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MULTIDISCIPLINARY STUDIES OF THE GENUS *CYSTODYTES* (ASCIDIACEA): FROM MOLECULES TO SPECIES

Memòria presentada per **Susanna López-Legentil** per accedir al títol de Doctor en Ciències Biològiques al Departament de Biologia Animal (Unitat d'Invertebrats) de la Facultat de Biologia (Universitat de Barcelona), sota la direcció dels directors **Xavier Turon** i **Bernard Banaigs**.

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Als meus pares

A mes parents

MIQUEL & MARTINE

“La nuestra es una época de creciente especialización. De conformidad con esa tendencia, la erudición moderna pone un acento desmesurado en la especialización, lo cual como atestigua la universidad moderna, implica y entraña la segregación del conocimiento en “disciplinas” diferenciadas. [...] Pero la realidad, la historia y el conocimiento no pueden dividirse en segmentos y compartimientos. [...] Lo que se necesita es un enfoque interdisciplinario del material que se haya escogido, un enfoque móvil y flexible que permita moverse con libertad entre disciplinas dispares, a través del espacio y del tiempo”.

M. BAIGENT, R. LEIGH, H. LINCOLN

ABSTRACT

Intraspecies variability is widespread in marine invertebrates. Previous reports of *Cystodytes* (Ascidiacea, Polycitoridae) in the Mediterranean have been routinely attributed to the nominal species *Cystodytes dellechiajei*. This colonial species is widely distributed in both tropical and temperate waters. In the Mediterranean, even though zooid anatomy is remarkably uniform, the general morphology of the colonies varies greatly, especially in terms of color and spicular composition (observed under scanning microscopy). Two different chemotypes, according to their variability in alkaloid chemistry, were defined for 4 different color morphs (green, blue, brown and purple) using both HPLC and RNM techniques and MALDI-TOF analyses. The purple morph displayed a chemotype based on the sulfur-containing pyridoacridines shermilamine B, kuanoniamine D, and their deacetylated forms: deacetylkuanoniamine D and deacetylshermilamine B (new natural product). The blue and green morphs displayed a chemotype based on the C_9-unsubstituted pyridoacridines ascididemin and 11-hydroxyascididemin. No major alkaloid was found in the brown form. The intracolony localization of the major alkaloids (zooids vs. tunic) was studied by MALDI-TOF techniques, whose usefulness in the field of marine chemical ecology was therefore assessed. All of these alkaloids were present in both the tunic and zooids, with the exception of the purple morph, where shermilamine B and kuanoniamine D were found in the tunic, whilst their deacetylated forms were found in both tunic and zooids. Cellular localization of these compounds and ultra structural study of the tunic of the blue, purple and green morphs were attempted by electron microscopy and X-ray microanalysis. The main cell types identified were bladder cells, pigment cells, amebocytes, phagocytes, and morula cells. The sulfur-containing pyridoacridines appeared to be stored in the pigment cells. In addition, the anti-predatory properties of crude extracts, tunic acidity, and spicular contents were assayed in the blue and purple Mediterranean forms of *Cystodytes*, and a purple morph of Guam (USA), using three generalist predators (damselfishes, puffer fishes and sea urchins). Toxicity of crude extracts was also measured by the Microtox bioassay. All crude extracts, as well as ascididemin, were toxic and significantly deterred fish but not sea urchin predation.

In contrast, acidity by itself, and spicular shape and concentration did not deter feeding. To determine if the observed variation in the Mediterranean has a genetic basis, a fragment of the mithocondrial gene Cytocrome Oxidase I was sequenced and phylogenetic and population genetic analyses were performed. The results pointed towards the existence of at least 2 species within the 67 samples of 15 colors analyzed. The first well-defined group contained mainly blue colonies with the disc-shaped spicules typical of the genus. The second grouped all samples of a purple morph with sphere-shaped spicules in addition to the disc-shaped ones. When genetic variability was partitioned between color morphs and between geographic locations, the former component explained most of the variance. To assess whether these morphotypes are in fact sibling species, a study of the biological cycles and growth rates of both color morphs was carried out from July 2002 to February 2004. Both the reproductive and growth periods, although partially overlapping, showed significant temporal lags, reinforcing previous genetic and chemical results indicating that these morphs are reproductively isolated and represent distinct species. To determine whether there is a seasonal pattern in the production of chemical and physical defenses and, if so, whether it correlated with the reproductive and/or growth cycles, colonies of the blue Mediterranean morph were sampled for a year, starting in February 2003. Ascididemin was quantified by HPLC and the ash content was calculated as an estimate of the physical defenses. The results showed that when the ascidian invested in reproduction, the energy allocated to other life cycle parameters, such as growth, chemical and physical defenses, was significantly reduced. The reproductive cycle appeared, therefore, to drive the temporal course of the production of defensive secondary metabolites and inorganic material in this species. The multidisciplinary approach used to study the ascidian genus *Cystodytes* provided an interesting case study through which to develop a better understanding of the biology, ecology and secondary chemistry of marine invertebrates.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 GENERALITIES

Marine invertebrates are a rich source of novel compounds with potential pharmacological and biotechnological applications (Faulkner 2000). These compounds also fulfill diverse ecological roles in nature (e.g. Becerro et al. 1997, Thacker et al. 1998, Newbold et al. 1999) and are crucial factors in the evolutionary establishment of the life-history strategies of the producer organisms.

However, many marine invertebrate species that produce important bioactive compounds are currently identified only at the genus level (see Marinlit, Annex I), remain undescribed (e.g. Caroll & Scheuer 1990) or are incorrectly identified, resulting in a total confusion when the source organism needs to be retrieved. Without information about species' identities and interrelationships it will be more difficult if not impossible to understand the function of bioactive compounds in marine invertebrates and to enhance production of these compounds for medical and biotechnological applications. Species identification can result even more complicated and necessary if intra-species variability exists (including chemical differences). In fact, morphological observations alone are often insufficient to determine whether we are dealing with intra-species variability or with sibling species. From there the use of additional approaches such as molecular techniques may arise. Phylogeny, population genetics and phylogeography significantly contribute to understand speciation events and provide at the same time important ecological and evolutionary information about the species studied. In addition, the

study of reproductive cycles allows assessing species differentiation according to the biological and cohesion species concepts and provides further information about the ecology and biology of benthic invertebrates.

Furthermore, life cycles are closely related to allocation of resources to secondary metabolites production. Assuming that there is a cost in terms of energy investment in the production of natural products, it must be optimized with respect to the needs of the organism and its energy budget (growth, somatic maintenance and repair, and reproduction). The localization of the bioactive compounds within the organism and the identification of the cells or organs responsible for their production may also provide important information of their biological significance. Therefore, although a theoretic link exists between man-established disciplines approaching to the biology, morphology, ecology and chemistry of marine invertebrates (here-above some examples), little is known about how they are interconnected and how they work in nature. This kind of multidisciplinary approach, encompassing and relating information gleaned from very different fields, from molecules to populations, is necessary to fully understand the life histories of marine invertebrates, yet this information is available for virtually no species (for an exception see Becerro 1994).

The present work focuses on a model organism, the colonial ascidian *Cystodytes dellechiajei* (Della Valle 1877), of which we sought to determine the meaning and variability in the production of secondary metabolites, and to relate this variability to other aspects of its biology and ecology. To this end, we used methods borrowed from molecular biology, phylogeography, chemistry, ultrastructural techniques, field studies and experimental procedures. The target species was selected because it features an unique array of characteristics: it produces diverse and potentially redundant physical and chemical defenses, it presents morphological variability qualitative in the area studied (western Mediterranean), larval dispersal is presumably short-range (favoring genetic isolation by distance,) and its biological parameters are largely unknown. Our work aims to investigate and relate all these traits, and will therefore cut across several disciplines:

- **An interface between BIOLOGY and GENETICS**

Many marine invertebrates can vary in size, shape and coloration. However, some variants may in fact correspond to different species that have not been recognized by traditional methods. Indeed, with the advent of molecular techniques,

much variation (morphological, chemical or otherwise) attributed to instraspecies adaptations to local environmental conditions corresponded with different species (e.g. Klautau et al. 1999,Knowlton 2000, Miller et al. 2001, McGovern & Hellberg 2003, Meroz-Fine et al. 2003). Diversity in coloration has largely been described in nature as being associated with polymorphism or with divergence. It has been claimed that color differences may be maintained by disruptive selection through the effect of prey, predators, competitors or symbionts (Galeotti et al. 2003, Mackenzie et al. 2004). In ascidians variation in pigmentation is common, and chromatic varieties have not, in general, been given any valid taxonomic status (Kott 1990). Although color variation is the difference more often reported, other traits such as shape and spicular composition may vary from one organism or one place to another, sometimes in response to specific environmental conditions. However, most of the time the significance of such differences is unclear. For instance, in ascidians, spicular differences are a reliable and common character used to differentiate between Didemnidae species (Lafargue & Laubier 1980), but its applicability to other families is dubious (e.g. Monniot 1970).

Studies of population subdivision and speciation are crucial to our understanding and management of marine biodiversity and its biotechnological applications (Holland 2000). However, the very concept of species is open to debate (Hull 1997, Sites & Marshall 2003). New molecular techniques allow an independent approach to this issue. Aside of phylogenetic studies, Templeton (2001) showed how formal phylogeographic analyses of genetic data can provide useful insights at the interface between intra- and interspecific evolution, within the framework of the cohesion species concept (Templeton 1989). In addition, phylogeography provides useful clues to interpret the mode by which historical processes may have influenced population demography, and left evolutionary footprints on the contemporary geographic distribution of gene based organismal traits. Indeed, all species populations show some level of genetic structuring due to environmental barriers, historical processes and/or life histories (e.g. Gerlach & Musolf 2000, Tiedemann et al. 2000, Goldson et al. 2001). In addition, populations are often genetically differentiated through isolation by distance (i.e. populations in close proximity are genetically more similar than more distant populations). The number of alleles exchanged between populations is indicative of the genetic structure of a species. Therefore, understanding gene flow and its effects provide crucial information to many fields of research, including population genetics, populations

ecology, and conservation biology (e.g. Avise 1992, 1998, Holland 2000, Lockwood et al. 2002). Populations of colonial ascidians are likely to be highly genetically structured over short distances (tens of Km), as their larvae undergo a prolonged embryogenesis, hatch with juvenile rudimentary structures already formed and can settle within minutes (Svane & Young 1989, Young et al. 2002). Genetic evidence also shows that dispersal in colonial ascidians is more restricted than that of broad-casting solitary forms (Ayre et al. 1997).

On the other hand, the study of biological cycles remains a useful tool with which to assess speciation events according to the biological and cohesion species concepts (Templeton 1989), and provides crucial information about the ecology and biology of benthic invertebrates. In modular marine invertebrates, the interaction between genotype and environment often generates a distinct profile of growth, fission, fusion, regeneration, and senescence for each clone (reviewed in Jackson 1979, Caswell 1985, Buss 1986). In addition, seasonal patterns are common in temperate habitats (Osman 1977, Sutherland & Karlson 1977, Sebens 1986, reviewed in Coma et al. (2000), often related to temperature changes. Seasonal patterns can also be shaped by the trade-off between resource allocation to reproduction and growth. Due to such plasticity, and complex and heterogeneous life cycles, organisms of the same age diverge widely in their growth, survival and fecundity processes. Information on these important biological processes is scarce for modular benthic invertebrates (Jackson & Coates 1986, Turon et al. 1998), and in particular for colonial ascidians (Bak et al. 1981, Turon & Becerro 1992), hindering the development of demographic and life-history theories applicable to modular organisms (Caswell 1982, 1985, Williams 1986, Sebens 1987).

An interface between BIOLOGY and CHEMISTRY

Marine organisms are under intense competitive pressure for space, light, and nutrients. In addition, sessile organisms are an easy prey for predators. These organisms, therefore, have developed a range of defense mechanisms including behavioral (e.g. cryptic colorations, nocturnal behaviors), physical (e.g. spicules, tissue toughness), and chemical strategies to ensure survival. Therefore, it could also be that multiple defense mechanisms play redundant roles in protecting an organism from external aggressions. The relative importance of these mechanisms

and the potential interactions between them may depend on: the group examined (i. e. Pennings & Paul 1992, Hay et al. 1994); the inability of a single defense to deter all type of predators and/or competitors (Paul & Hay 1986, Hay et al. 1987, Pisut & Pawlik 2002, Tarjuelo et al. 2002, Burns et al. 2003); the life history stage (Uriz et al. 1996, Pisut & Pawlik 2002); whether structures or compounds are energetically expensive (Paul 1992, Pawlik 1993); the developmental or physiological constraints that could prevent a particular defense from being used in all parts of the organism (Harvell & Fenical 1989); and on evolutionarily established constraints (Schmitt et al. 1995).

In contrast, secondary metabolites may play several ecological roles (Schmitt et al. 1995, Becerro et al. 1997, Thacker et al. 1998, Newbold et al. 1999) and confer ecological advantages to their producers as a response to diverse environmental pressures. These ecological roles include anti-predation, prevention of fouling, mediation of spatial competition, protection from ultra-violet radiation and facilitation of reproduction (see McClintock & Baker 2001 for further details). Ascidians have been reported to present both physical (spicules, tunic toughness) and chemical defenses (Swinehart et al. 1974, Stoecker 1978, 1980, Pisut & Pawlik 2002, Tarjuelo et al. 2002). Secondary metabolites (Paul et al. 1990, Davis 1991, McClintock et al. 1991, Lindquist et al. 1992, Vervoort et al. 1998), high vanadium concentrations (Stoecker 1980a) and a low pH (Webb 1939, Thompson 1960, Stoecker 1978, 1980a, b, Pisut & Pawlik 2002) have all been proposed as defenses against predation and fouling in some ascidian species.

Optimal defense theory assumes that chemical and morphological defenses in living organisms are costly and that natural selection will favor an allocation of resources to defenses that optimize their cost/benefit ratio in terms of fitness (Rhoades 1979, Fagerström et al. 1987). Intraspecific variability in the production of bioactive compounds can be then expected to depend on the environmental constraints and on the physiological state of the source organism. Studies on intraspecific chemical variation are valuable for understanding the factors affecting the production of chemical defenses, as well as providing insights into the ecological consequences and evolutionary implications of such variation. Intraspecific patterns of variation have been reported over temporal (Turon et al. 1996), between-colony (Maida et al. 1993, Becerro et al. 1995), or within-colony (Paul & Van Alstyne 1988, Harvell & Fenical 1989, Van Alstyne et al. 1994, Turon et al. 1996, Becerro et al.

1998) scales. Most available data point to predation as one of the factors affecting intraspecific variation (Hay 1996).

Nowadays, several studies point to symbionts or bacteria inhabiting marine invertebrates as the producers of bioactive secondary metabolites (e.g. Unson & Faulkner 1993, Bewley et al. 1996, Piel et al. 2004). This possibility is being subject of intensive studies (Guyot & Tavares 2000) and new techniques are being developed to overcome clear limitations in bacteria cultivation.

Another actual controversy contemplates whether the chemical defenses are constitutive (constantly produced; e.g. Puyana et al. 2003), or else are induced (initiation of metabolite synthesis; e.g. Thompson et al. 1987) or activated (stocked, less active metabolites are converted to defensive ones; e.g. Paul & Van Alstyne 1992, Paul 1992, Cetrulo & Hay 2000) in response to changes in the environment.

Finally, secondary metabolites with close chemical structures were observed to be present in species of a same genus or family. Therefore, some studies have proposed the use of secondary metabolites as taxonomical markers in marine organisms (Valls & Piovetti 1995, Marin et al. 1999). Although chemotaxonomy has not often been used for taxonomic purposes, it remains as an alternative way to address inter or intraspecies variability (i.e. Valls 1993).

1.2 THE TARGET SPECIES, *Cystodytes dellechiajei*

F. Ascidiacea

O. Aplousobranchiata

F. Polycitoridae.

G. *Cystodytes* von Drasche 1883

Type species: *Cystodytes dellechiajei* (Della Valle 1877)

This genus is characterized by the presence of a capsule formed of layers of overlapping disc-shaped calcareous spicules that encases the abdomen of each zooid, and into which the whole zooid can withdraw when it contracts (f.i. because of any disturbance). Colonies are encrusting. Zooids are usually distributed in circles with the atrial apertures in the center, forming rudimentary systems. A general diagram of the zooid anatomy is shown in Fig. 1. There are 4 rows of stigmata (Fig.

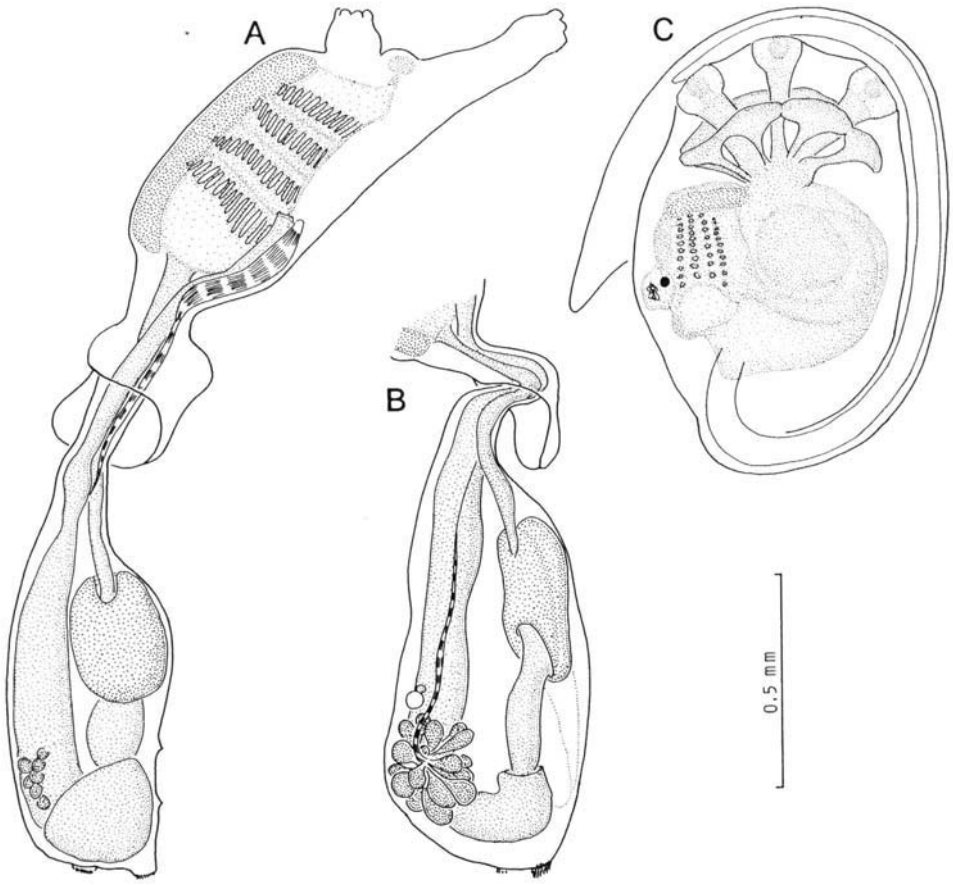


Fig. 1. *Cystodytes dellechiajei*. **A**. Zooid; **B**. Abdomen; **C**. Mature larva. From Monniot et al. (2001)

2). The round-shaped stomach is smooth and lies in the posterior half of the abdomen. Gonads, consisting of a circular arrangement of club-shaped testis follicles converging towards the vas deferens at the center of the circle, and an ovary containing generally only one oocyte, are in the gut loop. A single large embryo is incubated in the brood pouch, which is constricted off from the body wall at the top of the abdomen (Fig. 3). The larvae (Fig.4) has 3 stalked adhesive organs in the anterior mid-line surrounded by a circular fold of the larval ectoderm. As the larva matures, long radial grooves develop between the base of this fold and its anterior edge. Subsequently the grooves perforate leaving, embedded in the test around the adhesive organs, a ring of ectoderm attached by strands to the main part of the larval trunk (Kott 1990).

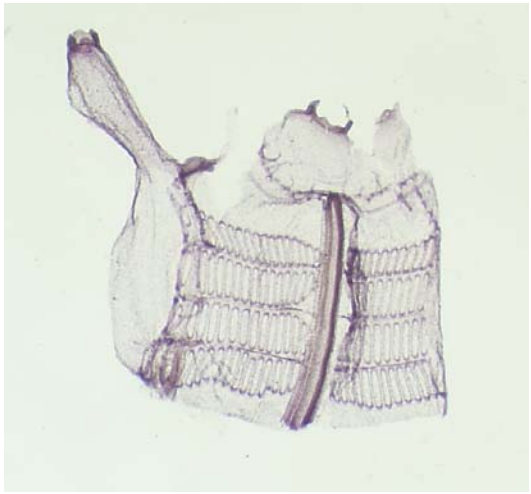


Fig. 2. Light micrograph of a dissected branchial sac showing the four rows of stigmata.

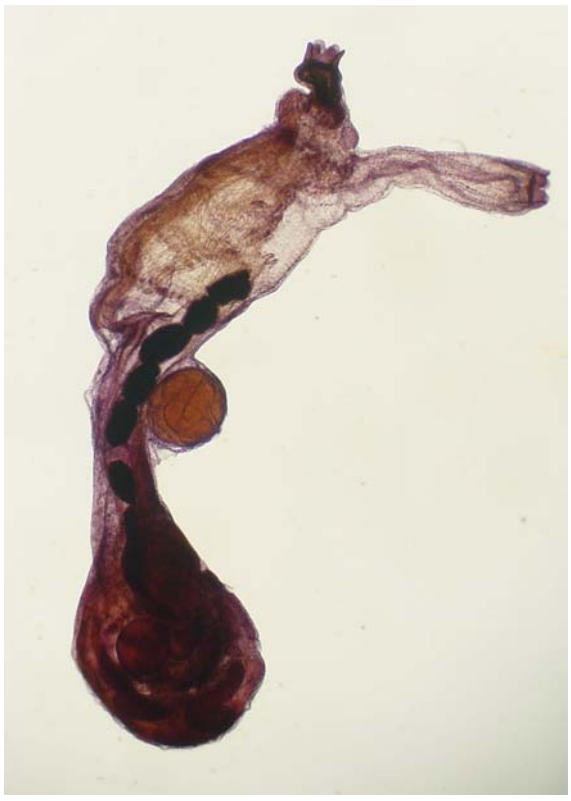


Fig. 3. Light micrograph of a zooid brooding an embryo.

Fig. 4. Mature larva with 3 adhesive organs after perforation of ampullary fold.

The taxonomic characters used in the assignment of species within this genus are unstable and largely influenced by authors' particular views, a problem derived from the morphological uniformity of the zooids and from their great contractility, which renders many characters difficult to observe (Van Name 1945, Monniot 1988, Kott 1990). On the other hand, color and colony size and shape show an amazing degree of variability (e.g. Harant 1929, Kott 1990, Monniot et al. 2001, see Fig. 5). Therefore, authors either tended to accept a restricted number of species while recognizing the difficulty of finding trustworthy characters at the specific level (Van Name 1945, Ärnbäck-Christie-Linde 1950, Kott 1990) or tried, on the basis of careful morphological examination, to disentangle the specific status within the genus in local areas (see Annex II for additional taxonomic information). Although Monniot (1970) concluded that spicular differences within the genus *Cystodytes* had no taxonomic value *per se*, they are still used in descriptions of new species, especially when the spicular features are distinctive (e.g. *C. ramosus* described by Kott 1992). Thus, the present specific diversity of the genus *Cystodytes* is largely a result of species assignment on the basis of color, shape, zooid morphology and spicular composition, on which there is no general agreement. Some of the observed morphological differences may also be due to intraspecies variability, as a result of genetic diversity or as a consequence of habitat, diet, chemistry or some combination thereof.

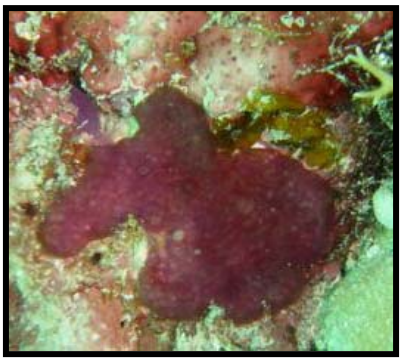
Cystodytes dellechiajei is distributed around the world in both tropical and temperate waters. Its distribution is also eurybathic, as some samples have been collected at a depth of 735 m (Monniot 1974). Color, texture, spicular composition, shape and zooid size may vary without any clear pattern of distribution (Turon 1987, Méliane 2002). As so many forms have been attributed to this taxon, it might well be a group of species (Monniot 1988). All Mediterranean specimens of the genus have been traditionally included in the species *C. dellechiajei* (Pérès 1958, Turon 1987, Brunetti 1994), but there may be other species as well (Brunetti 1994, Méliane 2002).

In addition, several pyridoacridines, a group of highly colored polycyclic aromatic alkaloids, have been reported in *C. dellechiajei* (Bonnard et al. 1995, Delfourne et al. 2000, Rottmayr et al. 2001). Among these are: ascididemin (Figure 6.1), first reported from the genus *Didemnum* (Kobayashi et al., 1988a) and *Eudistoma* (He & Faulkner, 1991); 11-hydroxyascididemin (Figure 6.2), first isolated from *Leptoclinides* sp. (Schmitz et al. 1991); cystodytins A-I (Kobayashi et

From Blanes (north Spain)

From Guam (USA)

From Gran Canaria (Spain)



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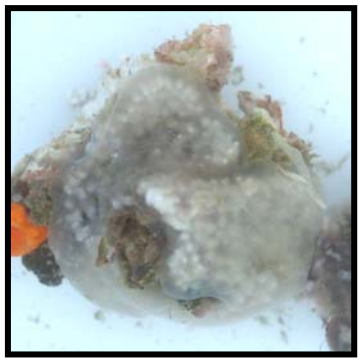

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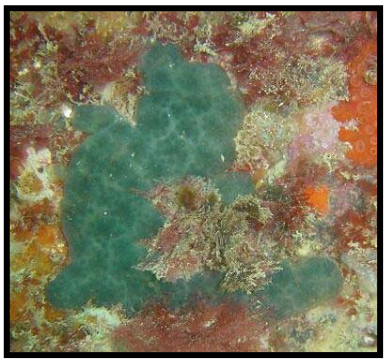
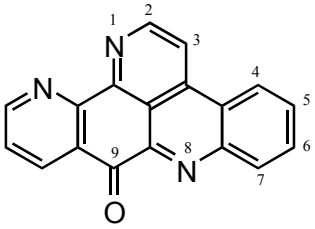


From Cabo de Gata (south Spain)

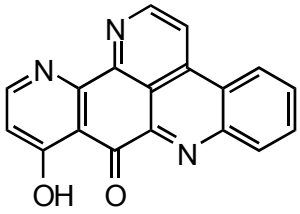
From Azores

From New Zealand

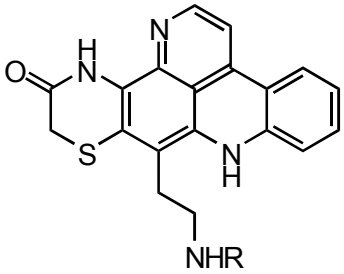
Fig. 5. Some color morphs of *Cystodytes* and their sapling location.



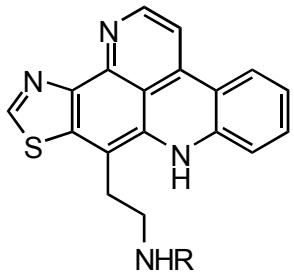
1: ascididemin



2: 11-hydroxyascididemin



3: R=$COCH_3$ shermilamine B



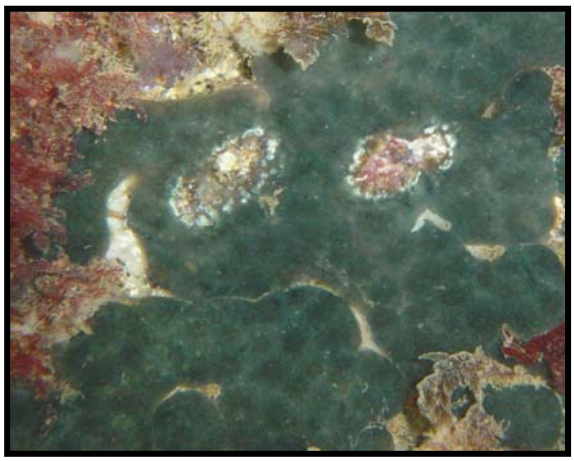
4: R=$COCH_3$ kuanoniamine D

Fig. 6. Structures of the six main alkaloids isolated from the studied specimens of *Cystodytes.* **1**) ascididemin, **2**) 11-hydroxyascididemin, **3**) shermilamine B, **4**) kuanoniamine D, **5**) deacetylshermilamine B, **6**) deacetylkuanoniamine D.

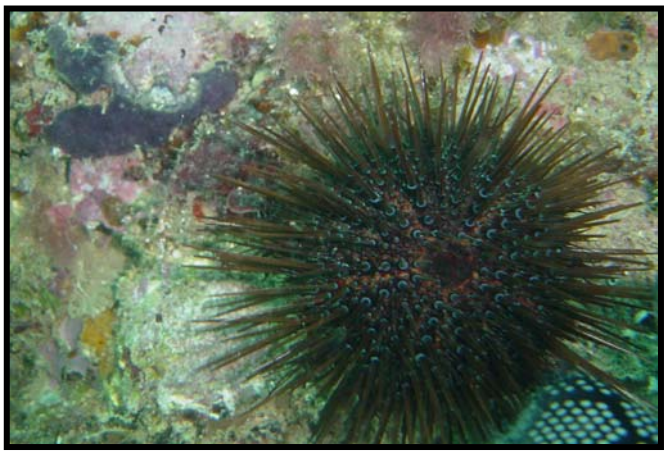
al. 1988b, Kobayashi et al. 1991); shermilamine B (Figure 6.3), first isolated from a *Tridemnum* species (Carroll et al. 1989); kuanoniamine D (Figure 6.4), first found in an unidentified ascidian (Caroll & Scheuer, 1990); and finally, sebastianines A and B (Torres et al. 2002). Some of these substances exhibit strong bioactivities and high cytotoxicity (Bonnard et al. 1995, Bowden 2000). Ascididemin also presents antibacterial and antifungal activities (Lindsay et al. 1995). To our knowledge, aside of a potential anti-fouling function (Debard et al. 1998), no ecological role of ascididemin has been described. In addition, *Cystodytes* colonies are also highly acidic (pH<2; Parry 1984, Tarjuelo et al. 2002). Colonies generally lack epibionts, present scarce signs of predation (Fig. 7), and are often observed competing for space with other species (Fig. 8). The vanadium concentration in this genus (≤ 10 ppm dry weight; Stoecker 1980a) is well under the concentration suggested to deter

predation in other ascidians (>1000 ppm dry weight; Stoecker 1980a). Tarjuelo et al. (2002) found a deterrent activity of the crude extract of a blue Mediterranean morph against two generalist predators. In contrast, the crude extract obtained from specimens collected at the same site showed no toxicity against sea urchin embryos and did not inhibit settlement of larvae of the bryozoan *Bugula neritina* (Becerro et al. 1997).

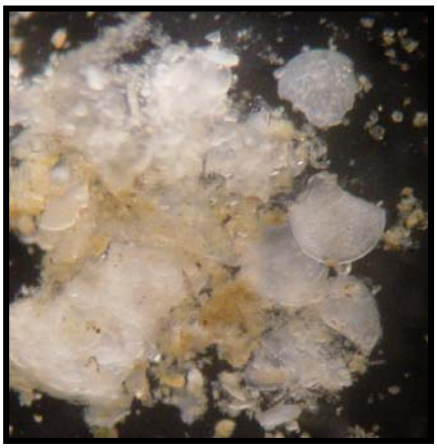
Hypselodoris orsinii on a blue colony



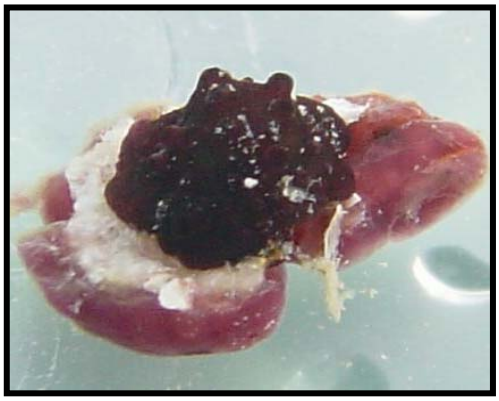
Sea urchin bites on a blue colony



Paracentrotus lividus bite on a purple colony



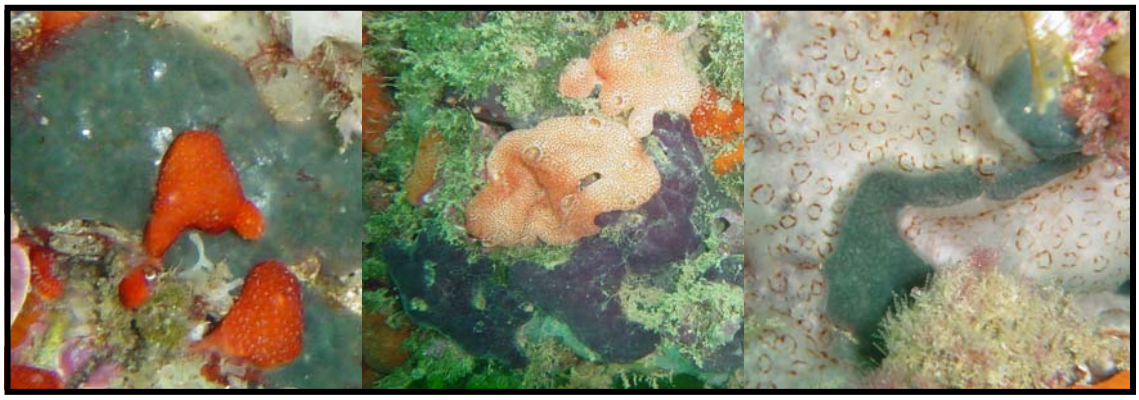
Cystodytes spicules in an urchin fesses



Coriocella sp on a purple colony from Guam (USA)

Fish bites on a purple colony

Fig. 7. Some potential predators and/or predator bites observed on *Cystodytes* colonies.



Didemnum sp. (red) *Didemnum* sp. (pink) *Cystodytes*

Calcareous sponge *Crambe crambe* *Ircina* sp.

Multiple competitors *Oscarella lobularis* *Diplosoma spongiformis*

Fig. 8. Some spatial competitors of *Cystodytes*.

1.3 OBJECTIVES AND STRUCTURE OF THE THESIS

Several studies have approached one or another aspect of the biology and ecology of marine organisms. Although these kind of studies increase general knowledge about a species or a phenomenon, they normally drive new questions that would generally need the use of a new methodology or discipline to get an answer. Although nowadays a panoply of techniques is available, their combined use remains often difficult. From there, the necessity of interacting with other researchers may arise. The main objective of the present work is to demonstrate that the application of several techniques, belonging to different fields, provides a better understanding of the biology and ecology of marine invertebrates. We chose the genus *Cystodytes* as a study case because previous work suggested intra and inter-species variability in biological features, a cosmopolitan distribution, multiple potential defenses against predation, and because it produces several pyridoacridine alkaloids of pharmaceutical interest.

Specifically, the objectives of this thesis are:

1. To identify the chemical variability between color morphs of *Cystodytes* occurring in the western Mediterranean.

2. To compare the cellular composition of the tunic between the most abundant color morphs, and to determine the secondary metabolites location and the presence of microsymbionts.

3. To determine, through experimental evidence, the ecological role of chemical and physical defenses in this genus.

4. To ascertain morphological and genetic variation among color morphotypes of *Cystodytes* and to relate them to chemical variation.

5. To analyze genetic variation and gene flow between populations of different color morphs.

6. To compare life-history patterns and reproductive strategies between the two most abundant color morphs in the area studied.

7. To ascertain the possible existence of trade-offs between allocation of resources to the production of secondary metabolites, structural material, and other biological functions (growth and reproduction).

In short, we wanted to combine chemical, genetical, ultrastructural and life-cycle information to better understand the defense mechanisms and biological strategies and interactions within this genus.

The thesis is structured in 7 main chapters that address the objectives pursued. Although all them are interconnected, each one was written as a whole unit to allow independent reading. Therefore, each includes an introduction, material and methods, results and discussion, and may occasionally contain cross-references to other chapters.

CHEMICAL VARIATION OF ALKALOIDS IN COLOR MORPHS OF *CYSTODYTES*

Most studies of secondary chemistry do not provide detailed information about the source organism. Therefore, it is currently impossible to match chemical with morphological variation in polymorphic species and genus. This problem hinders studies of potential biotechnological and pharmaceutical applications of these compounds. Nowadays, new techniques have been developed for the detection of substances in biological samples, including secondary ion mass spectrometry (SIMS) and matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF MS). These methods are highly sensitive, require only a few milligrams of sample material, and allow rapid detection of even small amounts of targeted substances.

The aim of this chapter is, first, to ascertain the alkaloid composition in the most abundant color morphs of *Cystodytes* from the western Mediterranean (green, purple, brown, and blue). Second, to determine the intraspecimen location of these alkaloids (tunic vs. zooids). Last, but not least, we wanted to assess the usefulness of MALDI-TOF MS techniques as a rapid and reliable tool in the detection of targeted substances, intraspecimen location, and chemotype assignment.

CELL TYPES, MICROSYMBIONTS AND PYRIDOACRIDINE DISTRIBUTION IN THE TUNIC OF *CYSTODYTES*

Studies on chemical variation of secondary metabolites in marine invertebrates are scarce and have mainly focused on ecological and seasonal variations. Less is known about the intraspecimen localization and production of these metabolites. Within-specimen location of bioactive compounds provides clues to the biological and ecological roles that these substances may play in the nature. Aside of secondary metabolite localization, investigations of morphology and classification of tunic cells are also necessary as a fundamental research in the group of ascidians.

The aims of this chapter are four-fold. First, we aim to characterize the ultrastructure of the main tunic cell types present in the most abundant color morphs of *Cystodytes* in the western Mediterranean (purple, blue, and green). Second, to determine whether differences in cellular composition exist between color morphs. Third, we study the presence and abundance of prokaryote symbionts in the tunic. Finally, we apply the X-ray microanalysis method to secondary metabolite location.

MULTIPLE DEFENSES AGAINST PREDATION

Defense mechanisms against predation in marine invertebrates can be structural, chemical or behavioral. However, and mainly because of experimental limitations, few studies have attempted to assess the importance of multiple defenses against predation in marine organisms. Ascidians have been reported to present both physical (spicules, tunic toughness) and chemical defenses. Secondary metabolites, spicular shape and concentration, high vanadium concentrations and a low pH have all been proposed as potential defenses against predation. Van Alstyne et al. (1994) pointed out the importance of considering all defense mechanisms that could be used by an organism when designing feeding experiments, rather than only addressing the effects of single defenses in isolation.

We studied 3 different morphotypes (two from the Mediterranean and one from the Pacific) belonging to the genus *Cystodytes*. Species of this genus present bioactive secondary metabolites, calcareous spicules and tunic acidity (pH < 1). The aim of this chapter was to determine whether these potential defense mechanisms

deterred feeding and whether they acted independently or synergistically in combination with each other.

HOW DO MORPHOYTPES AND CHEMOTYPES RELATE TO GENOTYPES?

Intraspecies variability in benthic invertebrates has been a long-standing source of taxonomic and biological controversy. Species with a large range of distribution may show morphological variants, usually related to their geographical or bathymetric distribution. Color, texture, general shape and other morphological characters may change from one organism or one place to another, sometimes in response to specific environmental conditions. Molecular approaches enable us to assess the genetic basis of such morphological variability. The correct assignment of species in benthic invertebrates is not merely a taxonomic issue, but has clear implications in applied aspects such as biodiversity management, tracing of invasions or determining sources of new pharmacologically active substances. The taxonomic characters used in the assignment of species within *Cystodytes* are unstable and largely influenced by authors' particular views.

The aim of this chapter is to determine how variability in traditional taxonomic characters such as color and spicular complement, relates to genetics and secondary chemistry in several *Cystodytes* morphotypes from the western Mediterranean. The morphology and spicular composition of 46 colonies of different colors and/or localities was examined by scanning electronic microscopy. A fragment of the mitochondrial gene COI was sequenced to define the haplotypes and to perform phylogenetic analyses.

POPULATION GENETICS, PHYLOGEOGRAPHY AND SPECIATION

Although studies of population subdivision and speciation are crucial to our understanding and management of marine biodiversity, the very concept of species is open to debate. In the former chapter we concluded that the morphological traits studied were not consistent enough to differentiate between *Cystodytes* species. Templeton (2001) showed how formal phylogeographic analyses of genetic data could provide useful insights at the interface between intra- and interspecific evolution, within the framework of the cohesion species concept. Although genetic

variability is expected, widely- distributed species must maintain a certain degree of genetic cohesiveness, mediated by gene flow, throughout their distribution range.

We have studied 7 populations of *Cystodytes* representative of the main color morphs and geographic areas of the western Mediterranean in an effort to explore the population subdivisions and phylogeographic relationships among them. MtDNA sequence data was used to analyze the degree of differentiation between these locations and to perform a phylogeographic analysis (NCA, nested clade analysis) of a haplotype network. We partitioned genetic variance into components associated with geographic origin and with color morphotypes and performed analyses of molecular variance (AMOVA). In this way, we wanted to clarify the relationship between the different color morphs and the genetic variation observed.

LIFE CYCLES AND GROWTH RATES OF TWO MORPHOTYPES OF *CYSTODYTES*

In modular marine invertebrates the interaction between genotype and environment often generates a distinct profile of growth, fission, fusion, regeneration, and senescence for each clone. The study of biological cycles in colonial ascidians, including growth, reproduction, and mortality, often reveals complex patterns. Temperate seas display marked seasonal fluctuations in environmental parameters that are reflected in many of the life cycle patterns observed in ascidians. Seasonality has commonly been linked to temperature, but other factors, such as nutrient availability, should also be considered. Although genetics and secondary chemistry provide important clues about species boundaries, the study of biological cycles remains essential to assess speciation events according to the biological and cohesion species concepts, and provides crucial information about the ecology and biology of benthic invertebrates.

The aims of this chapter are: to compare the reproductive traits, growth, and survival of the two most abundant morphotypes of the genus *Cystodytes* occurring in the western Mediterranean (purple and blue). To correlate these biological traits with other parameters, such as colony size, water temperature, and the presence or absence of nearby competitors. Finally, we wanted to ascertain whether previously found genetic and chemical divergence correlate with differences in biological parameters, providing clues as to the degree of reproductive isolation of these morphotypes and their taxonomic status.

ADJUSTING THE BUDGET: IS TEMPORAL VARIATION IN ASCIDIDEMIN PRODUCTION RELATED TO OTHER BIOLOGICAL PARAMETERS?

Little is known about the relationship between secondary metabolite chemistry, physical defenses and biological life cycles in marine invertebrates. Optimal defense theory assumes that allocation of resources to chemical and physical defenses must be optimized with respect to the needs of an organism and its energy budget (growth, somatic maintenance and repair, and reproduction). If so, we can expect to find a temporal variation in their production, depending on the environmental constraints and on the physiological state of the source organism. However, to our knowledge, no serious attempt to link this variability to life cycle features of the species exists.

The aim of this Chapter is to assess temporal variability in the production of defenses in the blue Mediterranean morph of *Cystodytes*. To study the variation in chemical defense production, we quantified ascididemin, the main pyridoacridine alkaloid found in this morph. Physical defense variability was estimated by calculating the colony ash content, as it mainly contains spicules and structural material. The main question is whether there is a predictable change in the amount of energy invested in chemical and physical defense production in relation to other biological parameters such as investment in reproduction and growth (analyzed in Chapter 7)..

The last part of the thesis is a general discussion of the results presented in previous chapters, here analyzed conjointly in an attempt to situate them within a single coherent global framework. A general bibliography is given at the end of the thesis in order to avoid repetitive references in the different chapters.

CHAPTER 2

CHEMICAL VARIATION OF ALKALOIDS IN COLOR MORPHS OF *CYSTODYTES*

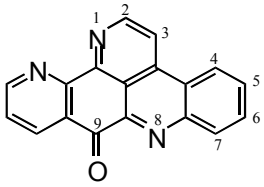
2.1 INTRODUCTION

Marine invertebrates are a rich source of bioactive secondary metabolites with cytotoxic, antimicrobial, antifungal, antiviral and antifouling activities (Ireland et al. 1988, Bhakuni & Jain 1990, Uriz et al. 1991, Becerro et al. 1997, McClintock & Baker 2001, Faulkner 2002). These secondary metabolites are assumed to perform ecological functions as chemical defenses against predators, pathogens, spatial competitors, ultraviolet radiation and fouling organisms (Bakus 1981, Coll et al. 1982, Paul 1992, Pawlik 1993, Hay 1996, Becerro et al. 1997, McClintock 1987, Lindquist 2002). Analysis of the variation in secondary metabolite production at several levels, ranging from the individual to the geographic area, is crucial to the understanding of their ecological relevance (Hay 1996). However, these studies are scarce and have mainly focused on geographical and seasonal variations (e.g. Harvell et al. 1993, Valls 1993, Maida et al. 1993, Turon et al. 1996, Fahey & Garson 2002, Martí 2002). Even less is known about the intraspecimen localization and production of these metabolites: studies at the intraorganismal level have usually addressed the role of endosymbionts in the production of chemical defenses (e.g. Unson et al. 1994, Turon et al. 2000, Salomon & Faulkner 2002).

