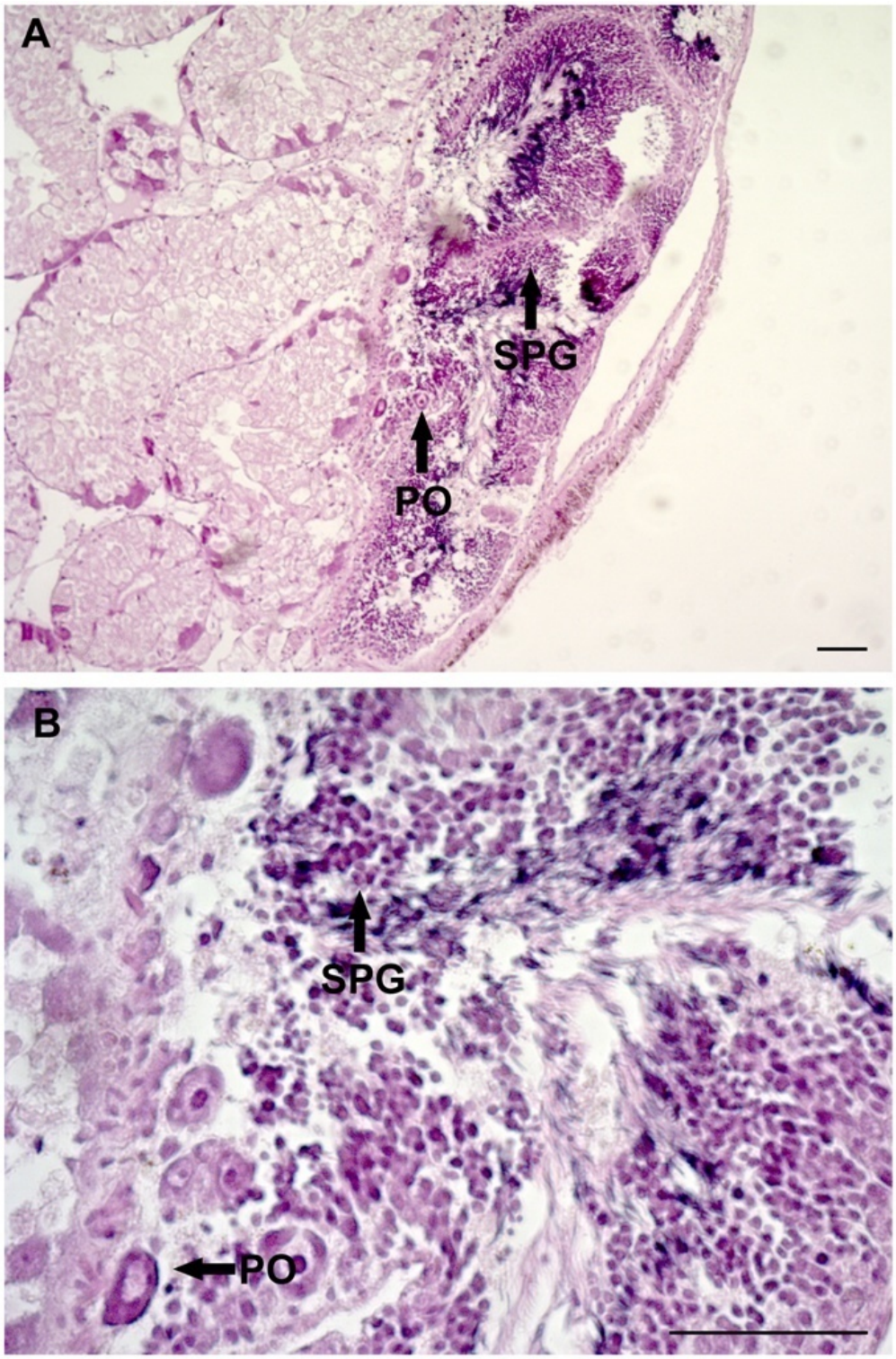

Figure 4.1.5.13 Light photomicrographs of *Patella rustica* hermaphrodite, magnification **A**: 100×; **B**: 400×. **PO** previtellogenic oocyte, **SPG** spermatogonia. Scale bar 50 µm.

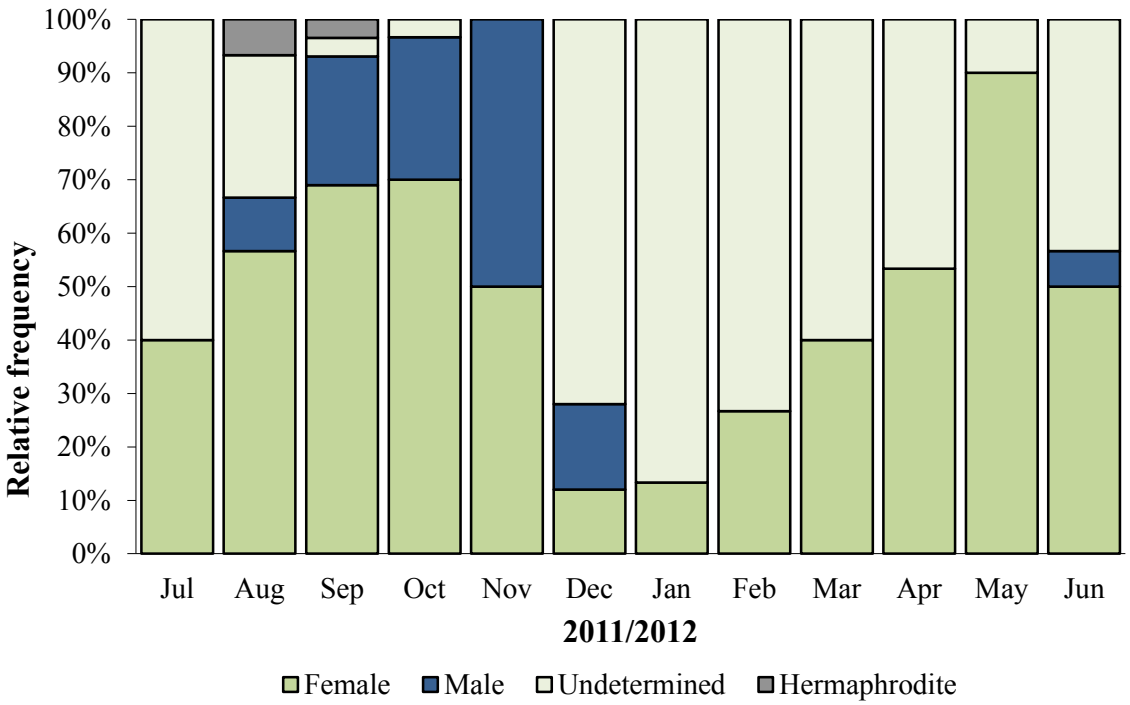

Figure 4.1.5.14 The relative frequencies of *Patella rustica* females, males, individuals for which it was not possible to determine sex and hermaphrodite, according to sampling month.

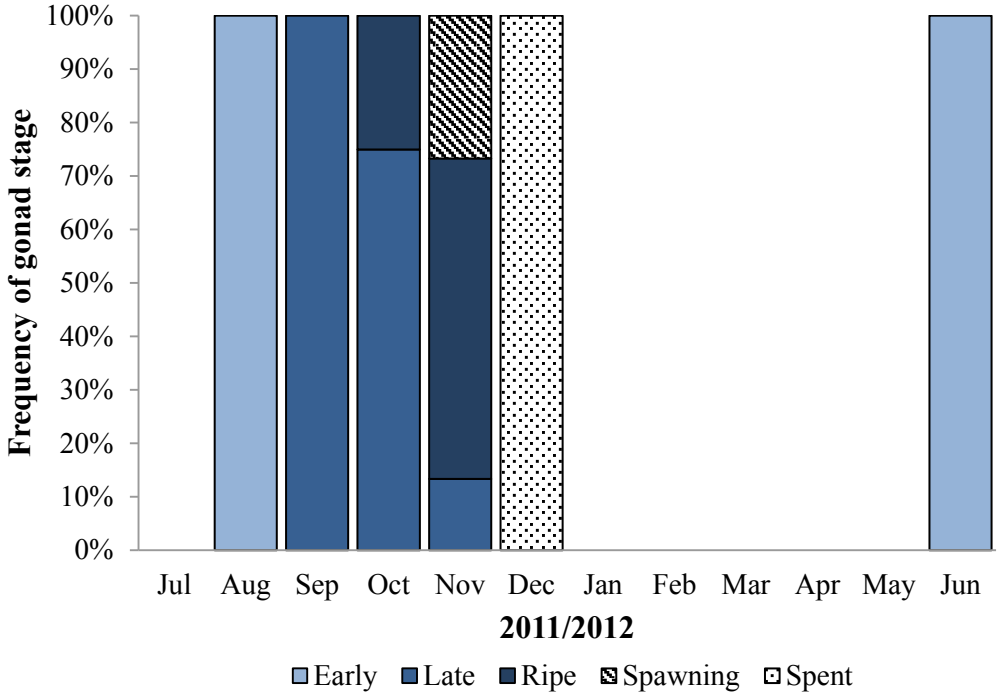

Figure 4.1.5.15 Monthly variations of frequencies of different gametogenic stages for *Patella rustica* males.

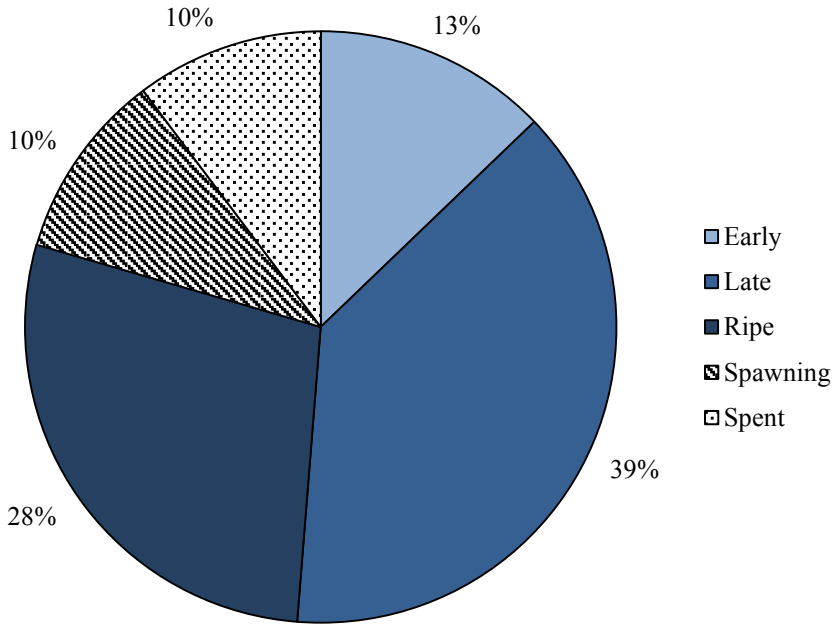

Figure 4.1.5.16 The overall frequency of each gametogenic stage for *Patella rustica* males.

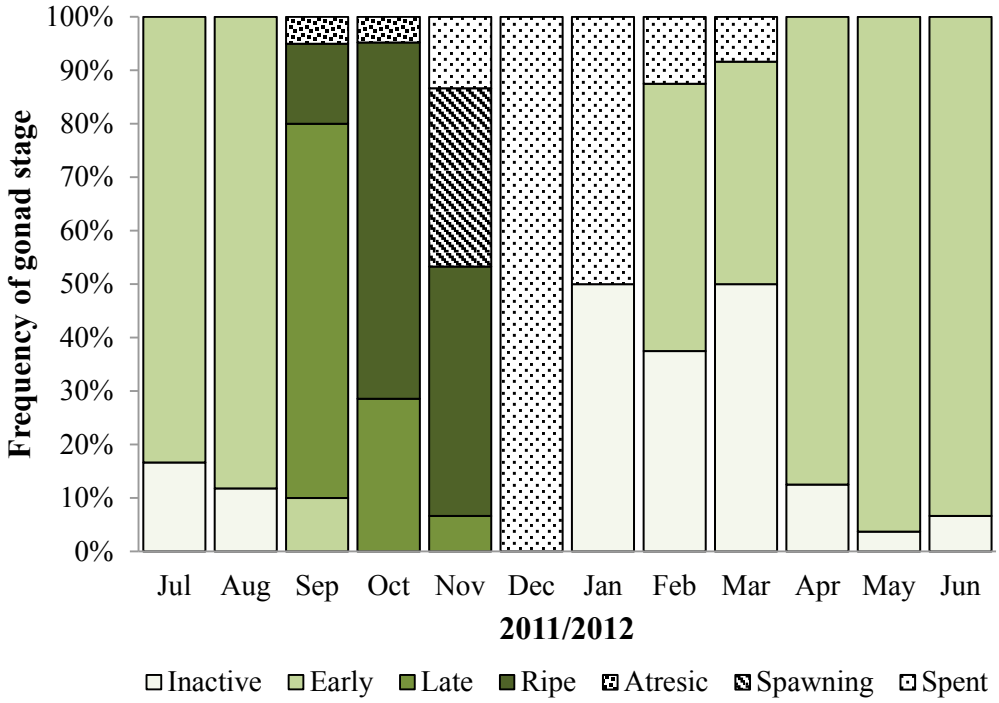

Figure 4.1.5.17 Monthly variations of frequencies of different gametogenic stages for *Patella rustica* females.

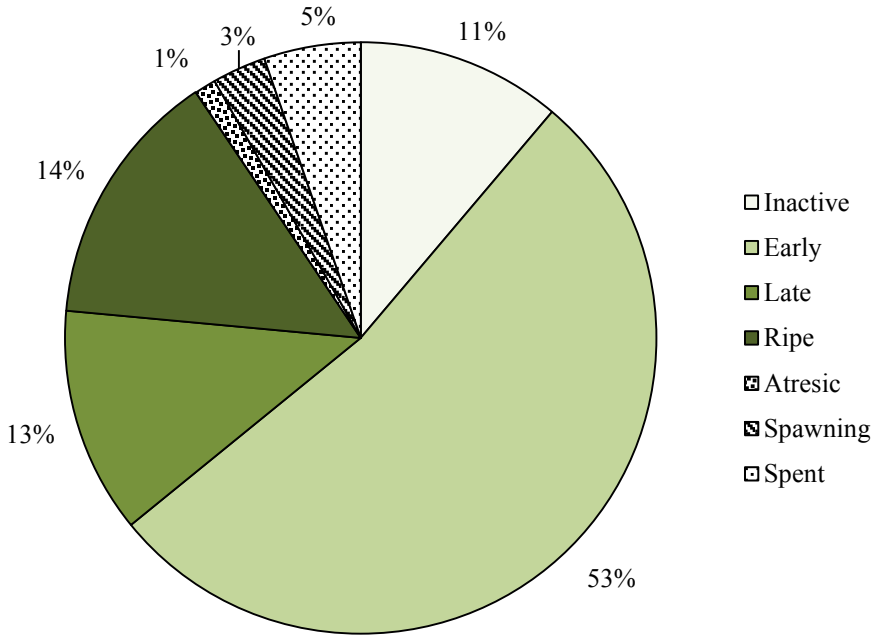

Figure 4.1.5.18 The overall frequency of each gametogenic stage for *Patella rustica* females.

Mean gonad index (MGI) for both sexes is shown in the Table 4.1.5.2 and also illustrated in the Figure 4.1.5.19. The highest values of MGI were recorded from August to November for both sexes. For females the highest value was recorded in October (4.6) and for males in November (4.1). This is in accordance with previously described gonads ripening and spawning peak. It was concluded that the spawning peak for both sexes occurred in November as all individuals (100%) collected in December were with spent gonads. This coincides with the lowest values of MGI in December for males and in January for females (both 1), when majority of individuals were already spent. In June, 100% of males and 93% of females had gonads in early developmental stage, and this was followed with the increase of MGI for both sex (2.8 for females and 3 for males).

Table 4.1.5.2 Mean monthly gonad index values (MGI) of *Patella rustica* females and males throughout the sampling period (absence of males in the sample is indicated with the slash).

Year	Month	MGI ♀	MGI ♂
2011	July	2.5	/
	August	2.6	3.0
	September	4.0	4.0
	October	4.6	3.6
	November	3.4	4.1
	December	1.0	1.0
2012	January	0.5	/
	February	1.6	/
	March	1.3	/
	April	2.6	/
	May	2.9	/
	June	2.8	3.0

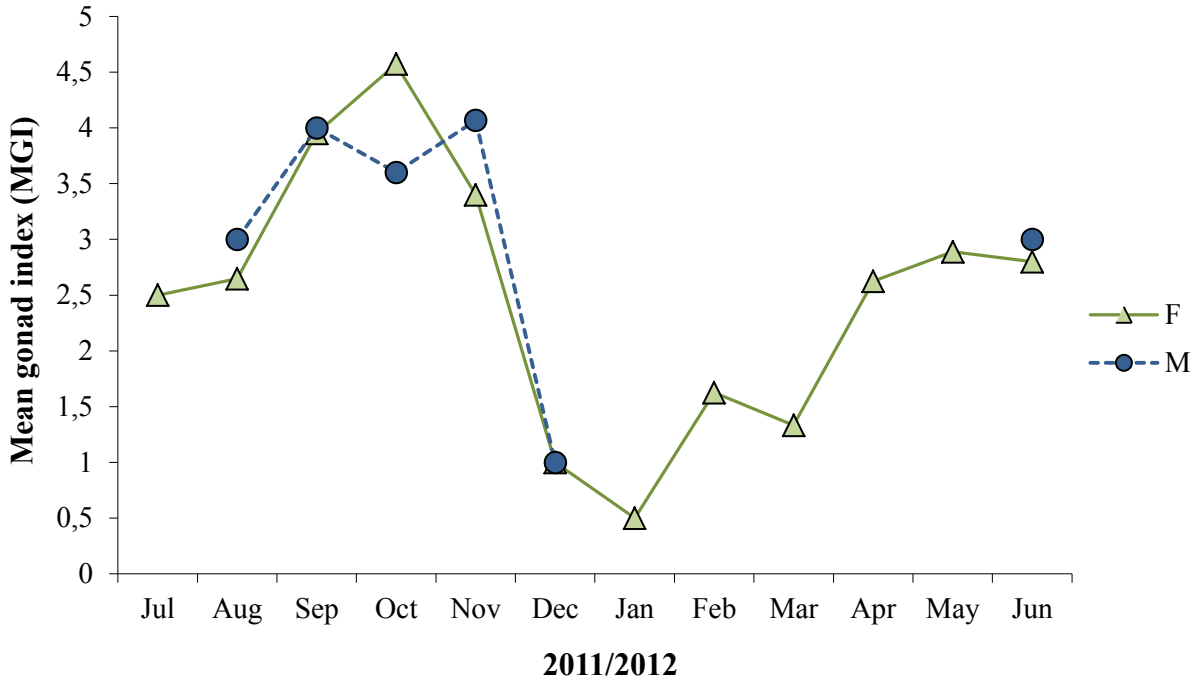

Figure 4.1.5.19 Mean monthly gonad index values (MGI) of *Patella rustica* females (F, green triangles) and males (M, blue circles) throughout the sampling period.

During the study period, total of 95 smaller individuals ranging in length from 10.1 to 22.4 mm were sampled in September (N=10), October (N=30), November (N=25), February (N=15), March (N=10) and May (N=5) and used for qualitative histological analysis. Out of

these 95 individuals, 40 were males (42.1%), 9 were females (9.5%) and 46 (48.4%) were marked as undetermined (Figure 4.1.5.20). Females had mean shell length of 16.7±2.4 mm, males 16.0±3.1 mm and undetermined individuals had mean shell length of 16.2±3.2 mm. The percentage distribution of different gametogenic stages for smaller males is shown in Figure 4.1.5.21 and for smaller females in Figure 4.1.5.22. *Patella rustica* individuals sampled during September 2011, including medium sized (27.3±1.5 mm), smaller sized (11.6±1.1 mm) and wider range of shell length (22.4±5.3), were used to estimate the size at which change of sex occurs. Males dominated the smaller size classes while females become more prevalent as size increased, from ~28 mm onwards (Figure 4.1.5.23).