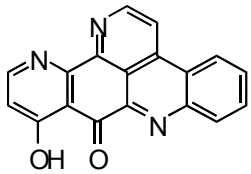
Ascidians, like other marine taxa, show some cases of high intraspecies variability (e.g. *Botryllus schlosseri*, *Ciona intestinalis*: Hoshino & Nishikawa 1985, Monniot et al. 2001, Stoner et al. 2002), color variation being the most frequently

described difference (e.g. Aron & Solé-Cava 1991, Dalby 1997, Monniot et al. 1991, Tarjuelo et al. 2004). Ascidians also exhibit one of the strongest antibacterial and antifungal bioactivities found among benthic invertebrates (Uriz et al. 1991), although the chemical compounds responsible for this activity have rarely been identified (Paul et al. 1990, Davis 1991, Vervoort et al. 1998).

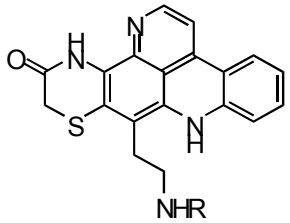
Cystodytes (Della Valle 1877) (Aplousobranchiata, Polycitoridae) is a colonial ascidian genus widely distributed in both tropical and temperate waters. In spite of a noticeable variability, previous reports of *Cystodytes* in the Mediterranean waters have been attributed to the widespread species *C. dellechiajei*. Color variation has been extensively reported (see review Kott 1990) in *C. dellechiajei* leading some authors to suggest that there may be several species present (Turon 1987, Brunetti 1994). In addition, several pyridoacridines, a group of highly colored polycyclic aromatic alkaloids, have been reported (Bonnard et al. 1995, Delfourne et al. 2000, Rottmayr et al. 2001). Among these are: ascididemin (Fig. 1.1), first reported from the genus *Didemnum* (Kobayashi et al. 1988a) and Eudistoma (He & Faulkner 1991); 11-hydroxyascididemin (Fig. 1.2), first isolated from *Leptoclinides* sp. (Schmitz et al. 1991); cystodytins A-I (Kobayashi et al. 1988b, Kobayashi et al. 1991); shermilamine B (Fig. 1.3), first isolated from a *Tridemnum* species (Carroll et al. 1989); kuanoniamine D (Fig. 1.4), first found in an unidentified ascidian (Caroll



1: ascididemin

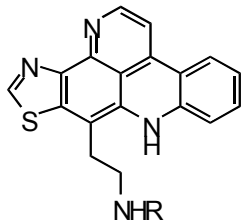


2:11-hydroxyascididemin



3: R=$COCH_3$ shermilamine B

5: R=H deacetylshermilamine B



4: R=$COCH_3$ kuanoniamine D

6: R=H deacetylkuanoniamine D

Fig. 1. Structures of the six main alkaloids isolated from the studied specimens of *Cystodytes.* 1) ascididemin, 2) 11-hydroxyascididemin, 3) shermilamine B, 4) kuanoniamine D, 5) deacetylshermilamine B, 6) deacetylkuanoniamine D.

& Scheuer, 1990); and finally, sebastianines A and B (Torres et al. 2002). Some of these substances exhibit strong bioactivities and high cytotoxicity (see Bowden 2000). Ascididemin also displays antibacterial, antifungal (Lindsay et al. 1995) and antifouling activities (Debard et al. 1998).

Most studies of secondary chemistry do not provide detailed information about the source organism, let alone an in-depth description of morphology and spicular types. Therefore, it is currently impossible to match chemical with morphological variation in this polymorphic genus. This problem hinders studies of potential biotechnological and pharmaceutical applications of these compounds, or of the systematics and taxonomy of *Cystodytes*.

Nowadays, new techniques have been developed for the detection of substances in biological samples, including secondary ion mass spectrometry (SIMS) and matrix-assisted laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF MS) (e.g. Pacholski & Winograd 1999, Todd et al. 2001, Stoeckli et al. 2001). These methods are highly sensitive, require only a few milligrams of sample material, and allow rapid detection of even small amounts of targeted substances. In particular, MALDI-TOF MS has become a technique of choice for the localization and characterization of peptides and proteins (Caprioli et al. 1997, Uttenweiler-Joseph et al. 1998, Stoeckli et al. 2001).

The aim of this study is firstly to ascertain the alkaloid composition in the most abundant color morphs of *Cystodytes* from the western Mediterranean (green, purple, brown, and blue). In addition, we analyze the intraspecimen location of these alkaloids (tunic vs. zooids), and assess the usefulness of MALDI-TOF MS techniques as a rapid and reliable tool in the detection of targeted substances, intraspecimen location, and chemotype assignment.

2.2 MATERIAL AND METHODS

2.2.1 Ascidian samples

Specimens of the ascidian *Cystodytes* were sampled in shallow water habitats (usually <7 m deep) by scuba diving in three different zones of the western Mediterranean in 2002 (Fig. 2): Catalonia (NE Spain), Cabo de Gata (SE Spain) and Ibiza-Formentera (Balearic Islands). Four main color morphs were found: blue, purple, green, and brown. The specimens were identified by the authors as belonging to the genus *Cystodytes*, based on the descriptions of Turon (1987) and Kott (1990). To determine the intraspecimen location of the alkaloids, we separated the zooids from the tunic and their spicular capsules using forceps under a binocular microscope. All samples were directly frozen and stored at -30ºC prior to lyophilization and further analyzes.

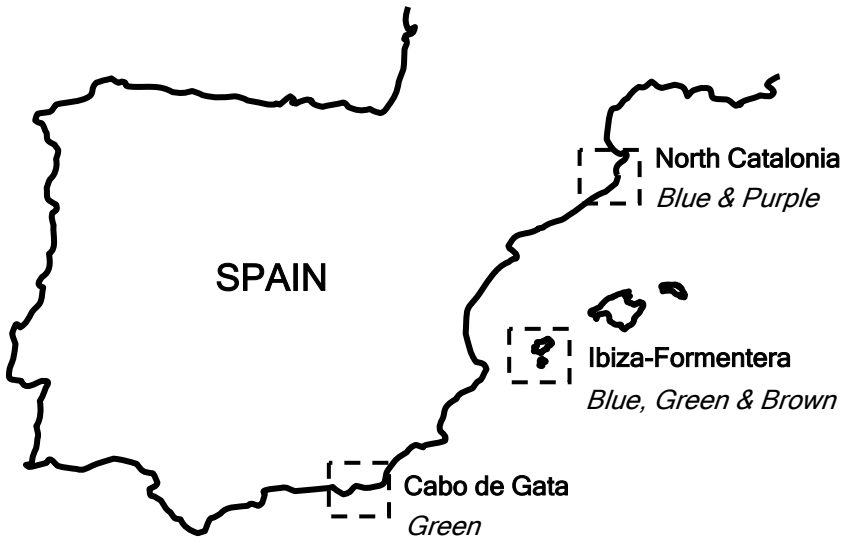


Fig. 2. Map showing the zones sampled: northern Catalonia, Ibiza-Formentera and Cabo de Gata and the color morphs present at each locality.

For MALDI-TOF MS, three colonies of each color morph were analyzed separately for each locality. We also separated tunic from zooids of the purple and blue Catalonia morphs, and the green Cabo de Gata morph and performed 3

separate replicate analyses for each using MALDI-TOF MS. For HPLC analysis, the extracts of several colonies from the same sampling site were pooled together to isolate the main pyridoacridines.

2.2.2 MALDI-TOF analysis

We followed a standard microextraction protocol to detect a broad pattern of metabolites, including peptides. For each colony, between 0.5 and 5 milligrams of freeze-dried material were mixed with solvent A [acetonitrile:methanol:water, 1:1:1, supplemented with 0.3 % TFA at a ratio of 1:5 (w:v)]. In a solvent mixture of dichloromethane:methanol (used for HPLC extraction), the alkaloids appear as free bases, whereas in solvent A they are extracted as TFA salts. Both methods allowed complete extraction of pyridoacridines. The use of a slightly more hydrophobic solvent (acetonitrile) is commonly applied in peptide detection, and does not interfere with pyridoacridine extraction. The supernatant (0.5 µl) was spotted onto a target well of a 100-position stainless steel sample plate and immediately mixed with 0.5 µl of the matrix solution (50 mg/ml 2,5-dihydroxybenzoic acid dissolved in solvent A).

Measurements were performed on a VOYAGER DE-PRO time-of-flight mass spectrometer from Applied Biosystems (Foster City, USA) equipped with a reflectron. No high-resolution imaging device was available. The acceleration voltage was set to 20 kV. Mass spectra obtained in delayed extraction mode allowed the determination of monoisotopic mass values (m/z; mass-to-charge ratio). For desorption of the components, a nitrogen laser beam (λ=337 nm) was focused on the template. Analyses were performed in positive reflector mode. A spectrum was compiled by averaging results from at least 150 shots taken across the width of the sample. After determination of monoisotopic mass values, post-source decay (PSD) measurements for recording fragment ions were performed.

2.2.3 Pyridoacridine alkaloid isolation and identification (HPLC and NMR)

We followed the optimized protocol for extraction, isolation and identification of pyridoacridine alkaloids of *C. dellechiajei*, described by Bontemps (1996). The freeze-dried colonies were extracted three times in a 1:1 (v:v) mixture of dichloromethane and methanol and then the combined extracts were concentrated under vacuum leaving a powdery organic residue. The different crude extracts were

compared by HPLC analysis (Alliance, Waters) with a photodiode array detector (PDA), and the resulting UV spectra of the different peaks were compared with the UV database available in our laboratory. To isolate and identify the major pyridoacridines, the organic extracts were submitted to successive chromatography. Each residue was mixed with RP-8 material (LiChroprep®, 40-63 μm, Merck) at a ratio of 1:6 (w:w), evaporated to dryness, and then subjected to RP-8 column chromatography using water and increasing proportions of methanol. Fractions displaying an orange coloration on TLC with Dragendorff reagent were purified by HPLC using Jasco 880-PU pumps under the following conditions: solvent, methanol:water 7:3 (v:v) with a flow rate of 2.5 ml/min; column, Uptisphere UP5ODB-25M; UV (Jasco 875 UV-vis detector) and light diffusion detector (Polymer Laboratories). Pyridoacridine alkaloids were characterized by proton and carbon nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C NMR; Jeol EX 400 spectrometer) and UV spectroscopy (Hewlett Packard diode array spectrophotometer). Spectral values of the purified metabolites were compared with reference compounds available in our laboratory and with published values from the literature.

2.3 RESULTS

2.3.1 MALDI-TOF MS results

Peak clusters at m/z 286 and m/z 301, corresponding to ascididemin (1) and 11-hydroxyascididemin (2) respectively, were found in the MS spectrum of the blue morphs from Catalonia and Ibiza-Formentera and the green form from South Spain and Ibiza-Formentera (Table 1). Ascididemin was also detected in the brown morph from Catalonia and Ibiza-Formentera. In the mass spectra obtained from the purple form, we detected peaks at m/z 361 and 391, corresponding to protonated molecular ions of shermilamine B (3) and kuanoniamine D (4) $[M+H]^+$ respectively. The PSD-fragmentation spectra of the peaks at m/z 319 and 349 were very similar to those of shermilamine B and kuanoniamine D but were shifted to a lower mass by 42 absolute mass units. Therefore, they were attributed to their deacetylated forms: deacetylshermilamine B (5) and deacetylkuanoniamine D (6), respectively.

alkaloid	mass observed (m/z)	purple	green	blue	brown
ascididemin	286	-	HPLC / MS	HPLC / MS	MS
11-hydroxyascididemin	301	-	HPLC / MS	HPLC / MS	-
deacetylkuanoniamine D	319	HPLC / MS	-	-	-
deacetylshermilamine B	349	HPLC / MS	-	-	-
kuanoniamine D	361	HPLC / MS	-	-	-
shermilamine B	391	HPLC / MS	-	-	-

Table 1. Main pyridoacridine alkaloids present in the four color morphs of *Cystodytes*.
HPLC: Compounds isolated and identified after HPLC/PDA analyses and characterized by NMR and UV spectroscopy.
MS: Compounds detected by MALDI-TOF spectrometry, with indication of their mass signal.

Independent analyses of the tunic and zooids revealed no major differences between body components in the green and blue morphs. In the tunic of the purple morph the four major compounds (3, 4, 5, 6) were found together, whilst in the zooids only compounds 5 and 6 were present (Fig. 3). No differences in alkaloid composition were found between the replicate analyses of the same color morph, locality, or intraorganism body component (n = 3).

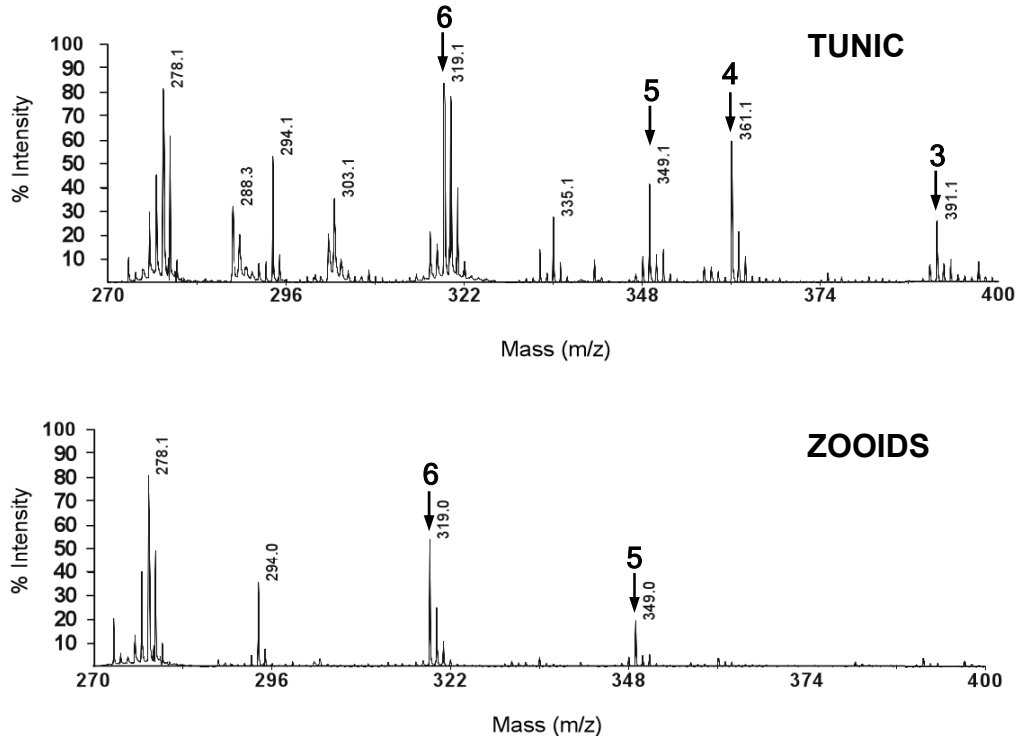


Fig. 3. Purple morph MALDI-TOF spectra of the intraspecimen location (tunic and zooids) of the major compounds. **3**) shermilamine B, **4**) kuanoniamine D, **5**) deacetylshermilamine B, and **6**) deacetylkuanoniamine D.

2.3.2 Pyridoacridine alkaloid isolation and identification (HPLC and NMR)

The major pyridoacridine alkaloids isolated from the different color morphs of *Cystodytes* are listed in Table 1. They belonged to two classes: C_9-unsubstitued pyridoacridines and sulfur-containing pyridoacridine ethylamine alkaloids. Although 1 and 2 were detected in all of the blue and green samples, 1 was isolated from the blue morph found in Catalonia and 2 from the green morph found in southern Spain. In the purple specimens collected in Catalonia four significant peaks were observed. The first two were identified as 3 and 4. The UV spectra of the other two peaks were identical to 3 and 4 and after structure elucidation by NMR were identified as their deacetylated forms: 5 (Fig. 1.5) and 6 (Fig. 1.6, Eder et al. 1998). No alkaloid was observed in the brown morph from Ibiza-Formentera using the same extraction and analytical procedures.

2.4 DISCUSSION

Our study revealed two major chemotypes within the four color morphs of *Cystodytes* studied. The first had sulfur-containing pyridoacridines and corresponded to the purple morph, whilst the second had C_9-unsubstituted pyridoacridine alkaloids and was found in the blue and green morphs. The purple morph contained 3 and 4, as noted previously by Rottmayr et al. (2001), together with their deacetylated forms (5 and 6). Eder et al. (1998) described 6 in the sponge *Oceanapia* sp. More recently, Skyler & Heathcock (2002) proposed a pyridoacridine family tree in which they predicted the existence of natural pyridoacridines such as 5, found here for the first time. To our knowledge, the coexistence of free ethylamine precursors and their acetamide forms has never been described before. Pyridoacridine alkaloids are pH-sensitive pigments. Compounds 3, 4, 5, and 6 are purple under acidic conditions. This helps to explain the purple color observed in the colonies, as the tunic of *Cystodytes* is highly acidic (pH<1; Tarjuelo et al. 2002). Similarly, the violet of another species, *Cystodytes violatinctus* Monniot 1988, is probably due to the presence of shermilamine analogs (Koren-Goldshager et al. 1998, 2000). Both compounds 1 and 2 are yellow under acidic conditions. The link between color and pyridoacridine composition is less evident in these latter cases, and may depend on other unknown molecules.

In the tunic of the purple morph the four major compounds (3, 4, 5, 6) were found together, whilst the apparent lack of 3 and 4 in the zooids, indicate two possible hypotheses: 1) The pyridoacridines are produced as free amines (5 and 6) in the zooids, and then distributed and stored in the tunic as acetylated forms (3 and 4), the tunic being the tissue most exposed to predators. 2) The most active metabolites are the free ethylamine compounds (5 and 6), present in both the tunic and the zooids. Particularly pertinent to this issue will be the identification and localization of the type or types of cells responsible for the production and/or storage of the active compounds in the different forms and the assessment of their ecological role. Underpinning most studies of chemical variation is the optimal defense theory, which assumes that chemical and morphological defenses in living organisms are costly, and that natural selection will favor an allocation of resources to defenses that optimize their cost/benefit ratio in terms of fitness (Rhoades 1979, Fagerström et al. 1987). Comparative studies of the bioactivity of the different forms,

as well as of their potential metabolic costs, will be needed in order to adequately place our findings within the framework of the optimal defense theory.

This study has shown the usefulness of MALDI-TOF MS as a quick and reliable tool for the detection of targeted low molecular mass compounds at both colony and intraspecimen levels. In addition, and due to its high sensitivity, the MALDI-TOF technique provided clues as to the presence of compound 1 in the brown morph that was undetected under the HPLC conditions used. Although in this study we applied MALDI-TOF techniques qualitatively to assess chemotypes among morphotypes (i.e. the presence/absence of already known alkaloids), further possible uses of this technique include the use of standards for quantification analyses and, especially, the co-application of imaging techniques. The full range of applications of this technique should improve our capacity for chemical detection and intraspecimen location of targeted compounds, which represents an important aspect of all descriptive and experimental work in the field of marine chemical ecology.

Finally, the chemical differences between color morphs raised concerns about the taxonomic status of the specimens routinely attributed to *C. dellechiajei* in the western Mediterranean. Some authors favor the view that several species have been grouped under the name *C. dellechiajei* (e.g. Monniot 1988), whilst others have found little basis for splitting this circumtropical species (e.g. Kott 1990). Whether the detected chemical variability reflects the existence of 2 chemotypes within a single species or whether we are dealing with a group of sibling species is still an open question. Careful compilations of morphological data coupled with biological studies, as well as an assessment of population genetic structure will be necessary to clarify the taxonomic status of this genus definitively. Our results highlight the importance of a detailed morphological description of the producer organism of marine natural products, especially for taxa in which the taxonomy is not well resolved, in order to fully understand the variation in secondary chemistry within and between species.

CHAPTER 3

CELL TYPES, MICROSYMBIONTS AND PYRIDOACRIDINE DISTRIBUTION IN THE TUNIC OF *CYSTODYTES*

3.1 INTRODUCTION

The ascidian tunic is a biologically active compartment that mediates many of the interactions between these animals and the environment. In particular, most defense interactions occur in the tunic; such interactions are either physically (toughness, spicules) or chemically (acidity, secondary chemistry) mediated. The tunic is analogous to a mesenchymatous tissue formed by a matrix of variable consistency and diverse cellular components (Hirose et al.1991, 2001). The tunic matrix contains fibres of cellulose-like polysaccharides associated with collagen and elastin-like proteins (De Leo et al. 1977, Patricolo & De Leo 1979, Van Daele & Goffinet 1987). The matrix contains a variety of free cells, most of them vagile, that perform multiple functions, such as pigmentation, acid storage, impulse conduction, contraction, and allorecognition (Mackie & Singla, 1987, Hirose et al. 1997, Hirose 1999, 2001). All these cell types seem to arise from blood cells that migrate into the tunic (Godeaux 1964, Cloney & Grimm 1970, Hirose et al. 2003). The tunic is also the site in which microsymbiont populations are found in many species (e.g. Lewin 1984, Hirose & Saito 1992, Groepler & Schuett 2003).

The tunic material, which makes up approximately 95 % of the total dry weight of the colonies in the genus *Cystodytes* (Tarjuelo & Turon 2004), is a cell-rich

covering (Lambert 1979, Rottmayr et al. 2001) that encases the zooids to form encrusting sheets 5-9 mm thick and up to several hundred cm^2 in area. The tunic is responsible for the various, apparently redundant, defense mechanisms described in *Cystodytes* colonies (Tarjuelo et al. 2002). These mechanisms include acid release, generation of calcium carbonate spicules that shield the zooids, and production of bioactive alkaloids (Kobayashi et al. 1988, Carroll & Scheuer 1990). In the western Mediterranean, colonies of the genus *Cystodytes* have usually been assigned to the circumtropical species *C. dellechiajei* (Della Valle, 1877) (Turon 1987, Brunetti 1994), in spite of a noticeable variability in terms of colony color, secondary chemistry, and spicule composition. In Chapter 2 we found two major chemotypes within the four color morphs studied, suggesting the existence of at least two different species in the Mediterranean. The first had the sulfur-containing pyridoacridines shermilamine B and kuanoniamine D (and their deacetylated forms) and corresponded to the purple morph, whilst the second featured the C_9-unsubstituted pyridoacridines ascididemin and 11-hydroxyascididemin and was found in the blue and green morphs.

Several pyridoacridine alkaloids have been isolated from sponges, ascidians, molluscs, and cnidarians (Molinski 1993, Salomon et al. 2001), and some biotransformation relationships have been hypothesized among them (Skyler & Heathcock 2002, Wenzel & Crews 2003). The wide taxonomical range of these compounds suggests the possibility that they might be produced by symbiotic bacteria. Alternatively, they may constitute a clear instance of convergent evolution of an efficient biosynthetic pathway (Salomon et al. 2001). Although the dichotomy between production of bioactive metabolites by the host or by prokaryote symbionts is a long-standing issue in sponge studies (e.g. Elyakov et al. 1992, Unson & Faulkner 1993, Faulkner et al. 1994, Uriz et al. 1996b), it has received much less attention in ascidians (e.g. Salomon & Faulkner 2002, Moss et al. 2003), and sometimes contrasting results have been obtained (Degnan et al. 1987, Biard et al. 2004). A comprehensive description of the cell types and microsymbionts present in the tunic and the cellular location of bioactive compounds could, therefore, yield important information about the source and biological roles of secondary metabolites in ascidians. To our knowledge, the only previous study of tunic morphology in *C. dellechiajei* was undertaken by Rottmayr et al. (2001) using light and confocal laser microscopy. In our study, we have also attempted to determine the within-organism alkaloid distribution by comparing the spectra obtained from energy dispersive X-ray

microanalysis of semithin sections (targeting the sulfur element of the purple morph alkaloids), a technique successfully used in sponges (Thompson et al. 1983, Turon et al. 2000) and never used in ascidians before.

The goals of this paper are four-fold. First, we aim to characterize the ultrastructure of the main tunic cell types present in the most abundant color morphs of *Cystodytes* in the western Mediterranean (purple, blue, and green). Second, to determine whether differences in cellular composition exist between color morphs. Third, we study the presence and abundance of prokaryote symbionts in the tunic. Finally, we apply the X-ray microanalysis method to secondary metabolite location.

3.2 MATERIAL AND METHODS

3.2.1 Sampling and preparation procedures

Specimens of the three color morphs of *Cystodytes* were sampled from the following sites on the Spanish Mediterranean coast: purple morph at Illes Medes (42° 02.5' N 3° 13.3' E), blue morph at Palamós (41° 50.4'N 3° 07.6' E), and green morph at Aguamarga, Cabo de Gata (36° 53.5' N 1° 57.9' W). Five colonies of each morphotype were collected by scuba diving and fixed immediately. In addition, some unfixed colonies were cut into slices and observed in vivo under stereo and light microscopes. Ultrastructural observation was performed on the three morphotypes, and microanalysis techniques were applied to the purple and blue forms.

Small fragments (about 1 mm^3) were cut from the distal and basal regions of the colonies and were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2; osmolarity adjusted to 980 mOsm with sucrose) for about 6 h at 4°C. They were then postfixed with OsO_4 (1% in the same buffer) for 2h at 4°C. To avoid problems in sectioning due to the presence of calcareous spicules, some of the samples were decalcified for 2 h in 1% acetic acid in cacodylate buffer. Several washes in the buffer were performed at each step of the fixation procedure. The samples were then dehydrated in a graded ethanol series and embedded in Spurr resin at room temperature. Semithin (0.3 µm) and ultrathin sections (ca. 60 nm) were cut with a Reichert Ultracut microtome. Ultrathin sections were contrasted with uranyl acetate and lead citrate for ultrastructural observation (Reynolds 1963). Semithin sections were stained with methylene blue for light microscopic observation, while others were left unstained for use in microanalysis. The latter were mounted on Cu grids and carbon-coated to reduce specimen charging under the beam. Microanalysis and ultrastructural examination were performed on Hitachi H800 MT and Hitachi H600 microscopes respectively (Hitachi Ltd., Tokyo); both belong to the Microscopy Unit of the Scientific and Technical Services of the University of Barcelona.

3.2.2 Energy dispersive X-ray microanalysis (ED-XRMA)

Under the impact of the beam in an electron microscope, electrons from the atoms in the sample are displaced from the inner orbital shells and replaced by electrons from outer shells. This last transition produces X-rays that can be

detected. The orbital shells are designated K, L, and M (from inner to outer), and the energy released by a replacement depends on the element involved and the electron jump, resulting in a series of signals that are characteristic for each element. These signals can be examined in a spectrum of X-ray energies obtained when the beam focuses on a particular structure.

For microanalysis, an acceleration of 100 Kv was used in STEM mode. Electron beam excitation was detected with a thin window (10 mm^2) Kevex detector (Kevex Corporation, California) connected to a Kevex 8000 analytical system with Quantex 6.13 software. The gain rate was adjusted to 1500-2000 $counts \cdot s^{-1}$ and acquisition time was set to 100 s.

The presence of signals corresponding to sulfur was examined in the spectra obtained. Since a certain amount of sulfur is present in all organic tissues, we looked specifically for structures giving a clear sulfur signal in the purple morph but not in the blue morph. Our rationale was that these structures are likely to represent storage sites of the alkaloid compounds, as the pyridoacridines of the two morphotypes differ in the presence of sulfur in the purple morph compounds. The energy peak corresponding to the signals Kα (peak at 2.3 keV) emanating from sulfur was easily discernible and did not overlap with other peaks in the samples. Therefore, the presence of this peak was preferentially used as a qualitative marker of the presence of sulfur. A second, quantitative measure used (following Turon et al. 2000) was the height ratio of the S Kα peak to the Cl Kα peak at 2.6 keV, as the ubiquitous chloride signal came mostly from the embedding Spurr resin and could be used as internal standard to control for the differences in the area scanned, magnification, and count rate (Thompson et al. 1983, Turon et al. 2000). The use of the Br/Cl ratio allowed formal statistical comparison (ANOVA) between morphotypes and between the compartments under study.

The X-ray analyses were performed at high magnification, producing spectra for small windows that covered one single cell, organelle, or bacterium. The electron beam covered the area studied in a raster pattern. Five to ten replicates were obtained from different sections for each of the main cell types, bacteria, and tunic ground substance. Spectra were also obtained from the Spurr resin outside the specimens to control for possible contamination.

3.3 RESULTS

The tunic organization and cell types in the three color varieties of *Cystodytes* studied are basically the same, with slight differences in the relative abundance of some cell types and in ultrastructural details. We will therefore present the results according to cell type and indicate differences between morphotypes where applicable. Different names have been given in the literature to the same tunic cell types in ascidians, many of them being derived from names applied to presumably equivalent cell types in the blood. We have chosen to use the names generally accepted by recent workers and, where possible, to keep the same names that were used by Rottmayr et al. (2001).

3.3.1 General structure of the colony and tunic

The tunic sections have a reticulated appearance due to the abundance of bladder cells (see below) that restrict tunic material and other cell types to the interstices between them (Figs. 1-2). These interstices are somewhat larger in the basal portions of the tunic (Fig. 3). The zooids are located in cavities whose basal half is surrounded by a layer of overlapping discoidal spicules (marked by empty spaces in the decalcified material, Fig. 4). In living specimens, the abdomens lie within this capsule of spicules, with the thoraces expanded in the distal half of the cavities and reaching the apical surface of the tunic where the two siphons open. When disturbed, the thorax retracts and both thorax and abdomen lie within the spicular capsule.

The tunic material consists of a clear matrix traversed by bundles of fibrils. These fibrils are more abundant in the distal-most region of the tunic, and they coalesce to form a thick cuticular layer over the surface of the colony (Fig. 5). Cyanobacteria, diatoms, and other microscopic epibionts are commonly found on the surface of the colonies (Fig. 5), which nevertheless lack macroscopic epibionts. A less defined cuticular layer also lines the upper half of the zooid cavities (where they lack the spicular envelope).

Figures 1-6

Fig. 1. Purple morph. Light micrograph of a semithin section of the distal part of a colony. The vacuoles correspond to bladder cells (BC), some pigment cells are indicated by asterisks. Other cell types are visible in the interstices left by the bladder cell. Methylene blue staining.

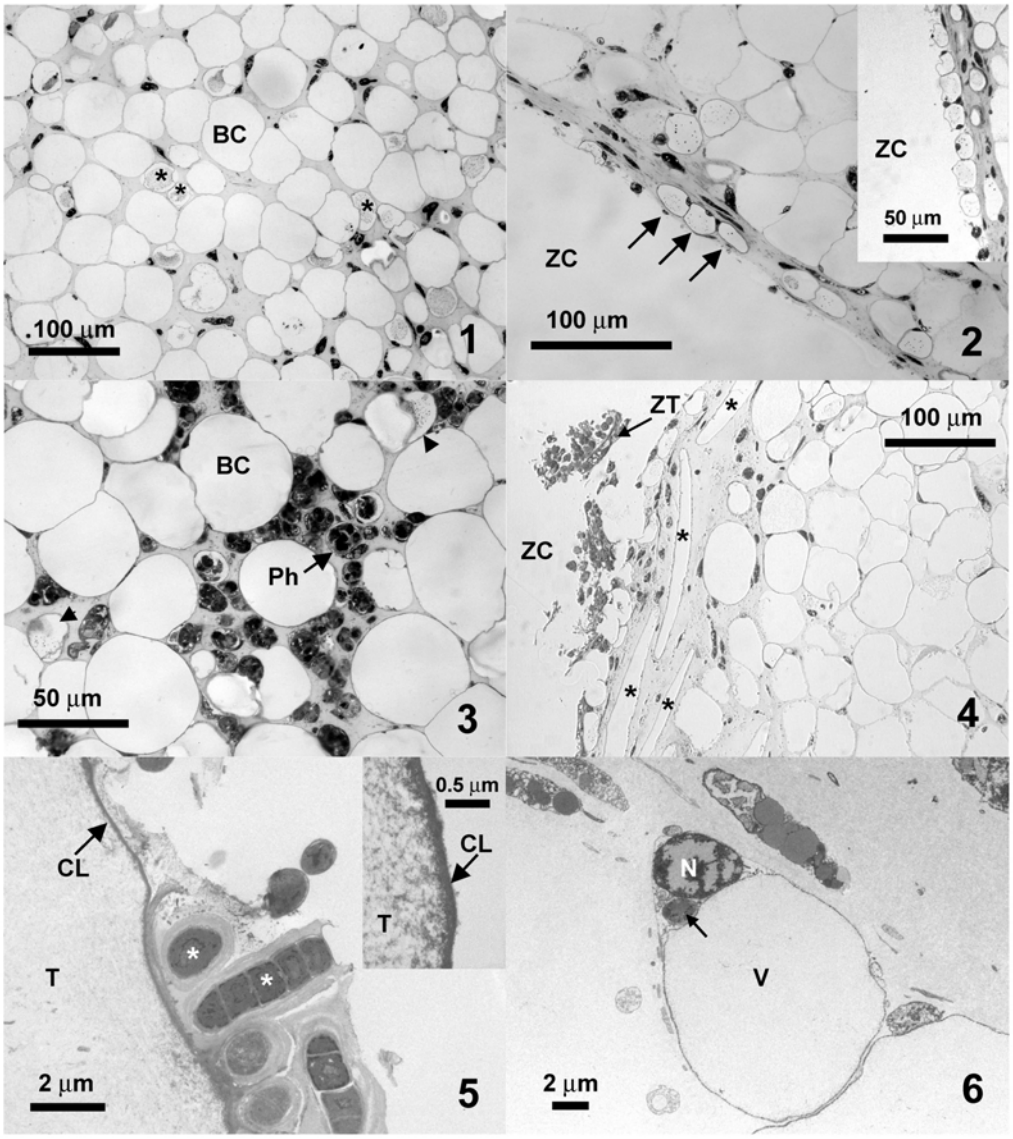
Fig. 2. Blue morph. Light micrograph of a semithin section of the wall of the thoracic part of a zooid cavity (ZC). Note the presence of pigment cells (arrows) in the lining of the cavity. Inset shows another area of the same zooid cavity wall where pigment cells are outside the tunic material. Methylene blue staining.

Fig. 3. Purple morph. Light micrograph of a semi-thin section of the basal part of a colony. The interstices between bladder cells (BC) are occupied by abundant phagocytes (Ph). Pigment cells (arrowheads) are scarce. Methylene blue staining.

Fig. 4. Blue morph. Light micrograph of a semithin section of the wall of the abdominal part of a zooid cavity (ZC). The lens shaped empty spaces (asterisks) correspond to the position of dissolved spicules. ZT: remnants of zooid tissue. Methylene blue staining.

Fig. 5. Blue morph. Image of the distal zone of tunic (T), showing the cuticular layer (CL) and the presence of several microepibionts (cyanobacteria, asterisks). Inset: detail of the cuticular layer of the tunic and the concentration of fibrilar material in it. TEM.

Fig 6. Blue morph. Small bladder cell with a big vacuole (V) of translucent material, and a nucleus (N) in a peripheral position. The small amount of cytoplasm surrounding the nucleus has a vacuole of heterogeneous contents (arrow). TEM.


BC
100 µm
1
ZC
100 µm
ZC
50 µm
2
BC
Ph
50 µm
3
ZT
ZC
100 µm
4
CL
T
2 µm
5
0.5 µm
CL
T
N
V
2 µm
6

3.3.2 Cell types

We have classified the main cell types into five distinct categories on the basis of morphology. Nevertheless, the relationships between these categories are complex, with intermediate forms and subtypes occurring in some of them.

3.3.2.1 Bladder cells (Figs 6-9)

This is by far the most abundant and conspicuous cell type, occupying most of the tunic sections. The diameter of fully developed bladder cells ranges between 30 and 50 μm. They are spherical or polygonal when closely packed together (Figs 1-4). They contain a single, giant vacuole with translucent contents that forces the cytoplasm to occupy a narrow peripheral band that is almost indiscernible in the majority of cases. The nucleus bulges out of this peripheral band, accompanied by a small amount of cytoplasm in which a vesicle with heterogeneous contents is often visible (Fig. 6). In the apical part of the tunic the bladder cells become disorganized in some areas and their position is only marked by strands of tunic. Developing bladder cells have smaller vacuoles in the periphery of the central one (Fig. 7), eventually, the nucleus seems to constrict off and disappear in fully formed bladder cells (Fig. 8). The wall of the fully developed bladder cell is made up of two closely adjoining membranes, the cell membrane and the vacuole membrane, with virtually no intervening cytoplasm in most of the cell perimeter (Fig. 9).

3.3.2.2 Pigment cells (Figs. 10-12)

Scattered among the bladder cells are smaller cells that also feature a large vacuole, but that contain granules of electron-dense material in their inner part. These cells are present throughout the tunic, but they appear to be concentrated in the distal region of the colonies, near the superficial layer. In some cases they are also seen lining the tunic region that surrounds the zooids' cavities (Fig. 2), and some images suggest that they can be released to these cavities and hence to the external environment (inset Fig. 2).

Pigment cells, as seen by examination of in vivo material, are chiefly responsible for colony coloration. Under the light microscope, they form a mass of

Figures 7-15.

Fig. 7. Green morph. A developing bladder cell. Note vesicles (arrow) that will presumably fuse with the big central vacuole (V). TEM.

Fig. 8. Blue morph. At later states of development, the nucleus of a bladder cell (BC) is constricted off from the remnants of cytoplasm and will eventually disappear. TEM.

Fig. 9. Green morph. A bladder cell (BC) and a pigment cell (PC) separated by a thin strand of tunic (T). The wall of both cells is formed by the cell membrane and the vacuole membrane with virtually no intervening cytoplasm (a small amount of cytoplasm is left in the area indicated by the arrow). Note also the dark material coating the vacuole membrane in the pigment cell from which dark filaments extend. TEM.

Fig. 10. Blue morph. Pigment cell (PC) with a big vacuole and a peripheral nucleus (N). Note small pigment granules and disperse filamentous material. TEM.

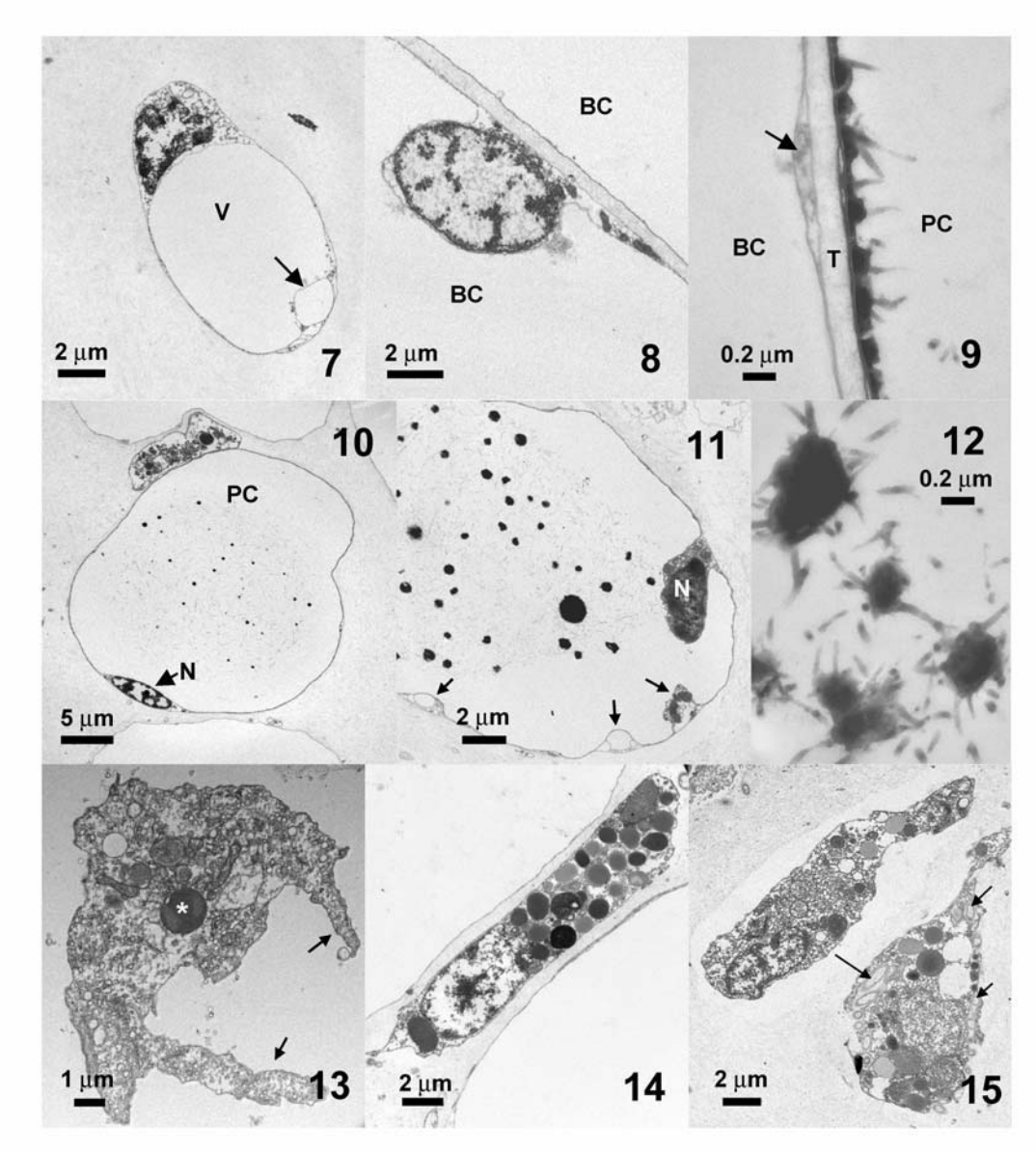
Fig. 11. Purple morph. Pigment cell showing the degenerating nucleus (N) about to be shed from the peripheral cytoplasm. Cytoplasmic remnants and vesicles are indicated by arrows. Note also that the size of the granules is bigger than in Fig. 10. TEM.

Fig. 12. Green morph. Detail of the granules of a pigment cell and the filamentous material that entangles them. TEM.

Fig. 13. Blue morph. Amebocyte with pseudopodia (arrows). The cytoplasm is filled with small secretory vesicles, mitochondria, and some bigger inclusions (asterisk). TEM.

Fig. 14. Purple morph. Elongated amebocyte with the cytoplasm filled with inclusions of varying density to electrons. TEM.

Fig. 15. Purple morph. Two amebocytes side by side. The one on the left has few inclusions and no bacterium appears associated with it. The one on the right has more inclusions of granular or uniform material and bacteria (arrows) appear attached to the cell membrane or engulfed in vacuoles.


V
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7
BC
BC
2 µm
8
BC
T
PC
0.2 µm
9
10
PC
N
5 µm
11
N
2 µm
12
0.2 µm
*
1 µm
13
2 µm
14
2 µm
15

reddish pigment made up of minute granulations in the purple morph, while those of the blue morph are visible as dark, more compact spots.

The pigment cells are 10 to 30 μm in diameter and, like the bladder cells, their cytoplasm is pulled into a narrow peripheral band from which the nuclear region bulges out (Fig. 10). In advanced states of formation, the nucleus disintegrates (Fig. 11). The inner zone of the vacuole features a variable number of dark granules from 0.2 to 2 μm in diameter and dark filaments (50 nm thick) that form a network, within which the pigment granules are entangled. The granules themselves are not bound by any membrane and seem to be continuous with the material in the filament network (Fig. 12). A layer of intensely electron-dense material with a beaded appearance and continuous with the filaments of the network lines the inner side of the membrane that surrounds the internal vacuole (Fig. 9). As in the bladder cells, the wall of developed pigment cells is made up of the cell membrane and the vacuole membrane with almost no cytoplasm in between (Fig. 9).

There are differences between color morphs in the amount of dark material concentrated inside the vacuole. The granules are bigger in the purple morph than in the equivalent cells of the blue and green morphs. Moreover, in the purple morph, besides larger granules, there is usually a more or less compact mass of smaller granules.

3.3.2.3 Amebocytes (Figs 13-20)

There is a variety of cell types of ameboid appearance that occupy the interstices left between the large bladder and the pigment cells. Although they differ in general appearance and, most likely, in function, we have observed many intermediate forms and are unable to make clear-cut distinctions between them. Consequently, we will refer to all them as ameboid cells and note their possible relationships.

The simplest type of ameboid cell is a pseudopodial cell, 10-15 μm in size, with clear cytoplasm filled with minute secretion vesicles of electron-translucent material and abundant mitochondria (Fig. 13); these cells seem to be in a process of active synthesis of substances and feature occasionally some inclusions (1-2 μm in diameter) with granular or uniform contents of moderate to strong electron density. In what is possibly a sequential process, these inclusions increase progressively in number. At more advanced stages, these inclusions and fill the cytoplasm and the

cells are elongated, up to 30 μm across (Fig. 14). Cells rich in inclusions are often associated with rod-like bacteria adhered to their membranes that are being actively phagocytosed (Figs. 15-16). A fibrilar material bounds the bacteria to the cell membrane (inset Fig. 16). Ameboid cells belonging to all developmental stages can be seen side by side (Fig. 15).

In some cases, the ameboid cells also contain inclusions with lamellate bodies (Figs. 16-17). Other cellular inclusions have membranes and dark spots of material (Fig. 18) that soon reorganize and form a vacuole, with the electron-dense material distributed close to the walls (Fig. 19). Ameboid cells at this stage are interpreted as precursors of the pigment cells, and the lamellate bodies may be the origin of the pigment vacuole (Figs. 18-19).

Other ameboid cells have phagosomes in their cytoplasm (Fig. 20) and, through an increase in size of the phagosomes, these cells may become, most likely, the phagocytes described below. Still another type of ameboid cell, that was rarely found, features a cytoplasm filled with fibrils oriented in parallel that may play a role in tunic contraction (see Fig. 23). Finally, near the surface layer of the colony, amebocytes and other cell types appear to be disintegrating, leaving only cellular “ghosts” in the tunic.

3.3.2.4 Phagocytes (Fig. 21)

This cell type is particularly abundant in the basal region of the tunic, where they cluster together in large interstices of tunic left by the bladder cells. The phagocytes are usually spherical cells 10-20 μm in diameter, with their cytoplasm occupied by one or several large phagocytic vacuoles in which remnants of other cells are visible (Fig. 21). At advanced stages of digestion, the phagosomes appear filled with membranous, myelin-like bodies.

3.3.2.5 Morula cells (Figs. 22-23)

Morula cells are readily distinguished by the presence of a few large (3 to 7 μm in diameter) vesicles containing granular or uniform material with variable electron density (from light to strong density, Fig. 22). These vesicles force the nucleus into a polygonal shape at the periphery of the cell. The cells are spherical or elongated (10-20 μm in size) and are scattered throughout the tunic, although they

Figures 16-24.

Fig. 16. Purple morph. Amebocyte with granular inclusions and surrounded by bacteria bound to its surface by a fibrilar material (inset). A lamellate body (LB) is found inside the cell. TEM.

Fig. 17. Purple morph. High magnification image of the lamellate body in Fig. 16. TEM.

Fig. 18. Purple morph. Amebocyte with granular inclusions, a lamellate body (LB) with some dense granulations, and an inclusion with dark granules and vesicular membranes (arrows). TEM.

Fig. 19. Purple morph. Amebocyte displaying a lamellate body (LB) in which vesicular membranes and dark granules are being formed, an a big vacuole with the membrane lined by dark material. TEM.

Fig. 20. Blue morph. An amebocyte with a phagocytic vacuole (asterisk) where an engulfed cell can be seen. TEM.

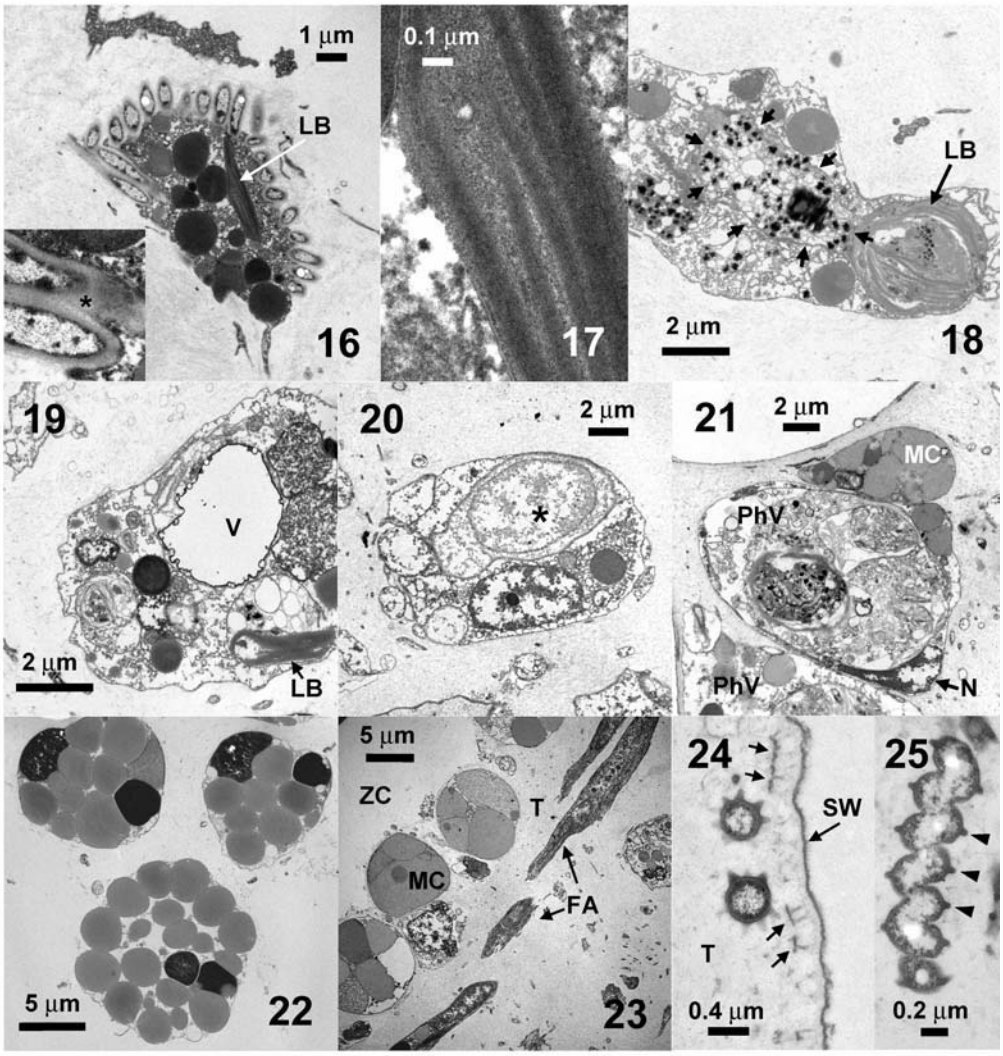
Fig. 21. Purple morph. A phagocyte found in the basal region of the colony. The nucleus (N) and the large phagocytic vacuole (PhV) are visible. Another phagocyte appears in the lower left corner and an morula cell (MC) lies close to the phagocyte. TEM.

Fig. 22. Purple morph. Morula cells with big vacuoles of different density to electrons. TEM.

Fig. 23. Blue morph. Lining of the thoracic part of a zooid cavity (ZC) showing morula cells in the limit of the tunic (T) around the cavity. Elongated, fibrilar amebocytes (FA) are also visible. Their microfibrils are oriented in parallel with the cavity wall. TEM.

Fig. 24. Green morph. Bacteria with dense, radiating coatings in the vicinity of a spicular cavity. Note dense, T-shaped formations (arrows) surrounding the spicular wall (SW). TEM.

Fig. 25. Purple morph. A screw-like bacterium. Note the single crest marked by arrowheads. TEM.


1 μm
LB
*
16
0.1 μm
17
LB
2 μm
18
19
V
2 μm
LB
20
2 μm
*
21
2 μm
MC
PhV
PhV
N
5 μm
22
5 μm
ZC
T
MC
FA
23
24
SW
T
0.4 μm
25
0.2 μm

seem to be more abundant around the lining of the zooid capsules, often in the outermost layer of tunic (Fig. 23). In the basal half (with spicules) of the zooid cavities they are found between the spicular layer and the limit of the tunic. This type of cell is more abundant in the purple than in the blue or green morphs.

3.3.3 Spicules

The spicules are abundant around the basal portion of the zooidal spaces, forming a shield that surrounds the zooids' abdominal region. In our decalcified material, they leave lens-shaped empty spaces in the tunic up to 200 μm in length (Fig. 4). The spicule wall is formed by a membrane with minute, T-shaped, dense bodies that are organized perpendicularly around it (Fig. 24). Occasionally, some amorphous material can be observed within the decalcified spicule spaces. Although there are always many cell types in proximity to the spicules, we have not seen any cells directly associated with them.

3.3.4 Bacteria

Three types of bacteria were present in the tunic of *Cystodytes*, but in low numbers. They generally accumulate near the limits of the zooidal spaces, either outside or inside the tunic. The main type are rod-like bacteria (up to 0.5 μm in diameter and 3 μm in length) that often appear "docked" in the ameboid cells until they are progressively engulfed (Figs 15-16). Much less abundant is a second type of bacterium, also rod-like, surrounded by a digitiform extracellular material that gave them a star-shaped appearance in transverse sections (Fig. 24). Finally, a third bacterial type was made up of elongated, screw-like bacteria that were found only rarely in the tunic. These bacteria feature a small crest that follows the coils (Fig. 25). There were no apparent differences in bacterial complement among the three morphotypes examined.

3.3.5 Microanalysis

To illustrate the coupling between ultrastructural observation and microanalysis, we show an example of spectra found for some of the structures and cell types present in the tunic of the purple and blue morphs (Fig. 26). The clarity of

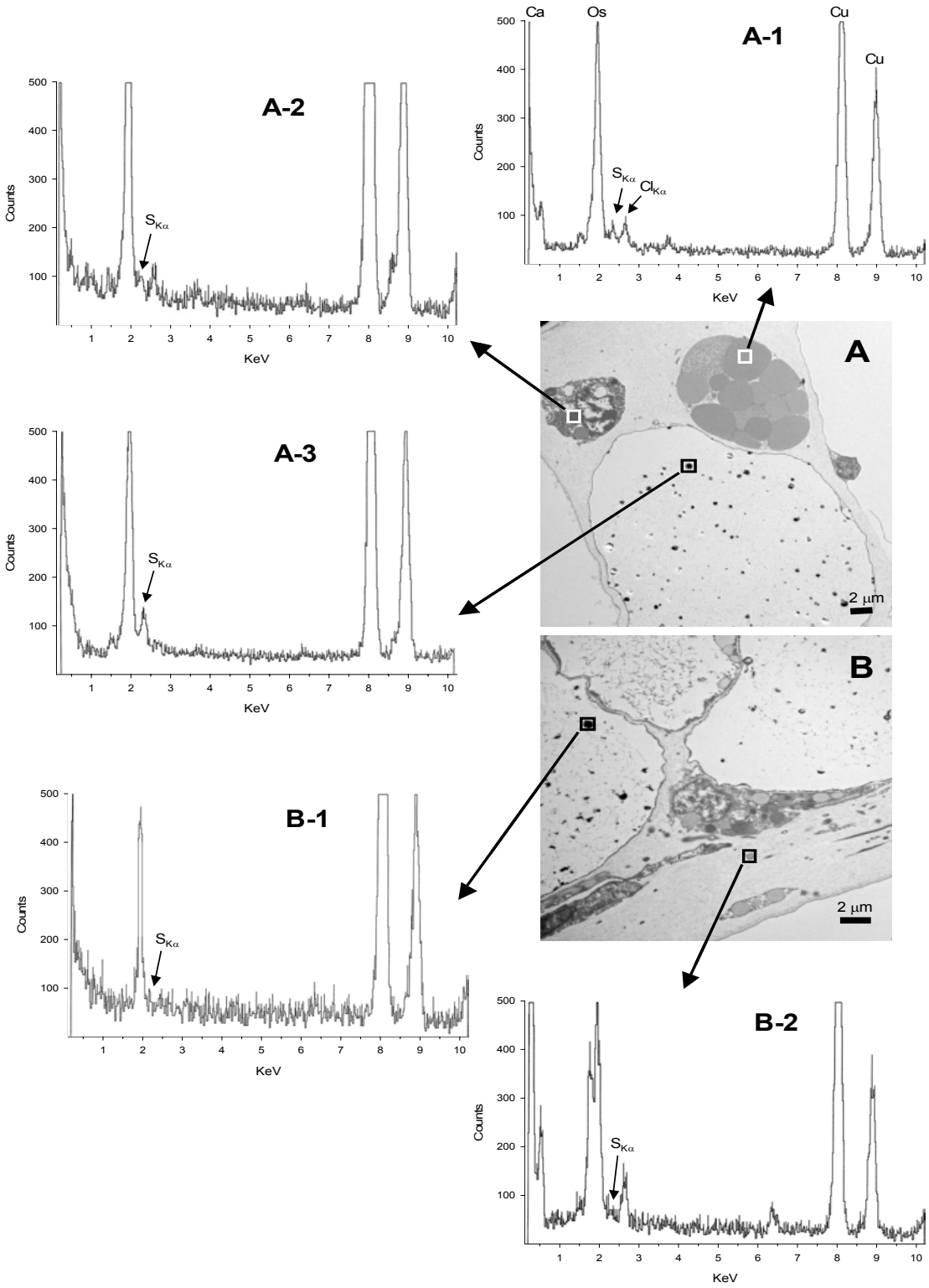


Fig. 26. Selected X-ray spectra from the purple morph (A) and the blue morph (B). A-1: morula cell; A-2: amebocyte; A-3: pigment cell; B-1: pigment cell; B-2: bacterium. In spectrum A-1 the main peaks obtained are labelled. In the remaining spectra only the peak corresponding to the S Kα signal is indicated.

the images depicted is affected by the thickness (0.3 μm) of the sections required for microanalysis and the lack of chemical contrast.

In terms of the location of sulfur in the sections, some counts are obtained in the zone corresponding to the S Kα peak in several cell types (particularly the ameboid cells and the morula cells), but the sulfur peak is only prominent in the granules of the pigment cells of the purple morph. The same granules in the blue morph scarcely gave any signal for sulfur above the background level.

Quantitative analysis based on the ratio of the sulfur peak to the ubiquitous chloride peak shows that sulfur levels are highest in the pigment cells of the purple morph, moderate in ameboid cells and morula cells, and lowest in pigment cells of the blue morph, bladder cells, bacteria, and tunic. The only structures in which sulfur is statistically more abundant (t-test) in the purple morph than in the blue morph are the pigment cells (Fig. 27). A two-way ANOVA analysis with color and cell type as factors shows that the interaction between them is significant, and post-hoc SNK tests at the 0.05 p-level reveal that the pigment cells of the purple morph have significantly higher levels of sulfur than any of the remaining compartments.

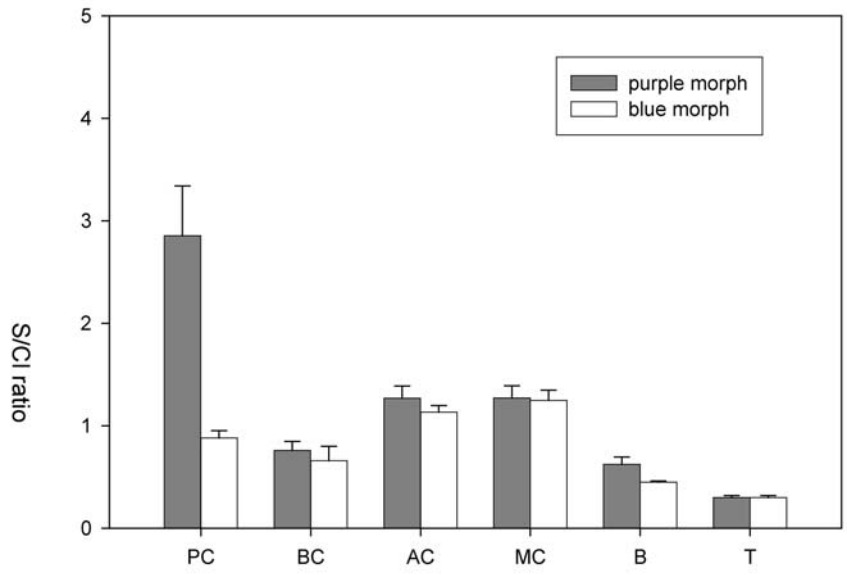


Fig. 27. Values of the ratio between the peaks S Kα and Cl Kα in the main compartments analysed in the purple and the blue morphs: PC: pigment cells; BC: bladder cells; AC: amebocytes; MC: morula cells; B: bacteria; T: tunic. Bars are standard errors.

3.4 DISCUSSION

The tunic of *Cystodytes* harbors a high diversity of cell types, as is typical of colonial ascidians, especially those without tunic blood vessels (Hirose 1992, Hirose et al. 1994b). Colonial ascidians with common vascular systems (e.g. Botryllidae, Hirose et al. 1991) and solitary ascidians (e.g. Smith 1970, De Leo et al. 1981, Di Bella et al. 1998, Hirose 1999) seem to display a lower richness of cellular types. In addition, our images suggest that the tunic is a dynamic system in which the different cell populations are in equilibrium and transformation processes from one cell type to another occur continuously.

Our results show little difference between the three color morphs examined. Tunic morphology may be quite uniform in the genus *Cystodytes*, as was found for zooid morphology (Kott 1990). The main differences relate to the pigment cells, which have more and bigger granules in the purple morph, and the morula cells, which are more abundant in the purple morph.

The main cell types found in the present study agree with the observations of Rottmayr et al. (2001) in *Cystodytes dellechiajei*, using light and confocal microscopy. However, the higher resolution afforded by electron microscopic techniques in this study allowed a more detailed morphological description. Rottmayr et al. (2001) distinguished five main cell types: bladder cells, pigment cells, granular filopodial cells, vacuolated filopodial cells, granular cells, and morula cells. We have introduced some modifications in this classification. First, we have grouped the filopodial cell types, together with other ameboid cell types found in our study, into a single group that we call amebocytes, as we consider that they all correspond, in fact, to different stages of development of a single cell type. Second, their granular cells are most likely to be macrophagic cells (see Fig. 17 in Rottmayr et al. 2001), which correspond to our phagocytes. Our results point to the amebocytes as a key cell type, evolving from true ameboid cells to bacteriocytes and phagocytes as they become laden with vesicles and phagosomes. Some amebocytes seem to give rise to pigment cells, and amebocytes may also be the origin of the bladder cells.

Bladder cells are known in ascidians to be acid containers (Hirose 1999, 2001) and this is most likely to be their function in *Cystodytes*. Although sulfuric acid is most frequently reported, *Cystodytes* may also contain hydrochloric acid (Lambert 1979, Rottmayr et al. 2001). The slightest rupture of the tunic of *Cystodytes* results in the release of strongly acidic fluids (pH 1; Tarjuelo et al. 2002). Bladder cells are

found in some groups of aplousobranch and phlebobranch ascidians (Hirose 2001). However, these cells tend to form a layer at the periphery of the tunic in other species, while in our case they are present everywhere from the base to the distal region of the colonies. The production of acidic substances is a widespread defense mechanism in marine invertebrates (Thompson 1988) and ascidians in particular (Stoecker 1978, 1980), although its effectiveness in this group has been disputed (Parry 1984, Davis & Wright 1989).

The pigment cells closely resemble the blood cell type with the same name described in botryllid ascidians (Hirose et al. 1998, Hirose et al. 2003; these cells have also been called nephrocytes, Milanesi & Burighel 1978). They have a large cytoplasmic vacuole as in our case, with electron-dense granules in it (although the granules can also have other morphologies). Some of these cells feature also a filamentous material similar to the one found in this work. However, the presence of this cell type in the tunic seems to be peculiar to *Cystodytes*. As in our case, this cell type determines colony color in botryllids (Hirose et al. 1998). Besides pigmentation, these cells seem to play a role in chemical defense in *Cystodytes* (see below). The pigment cells found in this study differ from those reported in the tunic of *Phallusia nigra* (Hirose 1999), the latter being ameboid cells with pigmented vesicles.

Amebocytes of many different kinds have been reported (under different names) both for the blood and the tunic of ascidians. They are probably undifferentiated cells that can give rise to many other types. In addition, they may play an important role in non-neural impulse conduction (Mackie & Singla 1987), have contractile fibrils that allow tunic contraction (Izzard 1974, Hirose 2001), phagocytose particles (Hirose et al. 1994a), and contain a variety of secretion granules. The length and width of the pseudopodia is highly variable in our sections. Therefore, it is possible that the more elongated amebocytes form a network, as described by Hirose (2001) for other species of the same group (Polycitoridae). The amebocytes found in the basal region of the tunic have become large phagocytes that engulf other cell types.

Finally, we have used the name morula cell to emphasize their similarity to the cells with the same name found ubiquitously in the blood of ascidians (Hirose et al. 2003), from where they may migrate into the tunic. Morula cells are known to play a role in allorecognition reactions in botryllid ascidians (Hirose et al. 1997, Rinkevich et al. 1998), and they can produce cytotoxic substances that contribute to the rejection reaction (Ballarin et al. 1998, Shirae et al. 2002). They have also been

reported to contain vanadium and/or iron (hence the names vanadocytes and ferrocytes sometimes given to these cell types; see Milanesi & Burighel 1978), but this view has recently been questioned (Ueki et al. 2002). *Cystodytes* is not a vanadium-rich ascidian, as shown by trace-metal analysis (Stoecker 1980) and confirmed in our X-ray study. Therefore, the role of morula cells in *Cystodytes* remains unknown.

We have not been able to observe the formation of spicules in the tunic of *Cystodytes*. Larfargue & Kniprath (1978) reported that spicules are formed in association with cells in special thoracic organs of didemnid ascidians, while spicules in solitary ascidians are formed extracellularly by sclerocytes (Lambert 1992, Lambert & Lambert 1997). Lambert (1979) found that spicule formation in *C. lobatus* begins at the junction between thorax and abdomen. Specific observations in this zone will be necessary to ascertain the mode of spicule formation in *Cystodytes*.

Although bacteria are present in the tunic of the three morphotypes, they are not abundant and their number is never comparable, for instance, to that found in some sponges (e.g. Uriz et al. 1996b, Turon et al. 2000). Furthermore, no sulfur signal corresponding to shermilamine B, kuanoniamine D, or their deacetylated forms, was detected in bacteria from the purple morph, suggesting that it is the host organism that produces these pyridoacridines. Salomon et al. (2001) also found that the related alkaloid dercitamide (kuanoniamine C) is produced by cells of the sponge *Oceanapia sagitaria*, and not by symbiotic organisms. These findings support the idea that the biosynthetic pathway of these products has been selected convergently in a variety of phyla (Salomon et al. 2001).

The structures that contain the highest amount of sulfur in the purple morph are the granules of the pigment cells. The same granules in the blue morph do not contain appreciable levels. This evidence, albeit indirect, strongly points to the pigment cells as the storage organs of the sulfur-containing pyridoacridine alkaloids characteristic of the purple morph but not of the blue morph. Rottmayr et al. (2001) also concluded that shermilamine B and kuanoniamine D are found in the pigment cells, because they appear reddish or purple in vivo (as free bases presumably under acidic conditions) and yellow in fixed material (presumably under neutral conditions), consistent with the behavior of these alkaloids in isolation. However, no measure of the intracellular pH was made (staining procedures show that acidic conditions are restricted to the interior of bladder cells in ascidians; Hirose 2001).

Consequently, evidence based on color alone is very circumstantial. Our results provide independent support to the suggestion that the pigment cells accumulate the pyridoacridines, and their higher abundance in the superficial zone of the tunic and surrounding the zooid spaces is in agreement with a defensive role of these substances. Indeed, chemical extract of the blue morph render artificial food highly unpalatable to the hermit crab *Cestopagurus timidus* (Tajuelo et al. 2002). Our observations indicate that pigment cells may be released through the zooid cavities' wall. Such a release would suggest a possible allelopathic role of these cells, as described for sponge cells with defensive chemical substances (Uriz et al. 1996a). Hirose et al. (1994b) similarly found release of granular cells through the tunic surfaces of the colonial ascidian *Aplidium yamazii* (Polyclinidae).

In Chapter 2 we found that whilst shermilamine B and kuanoniamine D were found only in the tunic, their deacetylated forms (potential precursors) were present both in tunic and zooids, as were ascididemin and hydroxyascididemin in the blue morph. Therefore, even if we have correctly located the storage compartment of these substances, it is still necessary to find out where synthesis occurs, where these molecules are located in the zooids, how they reach the tunic, and whether there is a change from a less-active precursor to a more active molecule. Clearly other cell types may be implicated in the biosynthetic pathway of these substances and further research should address these points in order to reach definitive conclusions about the pathways of production of these substances inside the organisms. This may be achieved through the use of cell separation and fractionation techniques for chemical analyses of particular cell types or precise spectrometric techniques for location of substances in thin slices of tissues (Todd et al. 2001).

In conclusion, we have characterized a highly diverse assemblage of cell types in the tunic of the colonial ascidian *Cystodytes* and identified potential sites of accumulation of bioactive molecules in this genus. The location of these substances in tunic cells indicates that they are not produced by symbionts, and their abundance near the colony surfaces is consistent with a defensive role. This work should serve as the basis for more detailed studies to link the biochemical pathways that produce chemical defenses with their cellular substrata in this genus.

CHAPTER 4

MULTIPLE DEFENSES AGAINST PREDATION IN *CYSTODYTES*

4.1 INTRODUCTION

Defense mechanisms against predation in marine invertebrates can be structural, chemical, or behavioral (Young & Chia 1987, Paul 1992, Pawlik 1993, Van Alstyne et al. 1994, Lindquist & Hay 1996, McClintock & Baker 2001). The relative importance and potential interactions between different defense mechanisms depend on: (1) the organism examined (e.g. Pennings & Paul 1992, Hay et al. 1994); (2) the inability of a single defense to deter all type of predators and/or competitors (Paul & Hay 1986, Hay et al. 1987, Schupp & Paul 1994, Pisut & Pawlik 2002, Tarjuelo et al. 2002, Burns et al. 2003); (3) the life history stage (Uriz et al. 1996, Pisut & Pawlik 2002); (4) the developmental or physiological constraints that could prevent a particular defense from being used in all parts of the organism, necessitating additional defenses to ensure protection of the whole organism (Harvell & Fenical 1989), (5) evolutionarily established constraints (Schmitt et al. 1995), and (6) whether structures or compounds are energetically expensive. Thus, they may perform multiple functions within the organism including ecological functions (Paul 1992, Pawlik 1993). For instance, in some sponges both large spicules and chemical defenses deter fish feeding (Pawlik et al. 1995, Burns et al. 2003, Burns & Ilan 2003). Soft corals and gorgonians can produce both chemical defenses and sclerites, which protect against predators (Pawlik et al. 1987, Harvell et al. 1988, Wylie & Paul 1989, Pawlik & Fenical 1992); however, the primary

function of sclerites may be structural rather than defensive (Koehl 1982, Lewis & Von Wallis 1991). In view of these and similar results, Van Alstyne et al. (1994) suggested that when designing feeding experiments all defense mechanisms against predation that could be used by an organism should be considered, rather than only addressing the effects of single defenses in isolation. However, mainly due to experimental limitations, few studies have attempted to assess the importance of multiple defenses against predation in marine organisms (Paul & Van Alstyne 1992, Schupp & Paul 1994).

Among benthic invertebrates, ascidians have been reported to suffer relatively little predation by generalist predators (Millar 1971, Goodbody & Gibson 1974, Stoecker 1980b), mainly fishes (Randall & Hartman 1968, Myers 1983) and, occasionally, urchins (Briscoe & Sebens 1988). Specialized predators on ascidians include mollusks, such as the lamellarians, cypraeids (Fretter & Graham 1962, Millar 1971, Lambert 1980) and many nudibranchs (Millar 1971, Paul et al. 1990), and polyclad flatworms (Millar 1971, Morris et al. 1980, Parry 1984, Schupp et al. 1999).

In addition, ascidians have been reported to present both physical (spicules, tunic toughness) and chemical defenses (Swinehart et al. 1974, Stoecker 1978, 1980, Pisut & Pawlik 2002, Tarjuelo et al. 2002). Secondary metabolites (Paul et al. 1990, Davis 1991, McClintock et al. 1991, Lindquist et al. 1992, Vervoort et al. 1998), high vanadium concentrations (Stoecker 1980a), and low pH (Webb 1939, Stoecker 1978, 1980a, b, c, Pisut & Pawlik 2002) have all been proposed as defenses against predation in some ascidian species. However, Parry (1984) suggested that neither the presence of vanadium nor an acidic pH prevented predation on ascidians. Another potential defense mechanism is by physical means. The physical defense theory holds that skeletal elements not only stabilize the structure of an organism (Koehl 1982, Lewis & Von Wallis 1991), but can also play a role in its defense. In some cases, spicules and sclerites have been shown to deter fish feeding on some marine invertebrates (Harvell et al. 1988, Van Alstyne et al. 1994, Koh et al. 2000, Burns & Ilan 2003) but not in others (Meylan 1988, Wylie & Paul 1989, Lindquist et al. 1992, Chanas & Pawlik 1995, 1996). The interaction of defense mechanisms against predation in ascidians remains largely unexplored and is limited by the difficulty of obtaining sufficient material for extraction and experiments.

The ascidian genus *Cystodytes* was chosen to assess the relative importance of multiple defenses against predation in ascidians. In Chapter 2 we

found 2 chemotypes within Mediterranean specimens of this genus, traditionally attributed to *Cystodytes dellechiajei*. The first chemotype was characterized according to the presence of C_9-unsubstituted pyridoacridines such as ascididemin (Fig. 1; Kobayashi et al. 1988) and 11-hydroxyascididemin (Schmitz et al. 1991), which were present in blue and green color morphs from the western Mediterranean. The second presented the sulfur-containing pyridoacridines shermilamine B (Carroll et al. 1989), kuanoniamine D (Caroll & Scheuer 1990), and their deacetylated forms (Chapter 2 and Eder et al. 1998, respectively). These pyridoacridines were present in the purple morph from the western Mediterranean, and were also found in a purple morph from Guam. Some of these substances exhibit a strong bioactivity and high cytotoxicity (Dassonneville et al. 2000, Bowden 2000). Ascididemin also displays antibacterial and antifungal activities (Lindsay et al. 1995). To our knowledge, apart from a potential anti-fouling function (Debard et al. 1998), no ecological role has been described for ascididemin. In addition, *Cystodytes* colonies are highly acidic (pH < 2; Parry 1984, Tarjuelo et al. 2002) and contain calcareous spicules encasing the zooids (Turon 1987, Kott 1990). As colonies generally lack epibionts and show scarce signs of predation, these potential defenses seem to result in an effective defense mechanism, although their relative importance and interactions are not known. The vanadium concentration in this genus (≤ 10 ppm dry weight, Stoecker 1980a; ≤ 3 ppm dry weight, this study) is well below the concentration suggested to deter predation in other ascidians (> 1000 ppm dry weight, Stoecker 1980a). Tarjuelo et al. (2002) observed a deterrent activity of the crude extract of a blue Mediterranean morph against the hermit crab *Cestopagurus timidus*. In contrast, the crude extract obtained from specimens collected at the same site showed no cytotoxicity against sea urchin embryos and did not inhibit settlement of larvae of the bryozoan *Bugula neritina* (Becerro et al. 1997).

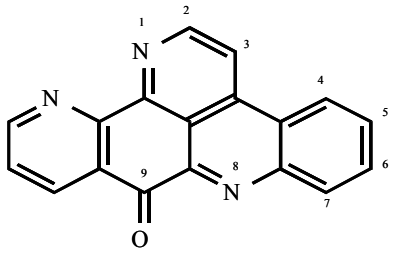


Fig. 1. Chemical structure of ascididemin, the major alkaloid of the Mediterranean blue morph.

To assess the relative importance of multiple defenses against predation in ascidians, we have studied a purple and a blue morph of *Cystodytes* from the western Mediterranean and traditionally assigned to *C. dellechiajei* and a purple morph from Guam (USA), assigned to *C. violatinctus* (Lambert 2003). Crude extracts, ascididemin (the major alkaloid of the blue morph), spicules, and tunic

acidity, as well as combinations thereof, were tested against three generalist predators: the damselfish *Abudefduf vaigiensis* and *A. sexfasciatus*, the urchin *Diadema savignyi*, and the puffer fish *Canthigaster solandri*.

4.2 MATERIAL AND METHODS

4.2.1 Ascidian samples

Specimens of the purple and blue morph of the ascidian *Cystodytes* were sampled by scuba diving in Catalonia (NE Spain, western Mediterranean) in 2002 and 2003. Another purple form was collected in Togcha Channel on Guam (N Pacific, USA) in 2003. The specimens were identified by the authors as belonging to the genus *Cystodytes*, based on the descriptions of Turon (1987), Kott (1990), and Lambert (2003). Morphologically, the only differences between these forms were the color and the presence of small, spherical spicules (in addition to the large, disc-shaped spicules characteristic of the genus) in the purple form from the Mediterranean (see Chapter 5).

4.2.2 Chemical extraction

Biologically active compounds from the cosmopolitan ascidian *Cystodytes dellechiajei* (the most abundant species of *Cystodytes*) are restricted to the lowest polar fraction (Becerro et al. 1997), and mainly correspond to pyridoacridine alkaloids (Bontemps 1996). The freeze-dried colonies were extracted three times in a 1:1 (v:v) mixture of dichloromethane and methanol and then the combined extracts were concentrated under vacuum to leave a powdery organic residue. The mean naturally occurring concentration of crude extract found in at least 5 independent extraction sets per color morph was 13% per dry mass of the ascidian, irrespective of color. This percentage was used, therefore, to run all the feeding trials. We also tested ascididemin (the major alkaloid of the blue morph) synthesized in the LCBE laboratory (University of Perpignan) following Bracher (1989) because it was the only compound available in enough quantity. We used the minimal concentration found after monitoring a natural population for 2 years (0.01% dry weight, see Chapter 8) in all feeding assays.

4.2.3 Spicular isolation and concentration

Spicular weight per dry mass was calculated by averaging the concentration found in 4 independent colonies per morphotype. Small pieces of the tunic were

removed, freeze-dried, weighed and boiled in commercial bleach for several minutes until complete dissolution of the tissue was achieved. Then, spicules were washed several times in water to remove organic remains, dehydrated in absolute ethanol and finally dried in an oven at 60°C. The average spicular concentration found and used in our assays was 4% of the dry mass of the ascidian.

4.2.4 Toxicity assays

The general toxicity of *Cystodytes* crude extracts and ascididemin was assessed using the Microtox method (Ribo & Kaiser 1987), based on reduction of the bioluminescence of the marine bacterium *Vibrio fischeri*. This method has proven to be precise and reproductible, and it correlates well with other common toxicity tests (Becerro et al. 1995). Three colonies were independently analyzed in each morphotype. The chemical extractions were performed separately following the protocol described above. Ascididemin samples were also tested in triplicate. Assays were performed by resuspending the extracts in artificial seawater in an ultrasonic bath. The Microtox standardized test uses 4 increasing concentrations of the sample solution (Ribo & Kaiser 1982). In our case, the concentrations of extract and ascididemin (relative to dry sample weight) tested were 125, 250, 500 and 1000 ppm. Based on reduction of bioluminescence, the Microtox device calculates a regression line and the EC_{50}, the effective concentration at which bioluminescence is reduced by 50%. ANOVA was performed to test for significant differences in toxicity between the crude extracts and ascididemin.

4.2.5 Field assays – Generalist predators

Field bioassays were conducted at a depth of 8 m at Gun Beach (Guam, USA). We conducted a field feeding deterrence experiment using a similar method to the one described by Schupp & Paul (1994). The following components were added at the concentrations mentioned above to an artificial diet (see Table 1): crude extract, ascididemin, disc-shaped spicules alone or with spherical spicules, sulfuric acid to obtain a pH < 1, and combinations thereof to test the hypothesis of a cumulative deterrent effect. When solvents were required to dissolve crude extracts or ascididemin in the treated food, the same amount of solvent was added to the control. The mixture was poured into 1 cm^3 molds containing a rubber O-ring, which

allowed the use of safety pins to attach cubes to ropes. Each rope contained either 4 controls or 4 treated food cubes. We placed a total of 24 pairs of control and treated ropes on the reef, one at a time. The pairs were removed when approximately half of the cubes were eaten in any of the treatments. We used Wilcoxon's signed-rank test for paired comparisons to test for significant differences in the number of cubes eaten between control and treated food. For the acid treatments, the whole experiment was limited to 35 minutes and a mean of 20 pairs of ropes, because the acidity of the cubes changed from pH 1.5 at time 0 to pH 3.5 after 40 minutes in seawater. The addition of crude extract or ascididemin to the artificial food did not have a significant effect on the original color of the food. The artificial diet had a mass/volume ratio of 1.06 $g \cdot ml^{-1}$, which approximates to the mass/volume ratio of the ascidian, 1.23 $g \cdot ml^{-1}$. We chose to work with weight concentrations instead of volumetric concentrations because preliminary tests using ascididemin at both volume and mass ratios yielded similar results (see Results) and, in any case, results obtained with mass ratios were more conservative (given the slightly lower density of the artificial food, if we had worked with volumetric concentrations we would have needed to include 16% more of each extract in the cubes).

Table I. Artificial food recipes for damselfishes, puffer fishes and sea urchins. Agar (Sigma nº A-6924), carageenan (Sigma nº C-1013), Kruse's Perfection Brand catfish and freeze-dried *Enteromorpha* sp.

RECIPES	Damselfishes	Puffer fishes	Urchins
Agar	1.25 g	0.15 g	0.25 g
Carageenan	1.25 g	0.15 g	0.25 g
Catfish	5 g	2 g	2 g
Enteromorpha	-	-	1 g
Water	80 ml	18 ml	25 ml

4.2.6 Laboratory feeding assays – Benthic predators

The sea urchin *Diadema savignyi* is very common on coral reefs around Guam and is normally found in shallow waters (0.5 m to 3 m). Sea urchins are mainly grazers that feed on algae, but are usually referred to as generalist predators that also feed on a variety of invertebrates (Barnes 1987, Briscoe & Sebens 1988). *D. savignyi* adapts poorly to laboratory conditions and has to be kept in separated

tanks to avoid mortality (up to 75% if kept together). However, after a few weeks of adaptation in separate aquaria, no significant mortality was recorded and, regular feeding behavior was observed for all specimens. Artificial food was prepared as indicated in Table 1. Treatments were prepared by addition of one of the following: crude extract, ascididemin, and disc-shaped spicules at the concentrations mentioned above. The mixture was heated and poured into a mold backed with aluminum window screening to form a strip that covered a mean of 325 squares of the window screening. Sea urchins were placed in 30 liters flow-through tanks and repeatedly offered artificial food until feeding started. The tanks were regularly cleaned to prevent algal growth. Prior to experiments, the urchins were starved for 24h. Each treatment consisted of seven urchins that were offered either treatment/s or control screens. Urchins were allowed to feed for one day and two nights. After each experiment, urchins rested 24h and were then offered untreated artificial food and allowed to feed for one day and two nights. Only the urchins that showed a normal feeding behavior were selected for subsequent testing. We used either t-test or Mann-Whitney analyses (if normality or heteroscedasticity failed) to identify significant differences between the number of squares eaten of the control and of the treatment. The artificial diet had a mass/volume ratio of 1.04 $g \cdot ml^{-1}$.

The puffer fish *Canthigaster solandri* is a benthic predator that feeds on benthic algae, ascidians, and other invertebrates (Amesbury & Myers 1982). It adapts well to laboratory conditions and has been used previously for deterrence tests (Rogers & Paul 1991, Becerro et al. 1998). Artificial food was prepared as indicated in Table 1. Treatments were prepared by addition of crude extract, ascididemin, disc-shaped spicules, sulfuric acid to obtain a pH < 1, and combinations thereof. The mixture was heated and poured into a mold backed with fiberglass window screening to form a strip that covered a mean of 277 squares of the window screening. Puffer fish were placed separately in 30 liters flow-through tanks and normally started feeding on standard artificial food within one to two weeks. The tanks were cleaned regularly to prevent algal growth. Prior to the experiment, the fish were starved for 48h. Each treatment set was composed of 7 fish, and they were offered either treatment/s or control screens. The fish were allowed to feed for 1 hour, in which time approximately half of the squares were eaten in any of the treatments. If the treatments contained sulfuric acid (initial pH < 2), the fish were allowed to eat for 30 minutes, as the pH reached values of 3 after that time (the pH of 2 extra screens submerged in empty tanks was recorded at the

end of each test). The artificial diet used for these tests had a mass/volume ratio of 1 $g \cdot ml^{-1}$. After each experiment, the fish did not receive food for 24h, followed by feeding on untreated artificial food *ad libitum*. Only the fish that showed normal feeding behavior were selected for further experiments. To compare results between treatments, we standardized the consumption by dividing the amount eaten in each replicate by the mean consumption of the controls of the corresponding experiment, and then compared these values using t-tests or ANOVA.

4.3 RESULTS

4.3.1 General toxicity assays

Four different extract/compound concentrations were tested for the crude extracts and ascididemin (Fig. 2): 1000, 500, 250, and 125 ppm (relative to sample dry weight). Toxic levels (EC_{50}, the concentration at which bioluminescence of bacteria is reduced by half) varied from 77.6 ppm (purple Mediterranean morph) to 168.7 ppm (ascididemin). There were no significant differences in toxicity between the different crude extracts and ascididemin (ANOVA, $p = 0.112$). EC_{50} values for the blue extract (167.3 ppm) and its major alkaloid ascididemin were very similar, suggesting that this alkaloid accounts for most of the toxicity displayed by the blue morph.

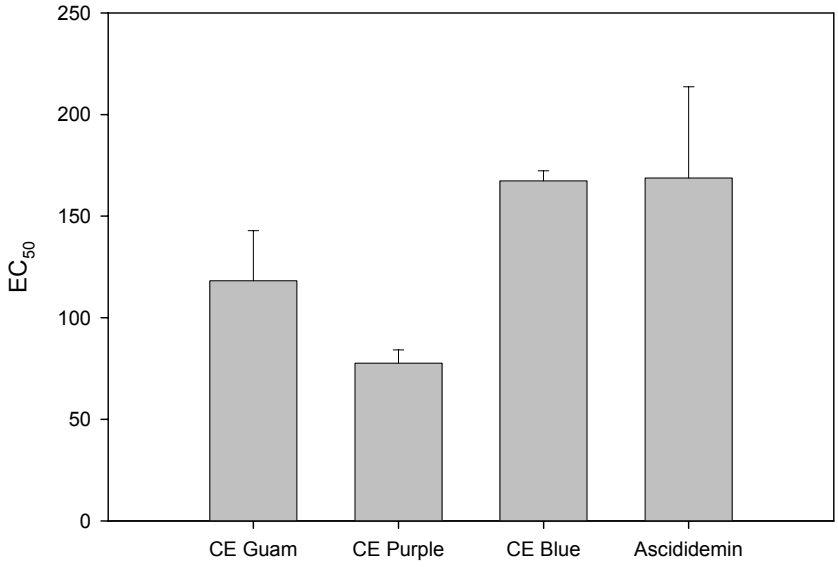


Fig. 2. Toxicity results (EC_{50} values) for the blue and purple Mediterranean forms, the purple Guam morph and ascididemin. Bars indicate standard errors.

4.3.2 Field assays – Generalist predators

At Gun Beach (Guam, USA) the damselfish *Abudefduf vaigiensis* and *A. sexfasciatus* were the main consumers during experiments, although occasional consumption by the wrasse *Thalassoma lutescens* and the triggerfish *Balistapus*

undulatus was also observed. When a new treatment set was placed, fish nibbled all the cubes presented to them; therefore, the final choice was not dependent upon which cube was tasted first. However, after some trials, the fish seemed to learn which cubes were treated and which were controls, as several treated cubes were left untouched. Ascididemin, which was tested in both volume and mass ratios, deterred feeding by reef damselfish (Fig. 3). Compared to controls, the three crude extracts significantly deterred fish feeding ($p < 0.001$ in all cases; Fig. 3). The disc-shaped spicules, which are typical of the genus alone, or combined with the spherical spicules found in the purple Mediterranean morph, did not deter predation ($p = 0.48$ and $p = 0.56$ respectively). Cubes with sulfuric acid or a combination of sulfuric acid and spicules did not deter fish feeding. However, when the cubes were treated with sulfuric acid, the fish displayed a flushing behavior in which they repeatedly swallowed and regurgitated the food cubes. When ascididemin was combined with spicules a significant difference was found ($p < 0.001$). Interestingly, when we added sulfuric acid to ascididemin, or to ascididemin and spicules, no significant deterrence was observed ($p= 0.25$ and $p = 0.07$ respectively).

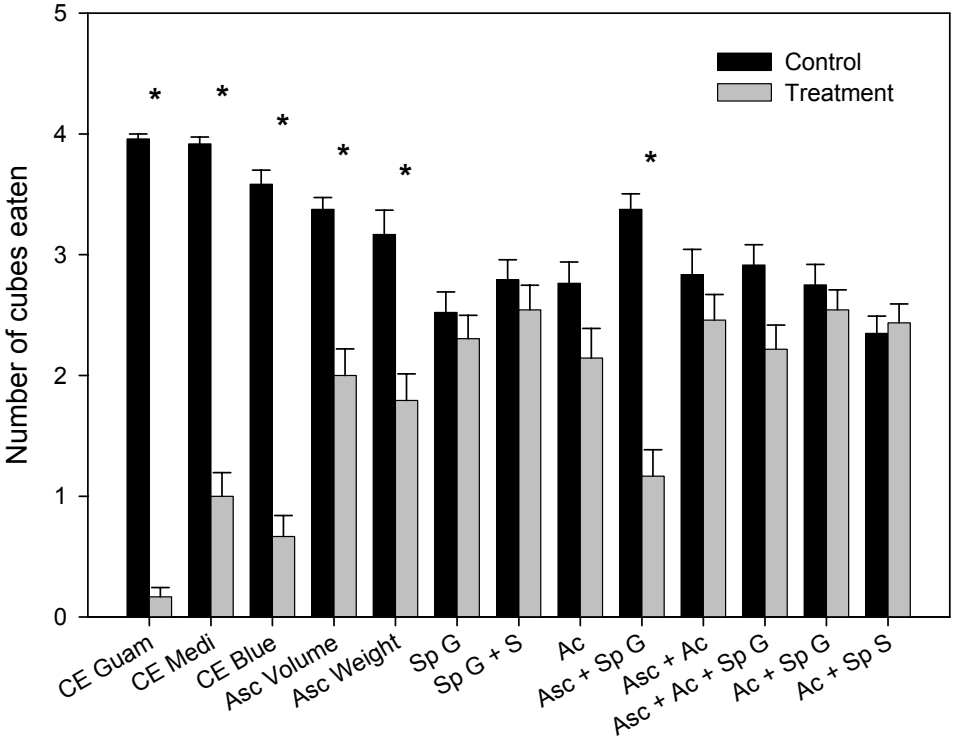


Fig. 3. Number of cubes eaten for each treatment by the damselfish *Abudefduf vaigiensis* and *A. sexfasciatus*. Codes for treatments: CE Guam indicates crude extract of the purple Guam morph; CE Medi, crude extract of the purple Mediterranean morph; CE Blue, crude extract of the blue Mediterranean morph; Asc Volume, ascididemin in a volume ratio; Asc Weight, ascididemin in a mass ratio; Sp G, disc-shaped spicules typical of the genus; Sp S, spherical spicules found in addition of the disc-shaped ones in the purple Mediterranean morph; Ac, acidified with sulfuric acid. Asterisks show significant differences. Bars indicate standard errors.

4.3.3 Laboratory feeding assays – Benthic predators

There were no significant differences between consumption of controls and of any of the treatments offered to *D. savigny* (Fig. 4). Although ascididemin seemed to be more deterrent than crude extracts, there were no significant differences in consumption between them (ANOVA, p = 0.078). In addition, the ascididemin standard error was the highest of all the treatments. Indeed, the urchins were observed to move randomly in their tanks during night time and eat whenever they found a screen, independently of its content. Aluminum screens were often damaged by the urchin bites. A period of two nights was necessary to ensure that at least 90% of the sea urchins encountered the screen.