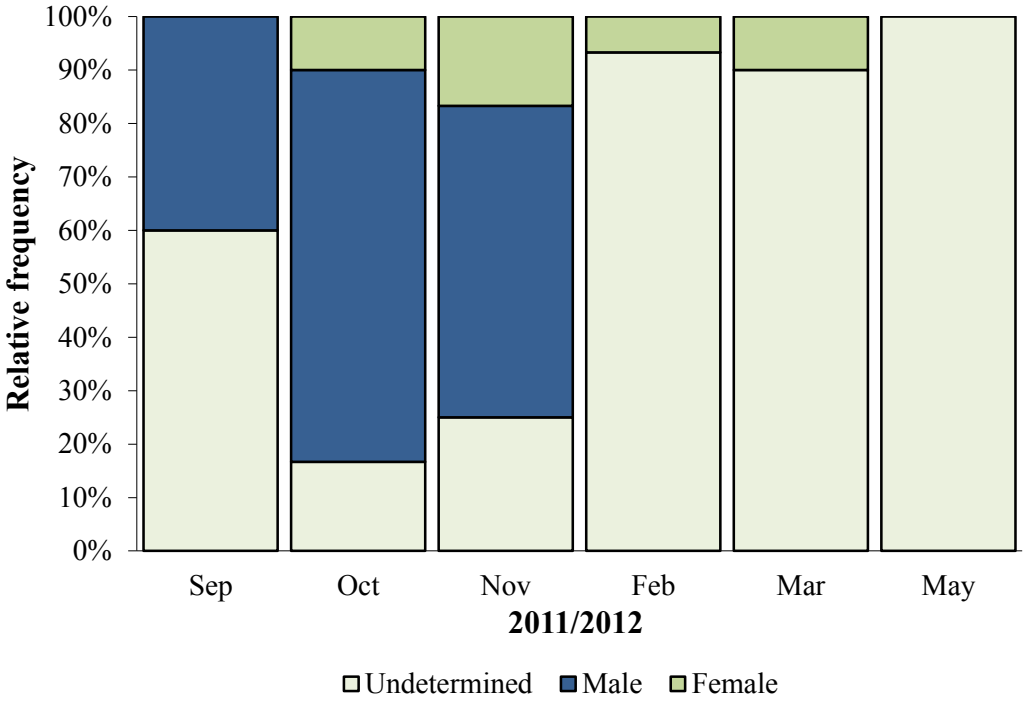

Figure 4.1.5.20 The relative frequencies of smaller *Patella rustica* females, males and individuals for which it was not possible to determine sex, according to sampling month.

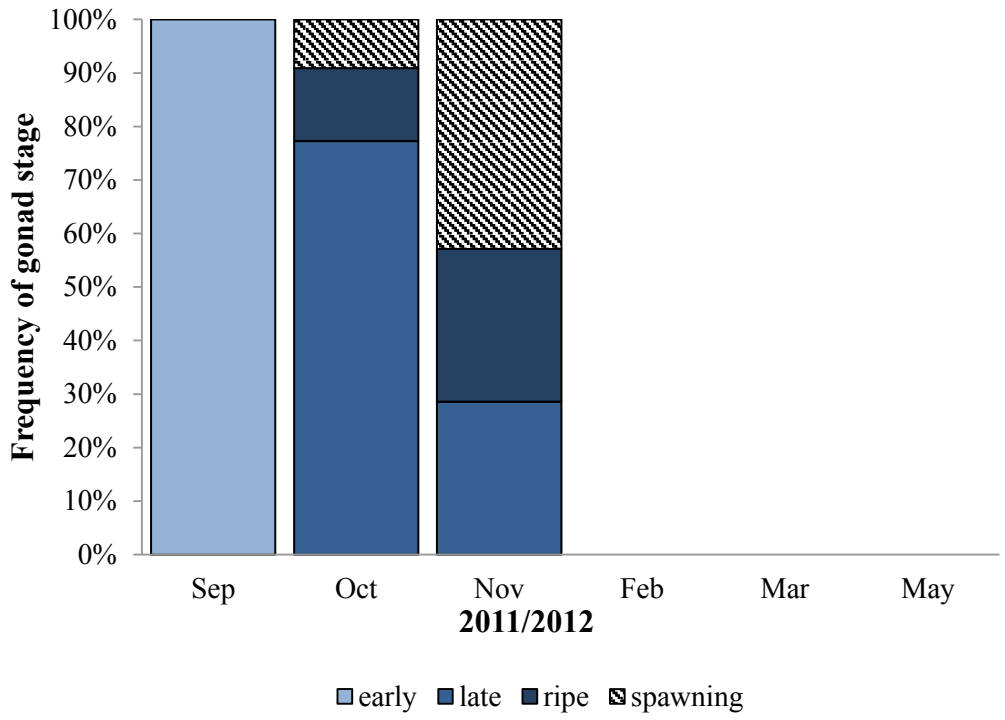

Figure 4.1.5.21 The frequency of each developmental stage of smaller *Patella rustica* males.

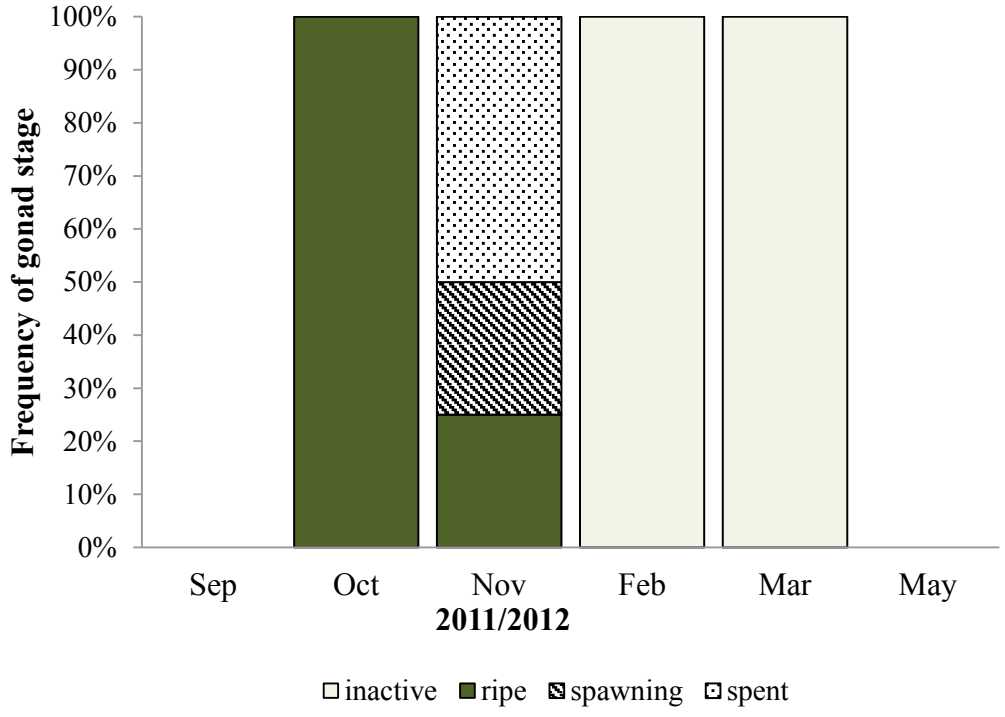

Figure 4.1.5.22 The frequency of each developmental stage of smaller *Patella rustica* females.

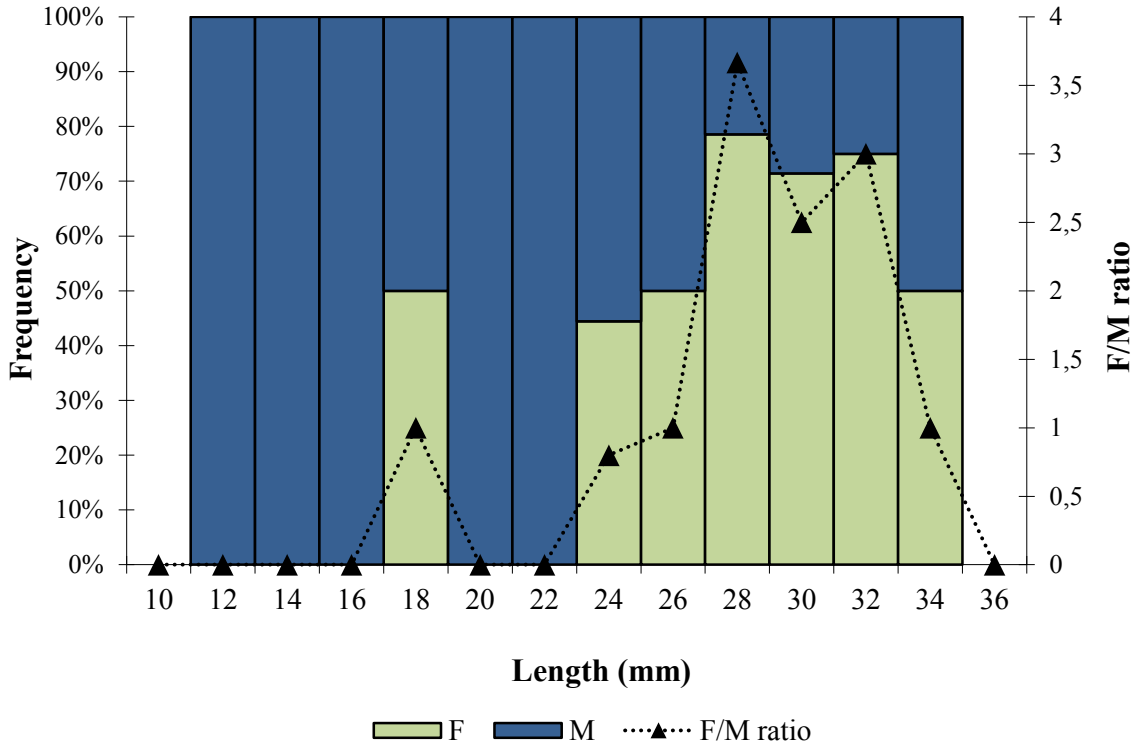

Figure 4.1.5.23 Female/male ratio and frequency distribution of male (M) and female (F) *Patella rustica* with respect to shell length.

Quantitative histological analysis. For each oocyte, size was measured and expressed as oocyte diameter and perimeter. Oocyte size was measured throughout the sampling period and for each developmental stage. Inactive stage was not included in quantitative analysis. Oocytes diameter ranged from 0.2 to 242.9 µm and oocytes perimeter ranged from 10.2 to 569.9 µm. Mean monthly values for diameters and perimeters, as well as the maximum and minimum values, are shown in Table 4.1.5.3.

Oocytes increased in size from September (63.8±40.1 µm and 176.9±111.1 µm for diameter and perimeter, respectively) to November (104.5±49.6 µm and 297.7±140.0 µm for diameter and perimeter, respectively). After April, the frequency distribution of oocyte size had a relatively uniform pattern until October, indicating a continuous process of maturation and finally release of gametes in November. The smallest oocytes were measured in individuals that were in early developmental stage (21.3±9.7 µm and 60.2±30.5 µm for diameter and perimeter, respectively), while the biggest oocytes were measured in individuals in ripe stage (115.6±44.1 µm and 329.0±125.4 µm for diameter and perimeter, respectively), when oocyte already went through vitellogenesis.

Table 4.1.5.3 Mean (X) oocyte size (diameter and perimeter) of *Patella rustica*, with standard deviation (SD), minimum (Min) and maximum (Max) values throughout the sampling period.

Year	Month	N	X diameter (µm)±SD	Min (µm)	Max (µm)	X perimeter (µm)±SD	Min (µm)	Max (µm)
2011	July	417	18.7±6.4	6.7	36.9	52.1±16.7	20.0	103.5
	August	1869	24.8±9.6	7.5	87.2	67.4±24.1	20.3	211.9
	September	947	63.8±40.1	8.5	204.8	176.9±111.1	29.7	516.1
	October	576	118.7±47.0	7.3	213.2	341.4±133.5	25.6	567.4
	November	356	104.5±49.6	5.7	242.9	297.7±140.0	17.9	570.0
	December	43	25.1±32.2	6.2	128.4	71.2±83.2	21.1	335.8
2012	January	133	8.1±10.2	3.2	110.1	24.1±29.0	10.2	313.2
	February	67	22.8±32.4	6.7	189.3	65.2±92.5	20.3	516.4
	March	232	15.4±20.9	5.3	171.7	44.7±62.9	15.0	520.1
	April	296	15.7±4.9	6.8	34.0	43.8±12.6	18.7	82.5
	May	1444	19.5±6.4	5.8	44.6	53.7±16.2	18.5	120.5
	June	1038	19.0±7.4	0.2	55.5	62.8±45.4	19.0	554.3
	Total	**7418**						

The oocyte size (diameter and perimeter) for each developmental stage is illustrated in Figure 4.1.5.24 and Figure 4.1.5.25. The smallest number of oocytes was measured in December (N=43) while the highest number was measured in August (N=1862) and this confirmed the pattern observed with qualitative staging of gonad development where 100% of individuals in December were with spent gonads while in August 88.2% were in early developmental stage. There was a significant positive correlation between mean gonad index and oocyte diameter (Table 4.1.6.1). The frequency distributions of oocyte diameter according to sampling months are shown in Figure 4.1.5.26 and the frequency distributions of perimeters in Figure 4.1.5.27.

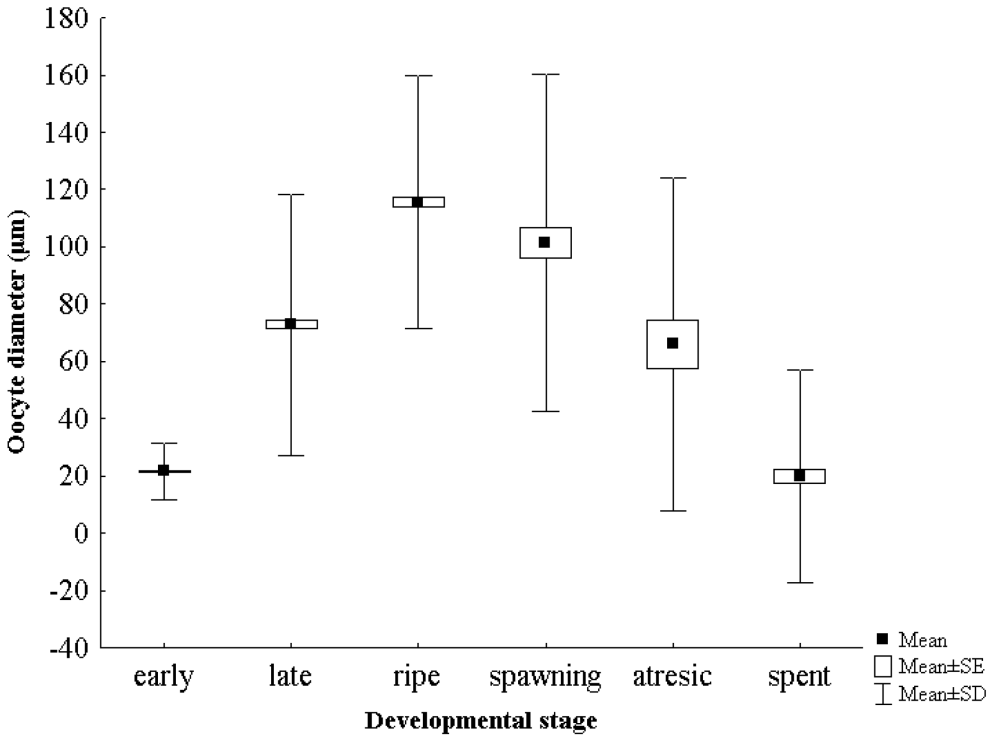

Figure 4.1.5.24 Mean oocyte diameter of *Patella rustica*, according to each developmental stage; SE-standard error, SD-standard deviation.

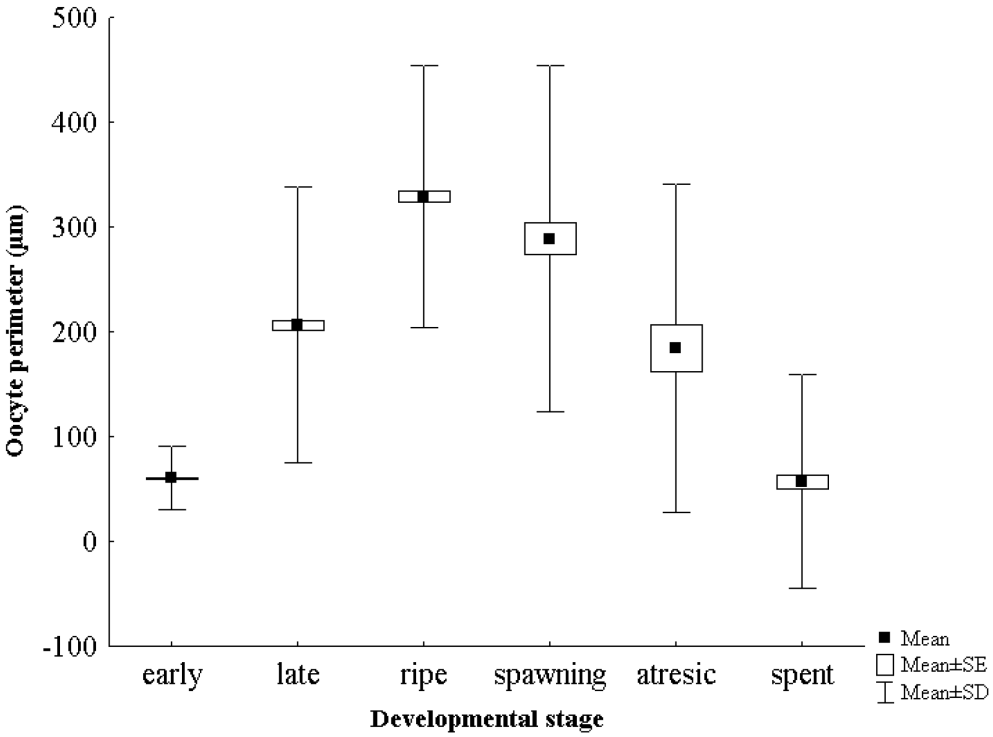

Figure 4.1.5.25 Mean oocyte perimeter of *Patella rustica*, according to each developmental stage; SE-standard error, SD-standard deviation.

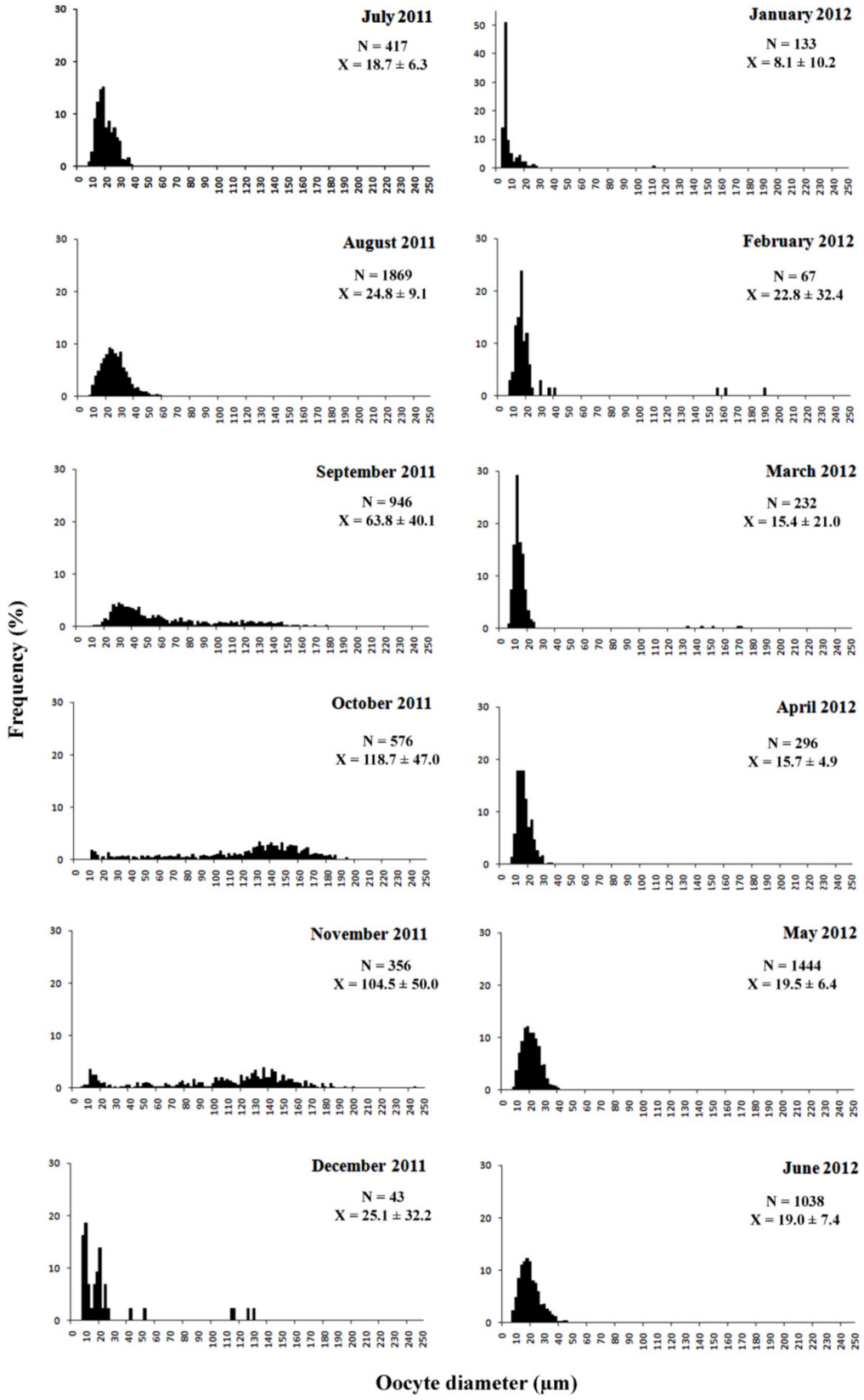

Figure 4.1.5.26 Size frequency histograms of oocyte diameter measured from monthly sampled *Patella rustica*.

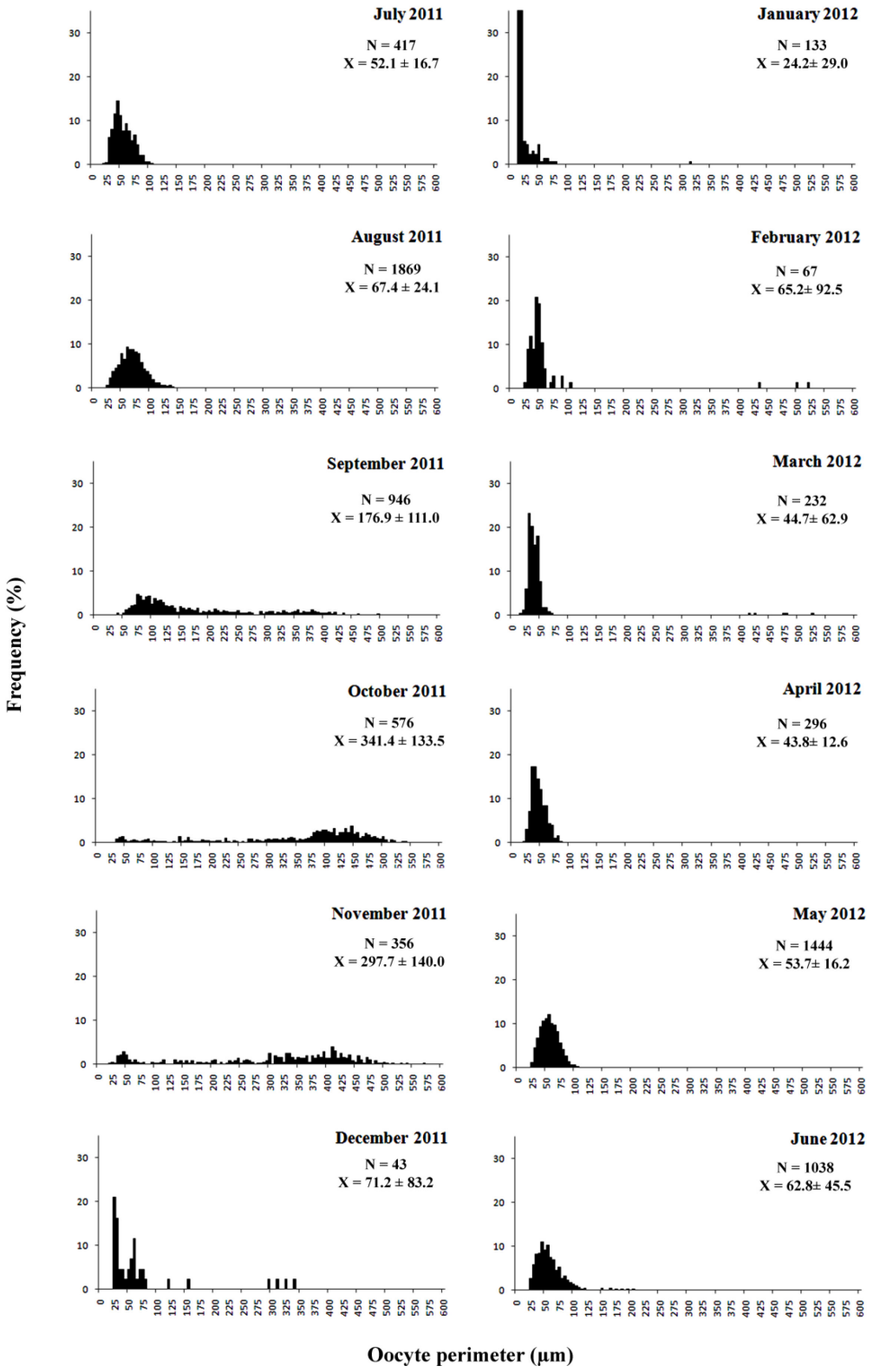

Figure 4.1.5.27 Size frequency histograms of oocyte perimeter measured from monthly sampled *Patella rustica*.

First sexual maturity. To determine first sexual maturity, 65 smaller individuals, ranging in length from 10.1 to 22.4 mm were collected during September, October and November, the period of late gametogenesis and maximal gonad activity. The estimated length where 50% of analysed males were sexually mature was 13.1 mm (Figure 4.1.5.28). However, for females it was not possible to estimate the length where 50% was mature since, in the total sample, only 9 individuals were females.

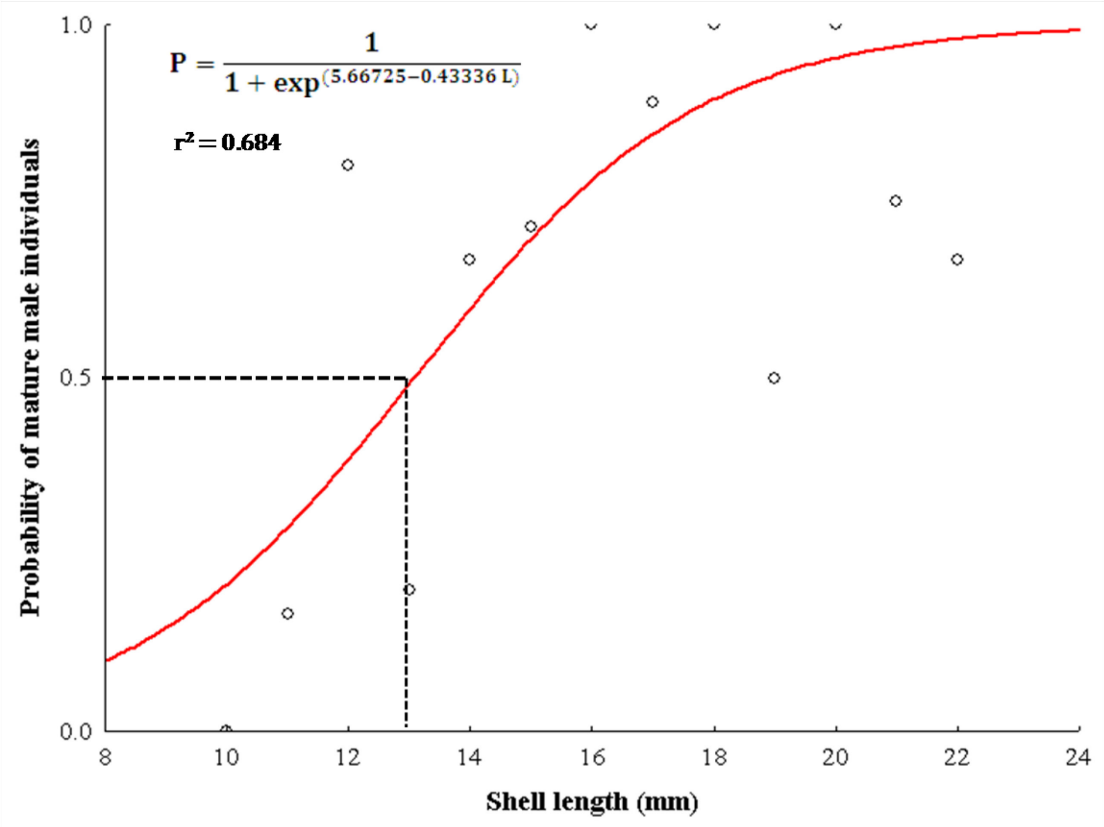

Figure 4.1.5.28 Ratio of mature males in September, October and November 2011 in relation to limpet's length (mm); dashed line represents the length of *Patella rustica* limpet (13.1 mm) at which 50% of analyzed males were mature.

4.1.6 Condition index analysis

The variation of condition index (CI) throughout the whole sampling year is shown in Figure 4.1.6.1. The monthly mean maximum value was recorded in May (179.6) and the minimum just after, in June (133.9). A statistically significant difference was noted in condition index values with respect to sampling month ($H=60.70$, $p<0.001$).

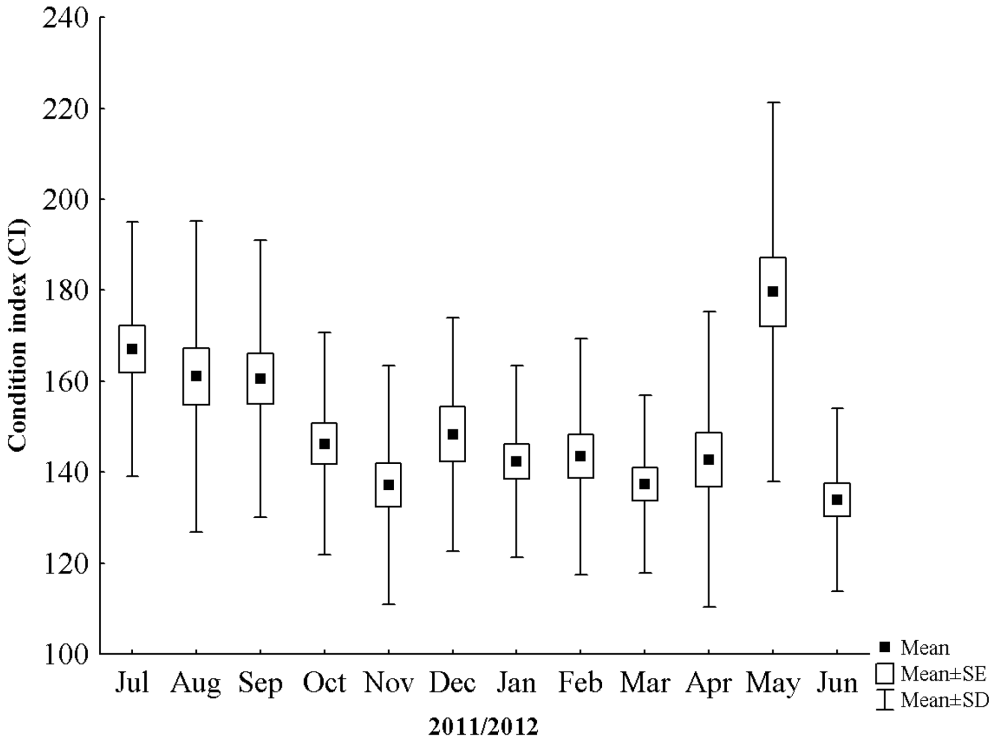

Figure 4.1.6.1 Mean monthly condition index of *Patella rustica*, according to sampling months; SE-standard error, SD-standard deviation.

Correlation between hydrographic parameters and *Patella rustica* biology. No significant correlations were found between condition index and mean gonad index (both for females and males). There was significant negative correlation between mean gonad index of males and chl *a* concentration (r=−0.812, p=0.050) and significant positive correlation between mean gonad index of females and air temperature (r=0.629, p=0.028). Between oocyte diameter and mean gonad index of females there was significant positive correlation (r=0.685, p=0.014). The results of Spearman's correlation analysis are shown in Table 4.1.6.1.

Table 4.1.6.1 The results of Spearman's correlation analysis to determine the degree of association between mean gonad index (MGI) of both females and males, oocyte diameter and female MGI, hydrographic parameters (sea surface and air temperature), chlorophyll *a* concentration (chl *a*) and condition index (CI); r - Spearman's correlation, p - p value (set at 0.05), significant correlations are denoted in bold.

	MGI ♀	**MGI ♂**	**Tair (°C)**	**Tsea (°C)**	**chl *a***
MGI ♂	r=0.754 p=0.084				
T_{air} (°C)	**r=0.629** **p=0.028**	r=0.087 p=0.870			
T_{sea} (°C)	r=0.545 p=0.067	r=−0.058 p=0.913	**r=0.895** **p<0.001**		
chl *a*	r=−0.378 p=0.226	**r=−0.812** **p=0.050**	r=−0.161 p=0.618	r=−0.329 p=0.297	
CI	r=0.140 p=0.665	r=−0.174 p=0.742	r=0.441 p=0.152	r=0.399 p=0.199	r=0.189 p=0.557
Oocyte diameter	**r=0.685** **p=0.014**				

4.2 Heat stress physiology of the Mediterranean patellid limpets

4.2.1 Arrhenius break point temperature

Limpets *Patella rustica*, *P. caerulea* and *P. ulyssiponensis* exhibited consistent differences in heart beat rates, which were not related to size differences between the three species as all experimental animals were of similar weight (2.5±1.0 g). Individual cardiac performance could be divided into two phases with increasing temperatures (Figure 4.2.1.1). All three species had regular heart beats at the beginning of the experiment (Figure 4.2.1.2) and in the first phase (23°C to 33°C) where heart rates increased in a log-linear fashion with temperature as described in the Arrhenius curve (Figure 4.2.1.1). In this phase, metabolic rates increased with temperature and this temperature range was used to calculate Q10 relationships. In the second phase (33°C to 45°C), after temperature increased continuously, all three species showed a steady phase, after which heart rates decreased considerably (Figure 4.2.1.1). In the last phase, Arrhenius plots of *P. ulyssiponensis* heart rates were qualitatively different from the other two species. The heart rates of *P. rustica* and *P. caerulea* decreased more gradually from their ABT point while that of *P. ulyssiponensis* had more irregular pattern, showing a levelling off in heart rate in the mid phase, after which heart rate decreased more abruptly.

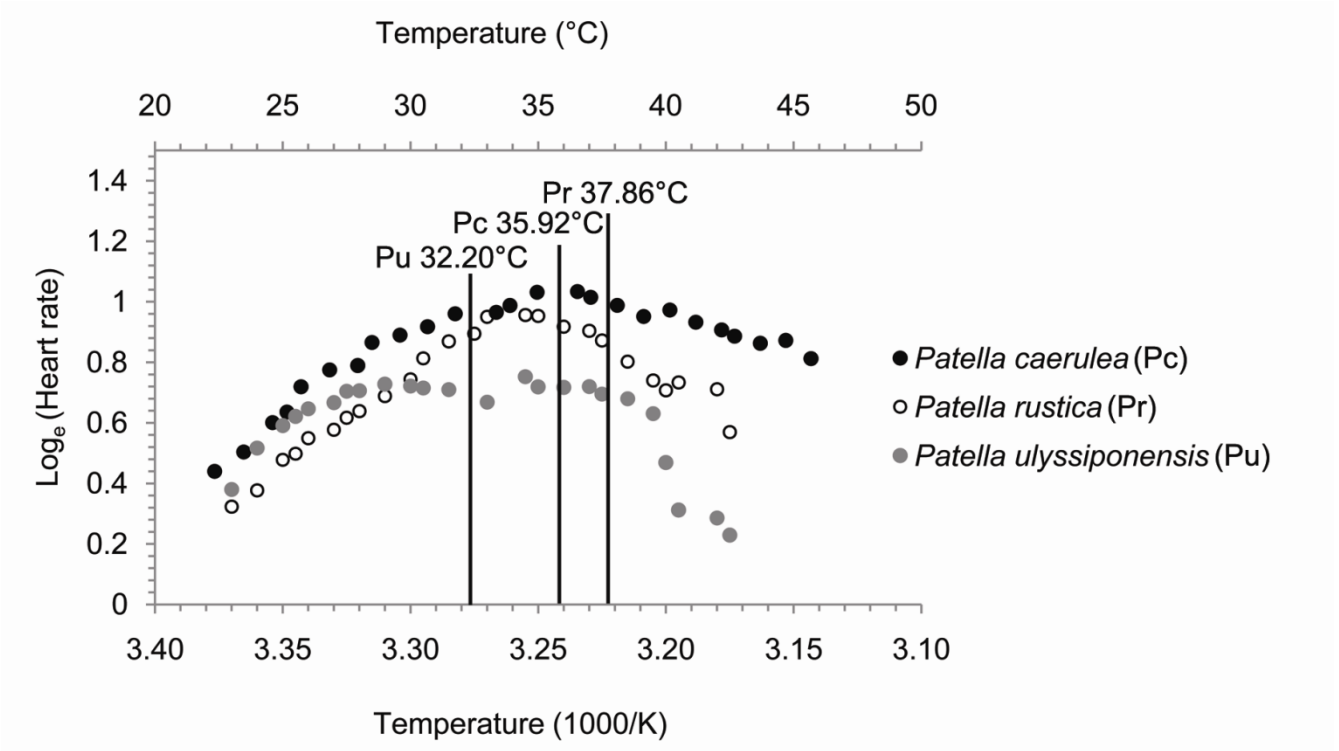

Figure 4.2.1.1 Arrhenius breakpoint temperatures (ABT) of heart rates for representative individuals of *Patella caerulea*, *P. rustica* and *P. ulyssiponensis*.

In general, *P. caerulea* had consistently faster heart rates throughout the experiment (2.4±0.4 Hz, mean±SD) than *P. rustica* (2.1±0.4 Hz), while *P. ulyssiponensis* had relatively slow heart rates (1.9±0.3 Hz). Based on two-phase regression, ABTs for the three species were all significantly different ($F_{(2,12)}$=7.58, p=0.007) with *P. rustica* having the highest ABT (37.9±2.07°C), followed by *P. caerulea* (35.9±2.6°C), and finally the low-zoned *P. ulyssiponensis* (32.2±2.3°C). The differences in heart traces at the point of their Arrhenius temperature is shown in Figure 4.2.1.3, and in Figure 4.2.1.4 these differences are even more expressed with prolonged heating time. The same pattern was found for Q10 relationships, which also differed between the three species ($F_{(2,12)}$=12.79, p=0.001). *Patella rustica* had the highest Q10 value (1.90±0.10, mean±SD), which was similar to *P. caerulea* (1.82±0.22), whereas the Q10 of both species differed from *P. ulyssiponensis* which had the lowest metabolic increase (1.33±0.23) in the range of 23°C to 33°C.

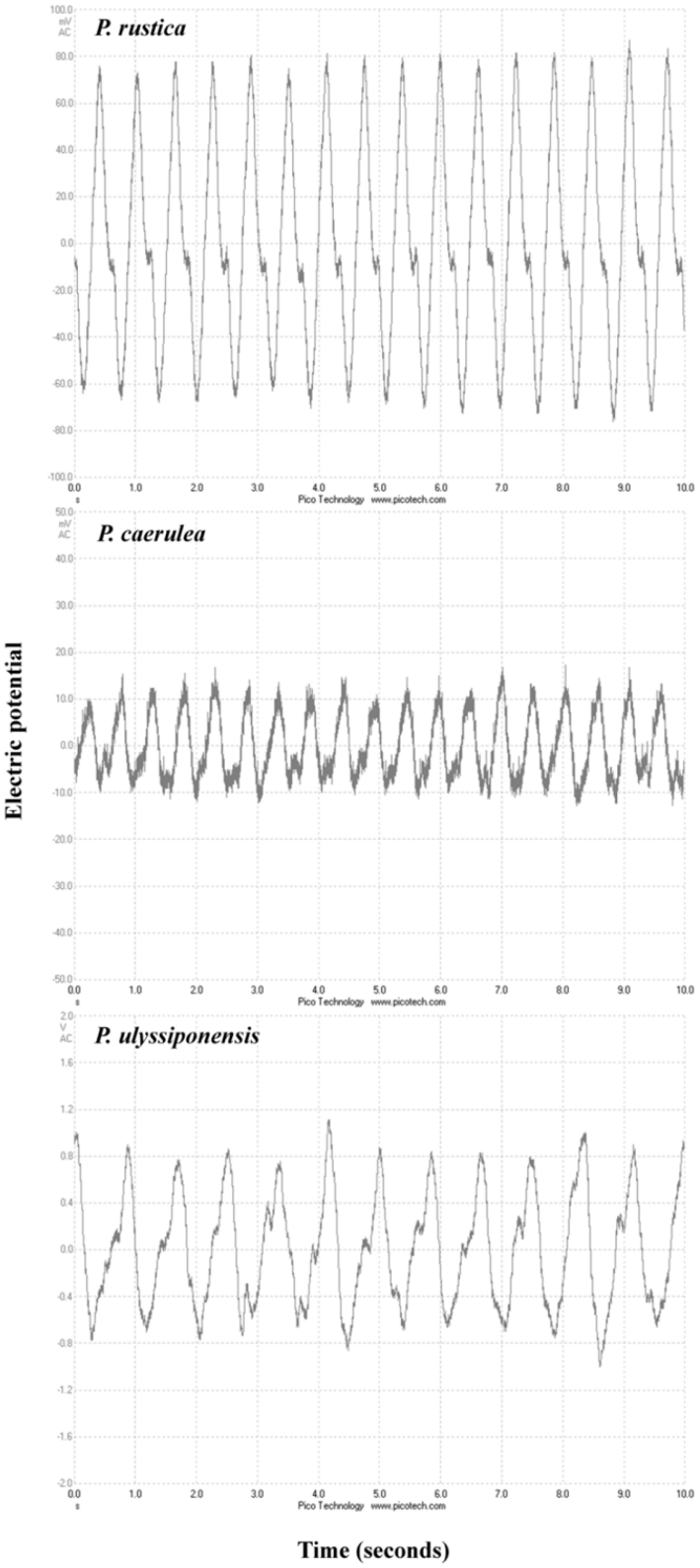

Figure 4.2.1.2 Heart beat traces recorded at the beginning (23°C) of the experiment for *Patella rustica*, *P. caerulea* and *P. ulyssiponensis*; time scale is 10 seconds and the y-axis vary with species.