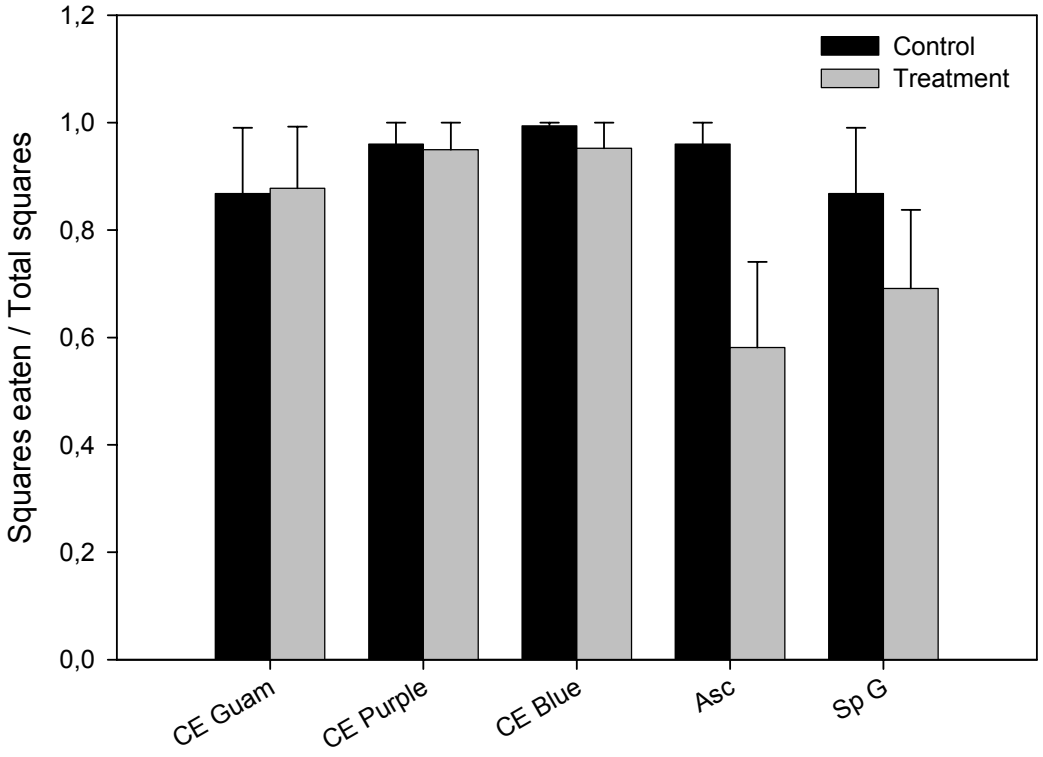


Fig. 4. Number of squares eaten by the sea urchin *Diadema savigny* per total number of squares. X-axis labels as in Figure 3. Bars indicate standard errors.

All crude extracts tested, alone or in combination with acid or spicules significantly deterred predation by the puffer fish *Canthigaster solandri* (Fig. 5). All fish sampled the food offered to them, meaning that no negative result was recorded

in any case. The combination of spicules with crude extract from either the purple or blue Mediterranean forms yielded similar results those obtained for the crude extracts alone. However, the combination of acid with crude extract from either the purple Guam morph or the purple and blue Mediterranean morphs yielded an increase in the deterrence when compared with acid alone (although this increase was only significant for the Mediterranean purple morph; t-test, $p = 0.012$). Ascididemin, alone or with spicules, also significantly deterred predation ($p = 0.023$ in both cases) although, as happened during field experiments with the damselfish, combination with spicules and sulfuric acid seemed to counteract somewhat the deterrent effect of the alkaloid ($p = 0.073$). No significant differences were found between controls and artificial foods treated with spicules, acid, or a combination of the two ($p > 0.1$ in all cases).

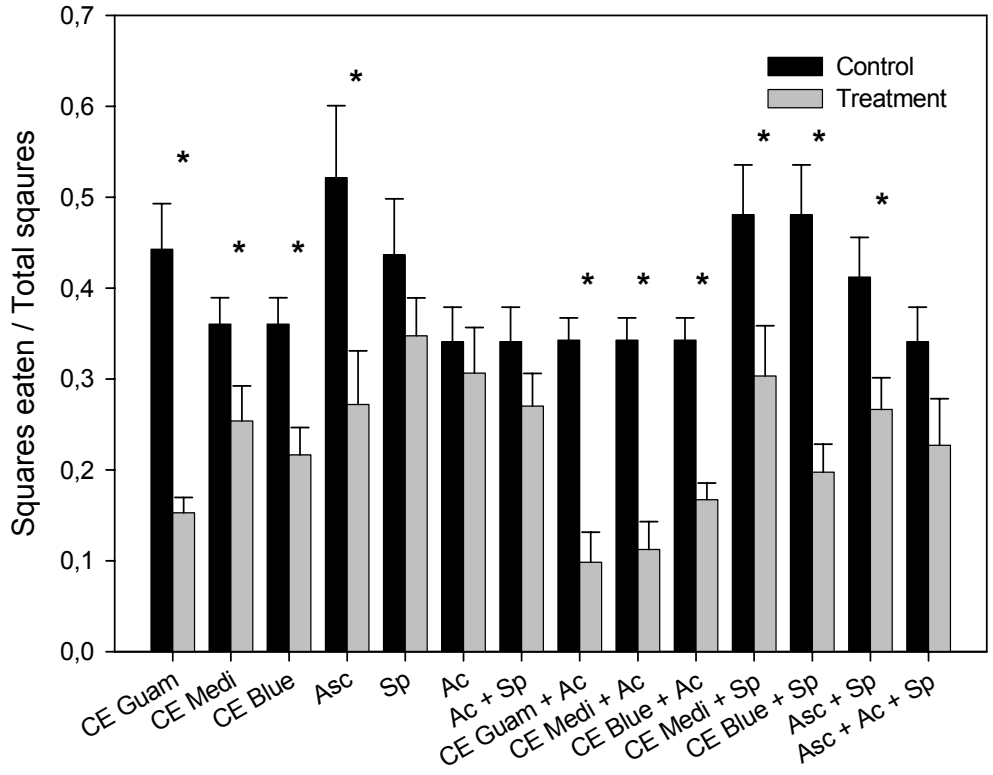


Fig. 5. Number of squares eaten by the puffer fish *Canthigaster solandri* per total number of squares. Sp indicates disc-shaped spicules typical of the genus, the other X-axis labels are as in Figure 3. Asterisks show significant differences. Bars indicate standard errors.

4.4 DISCUSSION

Our study showed that crude extracts from the purple and blue Mediterranean morphs and the purple Guam morph of the ascidian *Cystodytes*, as well as ascididemin, the major alkaloid of the blue morph, are toxic and significantly deter fish but not sea urchin predation. In contrast, acidity and spicule concentration by themselves do not deter fish or urchin feeding. All crude extracts yielded similar results, although the alkaloid composition of the purple forms (from Guam and the Mediterranean) and the blue Mediterranean morph are qualitatively different in terms of their pyridoacridine composition (see Introduction, Chapter 2). This suggests a parallel evolution of secondary metabolites as a defense mechanism against predation in this genus.

Crude extracts alone significantly deterred damselfish feeding in field assays and puffer fish feeding in the laboratory. However, whilst damselfish left many cubes untouched, puffer fish repeatedly sampled the offered food. Puffer fish are benthic predators and occasionally feed on ascidians (Amesbury & Myers 1982). Therefore, they may be more used to or tolerant of amino acid-derived metabolites such as alkaloids, the main defensive compounds in ascidians (Davidson 1993, Molinski 1993). On the other hand, the addition of acid to the crude extracts tended to increase the deterrent effect in puffer fish. In contrast, the combination of ascididemin and either sulfuric acid or spicules decreased the anti-predatory properties of ascididemin, with no significant deterrence of feeding observed. The loss of deterrence found here might be due to some kind of chemical interaction between sulfuric acid and ascididemin. Indeed, in the living ascidian, acidic vacuoles are located in bladder cells (Webb 1939, Hirose 1992), metabolites seem to be stored in pigment cells (Rottmayr et al. 2001, Chapter 3), and spicules are found in the tunic matrix encasing the zooids (Turon 1987, Kott 1990). Thus, all of the potential defense mechanisms are localized in separate compartments. In addition, acidity by itself or in combination with spicules did not deter fish feeding, although fish were observed to repeatedly regurgitate and swallow the food before they finally consumed it. When present, calcareous spicules may cause an extremely rapid neutralization of the acid and, even without spicules, seawater may be a sufficient buffer to quickly neutralize the acid (Parry 1984) and thus allow fish feeding.

Burns & Ilan (2003) found that deterrence in sponges was linked to spicule size and only those spicules larger than 250 μm deterred predation. Pawlik &

Chanas (1995) found no significant deterrence caused by siliceous spicules in sponges, and suggested a deterrent role of sclerites of calcium carbonate related to an alteration of the pH of the acidic gut of putative predators. A similar defense mechanism was previously suggested by Schupp & Paul (1994) for calcified algae. The disc-shaped spicules, up to 1mm long, typical of the genus *Cystodytes*, represent 4% of the dry weight of the ascidian. However, as observed by Lindquist et al. (1992) for other ascidian species, spicular composition and concentration did not significantly deter fish or sea urchin predation. In the artificial food, spicules are randomly distributed, whilst in the living ascidian they encase each zooid. In fact, spicules form a compact and relatively hard capsule into which the thorax can be retracted in case of attack by a predator. Therefore, it is probable that although the disc-shaped spicules do not act as a deterrent in the artificial food, they do play a protective role associated with the zooid in the living colonies. In contrast, no role could be attributed to the spherical spicules found in the Mediterranean purple morph, as they only measure ~70 μm and are scarce and often randomly distributed throughout the tunic (see Chapter 5). Calcareous spicules may also function as a shield to protect the zooids from the acid release that may occur during cell rupture. This suggestion is supported by stereomicroscope observation of bubbling in broken colonies as a result of calcium carbonate dissolution.

Urchin feeding seems to be directed by the probability of finding the screen rather than by any deterrence associated with the crude extracts, ascididemin, or the spicules. Secondary metabolites, therefore, do not deter all possible predators equally. Hay (1992) found that algal secondary metabolites that deter generalist fish predators are often consumed by mesograzers. Acidity is commonly found in combination with other defense mechanisms. Stoecker (1980b) suggested that acidity played an important deterrent role against crawling predators, because of the rupture of acid vesicles when they contact the ascidian surface. We could not test acidity with sea urchins because of methodological limitations (we could not maintain a pH < 2 for 2 nights). Therefore, a role for acidity in deterring sea urchin predation, as suggested by Stoecker (1980b) and Pelletreau & Muller-Parker (2002), cannot be ruled out. However, Pisut & Pawlik (2002) observed that the 9 ascidian species that exhibit an acidic pH (~1) tunic also yield deterrent organic extracts. In addition, we have often observed urchin bites in the purple and blue morphs of *Cystodytes* from the Mediterranean. Consumption by Mediterranean sea urchins

was confirmed by the observation of disc-shaped spicules in the gut of urchins close to grazed *Cystodytes* colonies.

The optimal defense theory assumes that chemical and morphological defenses in living organisms are costly and that natural selection will favor an allocation of resources to defenses that optimize their cost/benefit ratio in terms of fitness (Rhoades 1979, Fagerström et al. 1987). There is an apparent paradox between the postulates of this theory and the presence of potentially redundant defense mechanisms in organisms such as *Cystodytes*. A current hypothesis is based on the evolution of these mechanisms as a response to different predators and/or competitors. Previous studies found that chemical defenses deterred feeding by some, but not all, predators assayed (Paul & Hay 1986, Hay et al. 1987, Schupp & Paul 1994, Pisut & Pawlik 2002, Tarjuelo et al. 2002, Burns et al. 2003). Similarly, our results show that all crude extracts of *Cystodytes* deter fish feeding but not sea urchin grazing, while their combination with other potential defenses exhibit contrasting results, often depending upon the predator considered.

Alongside encompassing a wide range of predators, defense mechanisms often act at other levels, such as fouling avoidance or space competition (Stoecker 1980b, Schmitt et al. 1995, Becerro et al. 1997). Furthermore, they may act at different life history stages, as suggested by Uriz et al. (1996) and Pisut & Pawlik (2002). All these factors contribute to explaining the selection of apparently redundant defense mechanisms. At the same time, their multiple functions constrain their evolution, meaning that there is always scope for the evolution of specialized predators able to circumvent the defense mechanisms of a given species.

In conclusion, multiple defense mechanisms in the studied morphotypes of *Cystodytes* deter different predators and act independently or in combination. Our results highlight the importance of considering all potential defenses of an organism against as many potential predators as possible in order to reliably assess their ecological roles.

CHAPTER 5

HOW DO MORPHOTYPES AND CHEMOTYPES RELATE TO GENOTYPES?

5.1 INTRODUCTION

Intraspecies variability in benthic invertebrates has been a long-standing source of taxonomic and biological controversy. Species with a large range of distribution may show morphological variants, usually related to their geographical or bathymetric distribution. Thus, some degree of morphological differentiation within widely-distributed or cosmopolitan species is to be expected. Color variation is one of the most frequently reported (e.g. Aron & Solé-Cava 1991, Dalby 1997), but texture, general shape and other morphological characters may also change from one organism or one place to another, sometimes in response to specific environmental conditions (e.g. bryozoans, Harvell 1990). New molecular tools enable us to assess the genetic basis of such morphological changes (e.g. Miller et al. 2001, Howell et al. 2004, Mackenzie et al. 2004). Since the advent of molecular techniques, many cases of cryptic species have been uncovered in marine invertebrates (reviewed in Knowlton 2000). The correct assignment of species in benthic invertebrates is not merely a taxonomic issue, but has clear implications in applied aspects such as biodiversity management, tracing of invasions or determining sources of new pharmacologically active substances (Holland 2000, Sweijd et al. 2000, Féral 2002).

Ascidians, like other marine taxa, show some cases of high intraspecies variability, generally related to cosmopolitan species (e.g. *Botryllus schlosseri*,

Cystodytes dellechiajei, *Ciona intestinalis*; Hoshino & Nishikawa 1985, Kott 1990, Monniot et al. 2001, Stoner et al. 2002), which in fact may be instances of speciation events. Aron & Solé-Cava (1991) found a genetic differentiation between two varieties of the Brazilian *Botryllus niger*. Dalby (1997, 2000) concluded that two Australian morphs of *Pyura stolonifera* were reproductively isolated. More recently, Tarjuelo et al. (2001) studying the genetic structure of the ascidian *Clavelina lepadiformis* living inside and outside harbors suggested the existence of cryptic species.

We chose the genus *Cystodytes* as a case study to assess the significance of morphological variation. Species of this genus, of which there are at least 24 (Sanamyan 2002), are found all over the world, including the Antarctic (Van Name 1945, Millar 1968, Monniot & Monniot 1974). Of these, the best known is undoubtedly *C. dellechiajei* (Della Valle 1877), a colonial soft-bodied ascidian widely distributed in tropical and temperate waters.

The taxonomic characters used in the assignment of species within this genus are unstable and largely influenced by authors' particular views, a problem derived from the morphological uniformity of the zooids and from their great contractility, which renders many characters difficult to observe (Van Name 1945, Monniot 1988, Kott 1990). On the other hand, color and colony size and shape show an amazing degree of variability (e.g. Harant 1929, Kott 1990, Monniot et al. 2001). Therefore, authors either tended to accept a restricted number of species while recognizing the difficulty of finding trustworthy characters at the specific level (Van Name 1945, Ärnbäck-Christie-Linde 1950, Kott 1990) or tried, on the basis of careful morphological examination, to disentangle the specific status within the genus in local areas. For instance, Monniot (1988) described 8 different species of *Cystodytes* in the lagoon of New Caledonia, seven of them new.

The assessment of spicular differences in ascidians is a reliable and common character used to differentiate between Didemnidae species (Lafargue & Laubier 1980), but its applicability to other families is dubious. Although Monniot (1970) concluded that spicular differences within the genus *Cystodytes* had no taxonomic value *per se*, they are still used in descriptions of new species, especially when the spicular features are distinctive (e.g. *C. ramosus*; Kott 1992). Thus, the present specific diversity of the genus *Cystodytes* is largely a result of species assignment on the basis of color, shape, zooid morphology and spicular composition, on which there is no general agreement. Some of the observed

morphological differences may also be due to intraspecies variability, as a result of genetic diversity or as a consequence of habitat, diet, chemistry or some combination thereof. Intraspecies variability in *Cystodytes* is poorly understood; thus, molecular tools can undoubtedly cast some light.

Cystodytes dellechiajei is distributed around the world in both tropical and temperate littoral waters. Its distribution is also eurybathic, as some samples have been collected at a depth of 735 m (Monniot 1974). Color, texture, spicular composition, shape and zooid size may vary without any clear pattern of distribution (Turon 1987, Méliane 2002). As so many forms have been attributed to this taxon, it might well be a group of species (Monniot 1988). All Mediterranean specimens of the genus have been traditionally included in the species *C. dellechiajei* (Pérès 1958, Turon 1987, Brunetti 1994), but there may be other species as well (Brunetti 1994, Méliane 2002).

We used two molecular approaches to analyze variation in Mediterranean *Cystodytes*: genetics and secondary chemistry. Genetic data can provide information about clade relatedness, patterns of evolution and indirect estimates of gene flow. Among the panoply of genetic tools used in ascidians, some are more informative at the phylogenetic level, e.g. 18s rDNA sequences, (Swalla et al. 2000, Stach & Turbeville 2002), while others, such as allozymes (Aron & Solé-Cava 1991, Yund & O'Neil 2000, Dalby 2000) or microsatellites (Stoner et al. 1997, 2002) have been used at the population level. Mitochondrial sequence data is a bridge between these two approaches (Avise et al. 1987). Analysis of mtDNA sequence data has advantages, such as maternal inheritance without recombination, higher mutational rate, shorter coalescence times and more sensitivity than nuclear genes in tracing population subdivision over large geographical scales (Avise et al. 1987, Palumbi et al. 2001). MtDNA is commonly used in intraspecific phylogeographic studies (Avise 2000) and has been applied in ascidians to address cryptic speciation problems (Tarjuelo et al. 2001, Turon et al. 2003).

Chemotaxonomy can also be used to assess inter- or intra- species variability (Valls 1993, Miller et al. 2001).

The aim of the present study was to determine how variability in traditional taxonomic characters such as color and spicular complement, relates to genetics and secondary chemistry in several *Cystodytes* morphotypes from the western Mediterranean Sea.

5.2 MATERIALS AND METHODS

5.2.1 Ascidian samples

For morphological and genetic analyses, 46 colonies of *C. dellechiajei* were collected from eight Mediterranean sites: North Catalonia (NE of Iberian peninsula), Cabo de Gata, Cabo de Palos and Alborán Island (Southern Iberian coast), Mallorca-Menorca and Ibiza-Formentera (Balearic Islands), Tunisia and Sicily (Table 1, Fig. 1). A single black colony from Mayotte (Indian Ocean) was used for comparison. Samples were collected by SCUBA diving in 2001 and 2002. The original color of the colony was recorded before fixation in absolute ethanol, and general colony and zooid morphology were examined in the fixed material. Some of the samples were anaesthetized by cold exposure as described elsewhere (Turon 1987) and fixed in formaldehyde for examination of zooids in a relaxed state.

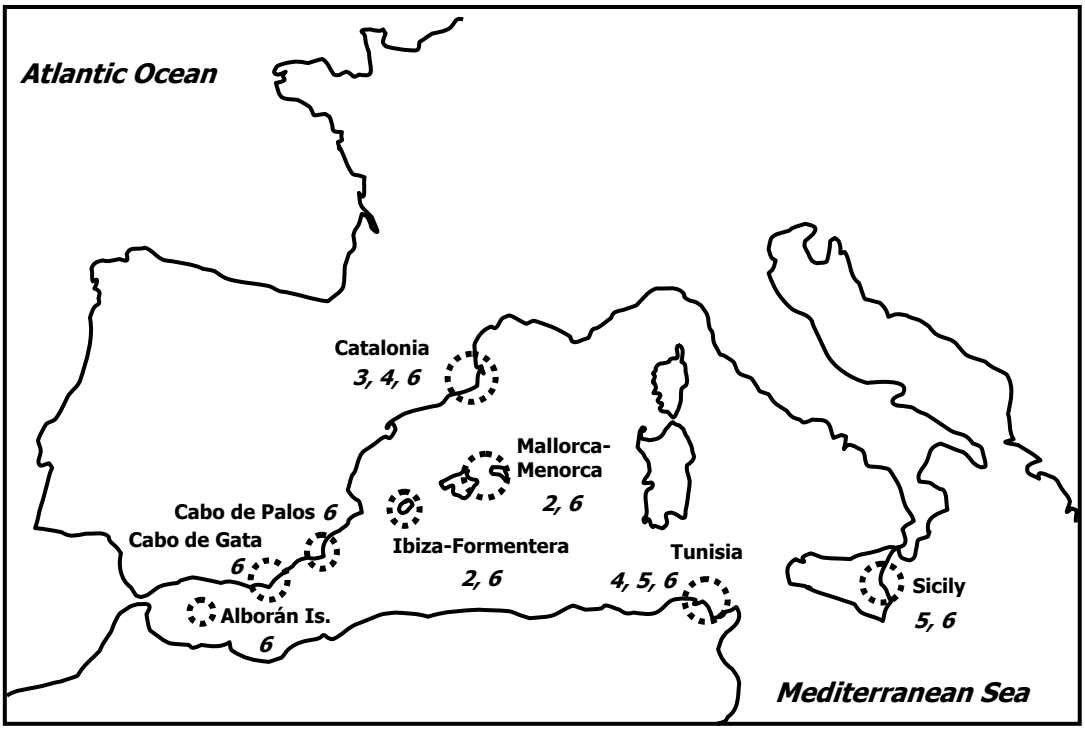


Fig. 1. Map showing the Mediterranean zones sampled: Catalonia, Cabo de Palos, Cabo de Gata, Alborán Islands, Ibiza-Formentera, Mallorca-Menorca, Tunisia and Sicily. Numbers refer to the presence of the corresponding clade (code of clades as in Fig. 5) in each area.

Table 1. List of haplotypes found, with sampling location, colour, major alkaloid known and spicular shape. GenBank accession numbers are also indicated

Haplotype	Location	Colour	Major alkaloid known	Spicular shape	GenBank #
S1	Ibiza-Formentera	Brown	-	Disc	AY523042
S2	Catalonia	Purple	Shermilamine B	Disc + Sphere	AY523043
S3	Catalonia	White	-	Disc	AY523044
S4	Mallorca-Menorca	Green	-	Disc + Sphere	AY523045
S5	Ibiza-Formentera	Blue	Ascididemin	Disc	AY523046
S6	Catalonia	Blue	Ascididemin	Disc	AY523047
	Alborán I.	Blue	-	Disc	
	Alborán I.	White-Grey	-	Disc	
S7	Catalonia	White, brown circles	-	Disc	AY523048
S8	Cabo de Gata	Green	Hydroxyascididemin	Disc + Sphere	AY523049
	Sicily	Pink	-	Disc + Star	
S9	Tunisia	Brown-Green	-	Disc + Sphere	AY523050
S10	Tunisia	Orange	-	Disc + Star	AY523051
	Catalonia	Purple	Shermilamine B	Disc + Sphere	
S11	Tunisia	Green, green circles	Hydroxyascididemin	Disc + Sphere	AY523052
S12	Tunisia	White, green circles	Hydroxyascididemin	Disc + Sphere	AY523053
S13	Tunisia	White, brown circles	-	Disc	AY523054
	Sicily	White, red circles	-	Disc	
S14	Sicily	Blue	-	Disc + Sphere	AY523055
	Sicily	White	-	Disc	
	Mallorca-Menorca	Blue	-	Disc	
	Mallorca-Menorca	Green	-	Disc + Sphere	
S15	Sicily	Yellow	-	Disc + Sphere	AY523056
S16	Catalonia	Green	-	Disc + Sphere	AY523057
	Mallorca-Menorca	Green	-	Disc + Sphere	
S17	Cabo de Palos	Green	-	Disc + Sphere	AY523058
S18	Mallorca-Menorca	Brown	-	Disc	AY523060
S19	Mallorca-Menorca	Brown	-	Disc	AY523061
S20	Catalonia	Purple	Shermilamine B	Disc + Sphere	AY523062
S21	Catalonia	Purple	Shermilamine B	Disc + Sphere	AY523063
S22	Catalonia	Purple	Shermilamine B	Disc + Sphere	AY523064
S23	Mallorca-Menorca	Green	-	Disc + Sphere	AY523065
S24	Catalonia	White, brown circles	-	Disc	AY523066
	Catalonia	White	-	Disc	
S25	Mallorca-Menorca	Blue	-	Disc	AY523067
	Mallorca-Menorca	Green	-	Disc + Sphere	
S26	Mallorca-Menorca	Blue	-	Disc	AY523068
S27	Mallorca-Menorca	Green	Hydroxyascididemin	Disc + Sphere	AY523069
S28	Ibiza-Formentera	Blue	Ascididemin	Disc	AY523070
S29	Ibiza-Formentera	Blue	Ascididemin	Disc	AY523071
S30	Ibiza-Formentera	Green	Hydroxyascididemin	Disc + Sphere	AY523072
S31	Ibiza-Formentera	Green	Hydroxyascididemin	Disc + Sphere	AY523073
S32	Cabo de Gata	Green	Hydroxyascididemin	Disc + Sphere	AY523074
S33	Cabo de Gata	Green	Hydroxyascididemin	Disc + Sphere	AY523075
S34	Mallorca-Menorca	Green	-	Disc + Sphere	AY523076
S35	Mayotte	Black	-	Disc + Toothed	AY523059

5.2.2 Scanning electronic microscopy (SEM)

To obtain the calcareous spicules, small pieces of the tunic were removed and boiled in commercial bleach for several minutes until complete dissolution of the tissue. They were washed several times in water to remove organic remains and dehydrated in absolute ethanol. Randomly pipetted samples were deposited on a stub covered with bi-adhesive tape, sputter-coated with gold, and studied under a Hitachi H1200 SEM.

5.2.3 DNA extraction and sequencing

To maximize DNA extractions, we separated the zooids from the tunic and their spicular capsules by forceps under a binocular microscope. The zooids were then kept in absolute ethanol at -25°C and later processed. Mitochondrial DNA was extracted using the Quiamp Mini Kit (Quiagen). Sequences were obtained for a segment of the Cytochrome *c* Oxidase subunit I (COI) mitochondrial gene. We used the universal primers HCO2198 and LCO1490 described in Folmer et al. (1994) to amplify the purple and brown forms and also to design more specific primers (AvA and AvB) for the blue and green morphs. The sequences of AvA (forward) and AvB (reverse) are 5'TTGGAATATGGTCCGCATTA3' and 5'ATGGCTGCAGCTAAAACTGG3', respectively.

Amplification was performed in a 20 μl total-reaction volume with 0.4 μl of each primer (25 μM), 0.5 μl dNTP's (10 mM), 2 μl 10x buffer containing 15 mM $MgCl_2$ (Promega), 1 U Taq polymerase (Promega) and 1 μl DNA. When using HCO2198 and LCO1490 primers, a single soak at 94°C for 2 min was followed by 35 cycles (denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 1.5 min), and a final extension at 72°C for 7 min, on a Perkin Elmer 840 DNA amplifier. The use of AvA and AvB primers followed an initial denaturation step at 94°C for 2 min and 35 cycles (94°C for 35 s, 45°C for 45 s and 72°C for 1 min), with a final extension at 72°C for 5 min, on a Perkin Elmer 9700 DNA amplifier.

The sequencing reaction was carried out using the same primers used for the amplification step and the ABI Big-Dye Ready-Reaction kit of Perkin Elmer. Sequences were obained on an ABI Prism 377XL automated sequencer.

5.2.4 Phylogenetic analysis

Sequences were aligned with SeqPup version 0.6 and alignments were confirmed by eye. All sequences have been deposited in the GenBank (accession numbers listed in Table 1). Tajima's D statistic was calculated with DnaSP version 3.51 (Rozas & Rozas 1999) in order to test whether mutations were selectively neutral or subject to selective pressures.

Relationships between haplotypes were established by using three methods: neighbour-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML). We used the COI sequence of another aplousobranch species, *Aplidium fuscum* (Polyclinidae), obtained by the authors in previous research, as an outgroup for the phylogenetic analyses.

The NJ algorithm, using the absolute number of nucleotide differences, was performed with the computer program MEGA version 2.1 (Kumar et al. 2001). The robustness of the NJ tree was evaluated by bootstrap analysis (Felsenstein 1985) with 1000 replicates.

MP cladograms were obtained using PAUP (version 4.0, Swofford 1998). Because of the large number of taxa, heuristic searches were performed (with TBR option). To minimize the risk of being trapped in local optima, searches were replicated 100 times with random addition of taxa. Bootstrap analysis was performed with 1000 replicates (Felsenstein 1985).

We used Modeltest 3.0 (Posada & Crandall 1998) to select the best-fit model of nucleotide substitution for our data set. Using PAUP, a haplotype tree was constructed under the maximum-likelihood criterion with the parameter estimates obtained under the best-fit model. A heuristic search was made with 100 replicates of random stepwise addition and tree bisection-reconnection (TBR) branch-swapping. Confidence in the nodes was assessed by 1000 bootstrap replicates of the above procedure but with fast-stepwise addition to keep computer time within reasonable bounds.

5.3 RESULTS

In all, 15 different colors or color combinations appeared in our sample set (Table 1). Their relative abundances varied greatly and, in most cases, only one or two color varieties were present at a single locality, although in some places three or even four color varieties were found, with overlapping ecological and bathymetric distribution.

5.3.1 Morphology of the zooids

We did not find any clearcut difference in the organization of the systems of zooids in the colonies or in the zooids themselves. However, detailed observation of zooids was only possible on the three main color morphs collected off the Catalan coast (purple, blue and green), for which we obtained relaxed material and observed ripe individuals and larvae. These forms were remarkably similar; the general colony shape varied only in thickness (from 4 to 8 mm, approximately), which correlated with certain characters of the zooids such as total length and the number of stigmata per row, while the remaining zooid characters were uniform. A short description of the zooids follows:

Thorax length in relaxed individuals from 1.15 mm (thin colonies) to 2.8 mm (thick colonies). Abdomen length from 1.15 mm to 3 mm, respectively. Siphons 6-lobed, the atrial one highly variable in length. Tentacles from 13-17 in small zooids and up to 35 in larger zooids. Four rows of stigmata, from 13 to 27 per half row as a function of zooid size (Fig. 2A). The first row bends anteriorly near the dorsal midline. The thorax musculature is strong and continues as a wide band over the right side of the abdomen. The band bifurcates at the level of the stomach and ends in two short protrusions at the end of the abdomen, possibly anchoring the zooid into the tunic (Fig. 2B). Short stolonic vessels arise from the bifurcation point in some zooids. A skirt of tissue emerges dorsally from the esophagial region, this skirt is only visible in specimens carefully removed from tunic (Fig. 2C). Abdomen twisted in most zooids, with stomach in a dorsal position. Stomach smooth, joined to a mid-intestine and a rectum. Anus opens between the third and four rows of stigmata. Testis formed by 6-8 pear-shaped follicles arranged radially on the left side of the abdomen (Fig. 2D). Sperm-duct originates in the center of the testis and runs

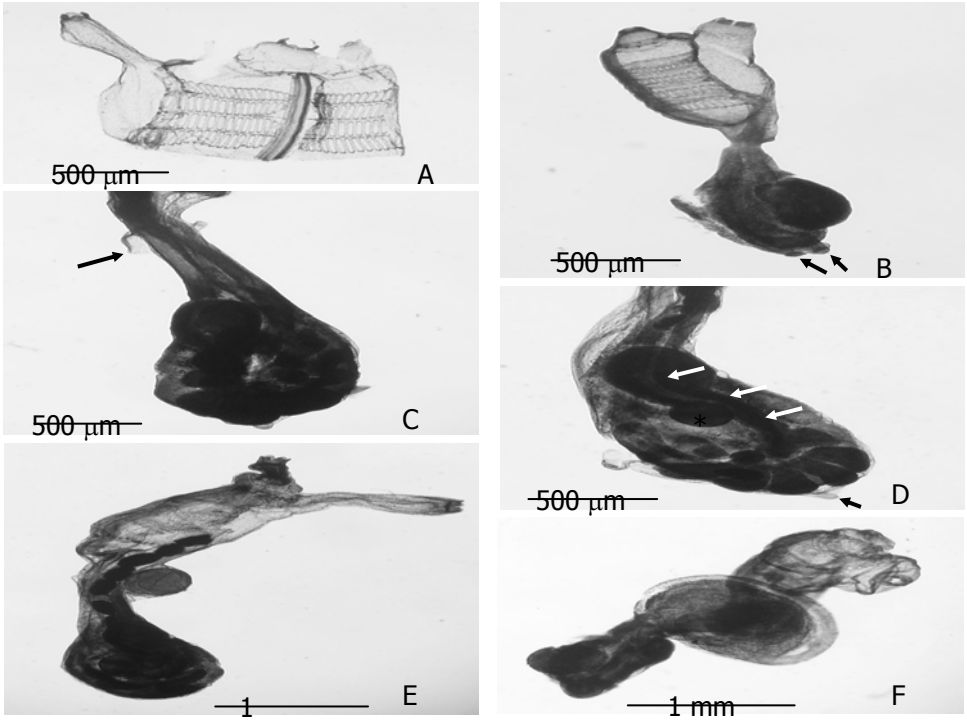


Fig. 2. A-F. Light micrographs of *C. dellechiajei*. Specimens from Catalonia – A. Green morph. Dissected branchial sac showing the four rows of stigmata. – B. Green morph. Juvenile zooid. The arrows point to the protruding end of the muscular processes on the right side of the body. – C. Purple form, abdomen of a zooid with male gonads, showing the characteristic dorsal skirt in the esophagial region (arrow). – D. Blue morph

straight anteriorly, except when oocytes are present, in which case the proximal part of the sperm-duct curves around them. Ovary slightly above the testis, formed, when mature, by one large ovum and one or two small oocytes. One larva can be brooded on the left side of the top of abdomen, where it protrudes with the dorsal side of the larva facing downwards (Fig. 2E-F). Occasionally a second embryo, much less developed, may be present under the first. Larvae, when mature, with a typical ring-shaped ectodermal expansion around the three adhesive papillae.

5.3.2 Morphology of the spicules

The genus is characterized by the presence of a capsule formed by layers of overlapping discoidal calcareous spicules, which encases the abdominal region of each zooid. When contracted, the branchial portion also retracts inside the spicular capsule. Small differences can be observed between these spicules, which vary from shield-shaped forms (with the center somewhat pointed, sometimes with a small hole in it) to flat, disc-shaped ones (Fig. 3A). In some cases, these spicules are also randomly distributed along the tunic, apparently “left behind” during budding (Lambert 1979).

Three additional spicule types were observed. The first were spherical (Fig. 3B), found either abundantly throughout the tunic or restricted to a few spicules concentrated in a portion of it, in which case they were easily overlooked. This variation in abundance showed no relationship with color. Within this spicular type, two morphologies were found: the first had concentric needles and the second was quite compact (Fig. 3C). As both types were found together in the same colony, no morphological importance is accorded to this difference, which may be the result of the preparation method. Although the spherical spicules were generally smaller than the discoidal ones, some small discoidal spicules were also observed (ca. 100 μm, Fig. 3D). Therefore, the flattened discoidal spicules do not seem to be the result of a two-dimensional growth of the spherical ones.

The second type, star-shaped spicules, was present in two specimens: one pink colony from Sicily (Fig. 3E) and an orange one from Tunisia. Finally, discoidal thick spicules with a toothed margin were found in the black morph from Mayotte (Fig. 3F).

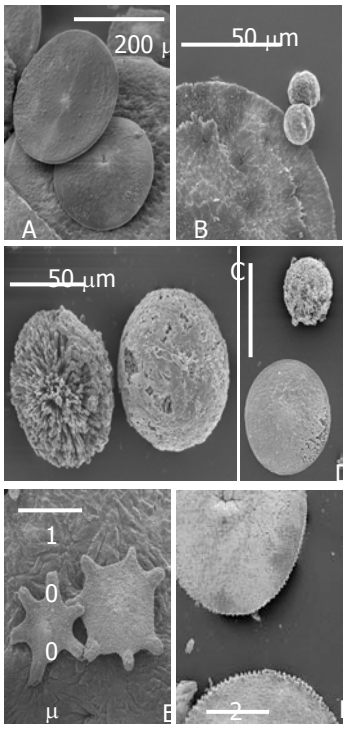


Fig. 3. A-F. SEM observations of *Cystodytes* spicules. – A. Blue specimen from Catalonia. Disc-shaped

5.3.3 Phylogenetic analysis

46 sequences were obtained for the COI mitochondrial gene with a final alignment length of 617 bp. No gap was needed. 35 haplotypes were identified (labeled S01 to S35) with a total of 160 (26%) variable sites (Table 1). Nucleotide variation was scattered across the entire sequenced region. As most variation was restricted to the third-base position of the codons, 157 (98.12%) of the nucleotide substitutions yielded synonymous changes. Replacement changes occurred only three times: two transversions affecting haplotypes S05 and S18, and one transition present in 14 of the haplotypes. Tajima's D was not significant (D=0.264, p>0.10) for our total data set, showing no evidence for selection in this gene and indicating that a neutral model cannot be rejected.

For the phylogenetic analyses we used the 35 haplotypes obtained (Table 1) with the sequence of *A. fuscum* as outgroup. The three different tree-construction methods used: neighbour-joining, maximum parsimony and maximum likelihood gave similar results. In Figure 4 the NJ cladogram is shown, with the support values obtained from bootstrap. The topology of MP and ML trees was essentially the same, so only the former is shown in Figure 5, which depicts the majority-rule consensus MP tree, as more than 60 best trees were obtained. Bootstrap support values from the MP and ML analyses are also shown in Figure 5. The comparisons between the different likelihood scores for each model of evolution showed that the K81uf+I model (K81 of Kimura 1981, but with unequal base frequencies) was the best-fit model for our data. It incorporates a substitution model and a proportion of invariable sites (I=0.678).

Six major clades appeared in all trees (indicated in Figs. 4, 5), differing only in the position of clade 3 (that clusters with clade 2 in the NJ tree and with clades 4, 5 and 6 in the MP and ML trees). The first clade (labeled 1 in Figs. 4, 5) is formed by the single sequence obtained from the Mayotte black form (S35) and stands in all analyses as outgroup to the remaining *Cystodytes* sequences. The second clade (2) groups three brown morphs from the Balearic Islands (with haplotypes S1, S18, S19), while the third (3) has only two haplotypes (S3 and S7), both white forms from Catalonia. Clade 4 contains the haplotypes from all the purple forms from Catalonia and one orange morph from Tunisia. It can be noted than in the ML tree the haplotype S22 stands outside this clade, as the sister group to the rest of clade 4 plus clades 5 and 6. Clade 5 is composed of haplotypes from three morphs from

Tunisia featuring either green circles or some green pigmentation and one yellow morph from Sicily (S15). Finally, clade 6 contains haplotypes coming from a mixture of different forms, colors and localities but is mainly dominated by green and blue morphs.

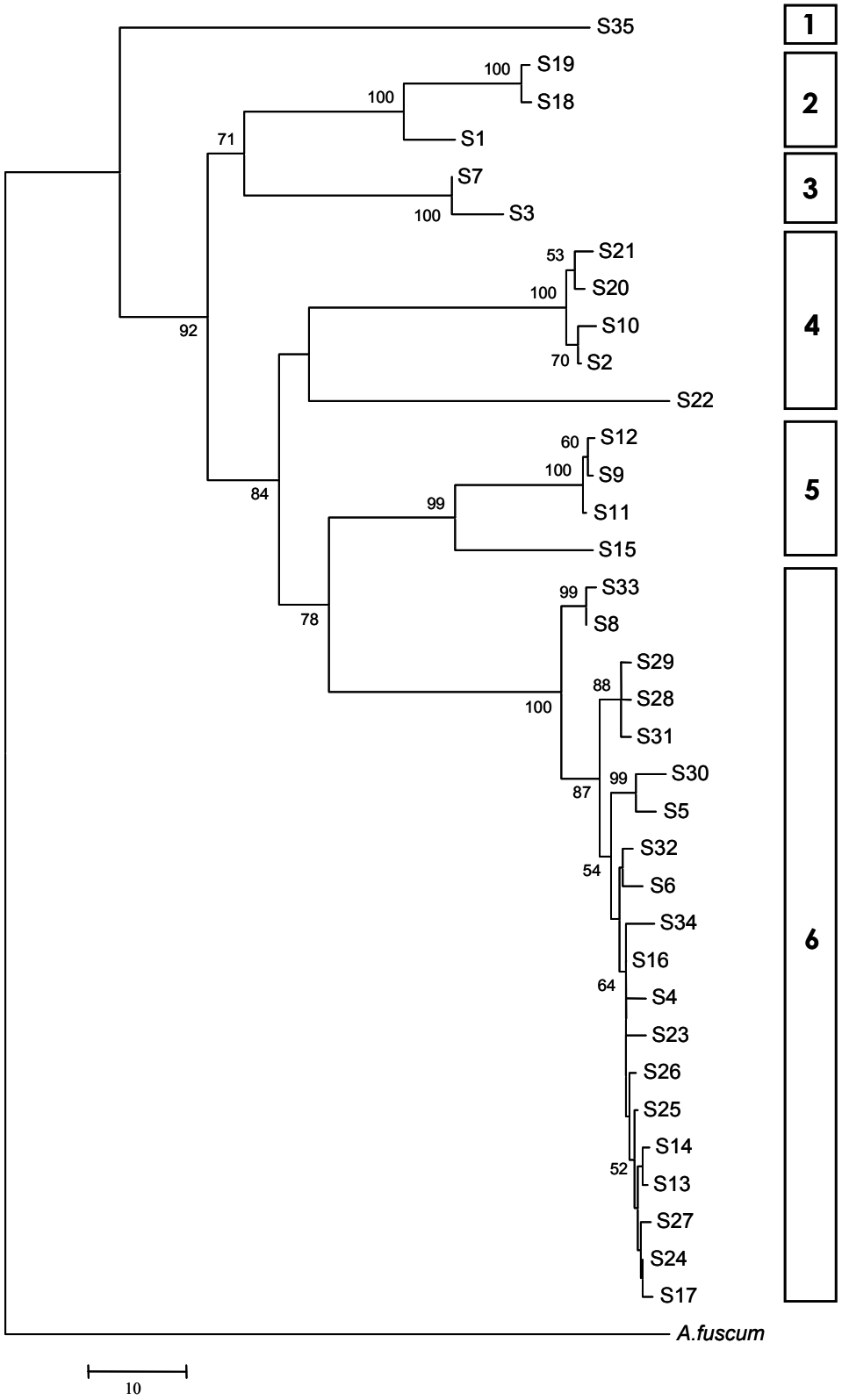


Fig. 4. Haplotypic neighbor-joining tree. Bootstrap values are shown for the branches with more than 50% support. The 6 recognized clades are signaled. Haplotype codes as in Table 1. Scale in distance units.

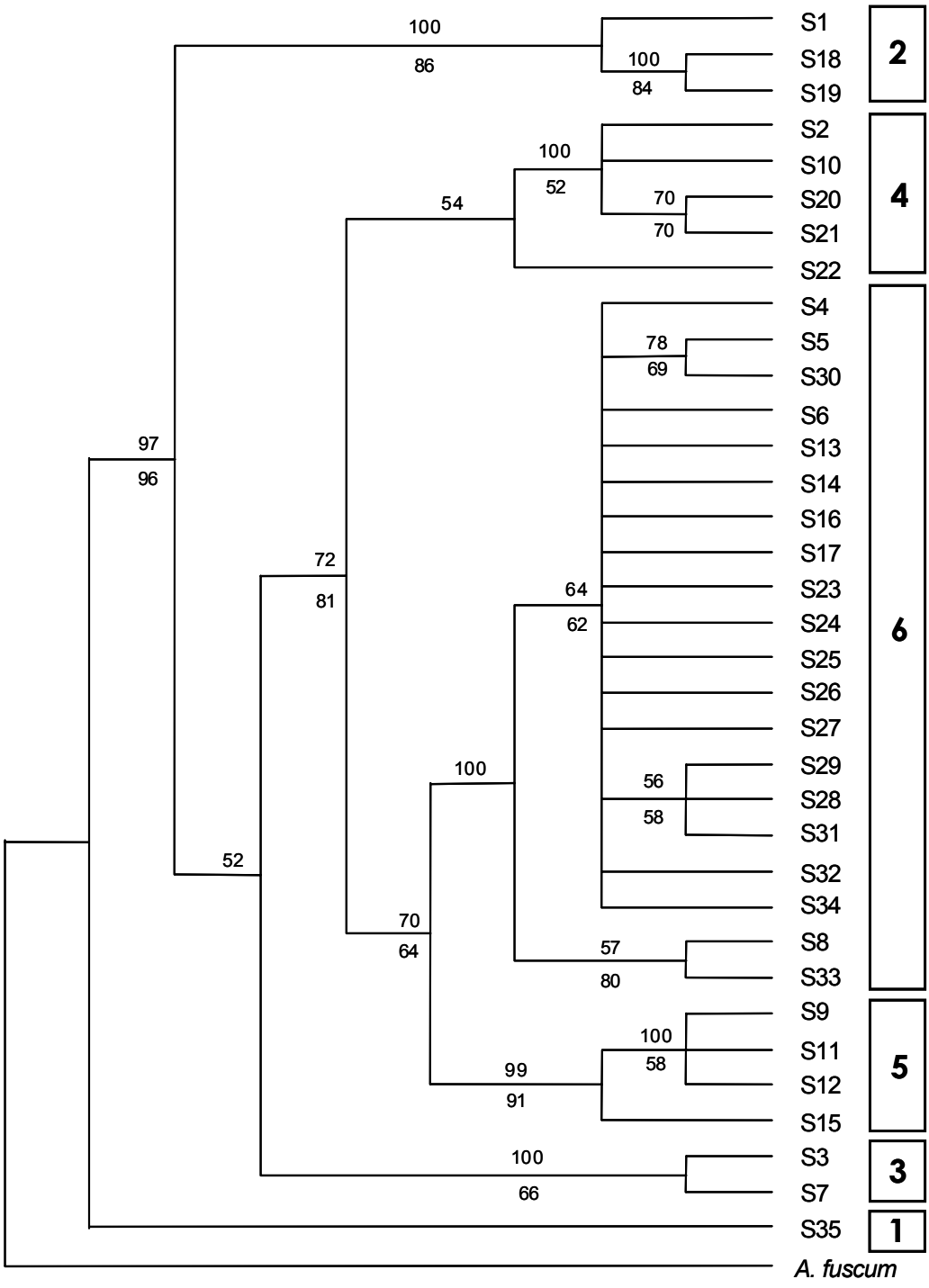


Fig. 5. Majority-rule consensus tree of the maximum-parsimony analysis. Bootstrap values are shown above the branches. Below the branches is indicated the support (if higher than 50%) obtained in the maximum likelihood analysis. The 6 recognized clades are signaled. Haplotype codes as in Table 1.