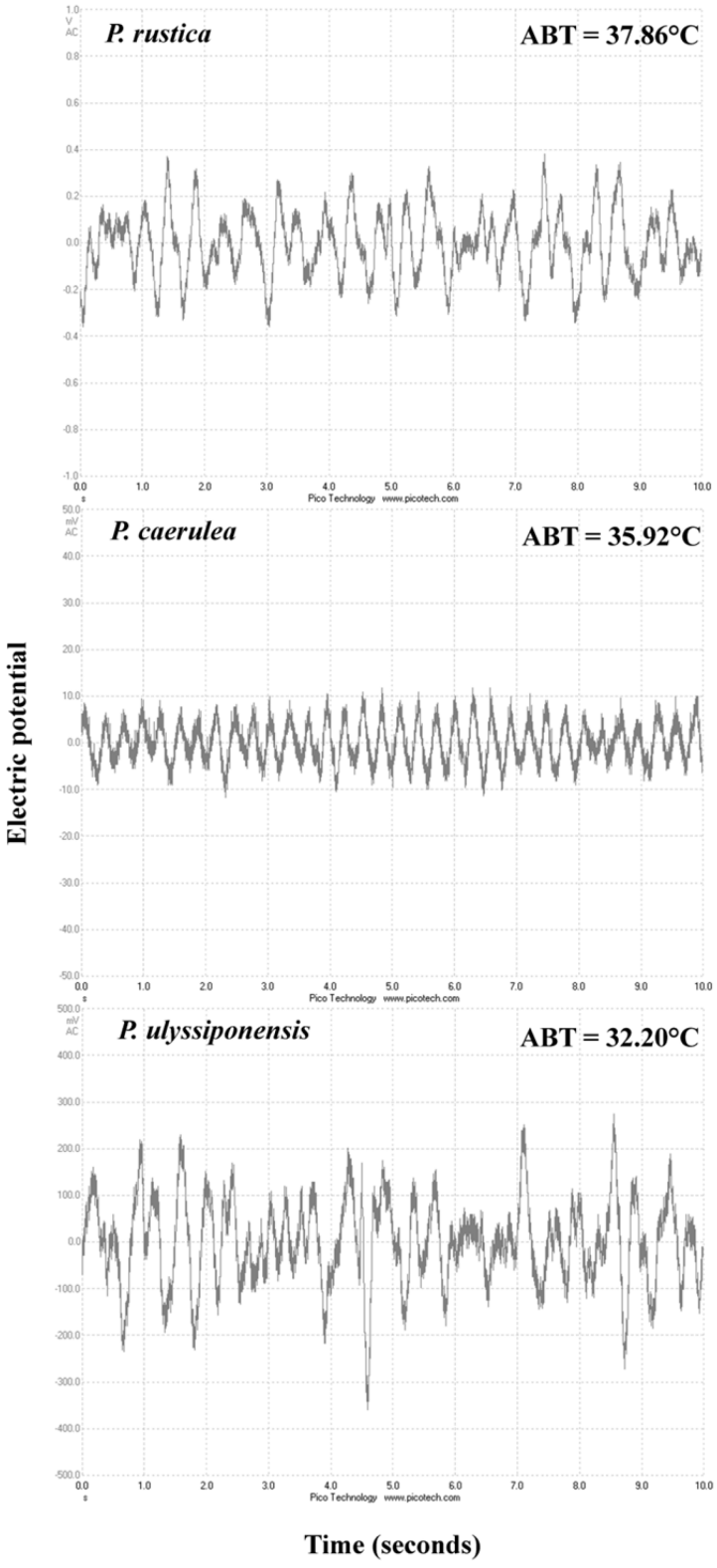

Figure 4.2.1.3 Heart beat traces recorded at the point of Arrhenius break temperature for three species: *Patella rustica* (after 75 minutes of heating), *P. caerulea* (after 65 minutes of heating) and *P. ulyssiponensis* (after 50 minutes of heating); time scale is 10 seconds and the y-axis vary with species.

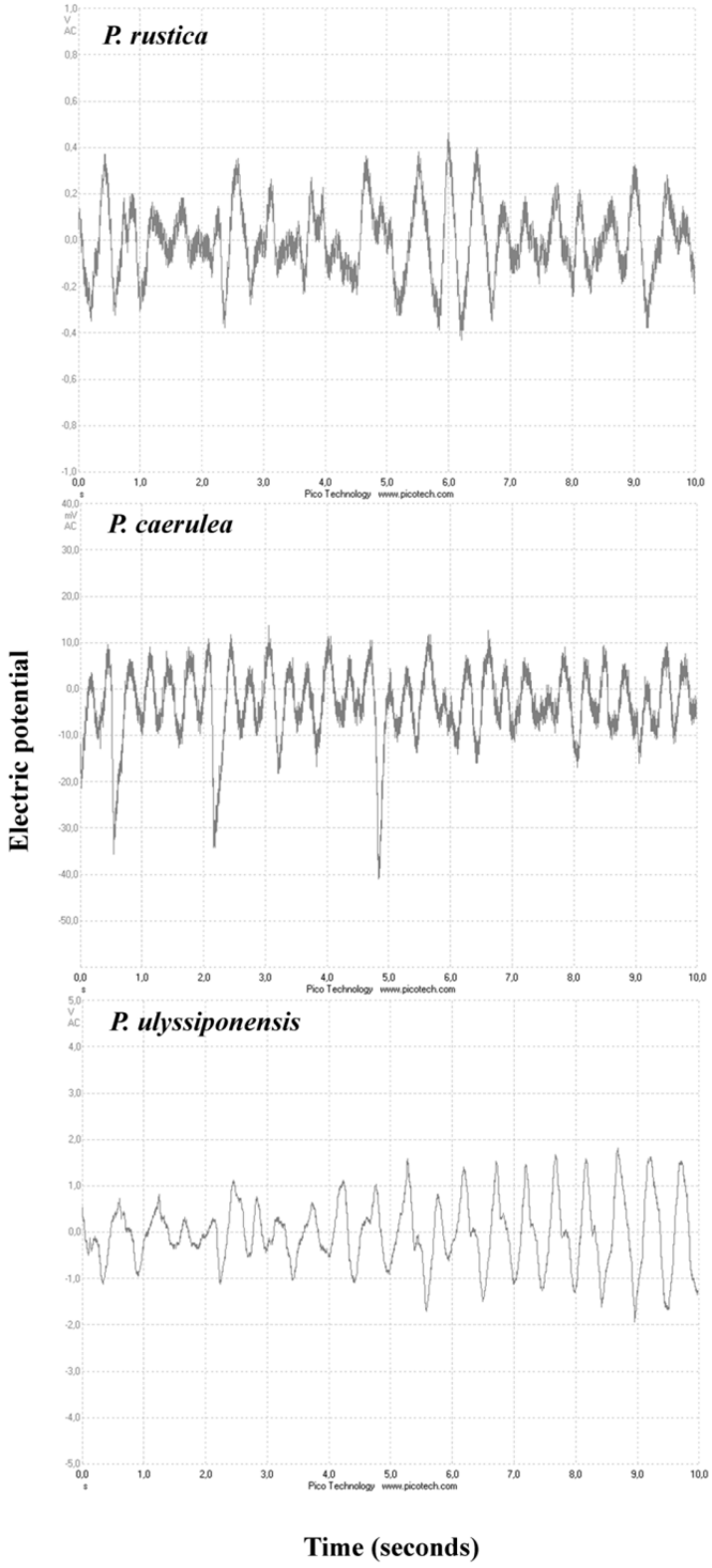

Figure 4.2.1.4 Heart beat traces recorded after 90 minutes of heating (39.5°C) for *Patella rustica*, *P. caerulea* and *P. ulyssiponensis*; time scale is 10 seconds and the y-axis vary with species.

4.2.2 Heat shock proteins

Patella rustica and *P. caerulea* showed different expression patterns of inducible *hsp70* mRNA (Figure 4.2.2.1). Both species had similar, low levels of *hsp70* mRNA at 20°C, which was the control temperature at the beginning of the experiment. After high temperature exposure, however, there were significant differences in *hsp70* mRNA levels among different temperatures and species (Table 4.2.2.1). In general, *hsp70* levels of *P. rustica* were greater than *P. caerulea*, especially at higher temperatures (Table 4.2.2.1, Figure 4.2.2.1). *Hsp70* levels of *P. rustica* varied with temperatures with upregulation occurring at 34°C (~36% RU), which continued at 38°C after 60 minutes (~143% RU) and 120 minutes of exposure (~194% RU). In *P. caerulea*, however, levels of *hsp70* mRNA (Figure 4.2.2.1) were relatively low at 34°C (~4% RU), but increased to reach maximum levels at 36°C (~71% RU), and remained at similar, but lower levels at 38°C (both after 60 minutes and 120 minutes exposure).

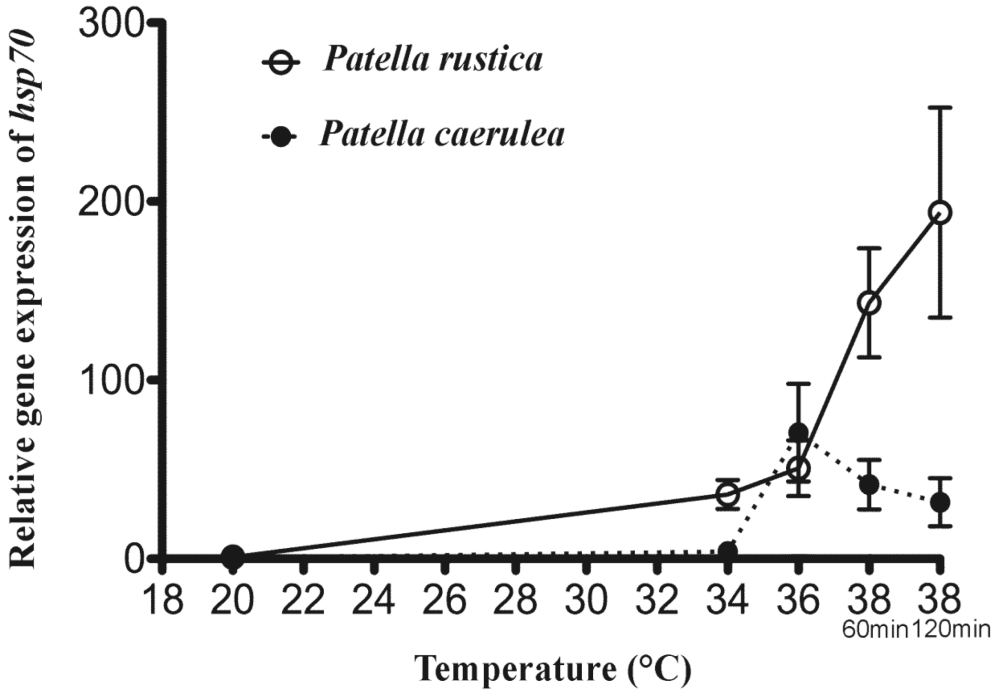

Figure 4.2.2.1 *Hsp70* mRNA levels (relative unit, RU %) in *P. rustica* and *P. caerulea* after exposure to different temperature regimes (20°C, 34°C, 36°C) and different durations at 38°C (60 and 120 minutes).

Table 4.2.2.1 Two factor PERMANOVA to investigate variation in *hsp70* gene expressions of the limpets *Patella rustica* (N=25) and *P. caerulea* (N=20) under different temperature treatments, followed by *post hoc* pairwise tests.

Source	df	SS	MS	pseudo - F	p
Species	1	2.762	2.762	27.879	0.001
Temperature	4	29.051	7.263	73.303	0.001
Species × Temperature	4	1.646	0.412	4.154	0.011
Residual	35	3.468	0.009		
Total	44	38.0280			
Temperature					
20°C *P. caerulea=P. rustica*					
34°C *P. caerulea<P. rustica*					
36°C *P. caerulea=P. rustica*					
38°C 60 min *P. caerulea<P. rustica*					
38°C 120 min *P. caerulea<P. rustica*					
Species					
P. rustica	20°C<34°C=36°C<38°C 60 minutes=38°C 120 minutes				
P. caerulea	20°C<34°C<38°C 120 minutes=38°C 60 minutes=36°C				

4.2.3 Neutral red uptake assay

Neutral red uptake assay showed that absorbance value of neutral red does change with different temperatures. There was significant differences in neutral red uptake between *Patella rustica* and *P. caerulea* (H=11.44, p=0.01). Higher values were recorded at 32°C for *P. rustica* (Figure 4.2.3.1) and at 38°C for *P. caerulea* (Figure 4.2.3.1), while both species had reduced values when returned again to spray seawater (T_3). There was no significant difference between different temperatures for *P. rustica* (H=5.97, p=0.113), while for *P. caerulea* there was significant difference between different temperature treatments ($F_{(3,8)}$=4.10, p=0.05; $T_0=T_1=T_2>T_3$).

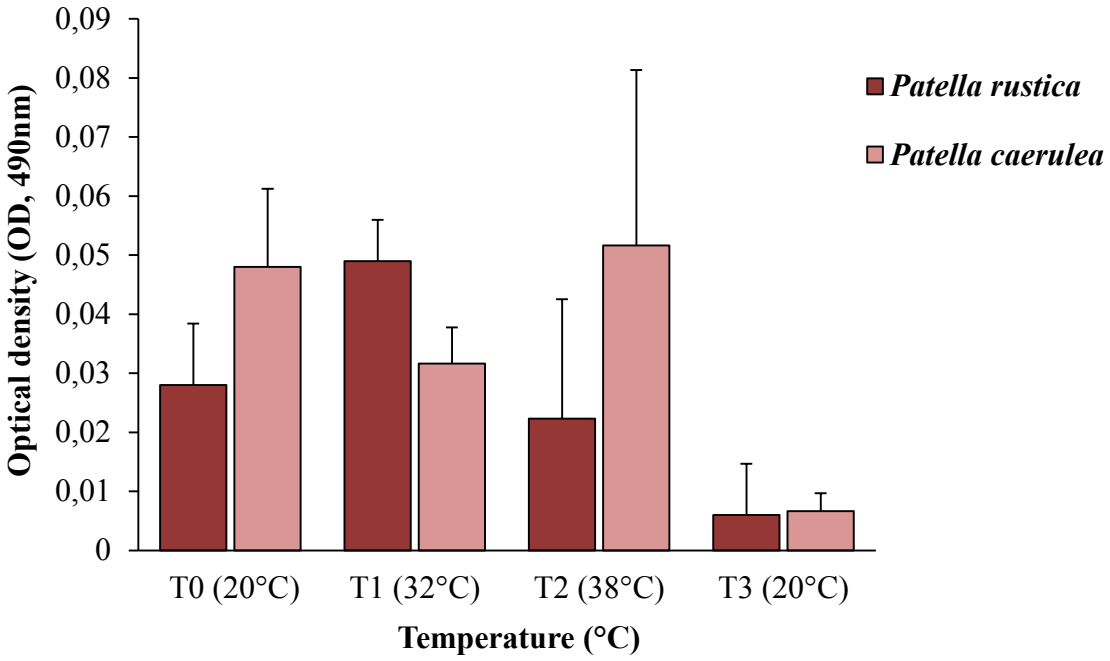

Figure 4.2.3.1 The absorbance value of neutral red (optical density) for *Patella rustica* and *P. caerulea* at different temperatures.

5. DISCUSSION

5.1 Age and growth of the limpet *Patella rustica*

Growth in shelled molluscs can be defined as an increase in shell size. This is a result of changes in mantle secretory activity and deposition of new rings/lines of conchiolin impregnated with calcium carbonate on the edge of the shell (Wilbur & Owen 1964; Fretter & Graham 1994). Growth patterns in bivalve shells have been widely used as the important measure of organism's vitality, while those in gastropod shells have mostly been understudied. The reason for this could be a spherical and/or spiral gastropod shell which is harder to orientate for shell sectioning (Richardson & Liu 1994), but also the fact that on outer shell surface for most gastropods there are no reliable patterns or they are weaker in definition compare to bivalves (Ekaratne & Crisp 1982; Richardson & Liu 1994; Richardson, 2001). Thus, the configuration of the gastropod shell is often considered challenging for growth studies, especially mesogastropod and neogastropod shells (Richardson, 2001). The exception is the limpet conic shape shell.

To determine limpets' growth and longevity, majority of the studies used mark-recapture (Kenny, 1977; Bretos, 1978, 1980; Kido & Murray 2003; Clark et al. 2004; Gray & Hodgson, 2003; Espinosa et al. 2008) or length frequency distributions method (Guerra & Gaudêncio 1986; Brethes et al. 1994; Khow, 2007). The first one is based on marked individuals released for recapture at regular intervals to obtain direct measurements of their shell increments, while latter one is depended on the population sampled at intervals where a measure of growth is obtained by changes in the mean size of cohorts (Gray, 1996). The analysis of inner growth lines is the most reliable method for age determination, but it requires more time and competence (Gosling, 2003). In intertidal molluscs, annual growth lines have to be distinguished from microgrowth lines forming daily or with tidal periodicity (Richardson, 1989, 1990; Richardson & Liu, 1994; Richardson, 2001). Branch (1974b) stated that in *Patella* limpets there are no obvious growth lines occurring within the shell and consequently, growth and age could not be directly obtained from the shells of individuals. This was refuted by Ekaratne & Crisp (1982) who were the first to describe periodicity of microgrowth patterns in gastropod shells; studying two intertidal grazers, *Patella vulgata* and *Littorina littorea* Linnaeus, 1758 and one intertidal carnivore, *Nucella lapillus* Linnaeus, 1758 they demonstrated that deposition of growth lines occurred during each emersion period. A similar tidal periodicity has been established in other limpets, mainly tropical species: *Fissurella crassa*

from North Chile (Bretos, 1978), *Siphonaria gigas* from Costa Rica (Crisp et al. 1990), *Cellana toreuma* from Hong Kong (Richardson & Liu 1994), *Scutellastra granularis* from South Africa (Vat, 2000), and *Helcion pectunculus*, also from South Africa (Gray & Hodgson 2003). The present study represents an application of use of inner growth lines for describing growth patterns in *Patella rustica*, Mediterranean limpet species.

The results from this study showed that in *P. rustica* shells one dark growth line is formed annually. Dark growth line representing slow shell growth was visible either at or very near the margin of shells collected between May and October. Based on these data, the growth line formation was set in May. This period coincides with the increase of air and sea temperatures. Generally accepted rule is that temperate limpets grow more rapidly in summer due to more food abundance (Brêthes et al. 1994; Jenkins & Hartnoll 2001; Vat, 2000). This however, may not be the case for *P. rustica*. The assumption is that the maximum growth occurs during winter months, in the period between December and March. Although marginal increment analysis is a good indicator of line formation, it does not allow a great deal of inference on growth dynamics. Therefore, further research needs to be conducted in order to confirm this. It is interesting to note that *P. rustica* has a longer breeding period during summer months and that the spawning peak occurs in November (see further down discussion on reproduction of *P. rustica*). This could indicate a possible mismatch between growth and reproduction, as has been documented for several other limpet species. The endemic Mediterranean limpet *P. ferruginea* on North Africa shores has faster growth during summer months while spawning peak occurs in autumn (Espinosa et al. 2008). Kido & Murray (2003) showed that fastest shell growth for *Lottia gigantea* on Southern California shores occurred in spring and early summer while slowest growth was recorded in autumn and winter, the period when limpets were believed to be accumulating gonadal material. The onset of sexual maturity has often been linked with a decrease in growth rate (Vahl, 1971), although this is not always a rule. The strong positive correlation between growth and reproduction was documented for seven South African *Patella* species (Branch, 1981), implying that although energy could be diverted from growth to reproduction and vice versa when food supplies are low, some species have strategies of low or high turnover (Branch, 1981). High shore species such as *P. rustica* are considered to be employing 'energy conservation' strategy (Newel & Branch 1980) because of their unstable environment and limited food resources (Sokolova & Pörtner 2003). In addition, according to the r-K theory (Stearns, 1976), species occupying such environments will often depend on recolonisation for their existence and will therefore benefit most from a

larger investment in reproduction (Branch, 1981). Hence, *P. rustica* will more likely channel abundant food supplies into energy for reproduction rather than growth. On the other hand, in winter months foraging is facilitated with rough sea conditions while desiccation stress is reduced to a minimum (Santini & Chelazzi 1995), suggesting that limpets could then obtain necessary conditions and energy for faster growth.