5.4 DISCUSSION

Morphological and genetic differences were found for *Cystodytes* inhabiting the Mediterranean Sea. Zooids, however, appear quite similar in most morphological characters. In particular, the muscle arrangement with two bands on the right side is the same as that found by Brunetti (1994) in Mediterranean specimens, but different from that found (one band at each side) in other areas (e.g. Van Name 1945, Kott 1990).

The mitochondrial gene studied is highly variable, with 35 haplotypes in 46 individuals. The different phylogenetic analyses reveal the same basic genetic structure, from which we determined the existence of six clades. The biggest genetic differentiation was found with the black morph from Mayotte, the only non-Mediterranean specimen analyzed. This single colony also had a specific kind of spicule (thick, disc-shaped, with a toothed margin), originally described in *C. aucklandicus* Nott 1892, and later found several times by Monniot (1988) and Monniot & Monniot (1996, 2001). Our data, therefore, support the validity of this species, to which our specimen is assigned. However, the black color of our sample seems an unreliable character, as Monniot (1988) and Monniot & Monniot (1996) found grey and brown *C. aucklandicus*, and many black *C. dellechiajei* were described by Brewin (1948).

No clear pattern of association between the various morphological parameters analyzed (color, spicular complement) or with any geographic area could be substantiated. Colors vary even between neighbouring colonies at the same site. Blue, green, white and brown morphs may be found together at the same locality (e.g. Blanes, northern Catalonia), and have several kinds of spicular composition. Conversely, the same spicular type can be found in several chromatic varieties (Table 1).

In addition, no relationship was found between spicule morphology and genetic clades. For instance, spherical spicules, which may be indicative of the species *C. philippinensis* Herdmann 1886 (Kott 1990, 2002), were found in individuals from three different clades (4, 5 and 6). The two colonies with star-shaped spicules were found in two separate clades (4 and 6), and the colonies with only discoidal spicules, which are the spicular complement of the typical *C. dellechiajei* (Kott 1990), appeared in clades 2, 3 and 6.

Color varieties seem to have a sounder genetic basis, although there are many exceptions. The main regularities observed are: the purple morph appears in our phylogram as a distinct group (clade 4, albeit this group includes also an orange form), while green and blue morphs constitute most of clade 6 and all the brown colonies from the Balearic Islands form clade 2. The lack of consistent correspondence between colors and genetic clades may simply reflect the fact that mtDNA can be carried across chromatic varieties as a result of hybridization, thereby suggesting that we are dealing with a single species. In another colonial ascidian, *Botryllus schlosseri*, polychromatism is the result of intraspecies variability controlled by a few nuclear *loci* (Sabbadin 1982, Yund & O'Neil 2000).

In Chapter 2 we described 2 major chemotypes within four *C. dellechiajei* color morphs (blue, green, brown and purple) from the north-western Mediterranean. The first had sulfur-containing pyridoacridines, corresponding to the purple morph. The second had C_9-unsubstituted pyridoacridines, found in the blue and green forms. A genetic basis for these two chemotypes seems substantiated. The purple morph (most of clade 4) was characterized by the presence of sulfur-containing pyridoacridines: shermilamine B (Carroll et al. 1989) and kuanoniamine D (Caroll & Scheuer 1990). The blue and green morphs (most of clade 6) presented the C_9 unsubstituted pyridoacridines: ascididemin (Kobayashi et al. 1988) and 11-hydroxyascididemin (Schmitz et al. 1991), respectively.

Our results show that groupings established from spicular composition or color alone are not supported by genetic or chemical information. Thus neither spicular composition nor color by itself is a sufficient criterion to discern between *Cystodytes* species. Some caution is necessary as we have only considered one mitochondrial gene and the study of others, especially nuclear genes, may yield different results. However, the COI gene seems well suited for discriminating between closely related species (Hebert et al. 2003).

Taken together, our results indicate that, if any distinction is to be made, it could be on the basis of the concordance of some genotypes with chemotypes that, in some cases, correlate with the color morph. For instance, specimens of the purple morph clustered together in the genetic analysis (clade 4) and feature sulfur-containing pyridoacridines. The association between secondary chemistry and color is not surprising in this case, as shermilamine B, the major alkaloid present in this form, is a purple compound (Banaigs & Bontemps-Subielos pers. comm.). However, we do not know the chemistry of all the varieties of *Cystodytes* studied, all other

characters are interspersed in our phylogram and there are no clear differences in zooid morphology. For the time being, therefore, it seems advisable to keep the Mediterranean specimens in a single species, *C. dellechiajei*. The study of the genetic structure of selected populations of Mediterranean *Cystodytes*, coupled with knowledge of their reproductive cycles and biological parameters, could provide valuable information about gene flow between populations and their degree of reproductive isolation.

CHAPTER 6

POPULATION GENETICS, PHYLOGEOGRAPHY AND SPECIATION

6.1 INTRODUCTION

The existence of cosmopolitanism and the boundaries between intra- and interspecies variability in benthic invertebrates have been a source of biological and taxonomic controversy. Species with a large distribution range may show morphological variants, usually related to their geographical or bathymetric distribution. However, the advent of molecular techniques has revealed a host of cryptic and sibling species in taxa formerly recognized as being widely distributed (Klautau et al. 1999, Knowlton 2000, McGovern & Hellberg 2003, Meroz-Fine et al. 2003). Therefore, morphology alone may be insufficient to assess species boundaries in marine invertebrates (Loukaci et al. 2004). Color variation in particular has often been reported in marine environments, and shows complex patterns of relationship with genetic differentiation, thus precluding any general assertion about the validity of color morphospecies (e.g. Wilson et al. 2000, McCartney et al. 2003, Mackenzie et al. 2004, Le Gac et al. 2004).

Although studies of population subdivision and speciation are crucial to our understanding and management of marine biodiversity (Holland 2000), the very concept of species is open to debate (Hull 1997, Sites & Marshall 2003). Templeton (2001) showed how formal phylogeographic analyses of genetic data can provide useful insights at the interface between intra- and interspecific evolution, within the framework of the cohesion species concept (Templeton 1989). Although genetic

variability is expected, true cosmopolitan species must maintain a certain degree of genetic cohesiveness, mediated by gene flow, throughout their distribution range. In marine environments, gene flow was thought to be secured by highly effective mechanisms of long-distance larval dispersal (Scheltema 1986, Russo et al. 1994). However, larvae of colonial invertebrates generally display limited dispersal capabilities (Jackson 1986, Hunt 1993, Hoskin 1997) and gene flow between populations may become seriously restricted to short geographic scales.

Cosmopolitanism in colonial ascidians poses a puzzling problem, as it seems to be at odds with their reproductive strategy. Larvae of colonial ascidians have short planktonic life-spans that can vary from minutes to hours (Millar 1971, Svane & Young 1989). In addition, evidence to date suggests that successful fertilization in colonial ascidians generally occurs over distances of only a few tens of centimeters (Grosberg 1991, Yund 1998, Bishop 1998). Therefore, the populations are mainly sustained by highly localized dispersal and recruitment, and are likely to display a marked genetic structure at a microgeographic scale (Yund & O'Neil 2000). Not surprisingly, instances of sibling speciation have shown up when ascidian species with variable morphology and wide distribution ranges have been studied using molecular markers (e.g. Aron & Solé-Cava 1991, Dalby 1997, Tarjuelo et al. 2001, 2004, Castilla et al. 2002). In addition, recent range expansions linked to human-mediated transport have added complexity to the issue of cosmopolitanism in ascidians (Lambert & Lambert 1998, Stoner et al. 2002, Castilla et al. 2002).

The general morphology of *Cystodytes dellechiajei* varies greatly. Although color variation is the most apparent feature reported, colony shape, texture, spicular composition, and zooid size may also vary, even within the same locality, without any clear distribution pattern (Turon 1987, Méliane 2002). In Chapter 5 we concluded that the morphological traits studied were not consistent enough to differentiate between *Cystodytes* species and we pointed out the need for formal population genetic analyses as well as biological studies in order to ascertain the status of the different forms attributed to *Cystodytes dellechiajei* in the western Mediterranean.

We have studied 7 populations representative of the main color morphs and geographic areas of the western Mediterranean in an effort to explore the population subdivisions and phylogeographic relationships among them. MtDNA sequence data was used to analyze the degree of differentiation between these locations, the main color morphs, and to perform a phylogeographic analysis (nested clade analysis;

Templeton 2004) of a haplotype network. In this way, we have sought to clarify the relationship between the different color morphs and genetic variation, and to determine whether *C. dellechiajei* should be split into several species.

6.2 MATERIAL AND METHODS

6.2.1 Ascidian samples

Sixty-seven individuals of *Cystodytes* were collected from seven Mediterranean sampling areas: northern Catalonia (NE Iberian peninsula), Mallorca-Menorca and Ibiza-Formentera (Balearic Islands), Cabo de Gata and Alborán Island (southern Iberian coast), Tunisia and Sicily (Fig. 1, Table 1). We considered each of these locations as populations because intra-zone distances were small and assumed to be within the range of larval dispersion of this species. Preliminary analyses of genetic variation (within color morphs) showed no significant intra-zone structure. Sampling was undertaken by scuba diving in 2001 and 2002. In order to avoid problems with colony fission and clonality, colonies were collected at least 5 m apart from each other. The specimens were identified as *C. dellechiajei* based on Turon (1987) and Kott (1990). The original color of the colony was recorded prior to fixation in absolute ethanol (Table 1).

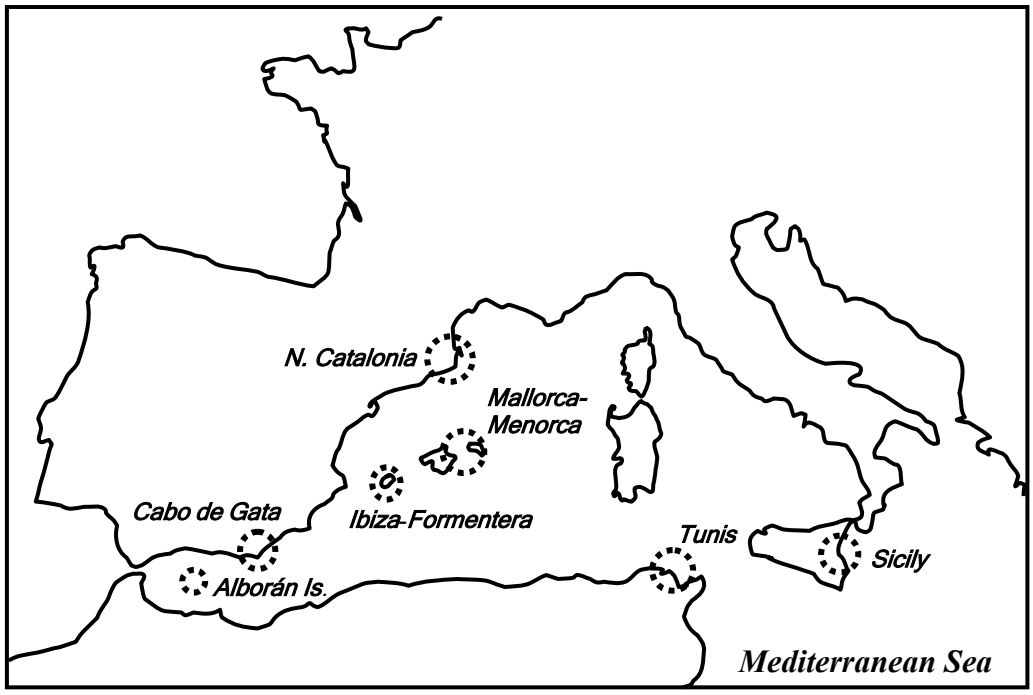


Fig. 1. Map showing the Mediterranean zones sampled: northern Catalonia, Cabo de Gata, Alborán Island, Ibiza-Formentera, Mallorca-Menorca, Tunisia, and Sicily.

Table 1. *Cystodytes dellechiajei* populations sampled in the western Mediterranean and their geographical location. The original color of the colonies, the sample size (N) and the haplotype distribution within locations and colors are also indicated.

Geographic locations	Color	N	Haplotypes
Northern Catalonia	Purple	10	H2, H10, H20, H21, H22
	Blue	4	H6
	White	4	H3, H7, H24
	Green	1	H16
Mallorca-Menorca	Brown	2	H18, H19
	Green	10	H4, H14, H16, H23, H25, H27, H34
	Blue	3	H14, H25, H26
Ibiza-Formetera	Brown	2	H1
	Green	4	H30, H31
	Blue	4	H5, H28, H29
Cabo de Gata	Green	6	H8, H32, H33
Sicily	Blue	1	H14
	Pink	1	H8
	Yellow	1	H15
	White	2	H14
Tunisia	Orange	1	H10
	Green	3	H9, H11
	White	2	H12, H13
Alborán Island	Green	2	H6
	Unknown	2	H10, H35
	White	2	H6

6.2.2 DNA extraction and sequencing

In order to maximize DNA extractions, we separated the zooids from the tunic and spicular capsules under a binocular microscope. The zooids were then kept in absolute ethanol at -25ºC until used. Mitochondrial DNA was extracted from the zooids using the 'Quiamp Mini Kit' (Quiagen). Sequences were obtained for a segment of the Cytochrome *c* Oxidase subunit I (COI) mitochondrial gene. The universal primers HCO2198 and LCO1490 described in Folmer et al. (1994) were used to amplify the purple, brown, white, and orange forms, whilst primers AvA and AvB described in Chapter 5 were more successful for the amplification of the blue, green, pink, and yellow color morphs. Amplification was performed in a 20 μl total-reaction volume with 0.4 μl of each primer (25 μM), 0.5 μl dNTP's (10mM), 2 μl 10x buffer containing 15mM $MgCl_2$ (Promega), 1 U Taq polymerase (Promega), and 1 μl DNA. When using HCO2198 and LCO1490 primers, a single soak at 94°C for 2 min

was followed by 35 amplification cycles (denaturation at 94ºC for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 1.5 min), and a final extension at 72°C for 7 min, in a Perkin Elmer 850 DNA amplifier. The AvA and AvB primers were used with an initial denaturation step at 94°C for 2 min and by 35 cycles (94°C for 35 s, 45°C for 45 s and 72°C for 1 min), with a final extension at 72° for 5 min, in a Perkin Elmer 9700 DNA amplifier.

The sequencing reaction was carried out with the ABI Big-Dye Ready-Reaction kit (Perkin Elmer) using the same primers as in the amplification step. Sequences were obtained on an ABI Prism 377XL automated sequencer. The nucleotide sequences obtained in this study were added to the extant genetic database created in Chapter 5 and were deposited in the GenBank, with accession numbers AY523042-AY523057, AY523060-AY523076 and AY821784.

6.2.3 Population genetic analysis

Sequences were aligned using SeqPup version 0.6 and alignments were confirmed by eye. Tajima's D statistic was estimated with DnaSP version 3.51 (Rozas & Rozas 1999) to test whether selective pressures were acting on the substitutions.

Further analyses where performed using Arlequin version 2000 (Schneider et al. 2000). Nucleotide diversity and haplotype diversity (Nei 1987) were calculated for each population. The exact test of population differentiation (Raymond & Rousset 1995) was used to test the null hypothesis of a random distribution of the different haplotypes among geographic locations and colors, and its significance was estimated from 10000 random permutations of the original data matrix. We calculated pairwise F_{ST} values and their significance through permutation tests (1000 replicates) for both the geographic location and the main color morphs (purple, blue, white, green and brown). To test the isolation by distance between populations (Rousset 1997), we performed a Mantel test with 10000 permutations. Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed to test hierarchical models of genetic variability using pairwise differences as a measure of divergence. We tabulated our data according to two grouping categories: per geographic location and per color morph. This data was then used to test two models. In the first model genetic variance was partitioned into the following levels: among geographic location, between morphotypes within geographic location, and

within morphotypes at each location. In the second model, we estimated the relative variance contribution of the following components: between color morphs, between locations within each color morphotype, and within locations for each color morph. Comparison of the results provided an estimate of the relative contribution of geographic distance and morphotype to the overall genetic variability.

6.2.4 Phylogeographic analysis

We estimated a haplotype network using the TCS version 1.18 program (Clement et al. 2000), which implements the statistical parsimony method described by Templeton et al. (1992). This method estimates an unrooted tree and provides a 95% plausible set for the relationships between the haplotypes. We solved ambiguities (loops) in the resulting network by applying the criteria summarized in Pfenninger & Posada (2002). We then nested the network by hand, using the procedures described in Templeton et al. (1987) and extended in Crandall (1996). Symmetrically stranded intermediate clades were nested according to the method described by Templeton & Sing (1993). Once the nesting design was complete, we performed a nested clade analysis (NCA; Templeton et al. 1987) on the clades with geographical and genetic information using the Geodis version 2.0 program (Posada et al. 2000). A matrix of geographical distances (in Km) between the localities was input into the program, and distance data were automatically coded as clade distances (Dc, a measure of geographical dispersion of individuals bearing haplotypes of a given clade) and nested clade distances (Dn, a measure of how far individuals with haplotypes belonging to a given clade are from individuals bearing haplotypes of the immediate higher-step clade). NCA aims to distinguish recurrent gene flow from historical events as determinants of the present-day distribution of haplotypes (Templeton 1998). The program looks for significant associations between genetic and geographical distances (Dc and Dn) using randomization procedures. Significant associations can then be interpreted in terms of biological processes using the inference key described in Templeton (2004).

A muldimensional scaling analysis (MDS) was also performed on a matrix of genetic distances estimated as the number of pairwise differences with the Mega version 2.1 program (Kumar et al. 2001). The MDS was run in two dimensions with the Kruskal linear algorithm using the statistical software Systat version 9.

6.3 RESULTS

A total of sixty-seven colonies of the ascidian *Cystodytes dellechiajei* were sampled in the western Mediterranean, and eight color morphs were recorded. Purple, green, blue, white and brown were the most abundant color morphs, whilst orange, yellow, and pink colonies were found only occasionally (Table 1). After alignment and trimming, we obtained sequences for the COI mitochondrial gene with a final length of 617 bp. Thirty-five different haplotypes were identified with a total of 154 variable sites (Table 1). Nucleotide variation was scattered across the entire sequenced region but was mainly restricted to the third base position of the codon. Thus, 153 (99.35%) of the nucleotide substitutions yielded synonymous changes, and only one was a transition (A-G) resulting in a non-synonymous substitution. Tajima's D statistic was not significant ($D = 0.48880$, $p > 0.10$) for the entire data set, showing no evidence of selection in this gene, and indicating that a neutral model cannot be rejected.

6.3.1 Population genetics

Nucleotide diversity (π), haplotype frequencies within populations (H), and haplotype diversity (h) as a measure of within-population variation (Nei 1987) are summarized in Table 2.

The results of the exact test for population differentiation based on haplotype frequencies between the different locations and between color morphs in both cases revealed significant heterogeneity in the distribution of haplotypes ($p < 0.05$). Pairwise tests for genetic differentiation among geographic locations based on F_{ST} values showed that all pairwise comparisons involving the Tunisian population displayed significant genetic divergence. Likewise, almost all comparisons of the Catalan population with the other locations were significant (Table 3). The F_{ST} analysis in which the samples were grouped according to the main color morphs (purple, blue, green, white, and brown), disregarding their geographic origin, showed a strong genetic differentiation of the purple and brown morphs from all the others. In contrast, differences between blue, green, and white morphs were not significant (Table 4). The mean pairwise F_{ST} value between geographic locations was significantly lower than between color morphs (0.21 ± 0.03 vs. 0.55 ± 0.11, respectively, mean$\pm$SE; t-test, $p < 0.01$).

Table 2. Parameters of the populations studied: Nucleotide diversity (π) and haplotype diversity (h) and its standard error (SE) are listed. Haplotype frequencies are also given.

	Mallorca-Menorca	Sicily	Ibiza-Formentera	N Catalonia	Tunisia	Cabo de Gata	Alborán Island
p	0,038	0,049	0,049	0,081	0,072	0,006	0,072
h	0,924	0,9	0,889	0,912	0,933	0,600	0,6
SE	0,053	0,161	0,075	0,04	0,122	0,215	0,215
H1	0	0	0,2	0	0	0	0
H2	0	0	0	0,105	0	0	0
H3	0	0	0	0,0526	0	0	0
H4	0,133	0	0	0	0	0	0
H5	0	0	0,2	0	0	0	0
H6	0	0	0	0,211	0	0	0,667
H7	0	0	0	0,0526	0	0	0
H8	0	0,2	0	0	0	0,667	0
H9	0	0	0	0	0,333	0	0
H10	0	0	0	0,211	0,167	0	0,167
H11	0	0	0	0	0,167	0	0
H12	0	0	0	0	0,167	0	0
H13	0	0,2	0	0	0,167	0	0
H14	0,267	0,4	0	0	0	0	0
H15	0	0,2	0	0	0	0	0
H16	0,0667	0	0	0,0526	0	0	0
H18	0,0667	0	0	0	0	0	0
H19	0,0667	0	0	0	0	0	0
H20	0	0	0	0,0526	0	0	0
H21	0	0	0	0,105	0	0	0
H22	0	0	0	0,0526	0	0	0
H23	0,0667	0	0	0	0	0	0
H24	0	0	0	0,105	0	0	0
H25	0,133	0	0	0	0	0	0
H26	0,0667	0	0	0	0	0	0
H27	0,0667	0	0	0	0	0	0
H28	0	0	0,1	0	0	0	0
H29	0	0	0,1	0	0	0	0
H30	0	0	0,3	0	0	0	0
H31	0	0	0,1	0	0	0	0
H32	0	0	0	0	0	0,167	0
H33	0	0	0	0	0	0,167	0
H34	0,0667	0	0	0	0	0	0
H35	0	0	0	0	0	0	0,167

Table 3. Pairwise F_{ST} values among locations. Significant values at P<0.05 are indicated with an asterisk.

	Mallorca-Menorca	Sicily	Ibiza-Formentera	N Catalunya	Tunisia	Cabo de Gata
Mallorca-Menorca	**0**					
Sicily	0	**0**				
Ibiza-Formentera	0.071	0.088	**0**			
N Catalonia	0.299*	0.219*	0.232*	**0**		
Tunisia	0.491*	0.314*	0.340*	0.246*	**0**	
Cabo de Gata	0.249	0.153	0.251*	0.330*	0.569*	**0**
Alborán Island	0.061	0.008	0.004	0.051	0.274*	0.18519*

Table 4. Pairwise F_{ST} values among main colors. Significant values at p < 0.05 are indicated with an asterisk.

	Purple	Blue	White	Green	Brown
Purple	**0**				
Blue	0.866*	**0**			
White	0.644*	0.127	**0**		
Green	0.759*	0.041	0.045	**0**	
Brown	0.809*	0.906*	0.579*	0.763*	**0**

There was no evidence of isolation by distance between the geographic locations (Mantel test, p = 0.66). In fact, whilst the most distant populations (Sicily and Alborán Island, separated by a distance of 1618.28 Km) were not significantly different, the reverse was true of the closest ones, Cabo de Gata and Alborán Island (144.64 Km apart).

The hierarchic AMOVA analysis (Table 5) based on geographic origin revealed a negligible and non-significant (0.59%) variance component associated with between groups (=geographic locations) differentiation. This indicates that inter-group differences do not explain any significant variance above that accounted for by comparisons between colors within geographic location (ca. 76%) and within colors within location (23%), the latter two components being significant. In contrast, if we partition our model to consider location nested within color morphs (Table 5), then most of the variance (ca 59%) is concentrated between color morphs, with a much smaller variance (27%) explained by comparisons between locations (within colors), and a still lower residual variance within locations and color morphs (14%). All components, however, were highly significant in a permutation test (p < 0.001).

Table 5. Two level AMOVA considering both the geographic localization and the colors. Sum sq: Sum of squares; Var com: Variance component; % Var: Percentage of variation

AMOVA	d. f.	Sum sq	Var com	% Var	p-value
Among locations	6	406.51	0,117	0.59	0. 595
Among locations within colors	10	520.48	15	76,02	<0.001
Within colors	45	207.8	4,62	23,39	<0.001
Among colors	4	636.53	12,28	56.11	<0.001
Among colors within locations	11	254.53	6,44	29.42	<0.001
Within locations	42	133.05	3,17	14.47	<0.001

6.3.2 Phylogeography

The parsimony haplotype network is presented in Figure 2. The maximum number of steps for a 95% parsimonious connection between haplotypes (i.e. with a 95% confidence level that no multiple substitution has occurred at some site) is 10 mutations. We obtained 8 groups of haplotypes that were separated by more than 10 changes. Group A was the only one in which we found loops (two of them) connecting haplotypes. These ambiguities were easily solved using the frequency criterion (Pfenninger & Posada 2002) that states that low-frequency haplotypes are more likely to be connected to haplotypes with high frequency. The different groups of haplotypes appeared well separated in the two-dimensional space (stress of the configuration = 0.17) of the MDS ordination based on a matrix of genetic distances (Fig. 3). In particular, the largest group (A, with 19 haplotypes) formed a cluster at the positive end of dimension 1, well separated from the remaining groups. Figure 3 shows a graphical representation of the color morphs that predominate in the eight groups. Note that whilst group A is mainly formed by the blue, green, and white morphs, only one or two colours were found in the remaining groups.

The eight groups of haplotypes found in the parsimony network were subsequently treated as independent cladograms. No attempt was made to group these terminal units because the number of changes in the shortest connections between them was generally well above the parsimony limit of 10 (e.g. the larger group, A, is separated from the other groups by no less than 50 changes). The nesting design obtained for each group of haplotypes is also depicted in Figure 2.

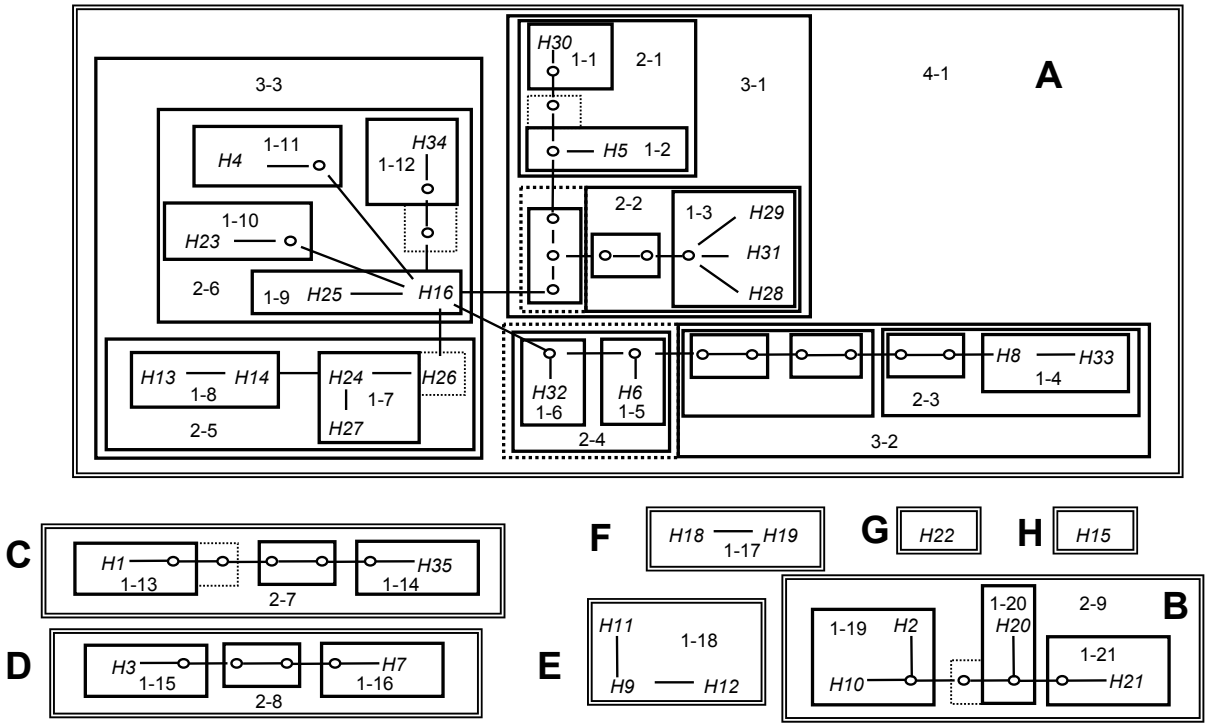


Fig. 2. Statistical parsimony cladogram obtained from the TCS program. Each line represents a mutational step. Missing intermediates are indicated by 'o'. The 8 groups that could not be connected unambiguously are designated by letters A to H. The nesting design adopted is shown with boxes, and boxes with dotted margins indicate the assignment of symmetrically stranded clades.

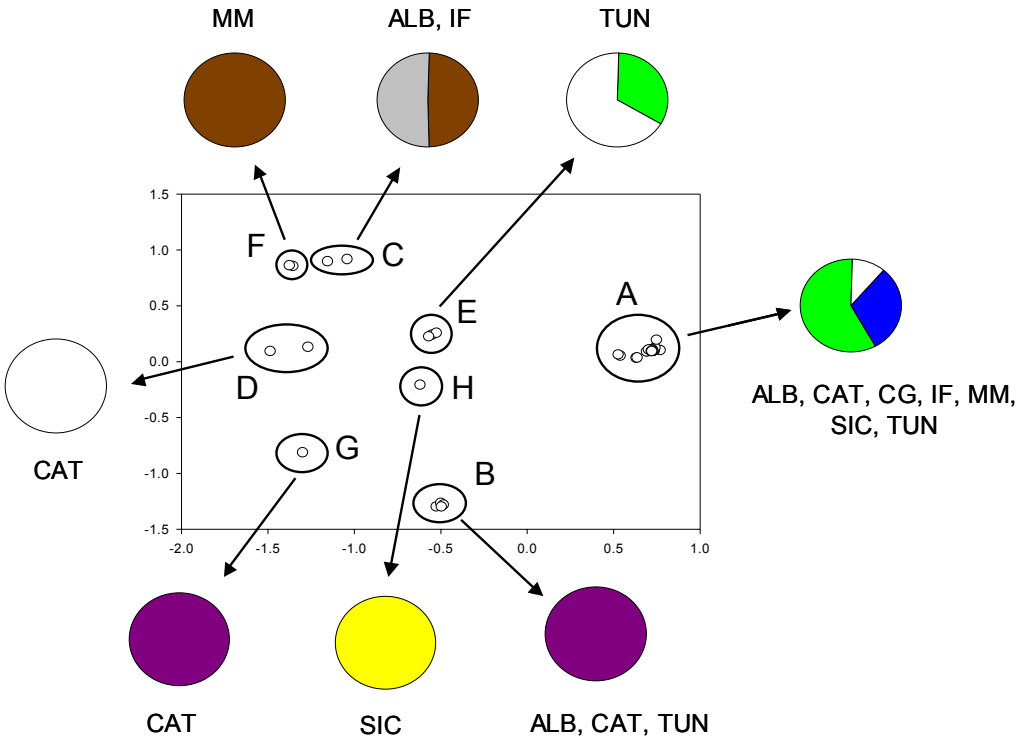


Fig. 3. Results of the MDS analysis, with the groups obtained in the haplotype cladogram overlaid. For each group the pie chart indicates the proportion of haplotypes featuring the main colors considered. When one haplotype contained representatives of more than one color morph, we assigned the color that was more often recorded in terms of number of individuals. The grey shading in the pie corresponding to group C indicates haplotypes whose color was not recorded. The initials below the pie charts indicate the geographical locations in which the haplotypes of that group have been found.

Only group A had a four-step nesting design; the other groups featured one- or two-level clades. From the nested cladograms, we obtained the corresponding clade and nested clade distances, and tested the significance of associations between geographic and genetic patterns. The results of NCA showed that most associations were not significant, indicating a lack of geographic structure in the data. Table 6 shows the relevant parameters for those clades that showed significant associations, as well as the corresponding inferences of the processes that generated the observed patterns. For Clade 2-5 the outcome was inconclusive, while for Clade 3-3 (that includes haplotypes found in Northern Catalonia, Balearic Islands, Sicily, and Tunisia) a contiguous range expansion was substantiated. For Clade 4-1 (the one that links the whole of

Table 6. Results of the nested clade analysis (NCA). Only clades with significant geographical associations as detected by the permutation test are included in the table. For each clade we list the members (either tips or interiors), the corresponding clade (Dc, in km) and nested clade (Dn) distances, and the Interior-Tip contrasts. We indicate significantly small (S) or large (L) distances. The "Inference" column describes the steps followed in the inference key of Templeton (2004) applied to that clade and the biological process inferred.

Clade	Clade members	Dc	Dn	Inference
2-5	1-7 (interior)	165.96	462.4	1-2-11-17
	1-8 (tip)	702.15	642.57(L)	Inconclusive Outcome
	I-T	-536.19	-180.16	
3-3	2-6 (interior)	82.98(S)	289.72(S)	1-2-11-12
	2-5 (tip)	621.09	503.52	Cont. Range Expansion
	I-T	-538.11(S)	-213.78(S)	
4-1	3-3 (interior)	483.75	559.48	1-2-3-5-15
	3-1 (tip)	0(S)	402.97(S)	Past Fragmentation
	3-2 (tip)	591.43	619.90(L)	
	I-T	98.02	15.03	
Whole cladogram	1-17 (tip)	0	1071.60(L)	1-2-3-5-15
	1-18 (tip)	0(S)	776.42(L)	
	1-19 (tip)	0	361.44	Past Fragmentation
	1-20 (tip)	0	419.03	
	2-7 (tip)	400.90	506.54	
	2-8 (tip)	0	397.67	
	2-9 (tip)	441.72	502.43	
	4-1 (tip)	557.43	549.37	

group A), a past fragmentation event explains the geographic distribution of the haplotypes. In agreement with Templeton (2004), a past fragmentation event is favored over a long distance colonization due to the number of steps (see missing intermediates in Fig. 2) separating the haplotypes found in Ibiza-Formentera (clade 3-1) from the other ones. Finally, at the level of the whole cladogram, another fragmentation event was inferred, this time being caused mainly by the separation of the haplotypes found in Tunisia (clade 1-18) from the remaining ones.

6.4 DISCUSSION

Our results show a noticeable variability among the samples in the gene studied. Although this variability seems to have the strongest component associated with the diverse color morphotypes, divergence according to location was also observed. Thus, F_{ST} values were low when comparing samples grouped according to geographic origin, although some comparisons were significant (e.g. most comparisons involving northern Catalonia and Tunisia). In contrast, the divergence between the main color morphs was significant in comparisons of the brown and purple morphs with the others. However, no significant genetic distance was detected between the green, blue, and white morphs. Overall, F_{ST} values were significantly higher between color morphs than between geographic locations. The AMOVA clearly illustrates this same pattern. The contrasting outcomes according to the partitioning of the model indicate that differences between color morphs are sufficiently large to blur any geographic differentiation when colors are combined. However, when variance due to color divergences is taken out, there remains a significant geographic variability between the geographic areas.

The haplotype network also shows a wide genetic variability, and up to 8 separate groups of haplotypes appeared that could not be connected parsimoniously. The MDS ordination matches the results of the haplotype network, and an important role of color morphs in the groupings seems evident. The largest group (A) shows a mixture of blue, green, and white colors, again indicating lack of differentiation of these particular morphs, whilst other groups appear more clearly linked to either a single or a few color morphs (purple, brown). The haplotypes occupying the innermost position in the nesting design of group A were found in northern Catalonia and Mallorca-Menorca; thus, any of these areas could have been the center of diversification of this large group of haplotypes.

When we performed the NCA, however, only a few of the clades showed significant associations between geography and genetic variability. This is surprising given the long branches that separate the diverse groups, which indicate a deep divergence. The pattern may be explained by secondary

expansions of the diverse groups in the areas studied after an initial, possibly allopatric, divergence. These range expansions would have erased most of the geographical signal. In this context, the maintenance of genetic differences strongly points to a reproductive isolation of at least some of these forms. Past fragmentation is a relevant biological inference when trying to infer species boundaries (Templeton 2001), as it falsifies the hypothesis that the samples came from a single evolutionary lineage. Our NCA shows that a past fragmentation event occurred that caused the divergence of some of the main groups, as well as within some clades (e.g. within group A). These results, coupled with some evidence of range expansion in other clades (e.g. clade 3-3), support the idea of a secondary expansion of already diversified lineages in the western Mediterranean.

Previous chemical analyses (see Chapter 2) revealed important qualitative differences in the alkaloid composition of some of the morphotypes studied here. The blue and green forms, corresponding to group A of the present study, featured C_9-unsubstituted alkaloid pyridoacridines, whilst purple forms, corresponding here to group B, had sulfur-containing pyridoacridines. This, along with the existence and maintenance of diverse morphotypes in the same locality (up to four color variants were found at the same place), strongly argues against the notions of ecological interchangeability and genetic exchangeability necessary for the establishment of a cohesion species (Templeton 2001). The shortest genetic divergence between haplotypes of groups A and B is 9.88% which, assuming a mutational rate for ectoderm mtDNA of 2.86/MY/locus (Morita 1999) gives us a scenario of at least ca. 3.5 millions of years of separation, probably more given that multiple substitutions must have surely occurred. Although this datum is only indicative because we do not have a calibrated molecular clock in this group, this time of divergence is coherent with the idea that these groups diverged after the Messinian salinity crisis (5-6 MY; Maldonado 1985) in the Mediterranean, coinciding with a period of ample scope for colonisation of new habitats and speciation in this Sea.

In conclusion, we found a complex distribution pattern of genetic variance mainly explained by a clear differentiation between some (but not all) color morphs. However, divergence among geographic localities was also evident when the different morphotypes were considered separately. Fragmentations and range

expansions seem to have shaped the present-day distribution of the haplotypes. Taken together with chemotype information, our results indicate that several species are present in the area, as already suggested by some authors (Turon 1987, Brunetti 1994, Méliane 2002). Clearly, previous reports of *Cystodytes dellechiajei* in the Mediterranean should be considered with caution. How many different species are present, and which one (if any) should keep the name *C. dellechiajei* will only be clarified after the analysis of more material from the Mediterranean and other seas.

The study presented here illustrates the importance of combining a variety of approaches in the study of species boundaries. This is particularly true in the case of species groups, such as ours, that produce active metabolites under investigation from the standpoint of their possible applications.

CHAPTER 7

LIFE CYCLES AND GROWTH RATES OF TWO MORPHOTYPES OF *CYSTODYTES*

7.1 INTRODUCTION

In modular marine invertebrates (reviewed in Larwood & Rosen 1979, Jackson et al. 1985, Harper et al. 1986), the interaction between genotype and environment often generates a distinct profile of growth, fission, fusion, regeneration, and senescence for each clone. Thus, the study of biological cycles in colonial ascidians, including growth, reproduction, and mortality, often reveals complex patterns (Bak et al. 1981, Ryland et al. 1984, Turon & Becerro 1992). In colonial ascidians, fusion and fission phenomena may occur frequently and cause rapid changes in original colony size (Mukai & Watanabe 1974, Bak et al. 1981, Stocker & Underwood 1991, Stocker 1991). On the other hand, environmental parameters, such as habitat boundaries, competition and partial predation, may cause a decrease in growth rate with size (Brunetti & Copello 1978, Brunetti et al. 1988, Stocker & Underwood 1991, Turon & Becerro 1992). For instance, Stocker & Underwood (1991) found that sexual and asexual reproduction rates of *Didemnum moseleyi* (Didemnidae) were lower in specimens found close to sponges. Due to the influence of such intricate and heterogeneous factors on life cycles, organisms of the same age can vary widely in their growth, survival, and fecundity parameters. Therefore, accurate measurements of relevant life-history traits can only be obtained by following the fates of individual colonies over as much of their life cycle as possible (Hughes & Cancino 1985, Stocker 1991). Such important biological

information is scarce for modular benthic invertebrates (Jackson & Coates 1986, Turon et al. 1998), and in particular for colonial ascidians (Bak et al. 1981, Turon & Becerro 1992), hindering the development of demographic and life-history theories applicable to modular organisms (Caswell 1982, 1985, Williams 1986, Sebens 1987).

Temperate seas display marked seasonal fluctuations in environmental parameters (reviewed in Coma et al. 2000) that are reflected in many of the life cycle patterns observed in ascidians (Turon 1988, Turon & Becerro 1992, Durante & Sebens 1994, Tarjuelo 2001, Caralt et al. 2002). Seasonality has commonly been linked to temperature, but other factors, such as nutrient availability, should also be considered (Coma & Ribes 2003). To date, water temperature changes (Berrill 1935, Millar 1971, Turon & Becerro 1992, Durante & Sebens 1994) and/or what Berrill (1935) called "sexual exhaustion" (when larval brooding has drained nutritive reserves) have been used to explain the continued alternation between phases of maximum asexual and sexual reproduction observed in many ascidians (Millar 1971, Morgan 1977, Brunetti et al. 1988, Durante & Sebens 1994). This trade-off suggests a partitioning of energy resources in the colony over the course of its life cycle that may be used alternatively for reproduction and growth. Nevertheless, few studies have addressed the existence of trade-offs in resource allocation in colonial ascidians (Yund et al. 1997, Newlon III et al. 2003, Tarjuelo & Turon 2004). In the Mediterranean, high water temperatures may also induce resistance forms and aestivation phenomena in some species (Coma et al. 2000). For instance, aestivation has been observed in *Clavelina lepadiformis* (Clavelinidae) (Caralt et al. 2002), whilst resistance forms have been reported in *Pseudodistoma crucigaster* (Pseudodistomidae), *Polysyncraton lacazei* (Didemnidae) (Turon 1988, 1992, Turon & Becerro 1992), and *Botrylloides leachi* (Styelidae) (Brunetti 1976).

Cystodytes dellechiajei (Della Valle, 1877) is a colonial ascidian widely distributed in both tropical and temperate waters. In Chapter 5 we concluded, on the basis of morphological, chemical, and phylogenetic studies, that although genetic and biochemical data suggests the presence of several species, the morphological traits studied (color patterns and spicular composition) were not consistent enough to differentiate between *Cystodytes* species in the Mediterranean Sea. In that study, up to five clades were substantiated using mtDNA data. However, both in Chapter 5 and 6 we found that the most abundant color morphs reported in the Mediterranean were clearly separated into two groups: the first containing the purple forms, and the

second containing mostly blue and green morphs. Both clades occurred sympatrically at the same locality. In addition, in Chapter 2 we found chemical differences in alkaloid composition between the purple morph and the blue and green forms. These studies concluded that additional data on biological cycles was important to assess whether the two clades correspond to separate species.

The aims of the present work are: (1) to compare the reproductive traits, growth, and survival of the two most abundant morphotypes of the genus *Cystodytes* occurring in the western Mediterranean (purple and blue), (2) to correlate these biological traits with other parameters, such as colony size, water temperature, and the presence or absence of nearby competitors, and (3) to ascertain whether previously reported genetic and chemical divergence correlate with differences in biological parameters, providing clues as to the degree of reproductive isolation of these morphotypes and their taxonomic status.

7.2 MATERIAL AND METHODS

7.2.1 Sample collection and assessment of biological cycles

We monitored a population of the blue morph and a population of the purple morph of *Cystodytes* occurring in two localities of the same stretch of coast in the NE of Spain (Palamós and L'Escala respectively; Fig. 1). The specimens were attributable to *C. dellechiajei*, based on Turon (1987) and Kott (1990). Both populations were sampled monthly from July 2002 to February 2004. Morphologically, the only apparent differences between the two morphotypes were their color and the presence of small, spherical spicules (in addition to the large, disc-shaped spicules characteristic of the genus) in the purple morph (see Chapter 5). At each locality and on each sampling date, water temperature was recorded and 10 colonies (separated from each other by at least two meters) were collected by scuba diving and immediately fixed in 5% formaldehyde. Whenever possible, the two sites were sampled the same day or as soon as possible afterwards. Once in the laboratory, the colonies were dissected and at least 10 zooids per colony were observed. The reproductive state of each zooid was then visually assessed under a stereomicroscope and scored according to one of the following categories: immature colonies (1), presence of testes (2), presence of testes with a sperm duct filled with sperm (3), presence of oocytes (4), brooding embryos (5) and brooding mature larvae (6). Clearly, these categories (with the exception of the first) were not mutually exclusive. When a zooid could be scored in more than one category, we scored the highest. A maturity index (MI) was then calculated for each colony by

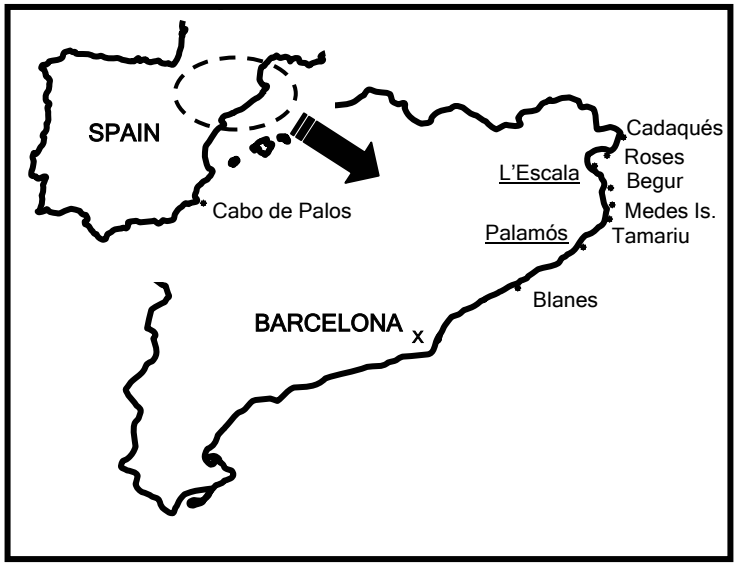


Fig. 1. Map of the localities studied. The two locations at which most of the monitoring was conducted (L'Escala and Palamós) are underlinied.

averaging the category number of the zooids examined. Monthly means were then calculated from the values of the 10 colonies studied.

As we monitored only one population per morphotype, we performed additional samples on a seasonal basis (4 per year) at the Medes Islands archipelago, which is located between the other two localities and contains both morphotypes (Fig. 1). In addition, we occasionally surveyed other populations distributed along the Spanish Mediterranean coast: Blanes, Tamariu, and Cabo de Palos for the blue morph, and Cadaqués, Roses, Tamariu, and Begur for the purple morph (Fig. 1). Furthermore, from July to September 2004 we repeated the sampling in Palamós and l'Escala, to verify that the pattern found in 2002/2003 was consistent between years.

7.2.2 Estimation of growth rates

Growth of the blue and purple colonies was monitored at Palamós and L'Escala respectively by means of a digital photographic study of marked individuals from September 2002 to January 2004. At both localities, walls of similar inclination, orientation, and depth were selected. The monitored colonies were mapped using nails driven into the rock and underwater sketches and measurements. As this species displays an encrusting form, changes in the surface area of the colonies provided a good estimate of growth. Colony outlines were added digitally to the pictures and areas were then measured using the SigmaScan Pro 5 program (Jandel). Fusions and divisions were also recorded. When fusion occurred, the areas of the colonies before merging were added together to give a single value per survey. When fission occurred, the areas of the resulting clone-mates were summed in subsequent measurements. Mortality of colonies was also recorded. We monitored 42 colonies for the blue morph and 39 for the purple form.

From changes in area over time, a monthly growth rate (GR) was calculated using the formula

$$GR_m = \frac{\left(A_m + A_{m-1}\right)}{A_{m-1}}$$

where A_m and A_{m-1} are the areas at month m and at the previous month (m -1) respectively. This growth rate represents the change in area over the course of a one month interval. The monthly growth rates presented here have been smoothed

using a weighted moving average [(current value x 0.5) + (previous value x 0.25) + (subsequent value x 0.25)] to avoid noisy fluctuations.

7.2.3 Data treatment

A two-way ANOVA with morphotype and month as main factors was used to ascertain differences between the maturity indexes. For the comparison of growth rates, a non-parametric repeated-measures analysis of variance (ANOVAR) using a randomization procedure based on Manly (2001) was performed. This consists of a two-stage permutation of the data: first, individuals were randomly reassigned to the two color morphs, and then readings for each individual were randomly rearranged among the observation times. The whole data series was randomized 4999 times (plus the observed one) to approximate the null hypothesis distribution of the sum of squares for each factor and their interaction. Finally, we examined how extreme were the observed values in the distribution generated. An effect was judged significant when the observed sum of the squares was exceeded by less than 5% of the corresponding values in the randomization series. The entire data set could be used, including colonies that died during monitoring. For colonies that were not present at all observation times, the permutation between times was carried out only within the period in which the colony was present in the study.

Survival of colonies was analyzed using the lifetime method, and hazard functions were computed for both localities. Survival of the two morphotypes was compared using a Wilcoxon-type test (Fox 1993). Cox's proportional hazards model (Fox 1993) was run to assess whether a relationship existed between survival time and maximal and final size of the colonies.

Spearman rank correlations were used to assess the relationship between size (mean of the study period) and mean and maximal growth rate of the colonies. Cross-correlation analyses (using the Pearson coefficient) were used to compare the cycles of reproduction and growth at both localities, as well as to assess relationships between these cycles and temperature.

Finally, we observed frequent contacts between some colonies of the purple morph and the toxic sponge *Crambe crambe* (Becerro et al. 1995). To determine whether the growth rates of these colonies are affected by the presence of this competitor, we performed a Mann-Whitney test on the average growth rate of colonies surrounded or not by this sponge.

The packages Systat version 9.0, Statistica version 4.0 and Sigmastat version 2.0 were used for the analyses. The randomization routine was written in Turbopascal version 6.0 by XT.

7.3 RESULTS

7.3.1 Reproduction and resistance periods

The time course of the main reproductive categories considered is shown in Figure 2 as the percentage of zooids (averaging values for 10 colonies) in each state considered. A seasonal reproduction is clearly substantiated for both populations, although there are also important temporal differences. Zooids with gonads were present throughout the year, but the sequence of reproductive stages exhibited a clear temporal trend. The reproductive state was markedly uniform within colonies. In most cases only a single or two different reproductive categories were observed within a given colony.

In the blue morph, immature zooids were only observed (although in small percentages) in summer, with testes appearing during this period and becoming ripe at the end of summer. Oocytes appeared in January-February, and by mid-spring there were embryos brooded in the zooids. Mature larvae were found in June, and the cycle was almost over (with only a few larvae remaining) in July. In the purple morph, immature zooids were more abundant (~40%) at the end of summer; by October all zooids had testes, and by January most of the testes had sperm ducts filled with sperm. Oocytes first appeared at this time and embryos became abundant in late spring to early summer. Well-developed larvae appeared in July through September, when the cycle restarted. Larval release, therefore, seemed to take place at the end of June in the blue form, and from the end of July to September in the purple morph. The difference appears to be due to a longer period of oocyte vitellogenesis in the latter (6 months) than in the former (4 months).

The evolution of the maturity index (MI) over time is shown in Figure 3A and 3B. Reflecting the temporal lag detected, the MI increased earlier in the blue population (from September onwards) and reached a peak in June, two months before the highest sea temperatures occurred, followed by an abrupt decrease as larval release proceeded. In contrast, for the purple population, the MI increased in winter and the breeding season occurred in late spring and summer, reaching a peak in July. Larval release occurred one month before the water temperature reached its maximum (Figs. 2, 3A). Although the study begun in July 2002, the same lag seemed to have occurred in 2002 (from July to September the MI was increasing in the blue morph and decreasing in the purple morph), and examination of the

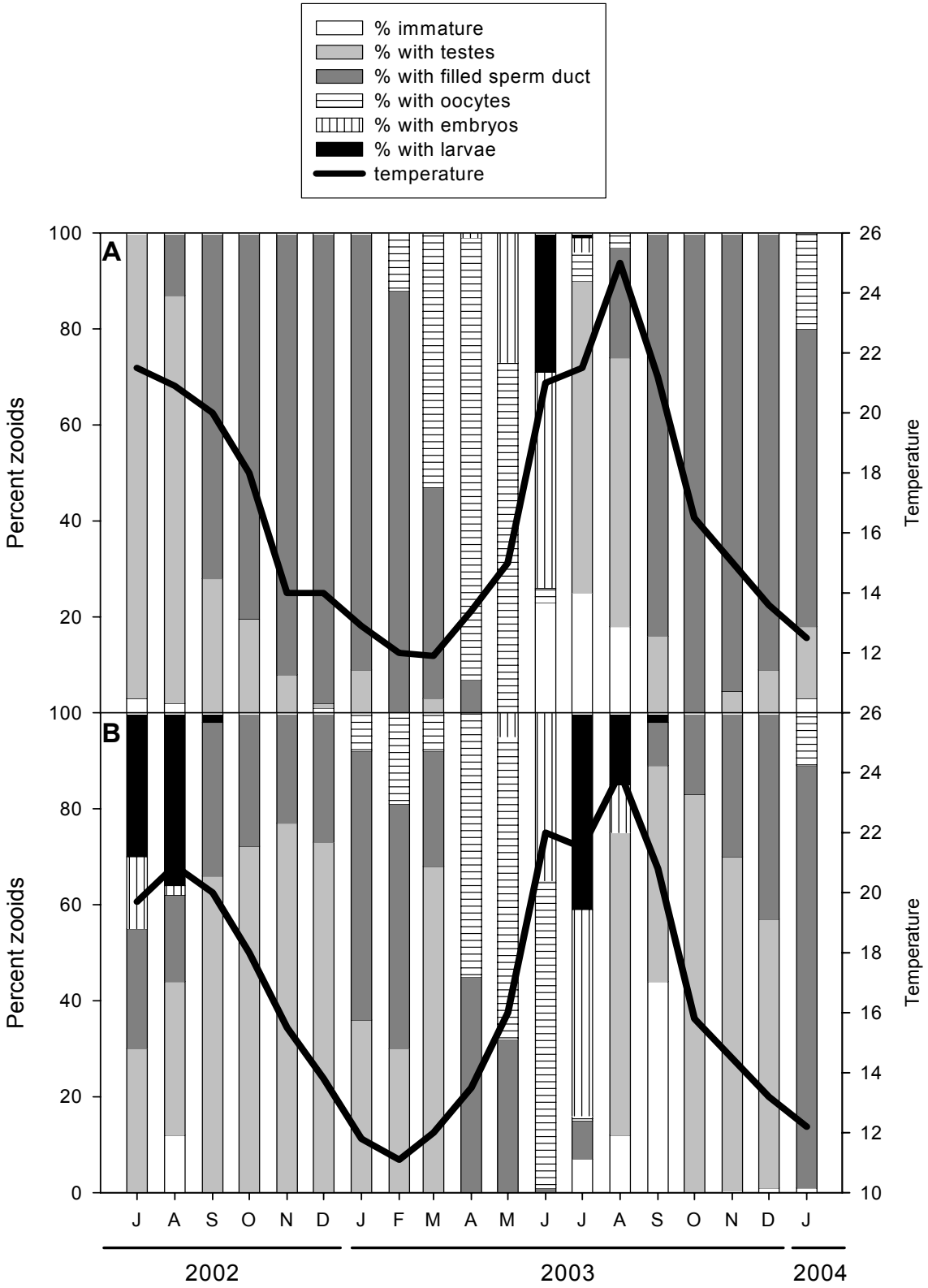


Fig. 2. Reproductive cycles in the blue (A) and purple (B) populations indicated as percentage of zooids (mean of 10 colonies) in the different states considered. Temperature values are superimposed.

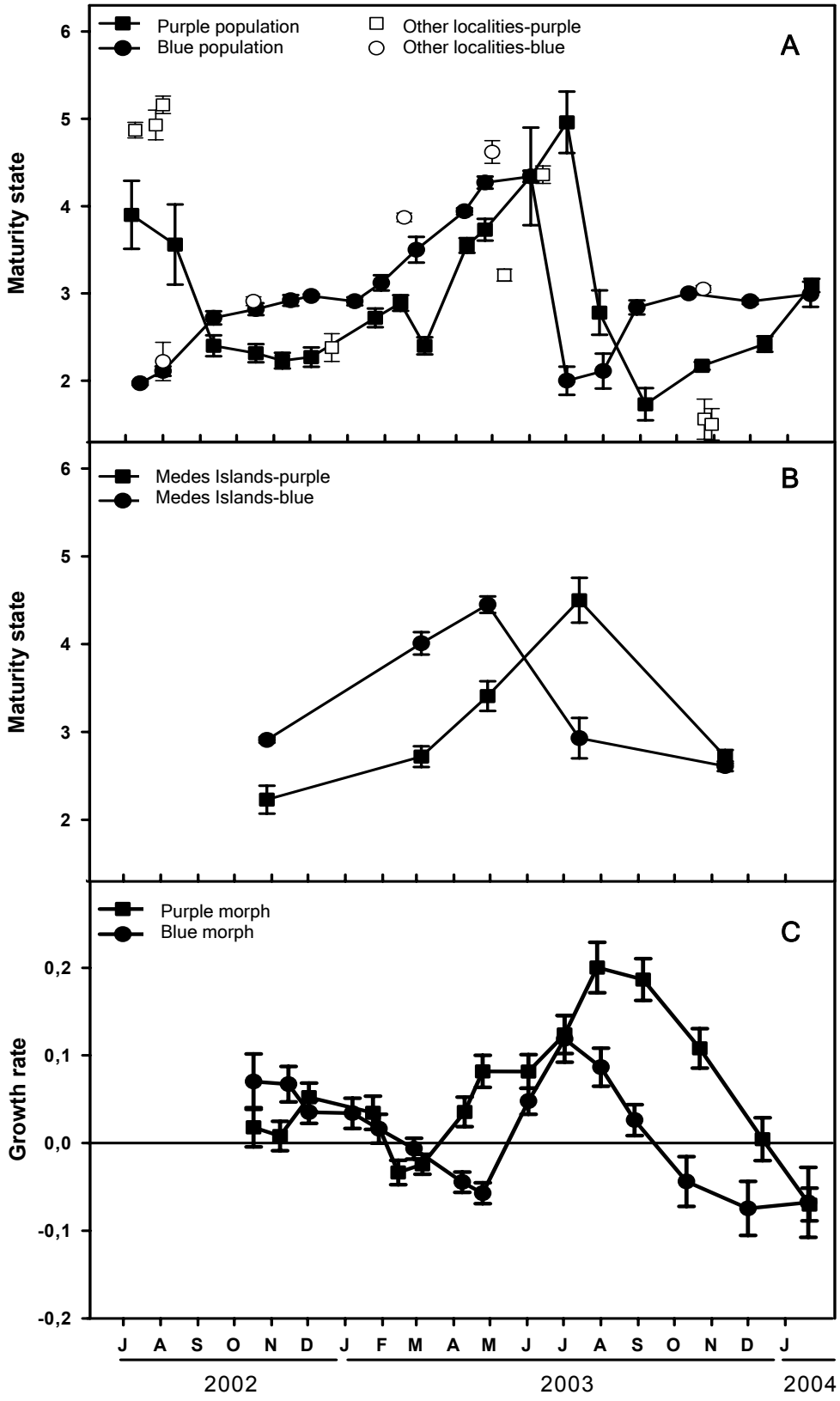


Fig. 3. (A) Time course of the maturity index (MI) in Palamós (blue population) and L'Escala (purple population). Open circles and squares indicate MI found in other localities inhabited by the blue and purple morphs respectively. (B) Seasonal time course of MI of both morphotypes at one locality in which they co-occur (the Medes Islands). (C) Mean growth rates obtained for the monitored colonies of the blue and purple morphs.

samples from July, August, and September 2004 (not shown) yielded exactly the same pattern as in the corresponding months of 2003. The maturity indices measured at the Medes Islands locality, where the two morphotypes coexist, showed the same temporal trend that was observed in L'Escalà and Palamós (Fig. 3B). Although we have less temporal precision due to the seasonal nature of the sampling, the MI increase in the blue morph occurred earlier than in the purple form. By late spring, the MI index was higher for the blue morph, whilst by mid August the MI had dropped in the blue population and was maximal in the purple morph, again indicating a temporal lag between the time courses of reproduction of the two morphotypes. As for other localities (Fig. 3A), these observations were consistent with the cycles observed in the monitored populations, indicating that they are a general feature of the two morphotypes in the area studied.

The two-way ANOVA on MI revealed that morphotype, time, and the interaction between the two were significant ($p < 0.001$ in each case), indicating a different time course of the reproductive cycle at both localities. Moreover, cross-correlation analyses of the evolution of MI showed that the reproductive cycle of the purple morph lagged one to two months behind that of the blue one, as indicated by a significant correlation at these time intervals (Fig. 4).

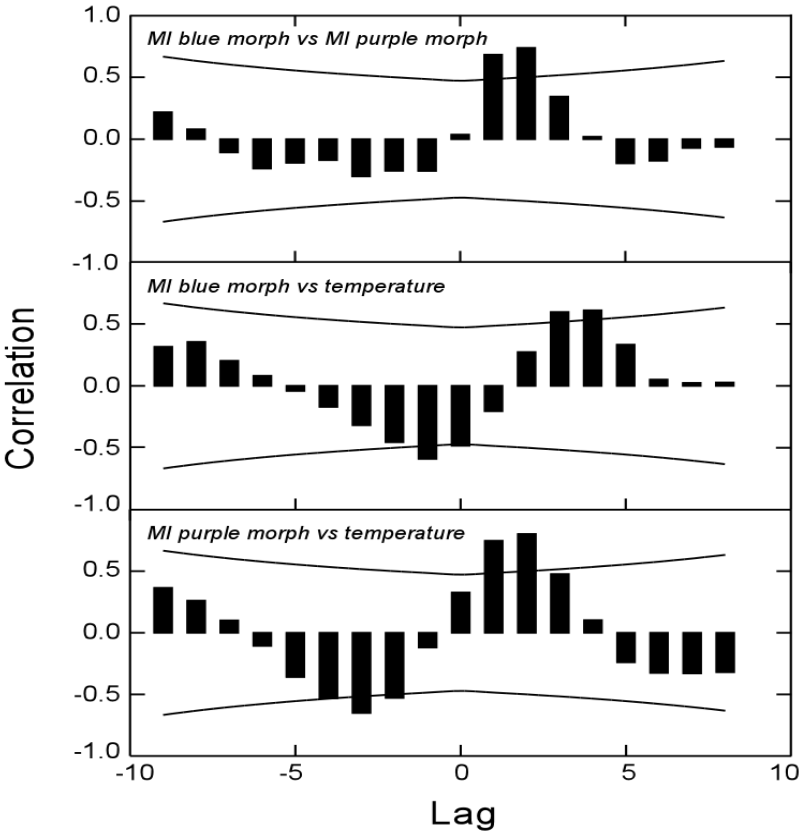


Fig. 4. Cross correlation analyses of the maturity indices of the blue and purple morphs, between them and with the temperature. Time lag is in months.

The cross-correlation analyses also showed

significant positive correlations between the maturity index and the temperature measured in the third and fourth subsequent months for the blue population and in the first and second subsequent months for the purple morph (Fig. 4). This result reflects the fact that the course of MI was some months advanced with respect to the temperature in the blue population, whilst this advancement was less marked in the purple morph.

A non-feeding state occurred in June 2003 in the majority of blue colonies. This phenomenon was very brief and did not last for more than a few weeks: at the next observation time the colonies were active again. The affected colonies were covered with a glassy cuticle that sometimes formed a reticulated net over the surface, and no siphonal aperture was visible. This phenomenon occurred when MI was maximal. No similar state was observed in the purple form.

Fusions and fissions occurred randomly in both populations, with no clear temporal pattern. Nine fusion and 23 fission events were recorded for the blue morph, whilst 13 fusions and 20 fissions were observed in the purple population.

7.3.2 Growth rates and mortality

The mean growth rates (GR) are also depicted in Figure 3C. The periods of growth fluctuated seasonally. The highest GRs were obtained during periods of elevated sea temperatures, whilst in winter the growth was restricted, or even negative. A lag similar to the one observed for the reproductive cycle was also evident in the growth rates, with peak values in the purple morph being reached one month later (August) than in the blue morph (July). Interannual differences were also observed. For the blue morph, GRs were lower during the second than the first autumn surveyed, whilst the reverse was true for the purple morph. The overall growth during the study period was higher for the purple population: the mean initial and final colony sizes for the blue morph were 948 and 1442 mm^2 respectively, whilst the purple morph displayed values of 973 and 2083 mm^2 respectively.

The results of the repeated measures ANOVA using the randomization test on the monthly GR showed that time, morphotype, and the interaction between the two were significant ($p < 0.001$, $p = 0.046$ and $p < 0.001$, respectively). A significant interaction term means that the time course of growth was different for the blue and purple colonies. The growth cycles followed the same general pattern, but were shifted by approximately 1 month. Accordingly, a cross-correlation analysis

comparing the two morphotypes showed the highest positive correlation between GRs at a one-month lag (Fig. 5). Cross-correlation analyses also indicated that monthly growth rates and temperature covaried positively over time for both morphotypes (Fig. 5).

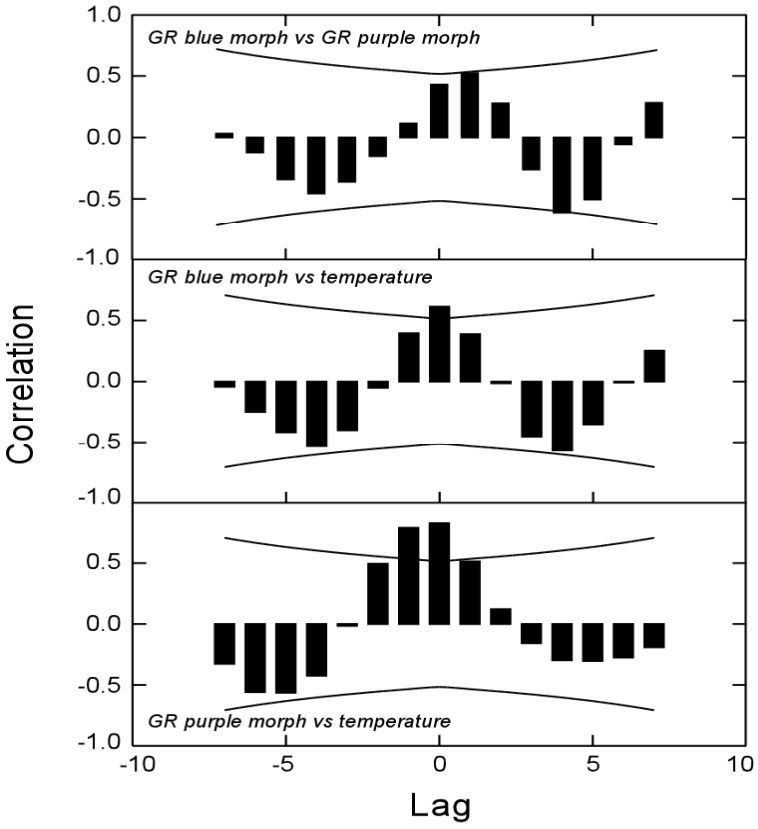


Fig. 5. Cross correlation analyses of the growth rates of the blue and purple morph, between them and with the temperature. Time lag is in months.

There were no significant differences in mortality between the 2 morphotypes considered (Gehan's Wilcoxon test p = 0.98). By the end of the study, 30 (72%) colonies of the blue morph and 30 (77%) of the purple form survived. In addition, Cox's regression model between hazard rate and maximal and final colony size reached, exhibited a significant negative correlation between mortality and size for both morphotypes ($p < 0.001$ in all cases). Dead individuals often corresponded to colonies smaller than 100 mm^2 in surface area, and no mortality occurred in individuals that attained sizes greater than 500 mm^2.

In terms of the relationship between size and GR, in all cases the Spearman correlation coefficient was negative, indicating a tendency towards less growth in larger specimens, but the relationship was only significant between size and maximal growth rate in the blue morph ($r_s = -0.392$, $p = 0.01$). The comparison of size and mean growth rate in the blue form, or between size and mean and maximal growth rate in the purple morph yielded non-significant correlation values ($p < 0.6$ in all cases). The Mann-Whitney test showed no significant differences between

growth rates of purple colonies surrounded by the toxic sponge *Crambe crambe* and those of colonies that were not (p = 0.801).

For both morphotypes, cross-correlation analyses (Fig. 6) showed a lag between maturity indices and growth rates. The lag was greater for the blue morph, and significant positive correlations were found between MI and GR in the third subsequent month, whilst in the purple morph significant positive relationships existed at lags of one to three months. At time lag 0, the usual Pearson correlation showed a significant negative correlation for the blue morph and a positive, but small and non-significant, one for the purple form (Fig. 6).

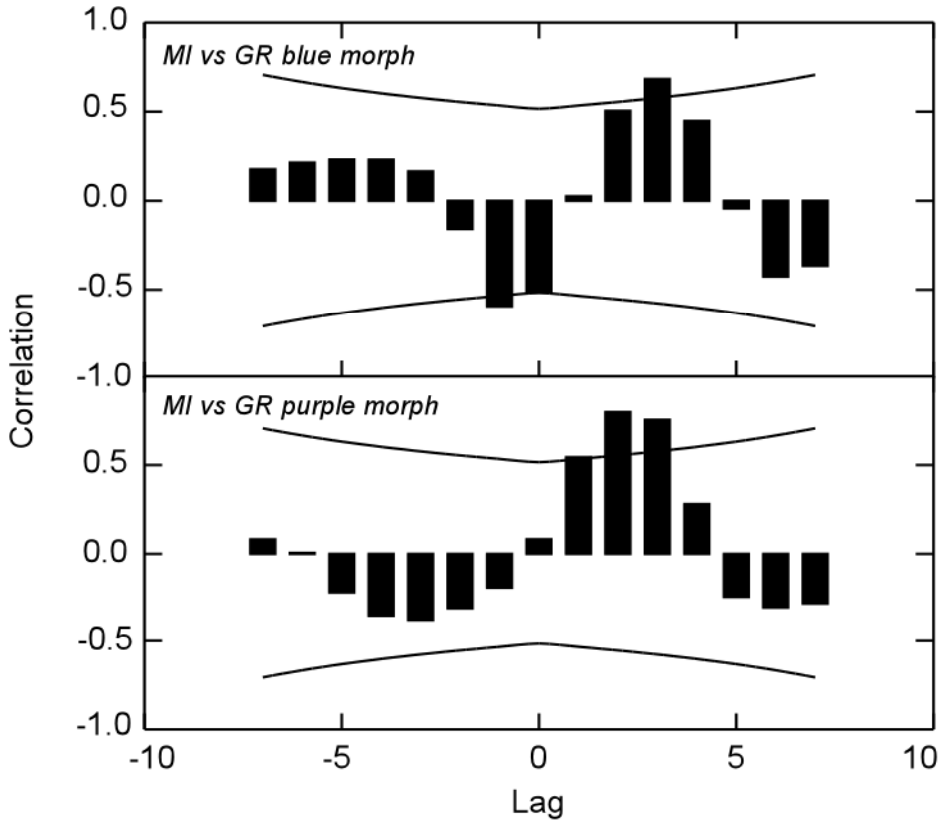


Fig. 6. Cross-correlation analyses between the maturity indices and the growth rates in the two morphotypes. Time lag is in months.

7.4 DISCUSSION

Both morphotypes showed a clear seasonal cycle for reproduction and growth. Larval release occurred in late spring and summer, followed by a period of asexual growth. Moreover, the present study revealed significant differences in growth rates and reproductive cycles between the blue and purple morphs, currently considered to belong to the species *Cystodytes dellechiajei*. The purple population lagged 1 to 2 months behind the blue one in the time course of both parameters, and the pattern was consistent between years. Although the two populations studied were in the same stretch of coast and they inhabit the same type of community, it may be argued that the differences found are the result of local conditions. However, the same lag in the reproductive cycle was observed at the Medes Islands, where specimens of the two morphotypes are found side by side. In addition, sampling of other populations showed that the biological cycles were highly coherent within color varieties, indicating that the pattern found is more likely to be the result of genetically fixed differences between these morphotypes.

There were no differences in the mortality observed between morphotypes, and a clear negative relationship with size was observed. Death occurred mostly in small colonies, which often resulted from fragmentation of larger ones. Similar patterns of reduced mortality with size have been reported for ascidians and other invertebrates (Turon & Becerro 1992, Turon et al. 1998), indicating that upon reaching a certain threshold size mortality is reduced in modular organisms (escape in size: Sebens 1982).

Turon & Becerro (1992) monitored a limited number (11) of small (less than 250 mm^2) colonies of the blue morph for 2 years in a nearby locality of the NE Spanish coast. These authors found a mortality of ~50% during the study period, and did not find a clear seasonal pattern of growth. A high mortality value in these small colonies was to be expected given the negative correlation found here between colony size and mortality. The lack of a seasonal pattern may be due to the small sample set studied and the high growth variability observed in this species. Indeed, modular organisms often present a high inter-individual variability (Ayling 1983, Todd & Turner 1988, Garrabou & Zabala 2001, Tanaka 2002). In our study, some colonies remained small throughout the study period whilst others exhibited marked changes in size. The majority of the colonies, however, displayed phases of rapid growth alternated with often significant size reductions. In addition, fusions and

fissions were not rare. In both morphotypes, fission was the most frequent phenomenon, with no clear temporal pattern. Fusions, on the other hand, mainly occurred between recently separated clone-mates as described in another ascidian species by Stocker (1991). Nevertheless, the fragment dynamics of this species is much lower than that reported for other colonial ascidians, such as some Didemnidae (Ryland et al. 1984, Stocker & Underwood 1991).

In the blue morph, a significant effect of size on maximal growth rates was evident, indicating less scope for growth once a certain area has been reached. This observation is in agreement with previous results in ascidians and other encrusting invertebrates (Turon & Becerro 1992, Garrabou & Zabala 2001). However, the same relationship was not significant in the purple morph, suggesting that whilst this effect is unlikely to be due to space saturation, it may represent an intrinsic characteristic of the blue morph.

Seasonal patterns are common in temperate habitats (Osman 1977, Sutherland & Karlson 1977, Sebens 1986, reviewed in Coma et al. 2000). In the Mediterranean, a variety of biological strategies for colonial ascidians regarding the duration and seasonality of reproductive periods have been described (e.g. Turon 1988, Turon & Becerro 1992, Tarjuelo 2001, Caralt et al. 2002, Molin et al. 2003). Temperature has been widely reported as the main factor controlling reproductive phases in ascidians (Berrill 1935, Millar 1971, Turon 1988, Durante & Sebens 1994, but see Coma & Ribes 2003), with many species reproducing in spring and early summer (Turon 1988), and also seen in this study. Seasonal patterns can also be shaped by the trade-off between resource allocation to reproduction and growth. The majority of temperate ascidians display alternating sexual and asexual phases (i.e. Millar 1952, 1974, Brunetti & Copello 1978, Turon 1988, Turon & Becerro 1992). In our study, the peak of growth rate always occurred after reproduction was over, which may induce a partitioning of the energy resources of the colony over the course of its annual cycle (Yund et al. 1997).

Periodic regression and regeneration phenomena have also been reported in colonial ascidians (Millar 1971, Turon & Becerro 1992, Tarjuelo 2001). Turon (1988, 1992) described a non-feeding state with regression of thoraces and appearance of a glassy pellicle over the surface of some Mediterranean colonial ascidians during the summer. In this study, a similar non-feeding form was observed in the blue population in June 2003. Although the maximal water temperatures were reached 2 months later (August), during June the seawater temperature increased by 6^{o}C,

representing the most significant increase recorded during the study. In addition, the presence of non-feeding forms coincided with the highest value of the maturity index. Therefore, the switch to a resting form does not seem to imply any change in the reproductive condition, as already observed in another colonial ascidian (*Polysyncraton lacazei*; Turon 1992). One month later, no resting forms were observed, the maturity index had dropped to its lowest value, and the growth rate was maximal. Although a temperature effect cannot be discounted, it is also possible that a resting period is necessary for zooid regeneration and to reallocate energy from sexual to asexual reproduction. No equivalent state was found in the purple population on the same dates.

Morgan (1977) observed that contact with other organisms inhibited growth of the colonial ascidians *Botrylloides nigrum* (Styelidae) and *Aplidium lobatum* (Polyclinidae). Subsequently, Bak et al. (1981) observed the same phenomenon in *Tridemnum solidum* (Didemnidae) overgrowing dead and/or living corals. Stocker & Underwood (1991) found that rates of sexual and asexual reproduction of *Didemnum moseleyi* (Didemnidae) were lower when specimens were found close to sponges. No relationship could be statistically assessed between the growth rates of the purple morph and the nearby sponge *Crambe crambe*, a chemically defended species known to be a good space competitor (Turon et al. 1996, Becerro et al. 1997). Other colonial ascidians (f.i. *Diplosoma spongiforme*; *Didemnum* sp.) and calcareous sponges were occasionally observed overgrowing some colonies of both morphotypes and may have punctually influenced their growth. Further and more specific work will be necessary to assess the influence of nearby competitors on the life cycle and ecology of *Cystodytes*.

Studies of morphological and ecological varieties of ascidians have often revealed a genetic differentiation, suggesting that formerly recognized species should be split (Aron & Solé-Cava 1991, Degnan & Levin 1995, Tarjuelo et al. 2001, Turon et al. 2003). In some cases, color varieties have corresponded to different species (Dalby 1997, 2000, Tarjuelo et al. 2004). In Chapter 2 and 5 we found that the blue and purple morphs belonged to two distinct genetic clades and featured two different chemotypes in terms of their alkaloid composition. Together with studies of gene flow, analyses of life cycle parameters can contribute to the establishment of species boundaries. Thus, the two morphotypes studied here featured significant differences in biological traits. Although the timing of male reproductive activity and appearance of oocytes is so broad that overlaps between these forms (meaning that

the possibility of cross-fecundation cannot be definitively ruled out), there is a lag in the reproductive periods that is maintained even in sympatric populations (e.g. the Medes Islands) and between years. Indeed, the larval release of one morphotype occurred almost exclusively after the release of the other morphotype was over. Most probably, fecundation also takes place at different times, all in all indicating that these two forms are reproductively isolated.

In conclusion, our results show that biological differences match previously reported genetic and chemical divergence between the two morphotypes analyzed. The present study, therefore, confirms the hypothesis that the two morphotypes in fact correspond to two sibling species. Although genetics and secondary chemistry provide important clues about species boundaries, the study of biological cycles remains a useful tool with which to assess speciation events according to the biological and cohesion species concepts (Templeton 1989), and provides crucial information about the ecology and biology of benthic invertebrates.

CHAPTER 8

ADJUSTING THE BUDGET: IS TEMPORAL VARIATION IN ASCIDIDEMIN PRODUCTION RELATED TO OTHER BIOLOGICAL PARAMETERS?

8.1 INTRODUCTION

Defense mechanisms are commonplace among sessile invertebrates; especially those that require exposed surfaces to acquire resources, such as many filter feeders. Defenses can be structural, associational, nutritional or chemical (Cronin 2001). Structural defenses include external shields (e.g. shells), sharp spines (e.g. urchin's test), support material that makes tissues too hard to be easily bitten (e.g. tough tunic of some ascidians), sclerites, and spicules (Harvell et al. 1988, Van Alstyne & Paul 1992, Van Alstyne et al. 1992, Koh et al. 2000, Burns & Ilan 2003). In addition, calcium carbonate structures can dilute nutritional value, raise the pH of the gut, and/or increase the efficacy of secondary metabolites (Pennings & Paul 1992, Hay et al. 1994, Schupp & Paul 1994, Pennings et al. 1996). Associational defenses imply seeking a protective partner or host (Hay 1986, Wahl & Hay 1995), while nutritional defense means that tissues have such low nutritive value that they will not be worth the effort (Hay et al. 1994, Duffy & Paul 1992). Finally, chemical defenses include secondary metabolites and acidity (e.g. Stoecker 1980). Potential benefits of secondary metabolites are not only defense against predation, but also against fouling organisms, bacteria, competitors, and the

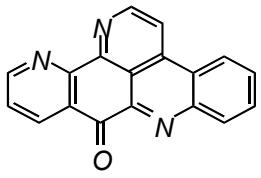
damaging effects of UV radiation; they may also provide cues for settlement or aggregation (see McClintock & Baker 2001).

Although little evidence is available, it is generally agreed that production, maintenance, transport and storage of secondary metabolites have associated costs in terms of trade-offs that can only be achieved by diverting materials and energy from other functions (Cronin 2001). Thus, natural selection acts to optimize the allocation of resources in the life history of an organism in a given environment and within evolutionary and ecological constraints (Cronin 2001). Likewise, optimal defense theory assumes that allocation of resources to chemical and physical defenses must be optimized with respect to the needs of an organism and its energy budget (growth, somatic maintenance, regeneration, and reproduction). If so, a temporal variation in defense production can be expected, depending on the environmental constraints and the physiological state of the source organism. However, attempts to link temporal patterns of production of secondary metabolites to life cycle features of marine invertebrates are scarce (e.g. Turon et al. 1996, Martí 2002).

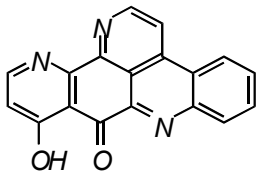
Studies of intraspecific chemical variation are valuable in order to identify factors that affect the production of chemical defenses, as well as to provide insights into the ecological consequences and evolutionary implications of such variation. Intraspecific patterns of variation have been reported over temporal (e.g. Hay & Steinberg 1992, Steinberg & Van Altena 1992, Yates & Peckol 1993, Turon et al. 1996, Martí 2002, but see Valls et al. 1993), between-organism (e.g. Yates & Peckol 1993, Harvell et al. 1993, Maida et al. 1993, Becerro et al. 1995), or within-organism (e.g. Paul & Van Alstyne 1988, Harvell & Fenical 1989, Van Alstyne et al. 1994, Turon et al. 1996, Becerro et al. 1998, Schupp et al. 1999) scales. Seasonality is particularly likely in temperate seas, where strong periodicities are imposed on the biological parameters of the organisms (Sebens 1986, Coma et al 2000).

The genus *Cystodytes* is a widely distributed ascidian that presents a noticeable morphological variability in terms of color and spicular composition (see Chapter 5). In Chapter 2, we described the identification of two chemotypes within this genus that, on the basis of genetic and reproductive studies, were revealed to correspond to sibling species (see Chapters 6 and 7). The first chemotype was characterized by the presence of C_9-unsubstituted pyridoacridines such as ascididemin (Fig. 2.1, Kobayashi et al. 1988) and 11-hydroxyascididemin (Fig. 2.2, Schmitz et al. 1991), and was found in blue and green color morphs from the

western Mediterranean. Ecological roles for ascididemin included potential anti-fouling (Debard et al. 1998) and anti-predatory functions (see Chapter 4).



1: ascididemin



2: 11-hydroxyascididemin

Fig. 2. Structures of the alkaloids isolated from the blue morph of *Cystodytes*. **1**) ascididemin and **2**) 11-hydroxyascididemin.

The aim of this Chapter is to assess temporal variability in the production of defenses in the blue Mediterranean morph of *Cystodytes*. To study the variation in chemical defense production, we quantified ascididemin, the main pyridoacridine alkaloid found in this morph. Physical defense variability was estimated by calculating the colony ash content, as it mainly contains spicules and structural material. The main question is whether there is a predictable change in the amount of energy invested in chemical and physical defense production in relation to other biological parameters such as investment in reproduction and growth (analyzed in Chapter 7).

8.2 MATERIAL AND METHODS

8.2.1 Ascidian samples

We monitored a population of the blue morph of *Cystodytes* occurring in Palamós (NE Spain, 41º 50.4'N 3º 07.6' E; Fig. 3). This population was sampled from February 2003 to February 2004. Five colonies (separated from each other by at least 2 meters) were collected monthly by scuba diving and kept alive in a cooler. They were then frozen within 8h.

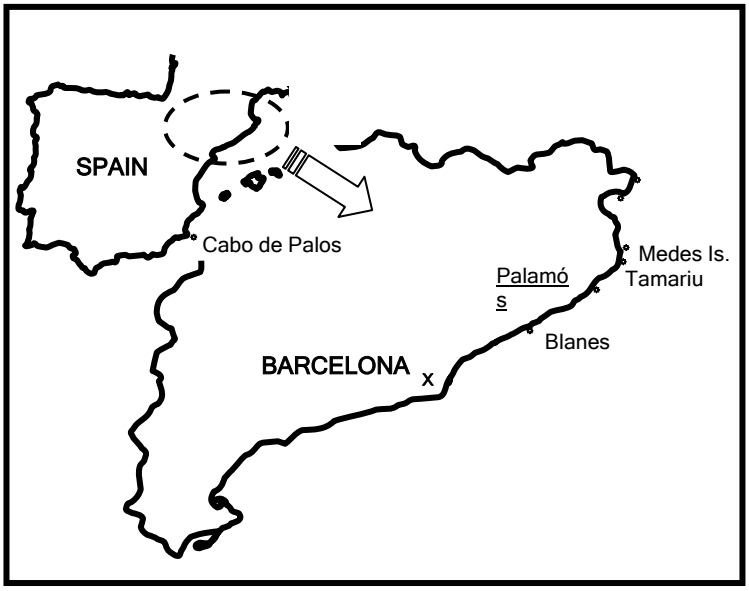


Fig. 3. Map of the localities studied. The main location at which most of the monitoring was conducted (Palamós) is underlinied.

As we monitored only 1 population, we performed additional samples on a seasonal basis (4 per year) at the Medes Islands archipelago and we occasionally surveyed other populations distributed along the Spanish Mediterranean coast: Blanes, Tamariu, and Cabo de Palos (Fig. 3). Furthermore, from July 2004 to January 2005 we repeated the sampling in Palamós to verify the pattern found.

8.2.2 Chemical extraction procedure

We followed the optimized protocol for extraction, isolation, and identification of pyridoacridine alkaloids from *C. dellechiajei* described by Bontemps (1996). Freeze-dried samples were extracted three times in a 1:1 (v:v) mixture of dichloromethane and methanol and filtered through a 20 µm PTFE filter in a vacuum chamber. The resulting solution was then dried by vacuum rotary evaporation, yielding a powdery organic residue. The different crude extracts were redissolved in MeOH three times. The final volume was adjusted to 10 ml and an aliquot of 1 ml

was then filtered through a 13mm, 0.20 µm PTFE syringe filter prior to HPLC injection. The injection volume was fixed at 10 µl.

8.2.3 HPLC analysis and quantification

Under initial HPLC conditions we observed significant peak tailing, resulting from cation-exchange interaction with positively charged basic analytes (pyridoacridines with the presence of two or three tertiary amine groups are basic compounds). Optimization of the elution conditions and the column packing and configuration allowed a suitable analytical separation of these alkaloids. The final elution conditions consisted of eluents A (water-methanol-acetic acid, 90:10:1) and B (100% methanol), an elution profile based on a linear gradient from 0% B to 100% B in 10 min, and a flow rate of 0.8 ml·min^{-1}, with a fixed temperature of 30°C. We used an Agilent Eclipse XDB-C8 (4.6 mm ID x 15 cm) analytical column. Analyses were performed using a Waters Alliance 2695 Separations Module with a Waters 996 photodiode array detector.

The ascididemin peak was detected at 380 nm and was integrated by applying the detector response based on peak area to a calibration curve obtained using synthetic ascididemin as an external standard. Ascididemin was synthesized in the LCBE laboratory (University of Perpignan) following the method described by Bracher (1989). The final amount of ascididemin was calculated by averaging three replicate injections. All analyses were performed using Empower software.

8.2.4 Storage considerations

Some preliminary studies were conducted to check that the mode and duration of the sample storage did not affect the extractability and stability of the secondary metabolites studied. It has been previously noted by Cronin et al. (1995) that extended maintenance of samples in the freezer before analysis, or the lyophilization process by itself could diminish the amount of secondary metabolites detected. We therefore performed two sets of experiments. In the first of them, 10 colonies were collected, divided in half, and one piece was freeze-dried immediately and kept in the freezer at -25°C. The second piece was kept frozen but not freeze-dried. After 5 months both halves were extracted and the amount of ascididemin quantified. In the second experiment, five colonies were collected and freeze-dried

immediately. One part of each colony was extracted and analysed immediately, while the remaining part was left at room temperature and extracted and analyzed after 10 months.

8.2.4 Quantification of ash content

After chemical extraction, the remaining colony pieces were oven-dried to a constant weight (60°C for at least 24h), placed in aluminum cups, weighed and burned in a furnace oven at 500°C for 12h. The resulting ashes were kept in a dry atmosphere until they were reweighed. Then, the ashes were observed under a stereomicroscope and all foreign material, such as small shells and worm tubes, was carefully removed and weighed. The ash content of the samples was corrected, if necessary, by subtracting the external calcium carbonate from the total.

8.3 RESULTS

8.3.1 Storage considerations

Yields of ascididemin did no differ significantly (paired sample t-test, p = 0.313) among the 10 colonies, of which one half was freeze-dried and stored at –25°C for 5 months and the other half was frozen but not freeze-dried (% dry weight: 0.00207±0.00016 and 0.00219±0.00016 respectively; mean±SE). In contrast, when we compared freeze-dried colonies of which one part was analyzed after collection and the other left at room temperature for 10 months prior to extraction and analysis, the amount of ascididemin detected was noticeably lower in the latter case (0.00222 ± 0.00012 and 0.00158 ± 0.00016 respectively; mean ± SE) and the difference was highly significant (paired sample t-test, $p < 0.01$). We conclude that the molecules studied are stable when kept at –25°C, either frozen or freeze-dried, but not at room temperature, even if freeze-dried. The storage strategy we used for the samples, therefore, consisted of freezing them immediately, freeze-drying within 1-2 months, and then keeping them until analyzed at –25°C.

8.3.2 Secondary metabolite quantification

All the samples studied showed a low molecular diversity, with one major peak (retention time: 9.3 min) identified as ascididemin and three minor peaks (6.3, 6.4, and 7.6 min) identified as cystodimine B (Jamme et al., in press), styelsamine C (Copp et al. 1998), and 11-hydroxyascididemin (Schmitz et al. 1991). As these minor compounds represented, in all cases, less than 10% of the total area of pyridoacridine composition, they were not quantified.