High shore species are more subjected to desiccation or wave sweeping and these two factors are known to influence the most their morphological characteristics (Tatarenkov & Johannesson 1994). Branch (1985b), comparing seven South African *Patella* species, stated that high shore limpets will exhibit allometric shell growth, with shell height increasing more rapidly than shell length, as an attempt to reduce evaporative water loss. *Patella rustica* investigated in this study followed this rule; shells were found to grow allometrically with allometry constant of α=1.66. This coincides with findings for other high shore limpet species: *H. pectunculus* showed to be increasing in height faster than length with α from 1.3 and 1.6, depending on location (Gray & Hodgson 2003), while *S. granularis* showed a great deal of variability in the degree of allometry, with α varying from 0.77 to 1.31, depending also on substratum and location. Location can also be the reason why Cabral (2006) stated that *P. rustica* shells on Portuguese coast exhibit isometric growth, i.e. shells parameters were changing proportionally during growth (α=1), as oppose to allometry recorded in this study. He did, however, demonstrate that *P. rustica* shells in comparison to its other congeners had the highest shell conicity related to its high shore zonation.

Estimated VBG coefficients for *Patella rustica* were L_∞=40.86 mm for length, W_∞=33.02 mm for width and H_∞=14.07 mm for height, while corresponding values of growth constant (K) were 0.23, 0.24 and 0.21 $year^{-1}$, respectively. Using these parameters and equation provided by Taylor (1958), the life span of *P. rustica* was estimated to be 12.7 years. However, the maximum longevity was mathematically defined here based on asymptotic length (40.86 mm) and does not allow a lot of inference to the actual longevity of the species, considering that in the current study only 2 individuals were more than 6 years old (6.75 and 7.75 years), with the oldest individual attaining a length of 33.5 mm. On the Portuguese shore, Lima et al. (2006) cautiously estimated maximum longevity of *P. rustica* to be 4-6 years and Ribeiro (2008) to a maximum of 5 years, but both of those studies used estimations and not data obtained from the actual growth patterns. In addition, variability in growth and the estimated maximum size is depended greatly on environmental conditions or substratum morphology within a locality (Branch, 1974b, Lewis & Bowman 1975; Gray, 1996; Vat, 2000). Since data obtained in this

study are the first to describe growth patterns of *P. rustica*, intra-specific comparisons of VBG parameters depending on different locations were not possible.

There is however a great body of literature dealing with growth patterns of different patellid species on different latitudes. Growth parameter K is related to the metabolic rate of an organism (Sparre & Venema 1998) but comparisons based simply on this parameter are not considered reliable because of the correlation of K and a L_∞; a high value of K responds to a low value of L_∞ and vice versa (Sparre & Venema 1998; Clarke et al. 2004; De Graaf & Prein 2005). Hence, in order to make inter-specific comparison, overall growth performance was calculated for majority of published studies of patellid limpets where estimates of K and L_∞ were available. Phi-prime index or growth coefficient (Ø') is derived empirically from the relationship between K and L_∞ (Ø'= log K +2log L_∞, Pauly & Munro (1984)) and is considered to be species specific and to represent the physiological capacity of the organism with genetically predetermined factors (Vakily, 1992). Growth performance data calculated for different patellid limpets based on K and L_∞ obtained from available literature are shown in the Table 5.1.1. The growth coefficient calculated for *P. rustica* in this study (0.24 $year^{-1}$) falls into the middle values reported for limpets, ranging from 0.06 to 1.40 $year^{-1}$. Growth performance of *P. rustica* (Ø'=2.60) is however, among the lowest range reported, ranging from 1.98 to 3.65. Observed longevity for *P. rustica* was 7.75 years and the calculated one according to von Bertalanffy parameters 12.7 years. Longevity is generally negatively correlated to growth rate (Ebert, 1975; Branch, 1981; Gray, 1996) since rapid growth has been found to be directly associated with early maturation, higher mortality and a short life span (Branch, 1981; Creese, 1981; Gray, 1996). This negative correlation is the most visible from the examples of *C. testudinaria* Linnaeus, 1758 that, in the comparison with other limpet species, had the highest growth coefficient (1.40 $year^{-1}$) but the lowest expected life span (2.1 years), while *P. ferruginea* had the lowest growth coefficient (0.06 $year^{-1}$) and among the highest expected longevity (23.8 years). Furthermore, Guerra & Gaudêncio (1986), based on length–frequency analysis, suggested a possible longevity of 3-4 years for *P. ulyssiponensis*, *P. depressa* and *P. vulgata* on the Portuguese coast. These species reach a maximum size of about 4 cm, implying that growth rates must be relatively high. Conversely, at higher latitudes (UK and France) *P. vulgata* and *P. depressa* exhibit slower growth rates of 1-5 mm per year, with a maximum life span of 5-16 years (Fischer-Piètte, 1948; Bowman, 1981). Antarctic species *Nacella polaris* is showed to have a very slow growth rate (0.08 $year^{-1}$) with observed longevity of ~14 years and calculated longevity of 38 years (Brêthes et al. 1994). Vat (2000) found that *S. granularis* on

the south-east coast of South Africa increased by 5.5-12 mm, depending on substratum, and reach longevity of 15 years, while calculated life span varied between 9 and 12 years. Findings from Vat (2000) for *S. granularis* differ greatly to its counterparts on the west coast of South Africa, growing 18 mm in the first year and reaching maximum life span of 7 years as reported by Branch (1974b).

From these data it is clearly visible that growth rate and longevity depend greatly on habitat conditions and reflect the true impact of environmental influences on limpet populations. Because of this, mathematically defined maximum life span has to be taken with caution. Nevertheless, it is clear that at the macroecological scale a broad relationship between observed growth rate and latitude does exist. The underlying causes are still not described completely. Growth rates can be highly variable and affected by numerous factors, such as temperature, seasonal food supply, shore height and substratum morphology, exposure to wave action or density of conspecific and other co-occurring invertebrates (Lewis & Bowman 1975; Underwood, 1979; Branch, 1981; Creese, 1988; Gray, 1996; Espinosa et al. 2008). Alterations in food availability and seasonal fluctuations in temperature are both known to decrease limpets' growth (Branch, 1981; Bosman & Hockey 1988a, b; Lasiak, 1993; Richardson, 2001). Seasonal changes in growth rates are differing latitudinally, and are considered to be primarily influenced by temperature (Ekaratne & Crisp 1982; Richardson, 2001). The general consideration is that limpets in tropics grow more rapidly than temperate or polar limpets, i.e. species from higher latitudes vs. species from lower latitudes (Brey & Clarke 1993; Clarke et al. 2004; Khow, 2007). It is not yet clarified whether physiological constraints, a reduced or prolonged growing season or combination of both might be the cause of dissimilar growth rates at oppositely differing latitudes (Clarke et al. 2004). However, the most apparent environmental factors that are changing with latitude and might be considered to influence growth rate are temperature, insolation and photoperiod (Clarke et al. 2004).

It is noteworthy to mention that during the analysis of inner growth lines, it was determined that *P. rustica* shells had been heavily infested with boring algae, and were in different degrees of degradation. Shell infestation resulted in difficulties to define the exact position of the first growth line in the majority of examined limpets. All identified algae were filamentous cyanobacteria that colonize as epiliths or penetrate inside the limpet shell (and shells in general) as endoliths in between periostracum and prismatic layer. Occurrence of shell-penetrating marine cyanobacteria has been widely reported since the end of the 19th century (Pantazidou et al. 2006). In limpets, the high degrees of shell erosion have been

reported for *S. granularis* and *C. toreuma* (Richardson & Liu 1994; Vat, 2000). In most cases, this infestation does not affect the organism although the shell may become weakened (Richardson & Liu 1994) and therefore pose a treat for limpets' survival, especially in harsh environments like intertidal.

This study provides an insight into the age and growth of *P. rustica*. Although it was performed only on one location, the results are valuable since it is the first description of this important life history trait for limpets in the Adriatic in general. This research represents a good base for further investigations on the effects of various environmental factors on shell growth rates and microgrowth pattern formation. Information like this can facilitate understanding of how environmental factors, food supply or (non)congeneric interactions act in controlling and determining latitudinal trends in limpets' shell growth throughout the Adriatic and the Mediterranean Sea.

Table 5.1.1 Growth performance data calculated according to Pauly & Munro (1984) for different patellid limpets based on K and L_∞ obtained from available literature. Longevity was calculated according to Taylor (1958) where t_0 was available, or obtained from the literature. Data arranged by latitude to ease comparison (N=northern hemisphere, S=southern hemisphere). These studies used different techniques and sample numbers, therefore the data must be regarded as indicative rather than definitive.

species	location	latitude	L_∞ (mm)	K year^{-1} (mm)	Ø'	longevity (years), according to	reference
Acmea persona	Newport, USA	44° N	41.6	0.26	2.65	unknown	Kenny, 1969
Patella rustica	**Zaton Bay, SE Adriatic**	**42° N**	**40.9**	**0.24**	**2.60**	**7.75 observed; 12.7***	**present study**
Patella ferruginea	Ceuta, North Africa (inside port)	35° N	119.6	0.14	3.30	8.9*	Espinosa et al. 2008
Patella ferruginea	Ceuta, North Africa (outside port)	35° N	113.6	0.06	2.90	23.8*	Espinosa et al. 2008
Lottia gigantea	Orange County, California	33° N	67.7	0.18	2.92	8.5	Kiddo & Murray 2003
Fissurella barbadensis	Barbados	13° N	33.9	0.13	2.16	2 observed	Ward, 1967
Cellana testudinaria	Ohoiwait, Indonesia	5° S	33.1	1.4	3.19	2.1*	Khow, 2006
Fissurella crassa	Huayquique, North Chile	20° S	94.5	0.16	3.15	16.8*	Bretos, 1980
Helcion pruinosus	SE South Africa	32° S	28.8	0.67	2.75	2.5 length-frequency data	Henniger, 1998
Scutellastra granularis	SE South Africa (mussel beds)	33° S	27.1	0.25	2.27	>15 observed; 12*	Vat, 2000
Scutellastra granularis	SE South Africa (aeolianite)	33° S	31.9	0.32	2.51	>15 observed; 9.4*	Vat, 2000
Scutellastra granularis	SE South Africa (quartzite)	33° S	33.0	0.33	2.55	>15 observed; 9.1*	Vat, 2000
Helcion penctunculus	SW South Africa	33° S	30.9	0.61	2.76	4.9*	Gray & Hodgson 2003
Cymbula granatina	SW South Africa	34° S	94.0	0.51	3.65	unknown	Branch, 1974b
Cymbula oculus	SW South Africa	34° S	79.0	0.58	3.56	unknown	Branch, 1974b
Helcion pruinosus	SW South Africa	34° S	29.6	0.69	2.78	2.9 length-frequency data	Henniger, 1998
Scutellastra cochlear	SW South Africa	34° S	49.0	0.33	2.90	6-7 observed	Branch, 1974b
Scutellastra granularis	SW South Africa	34° S	40.0	0.56	2.95	6-7 observed	Branch, 1974b
Scutellastra longicosta	SW South Africa	34° S	68.0	0.3	3.14	6-7 observed	Branch, 1974b
Nacella polaris	Signy Island, Antarctic	61° S	34.4	0.32	2.58	>10 observed	Clarke et al. 2004
Nacella polaris	Esperanza Bay, Antarctic	63° S	71.4	0.08	2.63	38*	Brêthes et al. 1994
Nacella polaris	Rothera Point, Antarctic, 1997	67° S	34.6	0.08	1.98	>10 observed	Clarke et al. 2004
Nacella polaris	Rothera Point, Antarctic, 1998	67° S	61.4	0.11	2.62	>10 observed	Clarke et al. 2004
Nacella polaris	Rothera Point, Antarctic, 1999	67° S	49.7	0.07	2.24	>10 observed	Clarke et al. 2004

*calculated longevity according to Taylor (1958)

5.2 Reproduction of the limpet *Patella rustica*

Males and females were found to differ in size; female to male ratio for the group of limpets with a mean shell length of 24.2 mm was 4:1, while the same ratio for the smaller size class of limpets with a mean length of 16.2 mm was inverse, 1:4. Sex ratio and frequency distribution of male and female *P. rustica* with respect to shell length showed that males dominated the smaller size classes while females become more prevalent as size increased, from ~28 mm onwards. The preponderance of larger females suggests that *P. rustica* is a protandrous hermaphrodite. Although in some previous studies larger females were explained with their faster growth rates, as suggested by Moore (1937), this was not the case for *P. rustica*. Protandrous hermaphroditism of *P. rustica* is explained with age-length key and histological analysis that showed the existence of individuals, with a mean shell length of 25.2 mm that had both male and female characteristics. A number of limpet species are thought to be hermaphrodites, and majority are considered to be protandrous. The first one to link change of sex with a shift in sex ratios with size was Orton (1920, 1928) for the case of *P. vulgata*, and later more detailed work confirmed this hypothesis (Dodd, 1956; Orton et al. 1956; Blackmore, 1969; Thompson, 1980; Le Quesne & Hawkins 2006; McCarthy et al. 2008). For similar reasons, other species are considered protandric hermaphrodites, including *P. caerulea* (Bacci, 1947a,b; Pellegrini, 1948; Belkhodja et al. 2011), *P. ulyssiponensis* (Thompson, 1979; McCarthy et al. 2008), *H. pectunculus* (Gray, 1996; Gray & Hodgson 2003), *L. gigantea* (Kiddo & Murray 2003) and *P. ferruginea* (Rivera-Ingraham et al. 2011). Since there was a lack of smaller females in the total sample of *P. rustica*, it was only possible to estimate the length at which 50% of analyzed males become mature; the calculated shell length was 13.1 mm and according to the age analysis these males were younger than 2 years. Similar findings were demonstrated for other protandrous limpet species. *Patella vulgata* was found to be sexually maturing as two year old male while in the third year females become more prevalent in the population (Blackmore, 1969). Gray & Hodgson (2003) reported for *H. pectunculus* that males become sexually mature after one year, at 10-12 mm shell length, and in their second year, at about 16 mm shell length, they change sex. In Mediterranean limpet *P. ferruginea* the size at which change of sex occurred was correlated with the local density of large individuals: change can be delayed in populations where abundant large females are present (Rivera-Ingraham et al. 2011). What causes change of sex and what is the adaptive significance of protandry is still not completely explained. Generally, protandry is considered to be beneficial since larger females would be able to produce a greater number of energetically more

expensive eggs (Hoagland, 1978), especially to broadcast spawners and species relying on r-strategy, as *P. rustica*.

Reproduction cycle of *P. rustica* was described in previous studies, based on macroscopical examination of gonads according to the scale established by Orton et al. (1956) for *P. vulgata*. The present study described reproductive cycle of *P. rustica* using qualitative and quantitative histological analysis as the primary methods of staging gonad development. The results showed that *P. rustica* gonads have only one reproductive cycle per year with almost uniform developmental patterns for both males and females. Female early development started in February 2012 while males were in early developmental stage in June 2012. It is important to stress that males were not recorded from February 2012 to May 2012 and in July 2011, and this may explain this gap in between early development of females and males. Maturation for females started in September 2011 and for males one month later, i.e. from October 2011. The spawning peak in November 2011 was however synchronous for both sexes, since in December 2011 100% of female and male gonads were spent. Starting from January 2012 both sexes were reproductively inactive, until the early development restarted. There were a significant number of individuals whose gender could not be determined. They were recorded throughout the year, except in November 2011 when spawning occurred. However, January 2012 was the month with the most undetermined individuals (87%) and that trend, although descending, continued in latter months until June 2012. This was obviously connected with the beginning of post spawning and inactive (resting) period until the early development. The assumption is that among these undetermined individuals, great number was males in inactive stage that could not be determined with certainty. This would explain the lack of male individuals in the samples from January 2012 to June 2012 mentioned before. McCarthy et al. (2008) also reported the existence of undifferentiated *P. vulgata* and *P. ulyssiponensis*, with higher percentage being present in the post spawning period than when gonads were maturing.

Findings from this study confirmed general reproductive pattern of *P. rustica*, described previously on Algerian (Frenkiel, 1975), Basque (Othaiz, 1994) and Portuguese coast (Ribeiro et al. 2009), with few marked differences. This study suggests that *P. rustica* has longer breeding period, as was suggested also by Frenkiel (1975) and Othaiz (1994), as oppose to shorter one reported by Ribeiro et al. (2009). There was however, no evidence of multiple spawning events between August and November reported by Frenkiel (1975) and Othaiz (1994). The conditions controlling reproductive cycle in *Patella* spp. are not entirely known,

although, according to Lewis (1986), spawning involves an environmental trigger. In general, the seasonality of spawning varies between species and with temperature, causing the timing of spawning to vary depending on latitude (Guerra & Gaudêncio, 1986). *Patella depressa* and *P. ulyssiponensis* at the northern part of their range are shown to exhibit summer spawning periods (Dodd, 1956; Orton & Southward 1961; Thompson, 1979; Bowman, 1985; Bowman & Lewis 1986; Delaney et al. 2002; Moore et al. 2007; McCarthy et al. 2008), while Ribeiro et al. (2009) recorded their almost continuous breeding in the northern Portugal, with slight asynchrony in gonad development and spawning.

The most obvious change with latitude and timing of spawning is related with *P. vulgata*: spawning in September was reported for species in northern Scotland and NE England (Bowman & Lewis 1986), in October and November in Ireland (Thompson, 1980; McCarthy et al. 2008), in November in SW Britain and northern France (Orton et al. 1956; Bowman & Lewis 1986; Fisher-Piètte, 1948) and in November and December in northern Spain and Portugal (Ibañez et al. 1986; Othaitz, 1994; Guerra & Gaudêncio 1986; Ribeiro et al. 2009). *Patella caerulea* investigated in the gulf of Tunis and on the Algerian coast is reported to have a spawning peak in spring and summer (May/July) with partial spawning occurring again in December (Frenkiel, 1975; Belkhodja et al. 2011). Temperature has often been linked to the reproductive cycles of *Patella* spp. (Orton et al. 1956; Fretter & Graham 1976), as well as strong wave action stimulated with high wind speed (Orton et al. 1956; Orton & Sothward 1961; Branch, 1974a). Breeding and spawning can be correlated with food abundance: breeding when there is enough food supply is correlated with necessary higher energy input while spawning just prior to the phytoplankton bloom ensures food for planktotrophic larvae (Branch, 1974a; Branch, 1981). Sea roughness as a trigger for spawning can be extremely important for high shore species (Rivera-Ingraham, 2011), like *P. rustica*. However, specific site variations are likely to contribute to the rate of gonad development and the timing of spawning (McCarthy et al. 2008). There was statistically significant positive correlation between mean gonad index of *P. rustica* females and recorded air temperatures, and also a significant negative correlation of mean gonad index of males and measured chl *a* concentrations. Breeding cycle of *P. rustica* started in February 2012, when minimum air (7.5°C) and sea temperatures (12.5°C) were recorded. Maturation started in September 2011, responding with maximum sea temperature (25.8°C) and extended to November 2011 when spawning occurred. The lowest concentration of chl *a* was recorded in September 2011 (0.03 μg/L) and the highest one in April 2012 (1.68 μg/L). In April 2012 individuals were recorded to be in early development while in September

2011 both males and females were with late developed gonads. The reproductive patterns obtained in the present study can generally fit the latitudinal trend previously described for *Patella* spp., comprising in longer breeding period and spawning occurring later in the year, possibly triggered with low temperatures and/or sea roughness. The sampling scheme conducted in this study does not allow any further conclusions on a possible role of these factors in the observed spawning patterns and a more detailed and specific analysis of these aspects is required to determine how each of them might be contributing to *P. rustica* reproduction cycle.