The evolution (mean and standard error) of ascididemin concentration is shown in Figure 4A as the percentage of the compound per dry weight of the ascidian. Superimposed are the data from other localities and from the samples collected between July 2004 and January 2005. The maturity index and the mean growth rate during the same period described in Chapter 7 are also depicted in Figure 4B. The percentage of ascididemin per dry mass fluctuated seasonally and ranged from 0.07% to 0.225%. The lowest values were found in April, while the highest ones appeared in September, just before and after larval release in June 2003 (Fig. 4B) respectively. The cycle of ascididemin is similar to that of the growth

rates, although the highest values of ascididemin concentration lagged approximately one month behind the peak of growth, and ascididemin values did not drop drastically after reaching the maximum value, as happens with growth rates. A similar temporal trend was observed in the additional locations sampled (Fig. 4A). Although we have less temporal precision due to the seasonal nature of the sampling, these observations are consistent with the cycle observed in the monitored population. Examination of the samples collected in Palamós between July 2004 and January 2005 (Fig. 4A) yielded the same pattern as in the corresponding months of 2003, with the exception of a secondary peak appearing in October 2004.

8.3.3 Quantification of ash content

The time course of the variation in ash content during 2003 is shown in Figure 4C as the percentage of ash per dry mass of the ascidian. Although replicates from the same month could vary greatly (see standard error bars), a seasonal trend was evident that was similar to the one observed for ascididemin and the growth rates recorded during the year studied. After the maturity index maximum, the ash content increased from July to September 2003 and decreased again during wintertime. A similar temporal trend was observed for the Medes Islands and the other additional localities sampled occasionally (Fig. 4C).

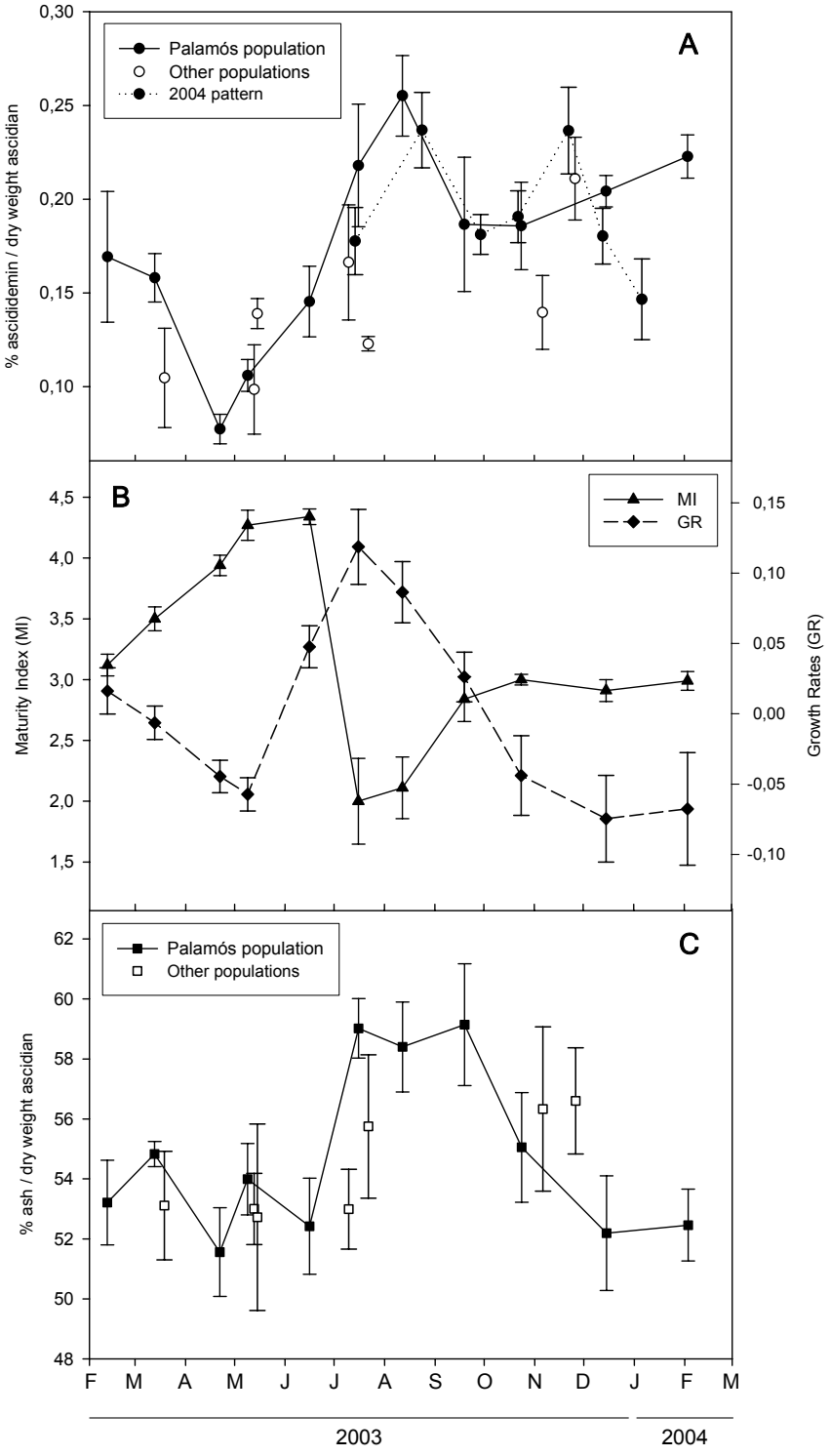


Fig. 4. (A) Time course of the percentage of ascididemin per dry mass of the ascidian in Palamós population from February 2003 to February 2004 (solid line) and to July 2004 to January 2005 (short dashes). Open circles indicate the amount of ascididemin found in other localities inhabited by the blue morph. (B) Seasonal time course of the maturity index (MI, solid line) and the growth rates (GR, short dashes) of the blue population described in Chapter 7. (C) Percentage of ash content per dry mass obtained for the monitored colonies. Open squares indicate the ash percentage found in colonies from other populations of the species in the same strench of coast. Bars indicate standard errors (SE).

8.4 DISCUSSION

8.4.1 Implications of storage procedures

Cronin et al. (1995) pointed out that methods of storing and extracting samples could significantly affect yields of biologically and ecologically important compounds. Specifically, these authors demonstrated that commonly used procedures for preserving samples had detrimental effects on algal chemical composition that were species and compound specific. Our extraction method for both the ascidian genus studied and for ascididemin (the major alkaloid of the blue morph) was already optimized by Bontemps (1996). Therefore, in the present study we only considered alteration of compound yield due to the duration and method of storage used prior to analysis. Both freezing and freeze-drying are methods commonly used to store samples, and it is assumed that no loss or conversion of compounds of interest occurs. However, this assumption is almost never tested experimentally. Ascididemin is a pyridoacridine alkaloid that is chemically very stable (Banaigs, unpublished data). Freeze-drying in itself does not affect the molecule, as extraction of fresh and freeze-dried material yielded no significant differences in ascididemin concentration (data not shown). However, after keeping freeze-dried samples at room temperature for 10 months, the ascididemin yield decreased by ~30%. Residual biochemical activity, heat, or light may have had an influence on the yields of ascididemin from these samples, as has been demonstrated for other compounds (de Scisciolo et al. 1990, Cork & Krockenberger 1991). On the other hand, samples kept in the freezer for 5 months, freeze-dried or not, yielded similar amounts of ascididemin. Therefore, in our case an optimized storage protocol consisted of freeze-drying the samples and keeping them in the freezer at -25°C prior to analysis.

Knowledge of how compounds vary qualitatively and quantitatively among parts of organisms, individuals, locations, and seasons is essential for our understanding of the ecological and evolutionary aspects of marine secondary metabolites. However, although analysis of metabolite variability relies entirely on accurate methods for sample preparation and conservation, there are few studies evaluating which methodological procedure should be employed (e.g. Norris & Fenical 1985, Hay et al. 1988, Cronin 1995, Becerro et al. 1997). Although it was not our main objective, this study adds new information to the issue and stresses the

importance of careful prior optimization of both the storage and extraction procedures for each compound studied to ensure reliable data in the field of chemical ecology.

8.4.2 Temporal variation of asciidemin production

Asciidemin concentration displayed a cyclic behavior that supports the hypothesis of an annual periodicity in the production of bioactive substances, with a minimum in spring and a maximum after the reproduction peak in late summer and autumn. The same pattern was observed for the ash content of the colonies, suggesting that an annual periodicity also exists for the production of inorganic contents, mainly spicules. The large standard errors found in both the production of asciidemin and the ash content indicated that there was a noticeable inter-individual variability; this is probably commonplace among invertebrates (e.g. Van Alstyne et al. 1994, Turon et al. 1996).

The temporal cycles observed could be determined by internal, physiological parameters or by external interactions (e.g. the rhythms of interacting species). According to the optimal defense theory, the presence of temporal patterns in the investment in chemical and physical defenses is to be expected if the production of defensive metabolites has a fitness cost. In addition, this investment has to be optimized in the context of the general energy budget of the species.

Our data show a clear temporal trade-off between investment in reproduction and the other biological parameters considered. This is in agreement with Tarjuelo & Turon (2004) who concluded, studying patterns of reproductive investment vs. tunic production in 11 colonial ascidians, that species with low fecundity that have large and complex larvae, such as *Cystodytes dellechiajei*, invest the most energy in reproduction. The energy value (in caloric content) of the reproductive material (testes and larvae) is of the same order or higher than that of the zooid itself [KJ (testes + larva)/KJ zooid is 1.14±0.19, mean±SE; Tarjuelo & Turon 2004]. The high investment in reproduction in this species may explain why the energy allocated to other life cycle parameters (e.g. growth, and chemical and physical defenses) was significantly reduced. Furthermore, Turon et al. (1996), in a study of the variation in toxicity of the sponge *Crambe crambe* occurring in the western Mediterranean, found similar seasonal trends attributable to a temporal trade-off between investment in reproduction and chemical defense production.

One month after larval release, the inorganic content (mainly spicules) of the colonies increased progressively and they grew rapidly for approximately one month. Both parameters may be linked, as during periods of rapid growth there is an increase in zooid budding, and spicules in this genus are formed surrounding the abdominal part of the zooids (Lambert 1979). Finally, lagging approximately one month behind maximal growth rate, the production of secondary metabolites peaks, and remains high for some months, long after growth rates have diminished. This pattern indicates that trade-offs may also be established between chemical defense production and growth rates, albeit less drastically than with reproductive effort. Possibly, once colony growth is over and the capsules around the zooids are formed, more energy is available for secondary metabolite production, which seems to be the most efficient defense mechanism against predation (Chapter 4).

External interactions could also account for the temporal variation observed. Indeed, interacting organisms presenting a seasonal variation should be taken into account. The highest concentration of ascididemin in autumn may be related to the reactivation of growth of competing animal species after the summer period, which is an unfavorable season at the end of the reproductive period for many sessile invertebrates in the Mediterranean (Boero et al. 1986, Turon & Becerro 1992, Coma & Ribes 2003). However, when collecting the samples for the present study, special care was taken to collect colonies that were not surrounded by known toxic species or/and space competitors.

Our results indicate, therefore, that the reproductive cycle probably drives the temporal course of the production of defensive secondary metabolites and inorganic material such as spicules, although “environmentally modulated” elements may also play a role. This is consistent with the hypothesis that secondary metabolite production is costly in metabolic terms (Fagerström et al. 1987) and must be optimized with respect to other factors, mainly biological parameters, to adjust the energy budget of a given species.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

It is clear that all the chapters presented here are interconnected and that there is an interrelation between the results of our studies on the genus *Cystodytes*. However, because of the strict formal structure imposed in scientific papers and to allow independent reading, we have presented here self-explanatory chapters. Taken individually, they do not provide a general overview of our findings and we may lose sight of the overall nature of the species we are studying. Thus, the main objective of this discussion is to put together all the relevant information gleaned from the different parts of this thesis in search of a general view and a better understanding of the whole.

This study started with the identification of two major chemotypes within the four color morphs of *Cystodytes* studied. The first produced the sulfur-containing pyridoacridines shermilamine B (Carroll et al. 1989) and kuanoniamine D (Caroll & Scheuer 1990), together with their deacetylated forms (deacetylshermilamine B and deacetylkuanoniamine D), and corresponded to the purple morph. The second produced the C_9-unsubstituted pyridoacridine alkaloids ascididemin (Kobayashi et al. 1988) and 11-hydroxyascididemin (Schmitz et al. 1991), and was found in the blue and green morphs. No major alkaloid was found in the brown morph.

Pyridoacridine alkaloids are pH-sensitive pigments. The compounds shermilamine B, Kuanoniamine D, and their deacetylated forms are purple under acidic conditions. This helps to explain the purple color observed in the colonies, as the tunic of *Cystodytes* is highly acidic (pH<1; Tarjuelo et al. 2002). Ascididemin and 11-hydroxyascididemin are yellow under acidic conditions. The link between the blue

and green color and the pyridoacridine composition is less evident in this latter case, and may depend on other unknown molecules.

The identification and localization of the cells responsible for the production and/or storage of the active compounds in the different forms revealed 5 main cell types in the tunic of *Cystodytes*. These cells were identified as bladder cells, pigment cells, amebocytes, phagocytes, and morula cells. The morphology of the tunic was basically the same in the three morphotypes studied (blue, purple, and green). Our results indicated that the tunic is a dynamic system in which the diverse cell populations are in equilibrium, and in which transformation processes from one cell type to another occur continuously. Indeed, amebocytes comprised several subtypes that may correspond to a sequence of developmental stages that probably originate other cell types. Three main types of bacteria were present in the tunic, but they were scarce and their number never comparable, for instance, to those found in some sponges (e.g. Uriz et al. 1996, Turon et al. 2000). MALDI-TOF mass spectrometric analysis revealed that the four major compounds of the purple morph were present in the tunic, whilst shermilamine B and kuanoniamine D were lacking in the zooids. No sulfur signal corresponding to shermilamine B, kuanoniamine D, or their deacetylated forms, was detected by X-ray analysis in bacteria from the purple morph, suggesting that it is the host organism that produces these pyridoacridines. Indeed, the only structures presenting a relatively high amount of sulfur are the granules of the pigment cells. The same granules in the blue morph did not contain appreciable levels of sulfur. This evidence, albeit indirect, strongly suggests that these cells are the storage organs of the sulfur-containing pyridoacridine alkaloids characteristic of the purple morph but not of the blue form. However, other cell types may be implicated in the formation of these secondary metabolites, and it is still necessary to find out where synthesis occurs, where the deacetlyated molecules are located in the zooids, how they reach the tunic, and whether there is a change from a less-active precursor to a more active molecule. Addressing these points would imply the use of techniques such as cell separation and fractionation for chemical analyses, or precise spectrometric techniques for localization of substances in thin slices of tissue (Todd et al. 2001). Although pyridoacridine alkaloids are present in a variety of animal phyla (Molinski 1993), their production does not seem to be due to commonly occurring microsymbionts, but rather, is the result of a convergent evolution towards a successful biosynthetic pathway (Salomon et al. 2001).

Amino acid-derived metabolites such as alkaloids are the main defensive compounds in ascidians (Davidson 1993, Molinski 1993). Indeed, crude extracts from the blue and the purple Mediterranean morphs and a purple morph from Guam, as well as isolated ascididemin, are toxic and significantly deter fish predation. All crude extracts yielded similar results, although the alkaloid composition of the purple morphs (from Guam and the Mediterranean) is qualitatively different from the blue morph in terms of its pyridoacridine composition. This suggests a parallel evolution of related secondary metabolites as a defense mechanism against predation in this genus. In contrast, acidity by itself or its combination with spicules did not deter feeding, although fish were observed to display a flushing behavior in which they repeatedly swallowed and regurgitated the food cubes. In addition, ascidians have been reported to present physical defenses (spicules, tunic toughness, Swinehart et al. 1974, Stoecker 1978). When present, calcareous spicules may cause an extremely rapid neutralization of the acid and, even without spicules, seawater may be a sufficient buffer to quickly neutralize the acid (Parry 1984). The disc-shaped spicules, up to 1 mm long, typical of the genus *Cystodytes* represent 4% of the dry weight of the ascidian. However, as corroborated by Lindquist et al. (1992) for other ascidian species, spicular composition and concentration did not significantly deter predation. In the artificial food, spicules were randomly distributed, whilst in the living ascidian they encase each zooid. In fact, spicules form a compact and relatively hard capsule into which the thorax can be retracted in case of attack by a predator. Therefore, it is probable that although the disc-shaped spicules do not act as a deterrent in the artificial food, they do play a protective role associated with the zooid in the living colonies.

Neither crude extracts nor ascididemin deterred sea urchin predation. Secondary metabolites, therefore, do not deter all possible predators equally, as already indicated by previous studies (Paul & Hay 1986, Hay et al. 1987, Hay 1992, Schupp & Paul 1994, Pisut & Pawlik 2002, Tarjuelo et al. 2002, Burns et al. 2003). The optimal defense theory assumes that chemical and morphological defenses in living organisms are costly and that natural selection will favor an allocation of resources to defenses that optimize their cost/benefit ratio in terms of fitness (Rhoades 1979, Fagerström et al. 1987). There is an apparent paradox between the postulates of this theory and the presence of potentially redundant defense mechanisms in organisms such as *Cystodytes*. A current explanation is based on the evolution of these mechanisms as a response to different predators and/or

competitors. Alongside encompassing a wide range of predators, defense mechanisms often act at other levels, such as fouling avoidance or space competition (Stoecker 1980, Schmitt et al. 1995, Becerro et al. 1997). Furthermore, they may act at different life history stages, as suggested by Uriz et al. (1996) and Pisut & Pawlik (2002). All these factors contribute to explaining the selection of apparently redundant defense mechanisms. At the same time, multiple functions of secondary metabolites constrain their evolution (Schmitt et al. 1995), and there is always scope for the evolution of specialized predators able to circumvent the defense mechanisms of a given species. Our results highlight the importance of considering all potential defenses of an organism against as many potential predators as possible in order to reliably assess their ecological roles.

Morphological and genetic differences were also found for *Cystodytes* inhabiting the Mediterranean Sea. Zooid morphology, however, appeared to be generally quite similar, as was true of tunic morphology. The different phylogenetic analyses revealed the same basic genetic structure, from which we determined the existence of six clades. The largest genetic differentiation was found with the black morph from Mayotte, the only non-Mediterranean specimen analyzed. This single colony also had a specific kind of spicule (thick, disc-shaped, with a toothed margin), originally described in *C. aucklandicus* Nott 1892, and later found several times by Monniot (1988) and Monniot & Monniot (1996, 2001). Our data, therefore, support the validity of this species, to which our specimen was assigned. However, in Mediterranean forms, no clear pattern of association between the various morphological parameters analyzed (color, spicular complement) or with any geographic area could be substantiated. Colors vary even between neighboring colonies at the same site. Blue, green, white, and brown morphs can be found together at the same locality (e.g. Blanes, northern Catalonia), and have several kinds of spicular composition. Conversely, the same spicular type was found in several chromatic varieties. On the other hand, no relationship was found between spicular morphology and genetic clades. For instance, spherical spicules, which may be indicative of the species *C. philippinensis* Herdmann 1886 (Kott 1990, 2002), and discoidal spicules, which are the spicular complement of the typical *C. dellechiajei* (see Kott 1990), were found in individuals from three different clades. Color varieties seem to have a sounder genetic basis, although there are many exceptions. The main regularities observed were that the purple and brown morphs appear in our trees as distinct groups, while green and blue morphs constitute most of another

clade. Therefore, a genetic basis for the previously described chemotypes seems to be substantiated. Taken together, our results show that if any distinction is to be made, it could be on the basis of the concordance of some genotypes with chemotypes that, in some cases, correlates with the color morph. Thus neither spicular composition nor color by itself is a sufficient criterion with which to discern *Cystodytes* species.

The genetic study of seven populations from the western Mediterranean revealed a noticeable variability among the samples. Although this variability seemed to have the strongest component associated with the diverse color morphs, divergence according to location was also observed. Analysis of molecular variance clearly revealed the same pattern. In addition, the divergence between the most abundant color morphs was significant in comparisons of the brown and purple forms with the other morphs (green, blue, and white). No significant genetic divergence was detected between the latter morphs.

The haplotype network and the muldimensional scaling analysis also showed a wide genetic variability, and again an important role of color morphs in the groupings seemed evident. The largest group showed a mixture of blue, green, and white colors, again indicating a lack of differentiation of these particular morphs, whilst other groups appear more clearly linked to either a single or a few morphotypes (purple, brown). When we performed the nested clade analysis only a few of the clades showed significant associations between geography and genetic variability. This is surprising given that the long branches that separate the diverse groups indicate a deep divergence. The pattern may be explained by secondary expansions of the diverse groups in the areas studied after an initial, possibly allopatric, divergence. These range expansions would have erased most of the geographical signal. In this context, the maintenance of genetic differences strongly points to a reproductive isolation of at least some of these forms. A past fragmentation event caused the divergence of some of the main groups, as well as within some clades, and some evidence of range expansion in other clades supports the idea of a secondary expansion of already diversified lineages in the western Mediterranean.

Although genetics and secondary chemistry provided important clues about species boundaries, the study of biological cycles remained essential to assess speciation events according to the biological and cohesion species concepts. Therefore, the reproductive traits, growth, and survival of the two most abundant

morphotypes of the genus *Cystodytes* occurring in the western Mediterranean (purple and blue) were compared. Both morphotypes showed a clear seasonal cycle for reproduction and growth. In addition, significant differences in growth rates and reproductive cycles between these color morphs were found. The purple population lagged 1 to 2 months behind the blue one in the time course of both parameters. This pattern was consistent between years and the same lag in the reproductive cycle was observed at the Medes Islands, where specimens of the two morphotypes are found side by side. In addition, sampling of other populations showed that the biological cycles were highly coherent within color varieties, indicating that the pattern found is more likely to be the result of genetically fixed differences between these morphotypes.

There were no differences in the mortality observed between morphotypes, and a clear negative relationship with size was observed. Death occurred mostly in small colonies, which often resulted from fragmentation of larger ones. Similar patterns of reduced mortality with size have been reported for ascidians and other invertebrates (Turon & Becerro 1992, Turon et al. 1998). Some colonies remained small throughout the study period, whilst others exhibited marked changes in size. The majority of the colonies, however, displayed phases of rapid growth alternated with often significant size reductions. In addition, fusions and fissions were not rare. In both morphotypes, fission was the most frequent phenomenon, with no clear temporal pattern. Fusions, on the other hand, mainly occurred between recently separated clone mates, as described in another ascidian species by Stocker (1991). Nevertheless, the fragment dynamics of this species is much lower than that reported for other colonial ascidians, such as some Didemnidae (Ryland et al. 1984, Stocker & Underwood 1991).

In the blue morph, a significant effect of size on maximal growth rates was evident, indicating less scope for growth once a certain area has been reached. This observation is in agreement with previous results in ascidians and other encrusting invertebrates (Turon & Becerro 1992, Garrabou & Zabala 2001). However, the same relationship was not significant in the purple morph. As this effect is unlikely to be due to space saturation, it may represent an intrinsic characteristic of the blue morphotype.

A non-feeding form was observed in the blue population in June 2003, coinciding with the highest value of the maturity index. The switch to a resting form did not seem to imply any change in the reproductive condition, as already observed

in another colonial ascidian (*Polysyncraton lacazei*; Turon 1992). Although a temperature effect could not be discounted, it is also possible that a resting period is necessary for zooid regeneration and to reallocate energy from sexual to asexual reproduction. No equivalent state was observed in the purple population.

In temperate seas, temperature has been widely reported as the main factor controlling reproductive phases in ascidians (Berrill 1935, Millar 1971, Turon 1988, Durante & Sebens 1994, but see Coma & Ribes 2003), with many species, such as ours, reproducing in spring and early summer (Turon 1988). However, seasonal patterns can also be shaped by the trade-off between resource allocation to reproduction and growth. In our study, the peak of growth rate always occurred after reproduction was over, possibly indicating a partitioning of the energy resources of the colony over the course of its annual cycle (Yund et al. 1997).

Although the timing of male reproductive activity and appearance of oocytes is so broad that it overlaps between these forms (meaning that the possibility of cross-fecundation cannot be definitively ruled out), there is a lag in the reproductive periods that is maintained even in sympatric populations (e.g. the Medes Islands) and between years. Indeed, the larval release of one morphotype occurred almost exclusively after the release of the other morphotype was over, indicating that, most probably, fecundation also takes place at different times. Together with previous studies of gene flow and chemical divergence, the differences found in the life cycles of these two morphotypes indicate that these forms are reproductively isolated and, in fact, correspond to two sibling species.

Although little evidence is available, it is generally agreed that production, maintenance, transport, and storage of secondary metabolites have associated costs in terms of trade-offs that can only be achieved by diverting materials and energy from other functions (Cronin 2001). We sought evidence of a temporal variability in the production of chemical and physical defenses related to the life cycle parameters of the blue morph from the Mediterranean. The asciddemin concentration (the major alkaloid of this form) showed a cyclic behavior that supports the hypothesis of an annual periodicity in the production of bioactive substances, with a minimum in spring and a maximum after the reproduction peak in late summer and autumn. The same pattern was observed for the ash content of the colonies, suggesting that an annual periodicity also exists for the production of inorganic contents, mainly spicules. Therefore, a clear temporal trade-off between investment in reproduction and the other biological parameters considered (e.g.

growth, and chemical and physical defenses) was observed. This is in agreement with Tarjuelo & Turon (2004), who concluded that species with low fecundity that have large and complex larvae, such as *C. dellechiajei*, invest the most energy in reproduction. Indeed, a month after larval release, the colonies increased their inorganic content progressively and grew rapidly for approximately one month. The two parameters may also be linked, as during periods of rapid growth there is an increase in zooid budding, and spicules in this genus are formed surrounding the abdominal part of the zooids (Lambert 1979). Possibly, once colony growth is over and the capsules around the zooids are formed, more energy is available for secondary metabolite production, which seems to be the most efficient defense mechanism against predation.

In addition, external interactions (e.g. the rhythms of interacting species) could also account for the temporal variation in the production of chemical and physical defenses observed. Indeed, interacting organisms presenting a seasonal variation should be taken into account. The highest concentration of ascididemin in autumn may be related to the reactivation of growth of competing animal species after the summer period, which is an unfavorable season at the end of the reproductive period for many sessile invertebrates in the Mediterranean (Boero et al. 1986, Turon & Becerro 1992, Coma & Ribes 2003). Our results indicate, therefore, that although the reproductive cycle probably drives the temporal course of the production of defensive secondary metabolites and inorganic material, "environmentally modulated" elements may also play a role. This is consistent with the hypothesis that secondary metabolite production is costly in metabolic terms (Fagerström et al. 1987) and must be optimized with respect to other factors, mainly biological parameters, to adjust the energy budget of a given species.

Finally, we want to highlight some other ecological features observed in *Cystodytes* during the realization of this thesis. Specimens of the crab genus *Dromia* sp. were found to carry purple colonies both in the Mediterranean (Spanish coast) and the Pacific (Guam) (Fig. 1.1). Also, cyanobacteria occasionally covered some colonies of the purple and blue Mediterranean forms (Fig. 1.2). During May 2003, we observed several slit-like scars on the surface of some purple colonies in L'Escala (North Spain). We sampled three of these colonies and observed them under stereomicroscope and found some exemplars of the amphipod *Tritaeta gibbosa* (Bate 1862) inside the scars (Fig. 1.3, 1.4). In summer, after the observation of a non-feeding form in a blue Mediterranean population (June 2003), some colonies

displayed what seemed to us the sloughing off of a mucus layer (Fig 1.5). Finally, an amazing event was observed in September and October 2004 in L'Escala. Some purple colonies presented long extensions of tunic stretching downwards away of the colony to form a kind of propagule (Fig 1.6). The same phenomenon has been reported in sponges (Wulff 1991), and was attributed to an asexual reproduction strategy. Although punctual, natural history observations on the studied species are worth drawing attention to, as they contribute to our knowledge of its biology and suggest future directions of study.

The multidisciplinary approach undertaken to assess the main biological features of what has traditionally been called *Cystodytes dellechiajei* in the Mediterranean has revealed the existence of several species. Their taxonomy can only be formally resolved after the study of additional material from other zones, in the context of a global revision of this genus. In addition, the biological cycles of two abundant color morphs of *Cystodytes*, although overlapping, are clearly distinct. We have found that the defense mechanisms of this genus act as efficient deterrents against some, but not all, predators, and that secondary metabolites are produced by the ascidian and not by microsymbionts. Resources allocation to reproduction seems to drive the other biological cycles (growth rates and secondary metabolite and spicular production), as they are depressed during periods of high investment in reproduction. As predicted by the optimal defense theory, allocation to defense needs to be optimized with respect to the other requirements of the energy budget.

In conclusion, the ascidian genus *Cystodytes* provided an interesting case study through which to develop a better understanding of the biology, ecology, and secondary chemistry of marine invertebrates. However, several unanswered questions still remain. These include, amongst others, the nature of the biosynthetic pathway that generates pyridoacridine alkaloids and its cellular basis, the intracellular localization of ascididemin, and the function of asexual propagules. Nevertheless, it is clear that the more we learn about an organism, the more new questions arise, necessitating the use of new techniques and further research…

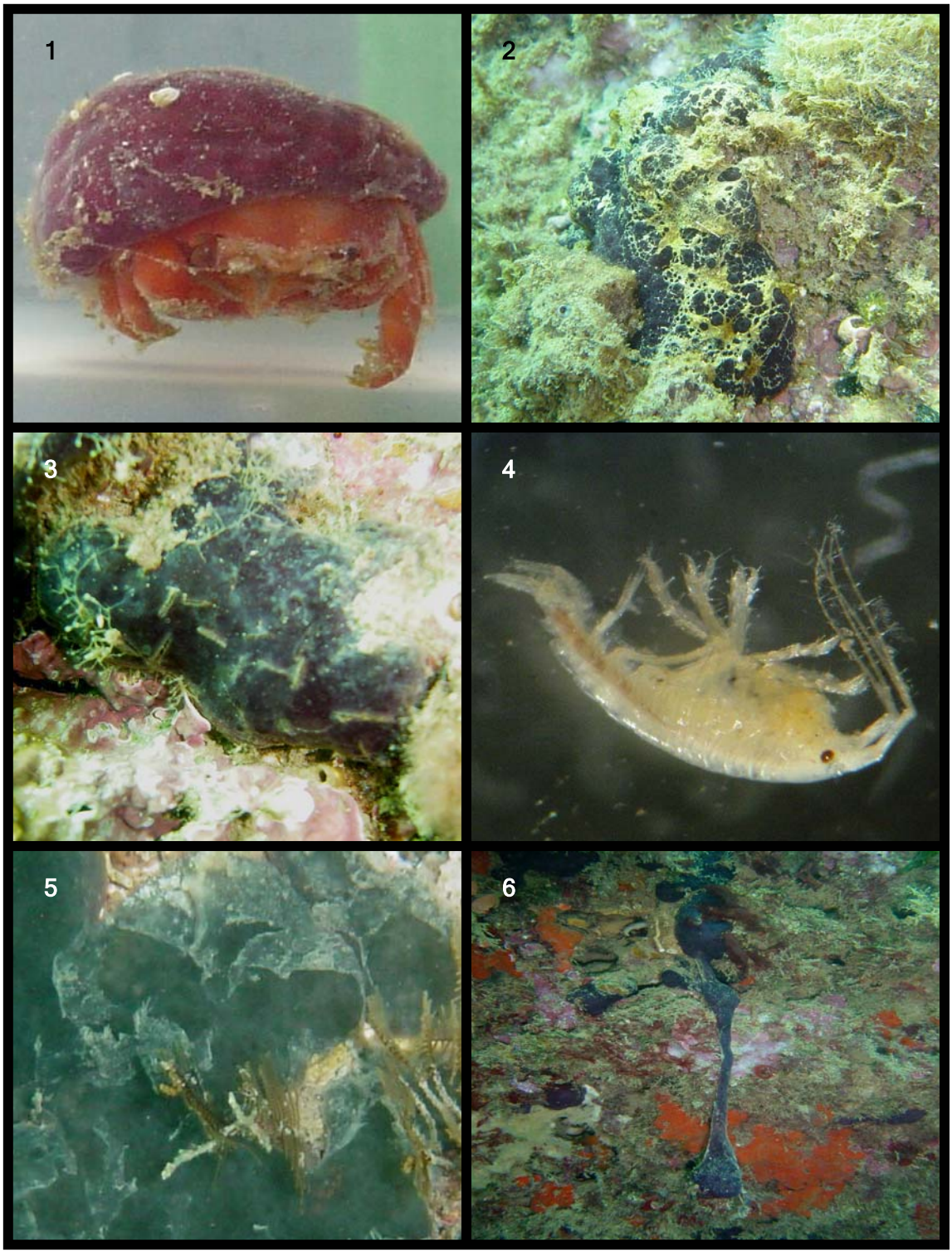


Fig. 1. Other ecological features observed in *Cystodytes* during the present study. **1**: The crab *Dromia* sp. carrying a purple colony from Guam (August 2003). **2**. Cyanobacteria covering a purple Mediterranean colony (September 2004). **3**: Scares in a Mediterranean purple colony caused by **4**. The amphipod *Tritaeta gibbosa* (May 2003). **5**. Mucus layer covering a blue Mediterranean colony (June 2003) and **6**. Propagule stretching downwards away of a purple Mediterranean colony (September & October 2004).

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ANNEXE I

KNOWN ALKALOIDS OF *CYSTODYTES*

NOTE: Names in italic mean that the compound was first described for another species (see reference).

Cystodytes dellechiajei

Bonnard et al. (1995)

Ascididemin Kobayashi et al. (1988)

Kobayashi et al. 1988

Cystodytins A, B, C

Kobayashi et al. (1991)

Cystodytins D, E, F, G, H, I

Steffan et al. (1993)

Shermilamine B Carroll et al. (1989)

Kuanoniamine D Caroll & Scheuer (1990)

Rottmayr et al. (2001)

Shermilamine B Carroll et al. (1989)

Kuanoniamine D Caroll & Scheuer (1990)

Delfourne et al. 2001

11-Hydroxyascididemin Schmitz et al. (1991)

Torres et al. (2002)

Sebastianines A, B

Cystodytes sp.

Plubrukarn et al. (1998)

Arnoamine A

Arnoamine B

McDonald et al. (1994)

Cystodytin J

Shermilamine C

Dehydrokuanoniamine B

Eilatin Rudi et al. (1988)

Cystodytin A Kobayashi et al. (1988)

Kuanoniamine D Carroll & Scheuer (1990)

Shermilamine B Carroll et al. (1989)

This study:

Shermilamine B Carroll et al. (1989)

Kuanoniamine D Caroll & Scheuer (1990)

Ascididemin Kobayashi et al. (1998)

11-Hydroxyascididemin Schmitz et al. (1991)

Deacetylkuanoniamine D Eder et al. (1998)

Deacetylshermilamine B

Jamme et al. in press

Cystodimines A, B

Deacetylshermilamine B

Bontemps-Subielos & Banaigs pers. com.

Styelsamine A, C Copp et al. (1998)

Cystodytes violatinctus

Koren-Goldshlager et al. (1998)

Shermilamine D, E

Tintamine

Segoline A Rudi & Kashman (1989)

Segoline B Rudi & Kashman (1989)

Isosegoline A Rudi & Kashman (1989)

Koren-Goldshlager et al. (2000)

Cycloshermilamine D

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Carroll AR, Cooray NM, Poiner A, Scheuer PJ (1989) A second Shermilamine alkaloid from a tunicate *Tridemnum* sp. J Org Chem 54:4231-4232

Caroll AR, Scheuer PJ (1990) Kuanoniamines A, B, C and D: Pentacyclic alkaloids from a tunicate and its prosobranch mollusc predator *Chelynotus semperi*. J Org Chem 55:4426-4431

Copp BR, Jompa J, Tahir A, Ireland CM (1998) Styelsamines A-D: New tetracyclic pyridoacridine alkaloids from the Indonesian ascidian *Eusynstyela latericius*. J Org Chem 63:8024-8026

Eder C, Schupp P, Proksch P, Wray V, Steube K, Müller CE, Frobenius W, Herderich M, van Soest RWM (1998) Bioactive pyridoacridine alkaloids from the micronesian sponge *Oceanapia* sp. J Nat Prod 61:301-305

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Rudi A, Benayahu Y, Goldberg I, Kashman Y (1988) Eilatin, a novel alkaloid from the marine tunicate *Eudistoma* sp. Tetrahedron Lett 29:6655-6656

Rudi A, Kashman Y (1989) Six new alkaloids from the purple red sea tunicate *Eudistoma* sp. J Org Chem 54:5331-5337

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Steffan B, Brix K, Pütz W (1993) Biosynthesis of shermilamine B. Tetrahedron 49:6223-6228

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ANNEXE II

TAXONOMY AND SYNONYMY Of THE GENUS *CYSTODYTES*

NOTE: The taxonomy and synonymy here reported are based on our literature search and on Sanamyan's Zoobase v. 2.20 (2001 & 2002): http://lithopssoft.com/zoo/index.html

Cystodytes antarcticus - SLUITER 1912

Millar 1968:

Cystodites antarcticus Sluiter 1912

Van Name 1945:

Cystodytes antarcticus Sluiter 1912, 1914

Kott 1969:

Cystodites antarcticus Sluiter 1912, 1914. Arnback 1950

Cystodytes antarcticus Van Name 1945

Cystodytes dellechiajei f. *antarctica* Millar 1960

Monniot & Monniot 1974:

Cystodites antarcticus Sluiter 1912

Cystodytes antarcticus Kott 1969

Monniot & Monniot 1983a:

Cystodites antarcticus Sluiter 1912

Cystodytes dellechiajei f. *antarctica* Millar 1960

Cystodytes antarcticus Arnback 1950. Millar 1968. Kott 1969, 1971. Monniot & Monniot 1973

Cystodytes aucklandicus - NOTT 1982

Monniot 1988

Monniot & Monniot 1996:

Cystodytes aucklandicus Nott 1982. Monniot 1988

Monniot & Monniot 2001:

Cystodytes aucklandicus Nott 1892. Monniot & Monniot 1996

Present work:

Cystodytes aucklandicus Nott 1892. Monniot & Monniot 2001

Cystodytes ceylonensis - HERDMAN 1906

Van Name 1918

Pérès 1949, 1954:

Cystodytes dellechiajei f. *ceylonensis* Herdman 1906

Cystodytes cretaceous - VON DRASCHE 1883

Herdman 1886

Ritter 1907

Salfi 1931:

Cystodites delle chiaiei var. *cretacea*. Drasche 1883

Lahille 1890

Cystodytes dellachiajei - DELLA VALLE 1877

Kott 1990:

Distoma dellachiajei Della Valle 1877

Cystodytes dellachiajei Hartmeyer 1912. Michaelsen 1915, 1923, 1930. Harant 1929. Van Name 1945. Brewin 1948, 1951, 1952, 1958, 1960. Kott 1954, 1972a, 1972b, 1981. Tokioka 1950. Millar 1953, 1960, 1962, 1963, 1964, 1966, 1978, 1982

Cystodytes dellachiaiae Van Name 1921. Berrill 1932. Kott 1957

Cystodytes Della Chiajei Pérès 1948

Cystodytes durus von Drashe 1833

Cystodytes draschii Herdman 1886. Van Name 1902. Michaelsen 1915, 1924 (*draschei*)

Cystodytes philippinensis Herdman 1886, 1891. Sluiter 1909. Caullery 1909. Hartmeyer 1909-1911. Van Name 1918

Cystodytes aucklandicus Nott 1892

Cystodytes perspicuus Nott 1892

Cystodytes violaceus Van Name 1902. Harant 1925.

Cystodytes ceylonensis Herdman 1906

Cystodytes hapu Monniot & Monniot 1996

***Cystodytes dellechiajei* - DELLA VALLE 1877**

Michaelsen, 1915, 1923,1930

Harant 1927:

Cystodytes cretaceous von Drasche 1883

Cystodytes durus von Drasche 1883

Cystodytes inflatus Heiden 1894

Cystodytes irregularis Heiden 1894

Cystodytes polyorchis Heiden 1894

Cystodytes philippinensis Herdman 1886

Harant 1929:

Distoma dellechiaiae Della Valle 1877, 1921. Michaelsen 1915

Cystodytes durus von Drasche 1883

Cystodytes draschii Herdman1886, 1891. Van Name 1902

Cystodytes durus Lahille 1890

Cystodytes violaceus Van Name 1902. Hartmayer 1909-1911, 1912 Harant 1925

Cystodytes draschei Seelinger 1893-1907. Hartmayer 1909-1911, 1912. Michaelsen 1915

Cystodytes violaceus Caullery 1909

Cystodytes dellachiajei Harant 1925

Millar 1953

Berrill 1932

Harant & Vernières 1933:

Cystodytes dellechiajei Harant 1925

Cystodytes cretaceous von Drasche 1883

Cystodytes durus von Drasche 1883. Lahille 1890

Cystodytes inflatus Heiden 1894

Cystodytes philippinensis Herdman 1886

Harant 1939

Van Name 1945:

Cystodytes dellechiaiae Van Name 1921. Berrill 1932

Cystodytes dellechiajei Michaelsen 1915, 1930. Harant 1929

Cystodytes draschii Herdman 1886. Van Name 1902. Michaelsen 1915, 1924 (*draschei*)

Cystodytes violaceus Van Name 1902. Harant 1925

Distoma dellechiajiae Della Valle 1877

Brewin 1948, 1951, 1952a, 1958, 1960

Tokioka 1950

Pérès 1953, 1957, 1958a, 1958b, 1959

Kott 1954:

Cystodytes dellechiaiae Van Name 1921, 1930. Berrill 1932.

Cystodytes dellechiajei Michaelsen 1915, 1930. Harant 1929. Van Name 1945

Cystodytes draschii Herdman 1886. Van Name 1902. Michaelsen 1915, 1924 (*draschei*)

Cystodytes violaceus Van Name,1902. Harant 1925

Distoma dellechiajiae Della Valle 1877

Gravier 1955

Millar 1960:

Cystodytes antarcticus Sluiter 1912, 1914. Arnback 1950

Millar 1962, 1963, 1964, 1966, 1970, 1975, 1977, 1978, 1988a, 1988b

Plante & Vasseur 1966

Monniot 1969, 1970, 1972, 1974, 1983

Kott 1972a:

Cystodytes dellechiajei Kott 1954. Tokioka 1950. Millar 1953, 1960, 1962, 1963, 1966

Distoma dellechiajiae : Della Valle 1877

Aplidium lobatum ?: NOT Savigny 1816

Cystodytes dellachiaiae Kott 1957

Cystodytes Delle Chiajei Pérès 1948.

Kott 1972b:

Cystodytes dellechiajei Kott 1972a

Fiala-Médioni 1974

Plough 1978

Bibiloni et al. 1980

Kott 1981

Millar 1982:

Distoma dellechiajiae Della Valle 1877

Cystodytes draschii Herdman 1886

Cystodytes auclandicus Nott 1892

Cystodytes perspicuus Nott 1892

Cystodytes draschei Michaelsen 1924

Cystodytes dellechiajei Brewin 1948, 1951, 1952a, 1952b, 1958, 1960. Millar 1960

Ramos 1982, 1984, 1987

Nishikawa 1984:

Cystodytes dellechiajei Millar 1975,1982. Kott 1981

Rios & Brito 1984

Monniot & Monniot 1985, 1986, 1994

Rios 1985

Lafargue et al. 1986

Turon 1987, 1988, 1990, 1993

Ramos et al. 1991

Brunetti 1994:

Distoma dellechiaie Della Valle 1877

Cystodytes durus von Drasche 1883

Cystodytes cretaceus von Drasche 1883

Cystodytes Dellechiajei ? Harant 1929

Cystodytes dellechiajei Van Name 1945. Tokioka 1950. Monniot 1972, 1983. Kott 1990

Naranjo & García-Gómez 1994

Monniot et al. 2001:

Distoma dellechiajei Della Valle 1877

Méliane 2002

Lambert 2003

Present study:

Cystodytes philippinensis Herdman 1886. Méliane 2002

Cystodytes tunisiensis Méliane 2002

Cystodytes denudatus - PÉRÈS 1953

Monniot 1969

Cystodytes draschei - HERDMAN 1886

Michaelsen 1924:

Cystodytes aucklandicus ? Nott 1892

Cystodytes perspicuus Nott 1892

Cystodytes draschii Herdman 1886. Van Name 1902

Cystodytes durus - VON DRASCHE 1883

Herdman 1886

Daumézon 1909:

Cystodites cretaceous von Drasche 1883

Cystodites Delle Chiajei von Drasche 1883

Cystodites inflatus Heiden 1894

Cystodites polyorchis Heiden 1894

Cystodites irregularis Heiden 1894

Cystodites durus var. *viridis*

Cystodites durus var. *didemniformis*

Lahille 1890:

Distoma Delle Chiajae Della Vale 1877

Cystodytes fuscus - MONNIOT 1988

Cystodytes guineensis - MICHAELSEN 1914

Sluiter 1927

Monniot & Monniot 1967, 1994

Monniot 1969

Monniot 1970:

Cystodytes guinensis Michaelsen 1914

Cystodytes hapu - MONNIOT & MONNIOT 1988

Monniot & Monniot 1996, 2001

Cystodytes inflatus – HEIDEN 1894

Cystodytes irregularis - HEIDEN 1894

Cystodytes jodomi - OKA 1929

Tokioka 1963

Cystodytes lobatus - RITTER 1900

Van Name 1945:

Cystodites cretaceous Ritter 1907 (apparently not *Cystodytes cretaceus* von Drasche, 1883)

Distoma lobata Ritter 1900

Eudistoma lobatum Hartmeyer 1909-1911

Tokioka 1963

Lambert 1979

Abbott & Newberry 1980

Sanamyan 1993:

Distoma lobata Ritter 1900

Cystodytes luteus - MONNIOT 1988

Cystodytes morifer - MICHAELSEN 1919

Monniot et al. 2001

Cystodytes mucosus - MONNIOT 1988

Cystodytes multipapillatus - MONNIOT 1988

Cystodytes philippinensis - HERDMAN 1886

Herdman 1891, 1906

Seeliger 1893-1907

Caulleby 1909

Hartmeyer 1909-1911

Sluiter 1909

Van Name 1918:

Cystodytes philippinensis Herdman 1886, 1891

Cystodites philippinensis Seeliger 1893-1907. Herdman 1906. Sluiter 1909. Caulleby 1909. Hartmeyer 1909-1911

Tokioka 1950

Méliane 2002

Kott 2003 :

Cystodytes philippinensis Herdman 1886. Tokioka 1950

Cystodytes hapu Monniot & Monniot 1987, 2001

***Cystodytes planus* - MONNIOT 1974**

***Cystodytes polyorchis* - HEIDEN 1894**

***Cystodytes punctatus* - MONNIOT 1988**

Monniot & Monniot 1996

***Cystodytes ramosus* - KOTT 1992**

Kott 2003

***Cystodytes roseolus* - HARTMEYER 1912**

Michaelsen 1914, 1915

Michaelsen 1919:

Cystodytes roseolus Hartmeyer 1912. Mcihaelsen 1914, 1915 (var. *greeffi*)

Pérès 1949

Millar 1962

Monniot 1969

Monniot & Monniot 1994

Monniot et al. 2001

***Cystodytes rufus* - SLUITER 1909**

Hartmeyer 1909-1911

Sluiter 1909

Van Name 1912:

Cystodites rufus Sluiter 1909. Hartmeyer 1909-1911

Van Name 1918

***Cystodytes semicataphractus* - SLUITER 1909**

Hartmeyer 1909-1911

Sluiter 1909

Van Name 1912:

Cystodites semicataphractus Sluiter 1909. Hartmeyer 1909-1911

Van Name 1918

Cystodytes senegalense – MONNIOT 1969

Monniot 1970

Monniot & Monniot 1994

Cystodytes solitus – MONNIOT 1988

Brunetti 1994

Monniot & Monniot 1996, 2001

Cystodytes sp.