Taking into consideration that qualitative histological methods of determining the reproductive stage may be to some extent subjective (Gosling, 2003), a quantitative method of measuring the size of oocytes was also employed. Oocytes size was expressed as both perimeter and diameter, since measuring only diameter may be unreliable due to occasional imperfect circular oocytes in gonad samples (Popović, 2012). Size of the oocytes was monitored from the early development to the maturation and is therefore representing an excellent display of reproductive cycle. In this study, quantitative analysis follows the pattern observed using qualitative histological methods. The distribution of oocytes, determined by oocytes size frequencies, showed bimodal pattern in which the first peak corresponded to a large number of immature oocytes, while the second peak was associated with greater number of vitellogenic oocytes. The frequency distribution of oocytes size had a relatively uniform pattern until September 2011, after which oocytes started to increase in size until November 2011, indicating a continuous process of maturation and finally release of gametes in November 2011. Parts of the oocytes undergo lysis and different stages of atresia during the whole period of development, and particularly at the beginning of spawning season, in September 2011 and October 2011. The atresic oocytes had an irregular shape, a hypertrophied chorion and were not included in oocytes size frequencies. Oocytary atresia is not unusual and has been reported before in different limpet species (see Morriconi, 1999; Belkhodja et al. 2011). The smallest number of oocytes was recorded in December 2011, when 100% of individuals had already gone through spawning process, while the highest number was recorded in August 2011 when 88% of individuals were in early development. Accordingly, the largest oocytes were measured in individuals in ripe stage (mean value of 116 μm and 329 μm for diameter and perimeter, respectively), when oocytes already went through vitellogenesis and accumulated glycogen supplies, lipid droplets and a fibrous jelly coat was formed between the vitelline envelope and the overlying follicle cells (Hodgson & Eckelbarger 2000). Similar

bimodal pattern of oocytes distribution was recorded for other limpet species, such as *N. deaurata* from Beagle Channel, whose mature oocytes had diameter from 120 to 150 μm (Morriconi, 1999), *S. granularis* from SE South Africa coast with diameter of mature oocytes ranging from 125 to 270 μm (Vat, 2000) or *H. pectunculus* from both the SW and SE coasts of South Africa, with recorded diameter of mature oocytes >120 μm (Gray & Hodgson 2003). Many *Patella* species are known to have high fecundity with the diameter of the oocytes not exceeding 180 μm, suggesting these species produce planktotrophic larvae (Branch, 1974). Therefore, spawning in such species is considered to be correlated and/or triggered with the peak of primary production (Brêthes et al. 1994; Morriconi, 1999), although some studies suggested that veligers are non-feeding, consequently the spawning and the phytoplankton bloom are not causally correlated (Hadfield et al. 1997). Spawning of *P. rustica* occurred during November 2011, and consequently in December 2011 planktotrophic larvae are supposed to be in the water column. Although there was no obvious correlation between spawning and recorded chl *a* concentration, in comparison with months throughout the sampling year (from 0.03 to 1.68 μg/L), December 2011 still had chl *a* concentration above the mean (0.29 μg/L).

In addition to mean gonad index as a direct measure of gonad activity (highest mean gonad index=maximum gonad activity), condition index was calculated as a concomitant parameter. Since the condition index depends primarily on reproductive activity and food availability, it is considered to be a good descriptor of the reproductive cycle (Ojea et al. 2004; Peharda et al. 2006). Although there was a slight following of condition index and gonad activity, there was no significant correlation between these two parameters. The highest calculated value in May 2012 (179.6) could be connected with the highest value of chl *a* concentration recorded a month earlier, since hepatopancreas and the gut occupies most of the visceral mass. In conclusion, although this parameter is shown to give accurate information on reproductive cycles, mainly of bivalve species, this does not seem to be the case for *P. rustica.*

This study provides first histological description of each developmental stage and first description of oocytes size and distribution in *P.* rustica, serving as a good baseline for further investigation. Furthermore, since *P. rustica* has a wider geographic distribution, from the Mediterranean to the Atlantic coasts of the Iberian Peninsula and northern Africa, it can serve as a good model species to study possible intraspecific biogeographic differences in reproductive strategy or growth performances.

5.3 Heat stress physiology of the Mediterranean patellid limpets

There were clear differences in ABT, Q10 relationships, *hsp* expression and lysosomal stability in the Mediterranean limpets, which were associated with their vertical distribution. The higher shore *Patella rustica* was able to tolerate higher temperatures than the other two, lower shore species, with Arrhenius breakpoint temperature being ~2 or 5°C higher than those of *P. caerulea* or *P. ulyssiponensis,* respectively. This suggests that *P. rustica* individuals should be able to maintain cardiac activity and normal oxygen supply at very high ambient temperatures, even above 37°C. Since rock temperatures on the Mediterranean shores are often above 37°C during midday at low tide in the summer (Sarà et al. 2013b), the proximity of maximal habitat temperature and upper thermal tolerance limits of *P. rustica* suggests this species is actually living close to its thermal limits and would face increased risk from further warming of environmental temperatures (Artale et al. 2010; Wethey et al. 2011).

Patella caerulea had faster heart rates as compared to *P. rustica* or *P. ulyssiponensis*. Heart rate is assumed to be a reliable indicator of metabolic rate in limpets (as indicated by Marshall & McQuaid 1992; Santini et al. 1999; Chelazzi et al. 2001), suggesting that *P. caerulea* has a higher metabolism than the other two species. Previous studies have shown that, in general, lower shore limpets have higher metabolic rates (Chelazzi et al. 2001, Dong & Williams 2011) than their higher shore counterparts. A lower metabolic rate in high shore animals has been interpreted as a mechanism to cope with more variable environmental conditions (Branch, 1981; De Pirro et al. 1999; Chelazzi et al. 2001; Marshall et al. 2011). In contrast, the lower zoned *P. ulyssiponensis*, which is usually confined to the sublittoral fringe (Sella et al. 1993; Boaventura et al. 2002), appears to be an exception to this generality, as this species has a very low metabolism (low heart rates) and the lowest Q10 relationship, but appears very sensitive to any variation in temperature; for example *P. ulyssiponensis* showed clear signs of bradycardia during exposure to elevated temperatures. This partially supports the findings of De Pirro et al. (1999) who analysed the effect of salinity variation on cardiac responses of *P. caerulea* and *P. ulyssiponensis.* De Pirro and co-workers (1999) concluded that when exposed to high and low salinity, the main response of *P. caerulea* was an initial increase in heart rate followed by decrease in cardiac activity. In contrast, *P. ulyssiponensis* exhibited bradycardia as a consequence of short term exposure to changes in salinity. Such findings support the idea that bradycardic patterns in response to stress factors might be adaptively used by limpets as a form of physiological isolation from stressful external conditions to reduce

blood flow through the gills or pedal sinus as proposed by Chelazzi et al. (1999) and De Pirro et al. (2001).

Previous studies on Mediterranean limpets demonstrated that the functional traits of the three studied species, e.g. foraging activities (Della Santina & Chelazzi 1991; Della Santina et al. 1993), physiological adaptations, such as energetic resources (Santini & Chelazzi 1995), respiration rates (Bannister & McQuaid 1974) and cardiac responses to different salinities or to copper pollution (De Pirro et al. 1999, 2001) are substantially different and related to their position on the shore, even though they can be found living within a few centimetres of each other. Santini & Chelazzi (1995) suggested that *P. rustica* has a more efficient mechanism of energy allocation during unfavourable conditions, resulting in a lower metabolism than *P. caerulea*. Such an 'energy conservation' strategy has been suggested to be important for high shore species such as *P. rustica* (Sokolova & Pörtner 2003), which are resource limited due to prolonged emersion periods. By contrast, lower shore species, such as *P. caerulea*, are proposed to adopt a more 'exploitative strategy', exploiting resources and increasing metabolic rates with increasing temperatures (Sokolova & Pörtner 2003).

Evolutionary adaptation to specific thermal niches has also resulted in species' specific capacities for passive heat resistance and consequently, different threshold temperatures for the induction of a heat shock proteins (see Hochachka & Somero 2002). More cold–adapted and/or lower shore species generally show upregulation of *hsp*s at lower temperatures than warm–adapted and/or high shore counterparts, as shown in *Tegula* snails (Tomanek & Somero 1999). In the high shore littorinid, *Echinolittorina malaccana* Philippi, 1847, Marshall et al. (2011) demonstrated that these gastropods exploit a strategy of metabolic depression when heated, with the onset of the heat shock response only occurring close to their breakpoint temperatures, after which aerobic scope becomes constrained and performance declines. These findings support results from this study, underlining the fact that upregulation of *hsp*s is closely related with the thermal tolerance limits of *P. caerulea* and *P. rustica.* Upregulation of *hsp70* occurs in *P. rustica* at 34°C, but increased at 38°C and kept increasing after prolonged exposure to the same temperature. Upregulation of the heat shock response in *P. caerulea* reaches a maximum level at 36°C. Hence, the onset of the heat shock response in both species is closely related with their thermal tolerance limits as represented by Arrhenius breakpoint temperature. The Arrhenius breakpoint temperature may in fact be correlative to the critical temperature, after which metabolism changes from being aerobic to anaerobic (Pörtner, 2012).

Lysosomal stability of the haemocytes was evaluated with the neutral red uptake assay. This test has been used as a measure of stress in various organisms under different environmental conditions or as a reliable indicator of toxic injury (Hauton et al. 1998; Camus et al. 2000; Brown et al. 2004; Zhang & Li 2006; Canty et al. 2009; Coughlan et al. 2009; Gopalakrishnan et al. 2009; Russo et al. 2009; Deschaseaux et al. 2011; Munari et al. 2011; Molnar & Fong 2012). The principle of the assay consists in the fact that neutral red, being positively charged dye, are able to absorb and bind only live cells while this ability declines in damaged or dead cells (Repetto et al. 2008; Russo et al. 2009). Measured absorption values are thus directly proportional to the amount of live cells (Russo et al. 2009). Results obtained demonstrated that temperature significantly influenced stability of haemocytes in both *P. rustica* and *P. caerulea.* In *P. rustica* the highest absorption value was measured at 32°C and in *P. caerulea* at 38°C. The first parameter to be disrupted with environmental changes is membrane stability of the cells (Brown et al. 2004). Since haemocytes mediate a series of immune reactions (Mello et al. 2012), the assumption is that at this temperature threshold (32°C for *P. rustica* and 38°C for *P. caerulea*) the haemocyte lysosomes will become more active (therefore accumulating more neutral red) in order to act as a 'distress signal'. These effects lead to changes in physiological processes (Brown et al. 2004) and therefore upregulation of *hsp70* is closely related with this threshold temperature: at 34°C for *P. rustica* and at 36°C for *P. caerulea*. However, production of *hsp*s continued in *P. rustica* with prolonged heat stress, while it declined in *P. caerulea*, making *P. rustica* adaptable to its microhabitat where severe thermal stress is occurring more often than at low shores. As a consequence of this adaptability, *P. rustica* under stressed conditions slows the metabolism, resulting in higher thermal tolerance limit (ABT=37.9°C) and longer survival. Nonetheless, production of *hsp*s holds great metabolic costs to an organism as well as suggesting that it has sustained some measure of cellular damage (Hofmann & Somero 1996; Feder & Hofmann 1999; Tomanek, 2002, Miller et al. 2009). This would explain inability of these species to recover after they have been returned to ambient temperature. Consequently, metabolic effects are apparent at the whole organism level (Brown et al. 2004) in the form of changes in the cardiac activity and related oxygen consumption. When temperature exceeds the pejus temperature (pejus meaning 'turning worse') the ability of animals to increase aerobic metabolism is reduced due to limited capacity to fulfill the oxygen demands of an organism (Pörtner, 2002; Pörtner et al. 2006; Sokolova et al. 2012).

The present study tested the response of the three Mediterranean intertidal limpets under abrupt experimental thermal exposure. Such a rapid increase of temperature has occurred frequently in the last decade in the Southern Mediterranean during heat waves (Cerrano & Bavestrello 2009). These events do not allow individuals the necessary time to acclimate their physiological and sub-cellular responses, and under such conditions, individuals may easily exceed their energetic limits (Abele, 2012). Even over the small tidal gradient, Mediterranean limpets still show thermal adaptations to specific, but perhaps more narrow, thermal windows which results in a very small optimal range of environmental conditions for these species (see Tewksbury et al. 2008). Maintenance of a narrow thermal window will minimize energetic costs which would be associated with species exhibiting thermal plasticity and having more wide thermal windows (Somero, 2002). The physiological mechanisms that allow species to extend their thermal windows are energetically demanding, and this cost is usually met at the expense of other critical functions such as growth and reproduction (Hofmann & Todghman 2010; Sarà et al. 2013a, b, c). There are, however, costs associated with having more narrow thermal windows, as suggested for tropical species where thermal stress is consistently high (Teweksbury et al. 2008). This lack of plasticity or capacity to tolerate wide thermal ranges could make Mediterranean intertidal organisms even more sensitive to increasing temperatures than corresponding species living along oceanic coasts worldwide where tidal amplitude is greater (e.g. Petes et al. 2007).

Failure of heart function in the Mediterranean limpets, for example, is very close to their upper thermal limits, and may become a weak link in denoting species' thermal tolerance, and consequently, could be a key determinant of their geographic range. Mediterranean limpets, therefore, already exist on the edges of their thermal tolerance windows, and any change in temperature, regardless of how small, is likely to have detrimental consequences to these species, with consequent impacts on community structure and functioning. Knowing how they are adapted to their present day microhabitats, give us a valuable insight in possible responses or adaptation ability to stress that each of these species will endure in the light of climate change scenarios.

Species scientific names used throughout this thesis are written according to World Register of Marine Species (http://www.marinespecies.org/).

6. CONCLUSIONS

This thesis provides an insight into the age, growth and reproduction of *Patella rustica* on south eastern Adriatic coast. Although this research was performed only on one location, the results are valuable since it is the first description of these important life history traits for *Patella* limpets in the eastern Adriatic in general. The thesis provides first histological description of each developmental stage and first description of oocytes size and distribution in *P.* rustica. Performed experimental research confirmed the hypothesis that the three congeneric Mediterranean limpet species *P. rustica*, *P. caerulea* and *P. ulyssiponensis* have different physiological response to thermal stress related to their vertical zonation on the shore.

The conducted research resulted in following conclusions:

- From the acetate peel replicas of *P. rustica* shell sections it is possible to read inner growth lines and determine age of specimens.
- The results from this study showed that one dark growth line is formed annually in *P. rustica*, and was deposited in May.
- Based on the reproduction data it was concluded that a first growth line actually represents nine months instead of one full year and this was taken into account when estimating the age and growth of *P. rustica*.
- More than 90% of collected individuals were less than 4 years old, while the mean shell length was 20.2 mm.
- Estimated VBG coefficients were L_{∞}=40.86 mm for length, W_{∞}= 33.02 mm for width and H_{∞}=14.07 mm for height, while corresponding values of growth constant (K) were 0.23, 0.24 and 0.21 $year^{-1}$.
- Growth performance of *P. rustica* (Ø'=2.60) is in the lowest range reported for other limpet species, indicating that *P. rustica* is growing relatively slow.
- The maximum longevity of *P. rustica* was mathematically defined to 12.7 years based on asymptotic length (40.86 mm), although only 2 analyzed individuals were more than 6 years old (6.75 and 7.75 years) with the oldest individual attaining a length of 33.5 mm.

- Shells were found to grow allometrically (α=1.66), confirming the rule that high shore species increase more rapidly in height than in length as an attempt to reduce evaporative water loss.

- Further investigation on the effects of various environmental factors is needed to conclude about growth dynamics of *P. rustica*.

- *Patella rustica* shells were in different degree of degradation as a result of boring activity of filamentous cyanobacteria. Shell infestation resulted in difficulties to define the exact position of the first growth line in the majority of examined limpets.

- Males and females were mainly found to differ in size, with females becoming more prevalent from ~28 mm onwards, suggesting that *P. rustica* is a protandrous hermaphrodite. Three hermaphrodites with a mean shell length of 25.19 mm were determined based on histological analysis.

- Estimated length at which 50% of analyzed males become mature was 13.1 mm and according to the age analysis these males were younger than 2 years. The same estimation was not possible for females due to their small number in the sample.

- *Patella rustica* has only one reproductive cycle per year with almost uniform developmental patterns for both males and females.

- It was determined that *P. rustica* has longer breeding period; from February to September for females and from June to October for males.

- The spawning was synchronous for both sexes and had only one peak in November. A month later, in December, all individuals had spent gonads.

- A significant number of individuals whose gender could not be determined were recorded throughout the year, with the biggest number reported in January, corresponding to the post spawning and inactive period. The assumption is that among these undetermined individuals, great number was males in inactive stage that could not be determined with certainty, explaining the lack of males in the samples from January to June.

- The reproductive patterns obtained in the present study generally fit the latitudinal trend previously described for *Patella* spp., however, a more detailed and specific analysis of environmental aspects is required to determine how each of them might be contributing to *P. rustica* reproduction cycle.

- This study presents first data on oocytes size of *P. rustica*, expressed as oocytes diameter and perimeter. The largest oocytes were measured in individuals in ripe stage when oocytes already went through vitellogenesis, while the smallest oocytes were recorded in individuals that were in early developmental stage.

- It was determined that *P. rustica* ocytes undergo lysis and different stages of atresia during the whole period of development, and particularly at the beginning of spawning season.

- Although condition index has been suggested to be an accurate parameter of reproductive cycle of many bivalve species, it was not the base for *P. rustica.* It is more likely that in this species condition index depends greatly on current food consumption, since visceral mass constitutes more than half of limpet's body.

- Experimental investigation demonstrated clear differences in ABT, *hsp* expression and lysosomal stability in *P. rustica*, *P. caerulea* and *P. ulyssiponensis* associated with their vertical distribution on the shore.

- Arrhenius breakpoint temperature for *P. rustica* was 37.9°C, for *P. caerulea* 35.9°C and for *P. ulyssiponensis* 32.2°C, indicating that the higher shore *P. rustica* was able to tolerate higher temperatures than the other two, lower shore counterparts.

- *Patella caerulea* had faster heart rates as compared to *P. rustica* or *P. ulyssiponensis,* suggesting that this species has a higher metabolism than the other two species.

- The onset of the heat shock response in *P. rustica* and *P. caerulea* is closely related with their thermal tolerance limits. Production of *hsp*s continued in *P. rustica* with prolonged heat stress, while it declined in *P. caerulea*, indicating adaptability of *P. rustica* to its high shore microhabitat.

- Temperature significantly influenced stability of haemocytes in both *P. rustica* and *P. caerulea,* leading to changes in physiological processes, such as upregulation of *hsp*,

after which metabolic effects are apparent at the whole organism level in the form of changes in cardiac activity.

7. LITERATURE

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8. PROŠIRENI SAŽETAK

UVOD

Najvažnija i najraznovrsnija skupina mekušaca su puževi s više od 62 000 opisanih živućih vrsta (Lindberg & Ponder 2001). Priljepci nesumnjivo pripadaju najprepoznatljivijim i najvažnijim puževima. Herbivori su i hrane se neselektivnim struganjem biofilma, uklanjajući tako rasplodne stadije makroalgi i ličinačke stadije beskralješnjaka te na taj način značajno sudjeluju u strukturiranju mediolitoralnih zajednica (Southward, 1964; Powel, 1973; Branch, 1981; Hawkins & Hartnoll 1983; Jernakoff, 1985; Hawkins i sur. 1992; Underwood, 2000; Paine, 2002; Jenkins i sur. 2005; Coleman i sur. 2006). Zajedno s organizmima filtratorima, priljepci sudjeluju u izmjeni organske tvari u mediolitoralu i sveukupnoj ekološkoj ravnoteži. Protok energije kroz jedinke, populacije i zajednice utječe na samu strukturu i funkcioniranje ekosustava te se stoga priljepci smatraju ključnim vrstama (engl. keystone species) mediolitorala (Power i sur. 1996).

Patella rustica Linnaeus 1758, *P. caerulea* Linnaeus 1758 i *P. ulyssiponensis* Gmelin 1791 su simpatrijske vrste priljepaka na stjenovitim sredozemnim obalama, različito raspoređene u mediolitoralnom pojasu. Vrsta *P. rustica* dominantna je u gornjem mediolitoralu, vrsta *P. ulyssiponensis* u donjem, a vrsta *P. caerulea* prisutna je u srednjem mediolitoralu, između biocenoza ove dvije prethodne vrste (Mauro i sur. 2003; Šimunović, 1995). Dok je vrsta *P. caerulea* endem Sredozemlja, ostale dvije su rasprostranjene duž sredozemne i atlantske obale. Vrstu *P. rustica* ili luzitanski priljepak vrlo je lako razlikovati po stožastoj ljušturi i smeđim točkama na njoj, ali vrste *P. caerulea* i *P. ulyssiponenses* su morfološki promjenjive ovisno o okolini te na nekim lokalitetima može doći do preklapanja u obojenosti ljušture i samom obliku iste (Cretella i sur. 1990; Sella i sur. 1993).

Različiti aspekti biologije sredozemnih priljepaka roda *Patella* istraživani su u posljednjih nekoliko desetljeća, ali još uvijek se relativno malo zna o njihovim temeljnim populacijskim procesima kao što su rast, starost i razmnožavanje. Poznavanje parametara rasta i razmnožavanja neophodno je za modeliranje populacijske dinamike, koja je u konačnici ključno kao potpora iskorištavanju i zaštiti određene vrste (Laudien i sur. 2003; Katsanevakis, 2007). Priljepci su poikilotermni organizmi i temperatura se smatra najvažnijim čimbenikom koji određuje njihovo preživljavanje i rasprostranjenost (Southward i sur. 1995; Denny & Wethey 2000; Helmuth & Hofmann 2001; Lima & Wethey 2009; Miller i sur. 2009; Somero, 2010). Sredozemno more karakterizirano je malim amplitudama morskih mijena, stoga

mediolitoralna stepenica obuhvaća pojas od svega šezdesetak centimetara. Unatoč razmjerno uskom pojasu, organizmi na ovom području žive na rubu kopnenog i morskog okoliša, okruženi promjenjivim i često nepovoljnim fizičkim uvjetima što podrazumijeva često i naglo mijenjanje ekoloških čimbenika kao što su temperatura, vlažnost, slanost i valovi. Jedina strategija preživljavanja u tim ekstremnim uvjetima je sposobnost jedinke da se prilagodi nastalim promjenama. Organizmi mediolitorala razvili su stoga morfološke (vanjska tjelesna građa), fiziološke (unutarnje funkcioniranje organa i stanica) te bihevioristične (kretanje) prilagodbe u svrhu ublažavanja stresa s kojim se svakodnevno susreću (Ngan, 2006; Harley i sur. 2009). Te iste prilagodbe zahtijevaju i određena energetska ulaganja što posljedično dovodi do stvaranja kompromisa (engl. trade-off). Prilagodbe vrsta roda *Patella* na temperaturne promjene nedovoljno su istražene. Želimo li razumjeti kako pojedine vrste odgovaraju na promjenjive okolišne uvjete, potreban je integrirani pristup koji će povezati višestruke stresore te njihov utjecaj na stanične i fiziološke odgovore (Maltby, 1999). Stanična i fiziološka prilagodba organizama postaje predmet sve većeg zanimanja znanstvenika, posebice promatrajući iste u svjetlu klimatskih promjena. Ravnoteža između unosa i potrošnje energije te troškova preživljavanja termalnog stresa ima sinergističku ulogu u određivanju vertikalne zonacije, tjelesne veličine kao i načina reprodukcije kod svih beskralješnjaka u području plime i oseke (Branch, 1981). Recentne klimatske promjene, posebice porast temperature i povećana insolacija, mogu značajno utjecati na navedenu ravnotežu i dovesti u pitanje opstanak pojedinih vrsta priljepaka.

Ova disertacija, iako nije strogo podijeljena, sastoji se od tri dijela. Prvi dio istražuje starost i parametre rasta vrste *P. rustica*. Ljušture su se uklapale u smolu, rezale po najdužoj osi rasta te su se iz izrađenih acetatnih preslika očitavale linije rasta (Richardson, 2001). Drugi dio disertacije istražuje reproduktivnu biologiju vrste *P. rustica*. Kvalitativne i kvantitativne histološke metode su korištene kako bi se pratilo sazrijevanja gonada tijekom reproduktivnog ciklusa. Treći dio disertacije odnosi se na laboratorijske pokuse kojima su se ispitivali fiziološki odgovori priljepaka vrsta *P. rustica, P. caerulea* i *P. ulyssiponensis* na toplinski stres. Pokusima se mjerila Arrheniusova prijelomna temperatura (ABT), koja ujedno predstavlja i metaboličko funkcioniranje organizma (vidi Stillman & Somero 1996), proizvodnja proteina toplinskog šoka (*hsp*) te lizosomalna stabilnost hemocita.

Svrha i ciljevi istraživanja. Unatoč ekološkoj važnosti, priljepci roda *Patella* do danas nisu bili objekt detaljnog istraživanja populacijskih procesa na hrvatskom dijelu Jadranskog mora. Stoga je ovo istraživanje provedeno kako bi se stekao uvid u dinamiku rasta, sastav

populacije i razvojni ciklus vrste *P. rustica* na južnom dijelu istočnog Jadranskog mora. *Patella rustica* nastanjuje pojas gornje granice plime i oseke, gdje su zalihe hrane ograničene, a dehidracija predstavlja gotovo svakodnevni stres. Kako i kada ova vrsta usmjerava svoju energiju na rast i razmnožavanje neka su od pitanja na koja se ovim istraživanjem želi odgovoriti. Osim toga, tri vrste sredozemnih priljepaka, *P. rustica*, *P. caerulea* i *P. ulyssiponensis*, predstavljaju izvrstan model za testiranje odnosa vertikalne zonacije i fiziološke prilagodbe na toplinski stres. Vrste koje naseljavaju različite visine na obali s obzirom na plimu i oseku, prilagođene su točno određenom mikrostaništu unutar kojeg razina i trajanje toplinskog stresa varira (Stillman & Somero 1996; Tomanek & Somero 1999). Povećanje temperature, podizanje razine mora i zakiseljavanje mora samo su neke od posljedica klimatskih promjena koje utječu na sve morske organizme. Kao njihova dugoročna posljedica predviđa se da će se raspodjela i broj vrsta promijeniti s obzirom na njihove sposobnosti prilagodbe novonastalim uvjetima. Naravno, naglasak je na brzoj prilagodbi organizama jer su promjene u okolišu brze i iznenadne te zahtijevaju neposredni odgovor. Neophodno je poznavati fiziološke odgovore organizma kako bi se ustanovilo kako su ove vrste prilagođene trenutnim okolišnim uvjetima te kolika je njihova sposobnost daljnih prilagodbi (Hochachka & Somero 2002). Uzimajući u obzir ulogu priljepka kao ključne mediolitoralne vrste, kao i interakcije na razini zajednice, utjecaj na individualne karakteristike imat će kaskadni efekt na dinamiku populacije priljepaka. Stoga će ovo istraživanje pridonijeti boljem razumijevanju posljedica narušavanja bioraznolikosti izazvanog klimatskim promjenama. Novija istraživanja (Lima i sur. 2006; Sousa i sur. 2012) pokazala su širenje rasprostranjenosti vrste *P. rustica* u sjevernom Atlantiku na kojem dosad nije bila prisutna. Opisom parametara rasta i razmnožavanja u istočnom Jadranu te fiziološkog odgovora iste na toplinski stres, ova disertacija može olakšati razumijevanje čimbenika koji utječu na širenje areala ove vrste te usporedbu s ostalim vrstama roda *Patella* u Sredozemlju.

Ciljevi ovog doktorskog istraživanja su:

- vrjednovanje formiranja linije rasta u ljušturama priljepka *P. rustica*
- odrediti starost i obrasce rasta priljepka *P. rustica* na jugoistočnoj obali Jadranskog mora
- opisati reproduktivni ciklus priljepka *P. rustica* na jugoistočnoj obali Jadranskog mora, koristeći se kvalitativnim i kvantitativnim histološkim metodama

- utvrditi pri kojoj dužini vrsta *P. rustica* postaje spolno zrela
- utvrditi omjere spolova u populaciji priljepka *P. rustica*
- utvrditi pri kojoj dužini se javlja promjena spola priljepka *P. rustica*
- laboratorijskim pokusima opisati fiziološke odgovore priljepaka *P. rustica, P. caerulea* i *P. ulyssiponensis* na toplinski stres
- mjerenjem otkucaja srca odrediti Arrheniusovu prijelomnu temperaturu vrsta *P. rustica, P. caerulea* i *P. ulyssiponensis*
- utvrditi da li se ekspresija proteina toplinskog šoka (*hsp70*) razlikuje između vrsta *P. rustica* i *P. caerulea*
- mjerenjem lizosomalne stabilnosti hemocita odrediti razinu staničnog odgovora na toplinski stres vrsta *P. rustica* i *P. caerulea*
- zaključiti da li se fiziološki odgovori priljepaka *P. rustica, P. caerulea* i *P. ulyssiponensis* na toplinski stres razlikuju s obzirom na njihovu različitu vertikalnu zonaciju na obali.

MATERIJALI I METODE

Područje i dinamika uzorkovanja. Za istraživanje rasta i razmnožavanja, uzorci priljepka *Patella rustica* prikupljani su od srpnja 2011. do lipnja 2012. godine u Zatonu na JI Jadranu. Zaton je zatvoreni zaljev stjenovitih obala, 8 km sjeverozapadno od Dubrovnika. Pokusi istraživanja fizioloških odgovora na toplinski stres izvodili su se u Laboratoriju eksperimentalne ekologije Sveučilišta u Palermu. Priljepci *P. rustica, P. caerulea* i *P. ulyssiponensis* uzorkovani su tijekom prosinca 2012. godine u zaljevima Addaura i Altavila u Tirenskom moru. Addaura i Altavilla su stjenovite obale sjeveroistočno i jugoistočno od Palerma na Siciliji.

Osnovni hidrografski parametri. Prosječne dnevne temperature površine mora i temperature zraka (°C) za dubrovačko područje, a za razdoblje od srpnja 2011. do lipnja 2012. godine dobiveni su od Hrvatskog hidrometeorološkog zavoda (http://meteo.hr). Salinitet i otopljeni kisik (mg/L) mjereni su od srpnja 2011. do lipnja 2012. godine uz pomoć YSI hidrografske sonde.

Koncentracija klorofila *a*. Mjesečni uzorci morske vode za određivanje fitoplanktonske organske tvari putem analize koncentracije klorofila *a* prikupljeni su u Zatonu od srpnja 2011. do lipnja 2012. godine. Neposredno nakon prikupljanja, uzorci morske vode volumena 500 mL su profiltrirani kroz staklene membranske filtre (Whatman GF/F) te pohranjeni u zamrzivač na −20°C do daljnje laboratorijske obrade. Koncentracija klorofila *a* je određivana fluorometrijskom metodom (Jeffrey & Welschmeyer 1997).

Analiza starosti i rasta. Prikupljenim jedinkama meso je pažljivo očišćeno od ljušture, a dužina (L), širina (W) i visina (H) svake ljušture izmjerena pomičom mjerkom preciznosti 0,1 mm. Prije određivanja starosti, potrebno je napraviti vrjednovanje linije rasta kako bi se ustanovilo koliko linija nastaje tijekom jedne godine. U ovom istraživanju je primijenjena metoda analize rubnog prirasta koja uključuje analizu manjih jedinki s višom stopom rasta tijekom razdoblja od godine dana. Svaki mjesec, 5 manjih jedinki (14,4±1,6 mm, srednja dužina±standardna devijacija) uklopljeno je u smolu. Pomoću STRUERS pile uklopljene ljušture su poprečno prerezane po najdužoj osi rasta. Presjeci ljuštura izbrušeni su brusnim papirom, ispolirani te ostavljeni 1 minutu u 0,1 M HCl. Iz tako pripremljenih presjeka izrađene su acetatne preslike prema Richardson (2001), a udaljenost zadnjeg prstena rasta od ruba ljušture mjerena je pomoću softvera Axio Vision Rel 4.8. Nakon vrjednovanja linije rasta, starost na 120 jedinki utvrđena je brojanjem unutrašnjih linija rasta. Acetatne preslike pripremljene su na prethodno opisani način. Iz dobivenih podataka starosti pri određenoj duljini izrađena je von Bertalanffy krivulja rasta (Sparre & Venema 1998). Maksimalni životni vijek izračunat je prema Taylor (1958).

Endobionti priljepka *Patella rustica*. Za određivanje skupine organizama u koje spadaju endobionti iz ljušture vrste *P. rustica*, ljušture su dekalcificirane u 8%-tnoj otopini kloridne kiseline (HCl). Dekalcifikacija je izvršena dva puta po 3 sata. Nakon dekalcifikacije ljušture, mekani organski dijelovi stavljeni su na predmetno stakalce i analizirani Zeiss AxioVision mikroskopom uz ukupno povećanje od 100×, 200× i 400×. Endobionti su slikani kamerom AxioCamera MRc5, a određeni prema Golubić i sur. (2005), Royer i sur. (2006) i Riascos i sur. (2008).

Histološka analiza gonada. Trideset jedinki približno istih dužina (24,2±2,9 mm) uzorkovano je od srpnja 2011. do lipnja 2012. godine, osim u prosincu kada je zbog nepovoljnih vremenskih uvjeta uzorkovano samo 25 jedinki. Dodatno su se uzorkovali priljepci različitih veličinskih kategorija. Ukupno 30 jedinki većeg raspona dužina, od 14,6 do 33,6 mm (22,4±5,3 mm) uzorkovano je u rujnu 2011. godine kako bi se procijenila dužina pri kojoj *P.*

rustica mijenja spol. Priljepci (N=95) raspona dužine od 10,1 do 22,4 mm (16,2±3,0 mm), uzorkovani su u rujnu 2011. (N=10), listopadu 2011. (N=30), studenom 2011. (N=25), veljači 2012. (N=15), ožujku 2012. (N=10) i svibnju 2012. godine (N=5) te su korišteni za kvalitativnu histološku analizu. Od ukupno 95 jedinki, njih 65 prikupljeno je u periodu maksimalne reproduktivne aktivnosti (rujan, listopad i studeni 2011. godine) s ciljem procjene minimalne veličine pri kojoj jedinke vrste *P. rustica* dostižu prvu spolnu zrelost (prema Sparre & Venema 1998). Iz svake jedinke odvojeno je gonadno tkivo zajedno s probavnom žlijezdom i fiksirano u 10%-tnoj otopini formalina. Za pripremu histoloških uzoraka gonada korištena je parafinska tehnika. Za kvalitativnu analizu, histološki preparati pregledani su na Zeiss AXIO Lab.A1 mikroskopu pri povećanju od 50×, 100× i 400×. Određen je spol i razvojni stadij prema McCarthy i sur. (2008) i Belkhodja i sur. (2011). Kod mužjaka je određeno pet razvojnih stadija: rano sazrijevanje (3), kasno sazrijevanje (4), zreli (5), mriješćenje (2) i izmriješteni (1) te sedam stadija u ženki: neaktivni (0), rano sazrijevanje (3), kasno sazrijevanje (4), zreli, (5), atretični (1.5), mriješćenje (2) i izmriješteni (1). Jedinke kojima nije bilo moguće odrediti spol označene su kao neodređene. Kvantitativna je metoda uključivala mjerenje broja, promjera i opsega oocita svih ženki priljepka *P. rustica* s vidljivom jezgrom unutar vidnog polja pri povećanju od 100×. Izračunat je srednji gonadni indeks (SGI) priljepka *P. rustica* kako bi se procijenio omjer razvitka, sazrijevanja, zrelosti i mriješćenja jedinki. SGI izračunat je zbrajanjem numeričkog broja pridruženog određenom razvojnom stadiju za sve jedinke, te dijeljenjem istog s brojem primjeraka u uzorku za svaki spol (Gosling, 2003).

Analiza indeksa kondicije. Za analizu indeksa kondicije uzorkovalo se mjesečno trideset jedinki (24,0±2.3 mm) od srpnja 2011. do lipnja 2012. godine, osim u prosincu kada je zbog nepovoljnih vremenskih uvjeta uzorkovano samo 18 jedinki. Dužina (L), širina (W) i visina (H) svakog priljepka izmjerena je pomičnim mjerkom preciznosti 0,1 mm, a mokra masa (w) digitalnom vagom preciznosti 0,01 g. Meso je pažljivo odvojeno od ljušture te je na analitičkoj vagi izmjerena mokra masa ljušture i masa mokrog mesa. Uzorci su zatim sušeni do konstantne suhe mase 24 sata u prethodno zagrijanoj pećnici na 105°C. Osušeni uzorci ponovno su izvagani kako bi se dobila masa suhe ljušture i masa suhog mesa. Indeks kondicije (IK) izračunat je prema Mann (1978).

Arrheniusova prijelomna temperatura. Testirane vrste u ovom pokusu su *P. rustica*, *P. caerulea* i *P. ulyssiponensis*. Otkucaji srca mjereni su pomoću neinvazivne metode koju su razvili Depledge & Anderson (1990), a prilagodili Chelazzi i sur. (1999). Na očišćenu ljušturu svakog priljepka fiksiran je infracrveni senzor pomoću trenutačnog ljepila. Položaj senzora na

vanjskoj strani ljušture odgovara položaju srca samog priljepka. Senzor se sastoji od diode koja emitira infracrvenu svjetlost, a signal se zatim filtrira, pojačava te snima pomoću prijenosnog osciloskopa (PicoScope 2203). Snimljeni zapisi analizirani su na prilagođenom softveru (PicoScope, ver. 6.6.13.15). Arrheniusovom prijelomnom temperaturom naziva se ona temperatura na kojoj se javlja diskontinuitet u padu Arrheniusovog grafa (Stillman & Somero 1996). Prijelomna temperatura označava točku na kojoj dolazi do naglog pada u funkcioniranju metabolizma određenog organizma. Ukupno 15 priljepaka (5 od svake vrste) nasumično je stavljeno u plastične posudice, a posudice uronjene u vodenu kupelj na 20°C. Temperatura se zatim povećavala svakih 15 minuta za 3°C dok god su se otkucaji srca mogli bilježiti. Temperatura na dnu plastičnih posudica (u kojima se nalaze priljepci) i u vodenoj kupelji mjerila se svake minute temperaturnim zapisivačima (termologeri, iButton Inc, ±0.5°C). Realno vrijeme stope otkucaja srca bilježilo se svakih 5 minuta, a njihove prijelomne temperature odredile su se regresijskim modelom (Dahlhoff & Somero 1993; Stillman & Somero 1996).

Proizvodnja proteina toplinskog šoka. Kako bi se odredila proizvodnja induciranih proteina toplinskog šoka (*hsp70*), 25 jedinki vrste *P. rustica* i 25 jedinki vrste *P. caerulea* izložene su različitim temperaturama i različitim trajanjem pojedinih temperatura. Dvadeset jedinki svake vrste stavljeno je u plastične posudice, a posudice uronjene u vodenu kupelj na 20°C. Temperatura se povećavala 3°C svakih 15 minuta do maksimalne temperature od 38°C koja se zatim održavala narednih 120 minuta. Po pet jedinki svake vrste uzorkovano je na 20°C kao kontrola. Temperatura na dnu plastičnih posudica i vodenoj kupelji mjerena je svake minute temperaturnim zapisivačima (termologeri, iButton Inc, ±0.5°C). Pet jedinki svake vrste nasumično je uzorkovano na 20°C, 34°C, 36°C, 38°C nakon 60 minuta i 38°C nakon 120 minuta. Uzorkovani priljepci su se iz vodene kupelji stavljali u akvarij u kojem ih je prozračena morska voda temperature 20°C prskala naredna 2 sata kako bi priljepci sintetizirali proteine toplinskog šoka (Dong i sur. 2008; Dong & Williams 2011). Količina gena *hsp70* određena je kvantitativnom lančanom reakcijom polimeraze u stvarnom vremenu.

Lizosomalna stabilnost hemocita. Da bi se utvrdila stanična razina odgovora na toplinski stres kod priljepaka *P. rustica* i *P. caerulea*, mjerena je lizosomalna stabilnost hemocita. Test s neutralnim crvenilom temelji se na činjenici da samo žive stanice apsorbiraju i vežu neutralno crvenilo te je stoga i količina akumuliranog crvenila unutar stanica direktno proporcionalna količini živih stanica (Repetto i sur. 2008). Po dvanaest jedinki svake vrste nasumično je stavljeno u plastične posudice, a posudice uronjene u vodenu kupelj na 20°C. Temperatura se povećavala jednakom stopom zagrijavanja kao u prethodno opisanom pokusu

(3°C svakih 15 minuta) do maksimalne temperature od 38°C koja se zatim održavala narednih 60 minuta. Po tri jedinke svake vrste uzorkovano je na 20°C kao kontrola. Tri jedinke svake vrste nasumično je uzorkovano na 20°C, 34°C, 36°C, 38°C nakon 30 minuta i 38°C nakon 60 minuta. Priljepci uzorkovani na 38°C nakon 60 minuta su se iz vodene kupelji stavljali u akvarij u kojem ih je prozračena morska voda temperature 20°C prskala naredna 2 sata. Na ovaj način testirala se mogućnost oporavka stanica nakon oštećenja. Otpuštanje neutralnog crvenila pinocitizirano unutar stanica mjereno je ELISA čitačem (Labsystem Uniskan®) na 490 nm. Vrijednosti su izražene kao optička gustoća (engl. optimal density, OD), a proporcionalne su količini crvenila koju apsorbiraju žive stanice.

Statistička obrada podataka. Statistička analiza provedena je koristeći se statističkim paketima Minitab v.16, Statistica v.8 (StatSoft Ltd.) i PRIMER (PRIMER-E Ltd.). Podatci su prvo testirani na homogenost varijanci primjenom Levenovog testa. Ovisno o rezultatima testa, korišteni su parametarski (jednosmjerna analiza varijanci - ANOVA) ili neparametarski (Kruskal-Wallis) testovi. Neparametarska Spearmanova korelacija je korištena kako bi se testirao odnos okolišnih parametara, srednjeg gonadnog indeksa mužjaka i ženki te indeksa kondicije. Omjeri spolova testirani su Chi-kvadrat testom. Razina statističke značajnosti iznosila je $p=0,05$.

REZULTATI

Analiza starosti i rasta. Vrjednovanje formiranja linije rasta provedeno je na 60 manjih jedinki, raspona dužina od 11,4 do 16,9 mm (14,4±1,6 mm). Tamni prstenovi rasta su bili prisutni na rubu ili blizu ruba ljušture na jedinkama prikupljenim od listopada 2011. do svibnja 2012. godine. Sukladno tome, određeno je da se linija rasta formira u svibnju. Analiza unutarnjih prstenova rasta iz acetatnih preslika napravljena je na ukupno 120 jedinki priljepka *Patella rustica*, uzorkovanih nasumično u rujnu 2011. godine. Minimalna zabilježena dužina bila je 8,1 mm, širina 6,2 mm, a visina 2,8 mm dok je maksimalna dužina iznosila 33,6 mm, širina 27,8 mm, a visina 11,8 mm. Srednja dužina analiziranih jedinki bila je 20,2±6,2 mm, širina 16,2±5,2 mm dok je srednja visina bila 6,6±2,1 mm. Budući da se mriješćenje ove vrste odvija u studenom, datum rođenja određen je kao 1. prosinca. Na temelju tih podataka zaključeno je da prva linija rasta predstavlja devet mjeseci umjesto jedne cijele godine. Procijenjena prosječna starost 120 jedinki iznosila je 2,9±1,4 godine. Izračunati asimptotski maksimumi su L_∞=40,86 mm za dužinu, W_∞=33,02 mm za širinu i H_∞=14,07 mm za visinu, dok su vrijednosti konstante rasta (K) bile 0,23 godina^{-1} za dužinu, 0,24 godina^{-1} za širinu i 0,21 godina^{-1} za visinu. Od ukupno obrađenih jedinki, 90,8% bilo je mlađe od 4 godine. Samo su

dvije (1,6%) jedinke bile starije od 6 godina (6,75 i 7,75 godina). Matematičkim izračunom maksimalni životni vijek procijenjen je na 12,7 godina.

Endobionti iz ljušture priljepka *Patella rustica.* Tijekom analize starosti i rasta, utvrđeno je da se većina ljuštura priljepaka nalazi u različitom stupnju erodiranosti. Stoga je bilo teško utvrditi točan položaj prve linije rasta na većini uzorkovanih priljepaka. Rezultat je to endobionata koji erodiraju između prizmatičnog sloja i periostrakuma ljušture. Većina određenih endobionata iz ljuštura vrste *P. rustica* pripada nitastim cijanobakterijama, a određeno je pet vrsta: *Mastigocoelus testarum* Lagerheim, 1886, *Hormathonema paulocellulare* Ercegović 1929, *Hyella caespitosa* Bornet & Flahault 1888, *Leptolyngbya* sp. Anagnostidis & Komárek, 1988 i *Calothrix* sp. Agardh, 1886.

Histološka analiza gonada. Uzorci gonada su prikupljeni na ukupno 355 jedinki (24,2±2,9 mm), od kojih je jedan uzorak (0,3%) izgubljen u obradi, 142 jedinke (40,0%) na kojima nije bilo moguće utvrditi spol su označene kao neodređene, a 3 jedinke (0,8%) su označene kao hermafroditi. Chi-kvadrat test pokazao je statistički značajnu razliku između broja mužjaka i ženki u srednjem dužinskom razredu (χ^2=82,1, p<0,001), dok je omjer ženki i mužjaka iznosio 4:1. Dodatno su obrađeni uzorci gonada 95 jedinki iz manjeg veličinskog razreda (16,2±3,0 mm), od čega su 40 jedinki (42,1%) bili mužjaci, 9 jedinki (9,5%) ženke, a na 46 jedinki (48,4%) nije bilo moguće utvrditi spol te su označene kao neodređene. Chi-kvadrat test je također pokazao statički značajnu razliku između broja mužjaka i ženki (χ^2=19,6, p<0,001) a omjer ženki i mužjaka iznosio je 1:4. Mužjaci dominiraju u manjim veličinskim kategorijama, dok ženke postaju brojem dominantnije pri dužinama većim od 28 mm. Procjena je da se pri toj dužini događa promjena spola kod većine jedinki priljepka *P. rustica.* Procijenjena dužina pri kojoj je 50% analiziranih mužjaka bilo spolno zrelo, iznosila je 13,1 mm. Kod ženki nije bilo moguće odrediti prvu spolnu zrelost zbog nedovoljnog broja u ukupnom uzorku priljepaka manje veličinske kategorije. Kvalitativnom histološkom analizom utvrđeno je da vrsta *P. rustica* ima jedan reproduktivni ciklus tijekom cijelog razdoblja istraživanja, od srpnja 2011. godine do lipnja 2012. godine. Obrasci razvoja su bili istovjetni kod mužjaka i ženki, sa samo nekoliko razlika. U lipnju i kolovozu 100% mužjaka bilo je u stadiju ranog sazrijevanja. Važno je naglasiti da u ukupnom uzorku od siječnja do svibnja i srpnja nije zabilježena prisutnost mužjaka. Od listopada do studenog gonade mužjaka prolaze kroz stadij kasnog sazrijevanja. U studenom je 60% mužjaka imalo zrele gonade, 27% ih je bilo u mrijestu dok je 13% jedinki još uvijek bilo u kasnom stadiju razvoja. Gametogeneza kod ženki započinje u veljači, dok se kasno sazrijevanje gonada odvija od rujna do studenog. U

studenom, 47% ženki je imalo zrele gonade, 33% ženki je bilo u mriješćenju, a 13% jedinki se već izmrijestilo. Tijekom rujna i listopada određeni postotak gonada ženki (5%) nalazio se u stadiju atrezije, gdje je većina previtelogenetskih i zrelih jajnih stanica bila u određenom stupnju propadanja. Najveće vrijednosti srednjeg gonadnog indeksa zabilježene su od kolovoza do studenog za oba spola. Za ženke, najviša vrijednost zabilježena je u listopadu (4,6), a kod mužjaka u studenom (4,1), što se podudara sa sazrijevanjem gonada i mrijestom. Mriješćenje se za oba spola odvija u studenom, a u prosincu i mužjaci i ženke imaju 100% izmriještene gonade. To odgovara i padu vrijednosti srednjeg gonadnog indeksa zabilježenog u prosincu za mužjake (1), a u siječnju za ženke (1). Izmjereni promjer oocita je bio u rasponu od 0,2 do 242,9 μm a opseg oocita od 10,2 do 569,9 μm. Veličina oocita povećavala se od rujna (promjer 63,8± 0,1 μm i opseg 176,9±111,1 μm) do studenog (promjer 104,5±49,6 μm i opseg 297,7 ± 140,0 μm). Nakon travnja, raspodjela frekvencije opsega oocita ima relativno jednoličan obrazac do listopada, što ukazuje na kontinuirani proces sazrijevanja i otpuštanje oocita u studenom. Najmanje oocite su izmjerene u stadiju ranog sazrijevanja (promjer 21,3± 9,7 μm i opseg 60,2±30,5 μm), a najveće u zrelim gonadama (promjer 115,6±44,1 μm i opseg 329,0±125,4 μm), kad su oocite već prošle kroz razdoblje vitelogeneze. Postoji pozitivna korelacija između srednjeg gonadnog indeksa i veličine oocita.

Indeks kondicije. Srednja mjesečna maksimalna vrijednost indeksa kondicije zabilježena je u svibnju (179,6), a minimum neposredno poslije, u lipnju (133,9). Statistički značajna razlika zabilježena je između indeksa kondicije i mjeseca uzorkovanja ($H=60,70$, $p<0.001$). Nije zabilježena značajna korelacija između indeksa kondicije i srednjeg gonadnog indeksa mužjaka i ženki. Značajna negativna korelacija zabilježena je između srednjeg gonadnog indeksa mužjaka i koncentracije klorofila *a* ($r=-0,812$, $p=0,050$), a značajna pozitivna korelacija između srednjeg gonadnog indeksa ženki i temperature zraka ($r=0,629$, $p=0,028$).

Arrheniusova prijelomna temperatura. Priljepci *P. rustica*, *P. caerulea* i *P. ulyssiponensis* pokazuju postojane razlike u srčanoj aktivnosti. Srčani ritam mogao bi se podijeliti u dvije faze s povećanjem temperature. Sve tri vrste imale su pravilne otkucaja srca u prvoj fazi, na početku pokusa (23°C-32°C) gdje se stopa otkucaja povećavala log-linearno s temperaturom. U drugoj fazi (33°C-45°C), nakon što se temperatura kontinuirano povećavala, sve tri vrste pokazuju znakove aritmije, nakon čega se stope otkucaja srca značajno smanjuju. Općenito, jedinke vrste *P. caerulea* imale su brže stope otkucaja srca tijekom čitavog pokusa (2,4±0,4 Hz, srednja vrijednost±SD) od jedinki vrste *P. rustica* (2,1±0,4 Hz), dok je srčani ritam kod jedinki vrste *P. ulyssiponensis* bio najsporiji (1,9±0,3 Hz). Arrheniusova prijelomna

temperatura značajno se razlikuje između tri navedene vrste (ANOVA, $F_{(2,12)}$=7,58, p=0,007). Kod vrste *P. rustica* zabilježena je najviša Arrheniusova prijelomna temperatura (37,9±2,07°C), nakon čega slijedi vrsta *P. caerulea* (35,9±2,6°C), a zatim vrsta *P. ulyssiponensis* (32,2±2,3°C).

Proizvodnja proteina toplinskog šoka. Priljepci *P. rustica* i *P. caerulea* pokazuju različitu razinu proizvodnje induciranih proteina toplinskog šoka, *hsp70* mRNA. Obje vrste imaju slične, niske razine proteina *hsp70* mRNA na početku pokusa pri kontrolnoj temperaturi od 20°C. Nakon zagrijavanja, razine proteina *hsp70* kod jedinki vrste *P. rustica* bile su veće u usporedbi s jedinkama vrste *P. caerulea*. Povećanje razine *hsp70* razine za vrstu *P. rustica* javlja se na 34°C (~36% RU), a povećanje se nastavlja i nakon 1-satnog (~143% RU) i 2-satnog (~194% RU) zagrijavanja na 38°C. Kod vrste *P. caerulea* razina *hsp70* je relativno niska na 34°C (~4% RU), raste do maksimalne razine zagijavanjem na 36°C (~71% RU), te se polako smanjuje zagrijavanjem na 38°C.

Lizosomalna stabilnost hemocita. Test bojanja hemocita neutralnim crvenilom dokazao je da se lizosomalna stabilnost hemocita mijenja zagrijavanjem. Između vrsta *P. rustica* i *P. caerulea* utvrđena je statistički značajna razlika u apsorbiranoj količini crvenila (H=11,44, p=0,01); veće vrijednosti zabilježene su 32°C kod jedinki vrste *P. rustica*, a na 38°C za jedinki vrste *P. caerulea*. Obje vrste su pokazivale smanjene vrijednosti apsorbiranog crvenila prilikom ponovnog vraćanja u morsku vodu sobne temperature, što upućuje na činjenicu da ove vrste nisu sposobne prevladati oštećenja lizosomalne membrane hemocita. Ne postoji značajna razlika između apsorpcije crvenila i različitih temperatura za vrstu *P. rustica* (H=5,97, p=0,113), dok za vrstu *P. caerulea* postoji značajna razlika između apsorpcije crvenila i temperature ($F_{(3,8)}$=4,10, p=0,049).

ZAKLJUČCI

Ova disertacija daje uvid u starost, rast i reprodukciju priljepka *Patella rustica* na jugoistočnoj obali Jadrana. Iako je ovo istraživanje provedeno na samo jednoj lokaciji, dobiveni rezultati vrijedni su jer predstavljaju prvi opis ovih važnih populacijskih procesa za vrstu *P. rustica* na istočnoj obali Jadrana. Istraživanje predstavlja prvi detaljni opis gametogenetskih stadija temeljen na kvantitativnoj histološkoj analizi, te daje prve podatke opsega oocita vrste *P. rustica*. Također, provedeni pokusi potvrdili su hipotezu da tri vrste priljepaka *Patella rustica*, *P. caerulea* i *P. ulyssiponensis* imaju različite fiziološke odgovore na toplinski stres kao rezultat njihove prilagodbe na različito zonirana mikrostaništa.

Zaključci ovog istraživanja su sljedeći:

- Moguće je odrediti unutrašnje linije rasta iz acetatnih preslika presjeka ljušture priljepka *P. rustica*.

- Priljepak *P. rustica* formira jedan prsten rasta godišnje, tijekom svibnja.

- Pri procjeni starosti vrste *P. rustica* uzelo se u obzir da prva linija rasta predstavlja 9 mjeseci umjesto jedne pune godine.

- Više od 90% analiziranih jedinki bilo je mlađe od 4 godine, dok je prosječna dužina ljušture iznosila 20,2 mm.

- Vrijednosti parametara von Bertalanffy krivulje rasta iznosili su: L_{∞}=40,86 mm za dužinu, W_{∞}=33,02 mm za širinu i H_{∞}=14,07 mm za visinu, dok su vrijednosti konstante rasta (K) iznosile 0,23 za dužinu, 0,24 za širinu i 0,21 godina^{-1} za visinu.

- Vrijednost phi-prime indeksa (Ø'=2,60) izračunatog za vrstu *P. rustica* spada među niži dio raspona vrijednosti zabilježenih za druge vrste priljepaka, što ukazuje da ova vrsta raste relativno sporo.

- Maksimalni životni vijek matematički je izračunat na temelju asimptotske duljine (40,86 mm) i iznosi 12,7 godina, iako su samo dvije analizirane jedinke bile starije od 6 godina (6,75 i 7,75 godina), dok je najstarija jedinka imala dužinu od 33,5 mm.

- Ljušture priljepka *P. rustica* pokazuju alometrijski rast (α=1.66), potvrđujući pravilo da vrste koje naseljavaju stijene gornje granice oseke rastu brže u visinu nego u dužinu kako bi smanjili gubitak vode isparavanjem.

- Potrebna su daljnja istraživanja kako bi se utvrdio utjecaj različitih okolišnih čimbenika na dinamiku rasta priljepka *P. rustica.*
- Tijekom istraživanja starosti i rasta, utvrđeno je da su ljušture priljepaka erodirane nitastim cijanobakterijama. Stoga je bilo teško utvrditi točan položaj prve linije rasta na većini uzorkovanih priljepaka.

- Utvrđeno je da se mužjaci i ženke uglavnom razlikuju u veličini. Mužjaci dominiraju u manjim veličinskim kategorijama, dok ženke postaju brojem dominantnije pri dužinama većim od 28 mm što upućuje na činjenicu da je *P. rustica* protandrični hermafrodit. Histološkim analizama zabilježen je hermafroditizam kod tri jedinke, prosječne dužine ljušture od 25,19 mm.

- Prema podatcima dobivenim u ovom istraživanju, duljina pri kojoj 50% mužjaka dostiže prvu spolnu zrelost iznosila je 13,1 mm, a prema starosnoj analizi ovi mužjaci mlađi su od dvije godine. Kod ženki nije bilo moguće odrediti prvu spolnu zrelost zbog njihovog nedovoljnog broja u ukupnom uzorku priljepaka manje veličinske kategorije.

- Kvalitativnom histološkom analizom utvrđeno je da vrsta *P. rustica* ima jedan reproduktivni ciklus godišnje. Obrasci razvoja su istovjetni kod mužjaka i ženki, uz manja odstupanja.

- Utvrđeno je produženo razdoblje sazrijevanja gonada, koje traje od veljače do rujna za ženke, a od lipnja do listopada za mužjake.

- Mriješćenje se za oba spola odvija sinkrono u studenom, dok u prosincu i mužjaci i ženke imaju u potpunosti izmriještene gonade.

- Tijekom istraživanja zabilježen je značajan broj jedinki čiji se spol nije mogao sa sigurnošću odrediti. Najveći broj neodređenih jedinki zabilježen je u siječnju, nakon mriješćenja, kad su gonade u inaktivnom stadiju. Pretpostavka je da su ove neodređene jedinke uglavnom bili mužjaci, što objašnjava njihov nedostatak u uzorku od siječnja do lipnja.

- Obrasci razmnožavanja zabilježeni u ovom istraživanju odgovaraju onima zabilježenim kod drugih vrsta roda *Patella* na sličnim geografskim širinama. Ipak, potrebna su detaljnija istraživanja okolišnih čimbenika kako bi se sa sigurnošću

moglo utvrditi kako svaki pojedinačno utječe na reproduktivni ciklus vrste *P. rustica.*

- Ovo istraživanje daje prve podatke opsega oocita priljepka *P. rustica* kroz različite gametogenetske stadije. Najmanje oocite su izmjerene u stadiju ranog sazrijevanja, a najveće u zrelim gonadama kad su oocite već prošle kroz razdoblje vitelogeneze.

- Utvrđeno je da oocite tijekom gametogeneze prolaze kroz različite stadije propadanja, a posebice na početku mriješćenja te neposredno nakon.

- Iako se indeks kondicije smatra pouzdanim parametrom praćenja reproduktivnog ciklusa kod mnogih vrsta školjkaša, ovim istraživanjem dokazano je da isto ne vrijedi za priljepke vrste *P. rustica.* Pretpostavka je da je indeks kondicije kod ove vrste bolji pokazatelj trenutne konzumacije hrane, budući probavna žlijezda zauzima značajni udio mesa priljepka.

- Provedeni pokusi dokazali su postojanost značajnih razlika u srčanoj aktivnosti, proizvodnji proteina termalnog šoka te lizosomalnoj stabilnosti hemocita kod vrsta *P. rustica, P. caerulea* i *P. ulyssiponensis*, a vezano za njihovu različitu vertikalnu zonaciju na obali.

- Arrheniusova prijelomna temperatura za vrstu *P. rustica* iznosila je 37,9°C, za vrstu *P. caerulea* 35,9°C, a za vrstu *P. ulyssiponensis* 32,2°C, što ukazuje na činjenicu da vrsta *P. rustica* ima višu granicu podnošljivosti temperaturnih skokova, za razliku od druge dvije.

- Pokusom je dokazano da vrsta *P. caerulea* ima veću srčanu frekvenciju u odnosu na vrste *P. rustica* ili *P. ulyssiponensis*, što dokazuje da ova vrsta ima i brži metabolizam.

- Inducirana proizvodnja proteina termalnog šoka kod vrsta *P. rustica* i *P. caerulea* je usko povezana s Arrheniusovom prijelomnom temperaturom tj. točkom na kojoj dolazi do naglog pada u funkcioniranju metabolizma. Kod vrste *P. rustica*

proizvodnja *hsp70* nastavlja se i nakon daljnjeg povećanja temperature, potvrđujući činjenicu da je ova vrsta prilagođena svom mikrostaništu na gornjoj granici oseke.

- Temperatura značajno utječe na lizosomalnu stabilnost hemocita kod vrsta *P. rustica* i *P. caerulea*, što uzrokuje promjene u fiziološkim procesima, kao što je proizvodnja *hsp70*, a u konačnici dovodi do promjena u srčanoj aktivnosti te funkcioniranju metabolizma.

9. BIOGRAPHY

Ivana Prusina was born on 19th of September 1981 in Dubrovnik, where she finished her elementary and high school education. In the academic year 2001/2002 she enrolled graduate study programme at the University of Split, University Department of Marine Studies. In October 2006 she was awarded a degree of master in marine biology and ecology.

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During her postgraduate study, she has participated in the workshops: ‘Intertidal organisms as a proxy for climate change’ organized by the University of Palermo and ‘The effects of climate change on vulnerable life traits of aquatic ectotherms: towards an integrated approach’ organized by the University of Florence and Alfred Wegener Institute in Bremerhaven. She also attended summer school ‘Challenges in Changing Coastal Seas’, organized by the Alfred Wegener Institute in Sylt.

She is a co-author of four scientific papers indexed in Current Content database. She has participated in several international scientific conferences with a total of 7 contributions.

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