Monniot 1983

Monniot & Monniot 1991

Cystodytes tasmaniensis – KOTT 1954

Kott 2001:

Lissoclinum tasmanense No *C. tasmaniensis*

Cystodytes tunisiensis – MÉLIANE 2003

Cystodytes variabilis - SLUITER 1???

Arnbäck 1950

Cystodytes violaceus - VAN NAME 1902

Van Name 1945:

Cystodytes dellechiajei No *C. violaceus*

Cystodytes violatinctus - MONNIOT 1988

Monniot & Monniot 2001

Lambert 2003

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RESUM - CATALÀ

ESTUDI MULTI DISCIPLINAR DEL GÈNERE *CYSTODYTES* (ASCIDIACEA): DE LES MOLÈCULES A LES ESPÈCIES

INTRODUCCIÓ GENERAL

GENERALITATS I ESPÈCIE D'ESTUDI

Els invertebrats marins produeixen abundants compostos nous d'interès farmacèutic i biotecnològic (Faulkner 2000). Aquests metabolits acompleixen també diverses funcions ecològiques a la natura (Becerro et al. 1997) i són factors decisius en l'establiment i l'evolució de les diferents estratègies de vida dels organismes productors. Tot i la importància d'aquests compostos, moltes de les espècies d'invertebrats marins que els produeixen només estan identificades a nivell de gènere (veure Marinlit, Annex I), romanen sense descriure (p.ex. Caroll i Scheuer 1990) o estan incorrectament identificades, resultant en una important confusió a l'hora de retrobar l'organisme productor. Sense informació sobre la identitat de les espècies i de les seves interrelacions, entendre la funció ecològica d'aquests compostos bioactius i intentar la producció d'aquest metabolits per a aplicacions biotecnològiques i/o medicinals resulta impossible.

D'altra banda, la identificació d'espècies es complica i resulta fins i tot més necessària si existeix una variabilitat a nivell intra-específic (incloent diferències químiques). De fet, les observacions basades únicament en la morfologia de l'espècie sovint resulten insuficients per a determinar si ens trobem davant d'un cas

de variabilitat intra-específica o un grup d'espècies críptiques. Es per això que sovint sorgeix la necessitat d'utilitzar tècniques alternatives com ara aproximacions moleculars. L'estudi de la filogènia molecular, la genètica de poblacions i la filogeografia contribueixen significativament a entendre els diferents processos d'especiació que han pogut tenir lloc i alhora aporten important informació ecològica i evolutiva sobre les espècies estudiades. A més a més, l'estudi dels cicles reproductors permet determinar el grau de diferenciació de les espècies d'acord amb el concepte biològic d'espècie, i incrementa la informació disponible sobre l'ecologia i biologia dels invertebrats bentònics.

D'altra banda, els cicles de vida estan estretament relacionats amb la distribució dels recursos destinats a la producció de metabolits secundaris. Si assumim que existeix un cost pel que fa a l'energia invertida en la producció de metabolits, aquests cost hauria d'estar optimitzat pel que fa a les necessitats de l'organisme i als altres paràmetres relacionats amb la seva biologia (creixement, manteniment somàtic i reparació, i reproducció). Aquesta optimització es pot posar de manifest estudiant els principals paràmetres biològics de les espècies i analitzant la variació quantitativa en la inversió en defensa en funció d'aquests paràmetres. D'altra banda, la localització d'aquests compostos bioactius dins d'un organisme i la identificació de les cèl·lules responsables de la seva producció també aporten informació sobre la seva significació biològica. Es per això que una aproximació multidisciplinar, englobant i relacionant informació de diferents camps, des de les molècules fins a les poblacions, és necessària per entendre l'estratègia biològica dels invertebrats marins. Tot i la rellevància d'aquest fet, aquest tipus d'informació multidisciplinar no és disponible per quasi cap espècie (per una excepció, vegeu Becerro 1994).

Aquest treball es centra en un organisme model, l'ascidi colonial *Cystodytes dellechiajei* (Della Valle 1877), un taxó polimòrfic del que s'ha discutit si és format per un grup d'espècies, per a intentar determinar el significat i la variabilitat en la producció de metabolits secundaris, i relacionar aquesta variabilitat amb altres aspectes de la seva biologia i ecologia. Per aquesta finalitat, hem utilitzat un conjunt de tècniques pertanyent a diferents disciplines com son l'ecologia molecular, la filogènia, la genètica de poblacions, la filogeografia, la química, tècniques ultra-estructurals i diversos estudis i experiments *in situ* i al laboratori. A més a més, aquesta espècie presenta un conjunt de característiques particulars: produeix defenses físiques i químiques que són potencialment redundants, s'ha descrit una

variabilitat qualitativa en la producció de metabolits secundaris, es coneixen diferències morfològiques en l'àrea d'estudi (Mediterrània Occidental), la dispersió larvària és suposadament curta (afavorint l'aïllament genètic per distància), i la seva biologia és poc coneguda.

OBJECTIUS I ESTRUCTURACIÓ DE LA TESI

Nombrosos treballs han estudiat un o altre aspecte de la biologia I l'ecologia dels organismes marins. Tot i que aquest tipus d'estudis contribueixen a augmentar el coneixement general que puguem tenir sobre una espècie o un fenomen, normalment el que fan es plantejar nous interrogants que demanaran la utilització d'una nova tècnica o disciplina per obtenir respostes. Tot i que actualment existeixen moltes i variades tècniques, la seva utilització soles o combinades amb d'altres encara resulta força complicada, ja sigui per la dificultat de la tècnica *per se* o perquè s'allunya molt de la nostra àrea de coneixement. D'aquí que moltes vegades neixi la necessitat de col·laborar amb altres investigadors. L'objectiu principal d'aquesta tesi és aplicar diferents tècniques pertanyent a diferents disciplines per assolir una millor i més complerta comprensió de la biologia i ecologia dels ascidis colonials del gènere *Cystodytes*.

Concretament, els objectius d'aquesta tesi són:

1. Identificar la variabilitat química en alcaloides de diversos morfotips (sobretot pel que fa el color) de *Cystodytes* de la Mediterrània Occidental.

2. Comparar la composició cel·lular de la túnica dels principals morfotips, determinar on es troben els metabolits secundaris, si existeixen microsimbionts i si aquests tenen algun paper en la producció de substàncies defensives.

3. Determinar a partir d'evidència experimental el paper que juguen a la natura tant les defenses químiques com físiques d'algunes espècies representatives d'aquest gènere.

4. Determinar si existeix una variabilitat genètica associada a les diferències morfològiques observades en *Cystodytes* i relacionar-la amb la variabilitat química.

5. Analitzar la divergència genètica i el flux genètic entre poblacions de diferents colors i localitats de la Mediterrània Occidental.

6. Comparar els cicles de vida i les estratègies reproductives dels dos morfotips mes abundants en l'àrea d'estudi.

7. Determinar si existeixen balanços ("trade-offs") al llarg del cicle biològic entre els recursos dedicats a la producció de metabolits secundaris bioactius i els dedicats a altres funcions biològiques (creixement i reproducció).

Així doncs, volem combinar informacions sobre la química, la genètica, la ultrastructura i el cicle de vida de *Cystodytes* per a entendre els mecanismes de defensa, les estratègies biològiques i ecològiques i les seves interaccions.

La tesi està estructurada en 7 capítols principals que, tot i estar inter-connectats, s'han escrit com unitats independents per a que es puguin llegir separadament i per facilitar-ne la publicació. Així doncs, cada capítol consta d'una introducció, material i mètodes, resultats i discussió, i poden contenir ocasionalment referències creuades a altres capítols. La darrera part de la tesi consta d'una discussió general sobre els resultats presentats en els capítols anteriors i situa les diferents parts en un marc global. Al final del treball, una bibliografia general agrupa totes les referències citades al llarg de la tesi per tal d'evitar redundàncies entre els diferents capítols.

RESULTATS I CONCLUSIONS

VARIABILITAT QUÍMICA DELS ALCALOIDES DELS PRINCIPALS MORFOTIPS DE *CYSTODYTES*

La majoria d'estudis sobre metabolits secundaris no proporcionen informació sobre l'organisme productor. Per tant actualment es gairebé impossible relacionar la química amb possibles variacions morfològiques a nivell d'espècies o gèneres polimòrfics. Aquest problema es fins i tot més greu quan es demostra que aquests compostos tenen un potencial biotecnològic o possibles aplicacions farmacèutiques. D'altra banda, avui dia s'estan desenvolupant noves tècniques per a la detecció de substàncies bioactives en mostres biològiques. Entre elles, l'espectrometria iònica de masses (SIMS) i l'espectrometria de masses "time-of-flight" acompanyada d'un làser de desorpció/ionització (MALDI-TOF MS). Aquests mètodes són altament sensibles, només necessiten uns pocs mil·ligrams de mostra i permeten una detecció ràpida i eficaç de quantitats minúscules de metabolits d'estructura i pes molecular coneguts.

L'objectiu d'aquest capítol és, primerament, determinar la composició en alcaloides dels morfotips més abundants de *Cystodytes* en la Mediterrània (verd, morat, marró i blau). Val a dir que fins ara aquests han estat assignats a varietats cromàtiques de l'espècie *C. dellechiajei.* En segon lloc, determinar la localització intra-espècimen d'aquests alcaloides (si estan a la túnica i/o als zooids). Finalment, determinar la utilitat de les tècniques MALDI-TOF MS com a eina ràpida i eficaç per a la detecció de compostos naturals coneguts i la determinació de quemotips. Per a comprovar la validesa dels resultats obtinguts, aquests es van comparar amb espectres UV obtinguts amb cromatografia líquida d'alta pressió ("HPLC") i utilitzant les substàncies pures com a estàndards. Per obtenir aquestes substàncies, es va haver de realitzar un treball previ de fraccionament amb "HPLC" per aïllar els diferents pics (=compostos naturals) i poder procedir al reconeixement de la seva estructura química a partir de ressonància magnètica nuclear ("NMR").

Les anàlisis químiques van permetre diferenciar 2 quemotips. El primer conté les piridoacridines (alcaloides) amb àtoms de sofre: shermilamine B, kuanoniamine D i les seves formes deacetilades: la deacetylshermilamine B (nou metabolit per la ciència) i la deacetylkuanoniamine D i correspon a les colònies de color morat. El

segon presenta 2 piridoacridines però amb l'àtom C_9 sense substitucions: l'ascididemin i la 11-hydroxiascididemin, que es troben als morfotips blau i verd. En el morfotip marró només es va detectar ascididemin en petites quantitats amb MALDI-TOF MS. Tots els alcaloides eren presents tant a la túnica com als zooids, amb l'excepció dels metabolits característics de la forma morada: la shermilamine B i la kuanoniamine D només es trobaven a la túnica mentre que les seves formes deacetilades eren presents a ambdós compartiments. S'ha pogut establir una clara relació entre aquest pigments (piridoacridines) i el color morat de les colònies; per als altres morfotips (blau, verd i marró), el color deu dependre d'altres molècules no identificades. La tècnica MALDI-TOF va resultar ràpida I eficaç per a la detecció de compostos diana de baix pes molecular a nivell tant inter- com intra-espècimen. Així doncs, aquesta tècnica resulta de gran utilitat per a la determinació ràpida de quemotips. Les diferències químiques trobades entre els diferents morfotips del que fins ara s'havia descrit com *Cystodytes dellechiajei*, planteja nous interrogants a nivell de l'estatus taxonòmic d'aquesta espècie en la Mediterrània Occidental. D'altra banda, els resultats obtinguts destaquen la importància d'una descripció detallada tant de la morfologia com del lloc de recol·lecció quan es treballa amb productes naturals. Aquesta consideració es especialment important per aquells grups que no tenen una taxonomia ben resolta i per tal de comprendre la variació en metabolits secundaris dins i entre espècies.

TIPUS CEL·LULARS, MICROSIMBIONTS I DISTRIBUCIÓ DELS ALCALOIDES EN LA TÚNICA DE *CYSTODYTES*

Existeixen molt pocs estudis sobre la diversitat de metabolits secundaris en els invertebrats marins, i els pocs que existeixen s'han centrat en variacions ecològiques i estacionals. Així doncs, gairebé no es coneix res sobre la localització intra-espècimen i l'indret de producció a nivell cel·lular d'aquests compostos. Aquest tipus d'estudi aporta valuosos indicis sobre el paper ecològic i biològic que aquestes substàncies bioactives poden tenir en la natura. A part de la localització de metabolits, és coneix molt poc sobre la morfologia i la classificació de les cèl·lules de la túnica d'ascidis. Així doncs, el seu estudi roman necessari com a part de la recerca fonamental en aquest camp.

Els objectius d'aquest capítol són, primerament, caracteritzar els principals tipus cel·lulars de la túnica dels morfotips blau, morat i verd de *Cystodytes* i determinar-ne la importància de la població bacteriana. I, en segon lloc, localitzar en quines cèl·lules de la túnica es troben els compostos biològicament actius descrits en el capítol 2. Per a respondre a aquestes qüestions, hem utilitzat tècniques de microscopia electrònica de transmissió i microanàlisis per raigs X.

Els principals tipus cel·lulars trobats són: cèl·lules vesiculars, cèl·lules pigmentàries, amebòcits, fagòcits, i cèl·lules en mòrula. El grup dels amebòcits compren diversos subtipus cel·lulars que segurament corresponen a una successió d'estadis de desenvolupament podent originar algun dels altres tipus cel·lulars. La morfologia de la túnica és bàsicament la mateixa en els tres morfotips estudiats. També es van identificar tres tipus diferents de bacteris. Tot i això, sembla que aquests no són els productors de la sehrmilamine B, kuanoniamine D i les seves formes deacetilades (alcaloides amb sofre), ja que el nombre total de bacteris trobat era sempre molt baix i les anàlisis de raigs X van resultar negatives. De fet, la tècnica de la microanàlisi mostra que l'únic tipus cel·lular que presenta un clar senyal de sofre són les cèl·lules pigmentàries al morfotip morat. Aquestes mateixes cèl·lules no mostren senyal al morfotip blau (alcaloides sense sofre). No descartem però, que altres tipus cel·lulars puguin estar també implicats en la formació d'aquests alcaloides. Tot i que els alcaloides de tipus piridoacridina estan presents en una gran varietat de tipus zoològics, la seva producció no sembla provenir de bacteris simbionts comuns, sinó més aviat d'una evolució convergent d'una via biosintètica exitosa.

MECANISMES DE DEFENSA CONTRA LA DEPREDACIÓ

Els mecanismes de defensa anti-depredació a l'abast dels invertebrats marins poden ser: estructurals, químics o de comportament. Tot i així, i sobretot a causa de limitacions experimentals, pocs estudis han intentat determinar la importància relativa dels diferents tipus de mecanismes defensius utilitzats pels organismes marins. En ascidis s'observen generalment molt poques marques de depredació. A més a més, semblen posseir mecanismes defensius tan físics (espícules, consistència de la túnica) com químics. Així doncs, aquests organismes

podrien utilitzar metabolits secundaris bioactius, espícules, altes concentracions de vanadi i/o un baix pH per a la seva defensa contra la depredació.

L'objectiu d'aquest capítol és determinar si els compostos secundaris bioactius, les espícules de carbonat càlcic i l'acidesa de la túnica (pH < 1) de *Cystodytes* actuen com a mecanismes d'anti-depredació i, si és el cas, si actuen de manera independent o combinant-se els uns amb els altres per augmentar-ne l'eficiència. Per a fer això, es van considerar 3 morfotips diferents de *Cystodytes*: un morat i un blau provenint de la Mediterrània Occidental i un morat de Guam (USA, Pacífic). De cadascun d'ells, es va obtenir l'extret brut i es van aïllar les espícules típiques de cada forma. També es va considerar l'ascididemin purificada (l'alcaloide majoritari de la forma blava), com a exemple de metabolit secundari bioactiu. Es van testar diverses combinacions d'aquesta substància amb àcid i/o espícules per saber-ne el funcionament i la importància a la natura. L'acidesa de la túnica es va imitar afegint àcid sulfúric als diferents tractaments i considerant els canvis de pH en funció del temps. També es van realitzar anàlisis de toxicitat dels extrets bruts i de l'ascididemin amb Microtox. Els tests d'anti-depredació es van realitzar a Guam amb els peixos *Abudefduf vaigiensis* i *A. sexfasciatus in situ* i amb el peix bentònic *Canthigaster solandri* i l'eriçó de mar *Diadema savigny* en aquari.

Tant els extrets bruts com l'ascididemin van resultar ser tòxics i van reduir significativament la depredació per part dels peixos *in situ* i en aquari. Per contra, cap dels abans esmentats va afectar la depredació per part d'eriçons. L'àcid sol o combinat amb els diferents tipus d'espícules no va impedir la depredació en cap cas. Així doncs, els nostres resultats indiquen que els diferents mecanismes potencials contra la depredació a l'abast de *Cystodytes* han estat evolutivament seleccionats per actuar, independentment o en combinació, contra un rang més ampli de depredadors. Fem èmfasi doncs en la importància de considerar tots els mecanismes defensius a la disposició d'un organisme a l'hora d'estudiar l'evolució i l'ecologia química dels invertebrats marins.

COM ES RELACIONEN ELS MORFOTIPS I ELS QUEMOTIPS AMB ELS GENOTIPS?

La variabilitat intra-específica és un fenomen comú entre els invertebrats marins. La talla, el color, la textura, la forma general, la química i altres

característiques poden variar bastant dràsticament d'un organisme a un altre. Aquesta variabilitat també representa un problema a l'hora de determinar l'estatus taxonòmic d'una espècie. Les espècies àmpliament distribuïdes solen presentar variacions morfològiques generalment atribuïdes a la seva distribució geogràfica o batimètrica. D'altra banda, la utilització de noves eines moleculars permet establir l'estatus taxonòmic d'espècies presentant certa variabilitat morfològica. S'ha demostrat que, tot i que en alguns casos existeix una veritable variació intra-específica, la majoria d'estudis han portat al descobriment d'espècies germanes o críptiques. La determinació d'espècies no es només important a nivell taxonòmic sinó que també comporta implicacions clares en el camp de la biologia aplicada, com ara en la gestió de la biodiversitat, el seguiment d'espècies potencialment invasores o per determinar els productors de noves substàncies bioactives d'interès farmacològic. *Cystodytes dellechiajei*, un ascidi colonial i suposadament cosmopolita, presenta una gran varietat de morfotips que generalment varien en el color, en la composició espicular i en la química secundària (veure capítol 2). Es per tot això que existeix una certa controvèrsia sobre si aquesta espècie és l'única present a la Mediterrània.

L'objectiu d'aquest treball és estudiar des del punt de vista genètic i morfològic una mostra representativa d'exemplars d'aquest gènere recollits a diverses localitats de la Mediterrània Occidental i que mostren un ventall de varietats cromàtiques. Volem determinar si la variabilitat en color i en composició espicular que s'han utilitzat tradicionalment per a la determinació taxonòmica d'espècies del gènere *Cystodytes* coincideixen amb la informació química i genètica.

Hem seqüenciat un fragment de 617 pb del gen mitocondrial Citocrom Oxidasa, subunitat I (COI) de 46 exemplars que provenien de les diferents localitats estudiades. Amb les seqüències obtingudes hem realitzat cladogrames utilitzant diversos criteris (distància, màxima parsimònia, màxima versemblança). D'aquestes mateixes mostres s'han estudiat caràcters morfològics com ara el color i la forma dels zooides i s'ha realitzat un estudi de les espícules amb microscòpia electrònica de rastreig. Aquesta informació s'ha combinat amb la informació de caire químic recollida al capítol anterior.

En total, es van trobar 15 colors diferents i 4 tipus d'espícules: discoidals, esfèriques, en forma d'estrella i discoidals amb el marge dentat. Els diversos arbres evolutius són força coincidents, i ens permeten diferenciar 6 clades que no es

corresponen amb els tipus espiculars i només parcialment amb els colors. Nogensmenys, alguns dels clades trobats es corresponen amb els quemotips descrits al capítol 2.

Tot i que els diferents colors i continguts espiculars semblaven indicar la presència de diverses espècies, concloem, en base als estudis genètics i químics, que les diferències morfològiques observades no son prou consistents per a subdividir el gènere *Cystodytes* de la Mediterrània en diverses espècies i que calen estudis de genètica de poblacions i de cicles biològics per confirmar la subdivisió en espècies de les varietats estudiades. Finalment, destaquem la utilitat de la genètica i de la química a l'hora d'establir l'estatus taxonòmic d'espècies morfològicament variables.

GENÈTICA DE POBLACIONS, FILOGEOGRAFIA I ESPECIACIÓ

Tot i que els estudis sobre les subdivisions a nivell poblacional i els fenòmens d'especiació són crucials per al nostre coneixement i gestió de la biodiversitat, el mateix concepte d'espècie és objecte de discussió. En el capítol anterior hem conclòs que els caràcters morfològics estudiats no eren prou consistents com per a diferenciar entre espècies del gènere *Cystodytes*. De fet, fins ara tots els morfotips observats a la Mediterrània Occidental s'han atribuït a l'espècie cosmopolita *Cystodytes dellechiajei*. Templeton (2001) va demostrar que estudis rigorosos filogeogràfics de dades genètiques podien aportar informació clau sobre l'interfase evolutiva inter- i intra- específica dins del concepte de la cohesió d'espècies. Tot i que les espècies àmpliament distribuïdes solen presentar una certa variabilitat genètica, les poblacions pertanyent a una mateixa espècie han de mantenir una certa cohesió a través de tota la seva zona de distribució.

En el nostre cas es van considerar 7 poblacions representatives del principals morfotips i de l'àrea geogràfica de distribució del gènere *Cystodytes* a la Mediterrània Occidental per a intentar determinar si existeixen subdivisions de les poblacions i si aquestes es poden relacionar des d'un punt de vista filogeogràfic (usant l'anàlisi de clades aniuats, NCA). Es van utilitzar 67 seqüències parcials del gen mitocondrial COI. La variabilitat genètica es va separar en components relacionats amb l'origen geogràfic i el color dels morfotips i a partir d'aquí es van

realitzar diverses anàlisis d'estructura genètica: F_{ST}, anàlisis de la variança molecular (AMOVA) i escalatge multidimensional, (MDS).

Totes les anàlisis emparades per a determinar l'estructuració genètica de les poblacions indiquen clarament que les diferències trobades entre els principals morfotips (pel que fa el color) són prou importants com per a emmascarar qualsevol diferenciació geogràfica. D'altra banda, la divergència genètica entre colors és significant quan es comparen les formes marró i morada amb les altres, però no entre les formes verda, blanca i blava. Tot i això, quan s'elimina la variabilitat deguda a la divergència entre colors, trobem una variabilitat significant entre localitats geogràfiques. Els estudis filogeogràfics (NCA) suggereixen que la distribució actual dels haplotips estudiats ve donada per fenòmens de fragmentació i d'expansió d'àrea. Tot plegat, i considerant també les diferències químiques trobades anteriorment, els nostres resultats indiquen que existeixen diverses espècies de *Cystodytes* en la Mediterrània Occidental i que és necessària una revisió taxonòmica, i que no es podrà basar únicament en caràcters morfològics (veure capítol 5), de les espècies pertanyents a aquest gènere.

CICLES BIOLÒGICS I TAXES DE CREIXEMENTS DE DOS MORFOTIPS DE *CYSTODYTES*

En els invertebrats marins modulars, la interacció entre genotip i medi sovint resulta en taxes de creixement, fissió, fusió, regeneració i senescència diferents per cada un dels clons. Els pocs estudis existents sobre els cicles biològics d'ascidis colonials incloent el creixement, la reproducció i la mortalitat mostren sovint cicles força complexes. D'altra banda, els mars temperats presenten una marcada fluctuació estacional dels paràmetres ambientals que es reflecteixen en els cicles de vida dels ascidis. Les variacions estacionals es relacionen sovint amb canvis en la temperatura, però altres factors, com per exemple la disponibilitat de nutrients, també s'haurien de considerar. Tot i que els estudis genètics i químics ens aporten informació important sobre els possibles processos d'especiació d'una població o conjunt de poblacions, l'estudi dels cicles biològics segueix essent necessari per a determinar si existeix un aïllament reproductiu d'acord amb els conceptes biològic i de cohesió d'espècies. Aquesta mena d'estudis ens aporta informació molt valuosa sobre la biologia i l'ecologia dels invertebrats bentònics.

L'objectiu principal d'aquest estudi és comparar els cicles reproductius, de creixement i de supervivència de les dues formes de *Cystodytes* més abundants en la Mediterrània Occidental (morada i blava) durant més de 20 mesos. Cites anteriors d'aquestes formes les van identificar com pertanyent a l'espècie *Cystodytes dellechiajei*. També s'han correlacionat aquests paràmetres biològics amb d'altres paràmetres com la talla de les colònies, la temperatura de l'aigua, i la presència o absència de competidors per l'espai. Finalment, s'han relacionat les divergències genètiques i químiques trobades durant els capítols anteriors amb les diferències biològiques trobades en aquest capítol per tal de determinar el grau d'aïllament d'aquests morfotips i el seu estatus taxonòmic.

Es va trobar un patró estacional dels cicles de vida d'aquests dos morfotips però presentant algunes diferències significatives. Les variacions estacionals tant en reproducció com en creixement de la forma morada tenien lloc aproximadament dos mesos més tard que per la forma blava. Per cada morfotip també existia un decalatge entre la reproducció i el creixement, indicant que existeix una repartició dels recursos o bé per a la reproducció o per al creixement. Més explícitament, el creixement d'ambdós morfotips és màxim una vegada passat l'alliberament de les larves. No es van trobar diferències significatives entre les taxes de mortalitat de les dues formes. Tots dos morfotips presenten una correlació significativa i negativa entre mortalitat i talla de la colònia. En el cas del morfotip blau, el creixement màxim estava negativament correlacionat amb la talla, mentre que per al morat no es va trobar cap relació significativa. Tampoc es van trobar diferències significatives entre el creixement de les colònies en contacte amb l'espècie tòxica *Crambe crambe* (Porifera) i les que no. Les diferències trobades en els cicles reproductius d'aquestes dues formes de *Cystodytes* coincideixen amb les divergències químiques i genètiques trobades anteriorment. Concloem, doncs, que aquests dos morfotips són, de fet, espècies diferents.

AJUSTANT EL PRESSUPOST ENERGÈTIC: VARIACIÓ TEMPORAL EN LA PRODUCCIÓ D'ASCIDIDEMIN EN RELACIÓ AMB ALTRES PARÀMETRES BIOLÒGICS

Es coneix molt poc sobre la relació que s'estableix entre la producció de defenses químiques com els metabolits secundaris, de defenses físiques com les

espícules i els altres paràmetres biològics d'una espècie, com el cicle reproductiu o el creixement. La teoria de la defensa òptima assumeix que la repartició dels recursos energètics per a la producció de defenses tant físiques com químiques, està optimitzada pel que fa a les necessitats d'un organisme (creixement, manteniment somàtic i reparació, reproducció) i el seu pressupost energètic. Si això es així, esperem una variació temporal en la producció de dites defenses, depenent de les limitacions ambientals i de l'estat fisiològic de l'organisme productor. Tot i la rellevància d'aquest tipus d'estudis, pràcticament no existeix cap treball aprofundit relacionant la variabilitat temporal en la producció de defenses i els altres paràmetres biològics d'una espècie.

L'objectiu d'aquest capítol és determinar si existeix aquesta variabilitat temporal en la producció de defenses i com es relaciona amb la variabilitat en altres paràmetres biològics. Per tal d'estudiar la variació en defenses químiques, es va quantificar l'ascididemin, que és la piridoacridina més abundant del morfotip blau de *Cystodytes*. Les defenses físiques es van estimar a partir de la variació en el contingut en cendres, ja que aquestes contenen principalment espícules i material estructural inorgànic. Es va mostrejar la mateixa població blava que al capítol anterior durant un any, per tal de poder relacionar directament els resultats obtinguts amb els cicles de reproducció i creixement estudiats en el capítol 7.

Per a quantificar correctament l'ascididemin, va ser necessari posar a punt un protocol de conservació de les mostres i optimizar les condicions d'anàlisi per cromatografia líquida d'alta pressió ("HPLC"). Aquesta consideració prèvia ens va permetre descobrir que les mostres liofilitzades conservades a temperatura ambient 10 mesos perdien fins a un 30% d'ascididemin. També ens va permetre comprovar que el fet de conservar mostres al congelador durant 5 mesos, no suposava una pèrdua significant de l'ascididemin que obteníem. Un cop ens vam assegurar que el procés de liofilització *per se* no influenciava la quantitat final d'ascididemin, les mostres utilitzades per a la realització d'aquest treball es van conservar liofilitzades al congelador.

L'estudi de la concentració d'ascididemin al llarg del temps va mostrar un patró estacional marcat, amb un mínim a primavera (just abans del començant del període reproductiu) i un màxim després de l'alliberament de les larves a finals de l'estiu i començament de la tardor. Es va observar el mateix patró per al contingut en cendres de les colònies, suggerint que també existeix una periodicitat anual en la producció del contingut inorgànic, principalment espícules.

Els resultats que hem obtingut indiquen doncs que existeix un cicle estacional en la producció de defenses i que el període reproductor és el que segurament condiciona la repartició de recursos disponibles per als altres paràmetres biològics (producció de metabolits secundaris, creixement, increment de material inorgànic). Aquests resultats són coherents amb la hipòtesi que la producció de metabolits secundaris té un cost metabòlic (Fagerström et al. 1987) i que aquest està optimitzat pel que fa les altres necessitats d'un organisme, majoritàriament reproductives, per tal d'ajustar eficaçment el pressupost energètic disponible d'una espècie.

En conclusió, l'aproximació multidisciplinar utilitzada per a estudiar el gènere d'ascidi colonial *Cystodytes* ens ha permès de conèixer millor la biologia, ecologia i química secundària d'aquest organisme. D'altra banda, ens ha permès de ressaltar la importància d'aquest tipus d'estudi a l'hora d'adquirir una visió més global, i segurament més propera a la realitat, de l'evolució dels invertebrats marins en el seu ambient. Tot i això, moltes qüestions romanen encara sense resposta, com ara

la localització inter-cel·lular de l'ascididemin, la biosíntesi de les piridoacridines i el nombre total d'espècies de *Cystodytes* a la Mediterrània. El que està clar és que com més en sabem sobre un organisme més preguntes noves ens fem, preguntes que necessitaran la utilització de noves tècniques i de més investigació si en volem trobar les respostes...

RÉSUMÉ - FRANÇAIS

ÉTUDE PLURIDISCIPLINAIRE DU GENRE *CYSTODYTES* (ASCIDIACEA) : DES MOLÉCULES AUX ESPÈCES

1. INTRODUCTION GÉNÉRALE

GÉNÉRALITÉS ET ESPECE ÉTUDIÉE

Les invertébrés marins sont à l'origine d'une grande variété de composés d'intérêt pharmaceutique et biotechnologique (Faulkner 2000). En milieu naturel, ces métabolites assurent diverses fonctions écologiques (Becerro et al. 1997) et sont des facteurs décisifs dans l'établissement et l'évolution des différentes stratégies de vie des organismes producteurs. Mais, malgré l'importance de ces composés, la plupart des espèces d'invertébrés marins producteurs ne sont identifiés qu'au niveau du genre (voir Marinlit, Annexe I), restent sans description (p. ex. Caroll et Scheuer 1990) ou sont incorrectement identifiés. Ceci a pour effet une confusion totale lorsqu'il faut retrouver l'organisme producteur. Le manque d'information sur l'identité des espèces et leur interrelation rend difficile la compréhension de la fonction écologique des composés bioactifs produits. Dans ce contexte, les applications biotechnologiques et/ou médicales semblent compromises.

L'identification des espèces se complique encore plus s'il existe une variabilité au niveau intra-spécifique (différences chimiques inclues). De plus, les observations basées uniquement sur la morphologie de l'espèce sont souvent insuffisantes pour déterminer si nous nous trouvons devant un cas de variabilité

intra-spécifique ou devant un groupe d'espèces cryptiques. C'est pour cela qu'il est souvent nécessaire d'utiliser des techniques alternatives appartenant à d'autres disciplines comme la biologie moléculaire. L'étude de la phylogénie moléculaire, la génétique des populations et la phylogéographie contribuent, de façon significative, à comprendre les différents procédés de spéciation qui ont pu avoir lieu, apportant ainsi une importante information écologique et évolutive sur les espèces étudiées. De plus, l'étude des cycles reproducteurs permet de déterminer le degré de différenciation entre les espèces en accord avec le concept biologique d'espèce et augmente l'information disponible sur l'écologie et la biologie des invertébrés benthiques.

Par ailleurs, les cycles de vie sont en étroite relation avec la disponibilité des ressources énergétiques qui peuvent être destinés à la production des métabolites secondaires. Si nous admettons le fait que la production de composés bioactifs a un coût (en terme d'énergie), ce coût devra être optimisé de façon à couvrir tous les besoins de l'organisme et ses paramètres biologiques (croissance, maintien somatique et réparation, reproduction). Cette optimisation peut être mise en évidence en étudiant les principaux paramètres biologiques d'une espèce et en analysant la variation quantitative de l'énergie investie en défense de l'organisme en fonction de ces paramètres. Il faut aussi remarquer que la localisation dans l'organisme de ces composés bioactifs et l'identification des cellules responsables de leur production, apportent une information très importante sur leur signification biologique. C'est pour cela qu'une approche pluridisciplinaire, englobant et mettant en relation l'information de différents champs, des molécules aux populations, est nécessaire pour mieux comprendre les stratégies biologiques adoptées par les invertébrés marins. Malgré son importance, ce type d'information n'est disponible que pour une seule espèce : l'éponge *Crambe crambe* (Becerro 1994).

Dans ce travail, l'ascidie coloniale *Cystodytes dellechiajei* (Della Valle 1877) sera étudiée en tant qu'organisme modèle. Il s'agit d'une ascidie très polymorphe et qui probablement renferme un groupe d'espèces. La variabilité de la production de métabolites secondaires ainsi que sa signification seront étudiées en corrélation avec d'autres aspects de la biologie et de l'écologie de cette espèce. Nous avons ainsi utilisé un ensemble de techniques appartenant à plusieurs disciplines comme la biologie moléculaire, la phylogéographie, la chromatographie, les techniques ultra-structurales et plusieurs études menés *in situ*.

Cette espèce présente un ensemble de caractéristiques particulières : elle dispose de systèmes de défense physique et chimique en place qui semblent redondants, une variabilité qualitative dans la production de métabolites secondaires, il existe des différences morphologiques dans la zone d'étude (Méditerranée occidentale) et sa biologie est peu connue. De plus, sa dispersion larvaire est supposée minime, ce qui favorise l'isolement génétique.

OBJECTIFS ET STRUCTURE DE LA THÈSE

De nombreux travaux font part exclusivement d'un aspect de la biologie ou de l'écologie des organismes marins. Bien que ce type d'étude contribue à augmenter les connaissances générales que nous possédons sur une espèce ou un phénomène, les interrogations soulevées sont nombreuses. Pour répondre à celles-ci il est nécessaire d'utiliser des techniques diverses propres à différentes disciplines. L'objectif général de ce travail est, par une approche pluridisciplinaire, de mieux comprendre la biologie et de l'écologie des ascidies coloniales du genre *Cystodytes*.

Les objectifs spécifiques de cette thèse sont de:

1. Identifier la variabilité chimique en alcaloïdes des divers morphotypes (surtout par rapport aux variétés chromatiques) de *Cystodytes* de Méditerranée Occidentale.

2. Comparer la composition cellulaire de la tunique des principaux morphotypes, localiser les métabolites secondaires dans les cellules, montrer l'existence ou non de microsymbiontes et si ces derniers jouent un rôle dans la production des substances de défense.

3. Déterminer expérimentalement la fonction, en milieu naturel, des défenses tant chimiques que physiques de quelques espèces représentatives de ce genre.

4. Étudier la variabilité génétique associée aux différences morphologiques observées chez *Cystodytes* et établir la relation avec la variabilité chimique.

5. Analyser la variabilité génétique ainsi que le flux génétique entre différentes populations de Méditerranée Occidentale.

6. Comparer les cycles de vie et les stratégies reproductives des deux morphotypes les plus abondants dans la zone d'étude.

7. Essayer de confirmer l'hypothèse d'une optimisation du bilan énergétique par l'analyse des ressources dédiées à la production des métabolites secondaires et celles consacrées à d'autres fonctions biologiques comme la croissance et la reproduction.

Ainsi donc, nous désirons combiner les informations issues de la chimie, la génétique, la structure cellulaire, et le cycle de vie de *Cystodytes* afin de mieux comprendre les mécanismes de défense, les stratégies biologiques et écologiques et leurs interactions.

La thèse est structurée en 7 chapitres principaux qui, malgré leur interaction, ont été écrits comme des unités indépendantes afin qu'elles puissent être lues séparément et faciliter leur publication. Ainsi, chaque chapitre contient une introduction, une partie « matériel et méthodes », une partie « résultats » et une discussion. Ces différentes parties peuvent contenir des références croisées avec d'autres chapitres. Le dernier chapitre de la thèse se compose d'une discussion générale sur les résultats présentés dans les chapitres antérieurs et situe les différentes parties dans un cadre global. A la fin du travail, une bibliographie générale regroupe toutes les références citées afin d'éviter des redondances entre les différents chapitres.

RÉSULTATS ET CONCLUSIONS

VARIABILITÉ CHIMIQUE DES ALCALOÏDES DES PRINCIPAUX MORPHOTYPES DE *CYSTODYTES*

La plupart des études sur les métabolites secondaires n'apportent pas d'information sur l'organisme producteur. En conséquence, actuellement, il est pratiquement impossible d'établir une relation entre la chimie et de possibles variations morphologiques au niveau d'espèces ou de genres polymorphiques. Le problème s'aggrave encore quand on démontre que ces composés possèdent un potentiel biotechnologique et des possibles applications pharmacologiques. Aujourd'hui des nouvelles techniques pour la détection de substances bioactives dans des échantillons biologiques se développent. Parmi elles, la spectrométrie de masses avec différents modes d'ionisation possibles comme l'ESI-MS (electro spray ionisation mass spectrometry) ou le MALDI-TOF MS (matrix assisted laser desorption ionisation time-of-flight mass spectrometry) sont de plus en plus utilisées. Avec seulement quelques milligrammes d'échantillon, ces méthodes permettent une détection rapide et efficace des métabolites connus présents en très faible quantité.

L'objectif de ce chapitre est, premièrement, de déterminer la composition en alcaloïdes des morphotypes les plus abondants de *Cystodytes* en Méditerranée (vert, violet, marron et bleu). Il faut signaler que, jusqu'ici, toutes ces formes ont été identifiées comme étant des variétés chromatiques de l'espèce *C. dellechiajei.* De plus, la localisation, au sein d'une colonie (dans la tunique et/ou dans les zoïdes), de ces alcaloïdes sera déterminée. La détection des métabolites secondaires et la mise en évidence des différents chemotypes sont dans un premier temps directement effectués sur les échantillons biologiques par MALDI-TOF MS. Ces résultats obtenus sur quelques colonies ont été confirmés et complétés par analyse des extraits en chromatographie liquide à haute pression (CLHP). Les composés majoritaires ont été purifiés en CLHP semi-préparative et caractérisés par spectrométrie de masse et résonance magnétique nucléaire (RMN).

Les métabolites secondaires présents dans les différents morphotypes de *Cystodytes* sont des alcaloïdes polyaromatiques appartenant à la famille des pyridoacridines pentacycliques. Les analyses chimiques ont permis de différencier

deux chemotypes. Le premier est constitué de pyridoacridines incluant un atome de soufre et une chaîne éthylamine comme la shermilamine B et la kuanoniamine D et leur forme déacétylée, la déacétylshermilamine B (nouveau métabolite identifié) et la déacétylkuanoniamine D. Ces alcaloïdes sont présents dans les colonies de couleur violette. Le second est constitué de pyridoacridines sans atome de soufre ni chaîne éthylamine comme l'ascididemine et la 11-hydroxyascididemine, qui se trouvent dans les morphotypes bleu et vert. Dans le morphotype marron a seulement été détectée l'ascididemine en petites quantités et ce grâce à la sensibilité du MALDI-TOF MS. Tous les alcaloïdes sont autant présents dans la tunique que dans les zoïdes, à l'exception des métabolites caractéristiques de la forme violette. La shermilamine B et la kuanoniamine D sont seulement localisées dans la tunique alors que les formes déacétylées l'étaient dans les deux. Une relation évidente a pu être établie entre ces pigments (pyridoacridines) et la couleur violette des colonies. Pour les autres morphotypes (bleu, vert et marron) la couleur doit dépendre d'autres molécules non identifiées. La technique MALDI-TOF est rapide et efficace pour la détection des composés ciblés de faible poids moléculaire au niveau inter et intra-spécimen. Cette technique est donc très appropriée pour la mise en évidence rapide de chemotypes. Les différences chimiques trouvées entre les divers morphotypes qui, jusqu'ici, avaient été décrits comme *Cystodytes dellechiajei* soulèvent de nouvelles interrogations sur le statut taxonomique de cette espèce de Méditerranée Occidentale. D'autre part, les résultats obtenus mettent en évidence l'importance d'une description détaillée de la morphologie et du lieu de récolte lorsque l'on travaille avec des produits naturels. Cette considération est particulièrement importante pour les groupes qui n'ont pas une taxonomie bien résolue et afin de comprendre la variation de la composition chimique en métabolites secondaires dans et entre les espèces.

TYPES CELLULAIRES, MICROSYMBIONTES ET DISTRIBUTION DES ALCALOIDES DANS LA TUNIQUE DE *CYSTODYTES*

Il existe très peu d'études sur la diversité des métabolites secondaires chez les invertébrés marins. Les seules études disponibles concernent principalement les variations écologiques et saisonnières. C'est la raison pour laquelle on ne connaît presque rien sur la localisation intra-spécimen et inter-cellulaire des substances

produites. Cependant, ce genre d'études apporte des indications très importantes sur le rôle écologique et biologique de ces composés dans la nature. L'étude de la morphologie et la classification des cellules de la tunique fait aussi partie de la recherche fondamentale sur les ascidies.

Les objectifs de ce chapitre sont, d'une part, de caractériser les principaux types cellulaires de la tunique des trois morphotypes les plus abondants de *Cystodytes* (vert, bleu et violet), et de déterminer l'importance de la population bactérienne. Puis, d'autre part, localiser les métabolites secondaires bioactifs trouvés (chapitre 2) dans la tunique. Pour répondre à ces questions, nous avons observé les échantillons en microscopie électronique à transmission et réalisé des microanalyses avec des rayons X.

Les principaux types cellulaires trouvés sont : des cellules vésiculaires, des cellules pigmentaires, des amébocytes, des phagocytes et des cellules en forme de morula. Les amébocytes contiennent plusieurs sous-types cellulaires qui correspondent, sûrement, à une succession de phases de développement et qui donnent lieux aussi probablement à quelques-uns des autres types cellulaires. La morphologie de la tunique des trois morphotypes étudiés est très similaire. On a trouvé trois classes de bactéries, mais toujours en nombre très faible, et il est donc peu probable qu'elles soient responsables de la production des métabolites secondaires bioactifs. En fait, les microanalyses sous rayons X du morphotype violet ont mis en évidence une concentration élevée de soufre dans les cellules pigmentaires. Il semblerait donc que les pyridoacridines « soufrées » se concentrent à cet endroit. Cependant, on n'exclue pas que d'autres types cellulaires puissent aussi être impliqués dans leur biosynthèse. Bien que les pyridoacridines soient présentes dans plusieurs organismes appartenant à des phylums différents, leur production ne semble pas liée à la présence des mêmes bactéries symbiotiques mais à une évolution convergente vers une voie biosynthétique efficace.

MÉCANISMES DE DÉFENSE CONTRE LA PRÉDATION

Les mécanismes de défense contre la prédation à la disposition des invertébrés marins peuvent être : structuraux, chimiques ou comportementaux. En raison des limitations expérimentales, très peu d'études ont essayé de déterminer l'importance relative de chacun de ces mécanismes défensifs utilisés par les

organismes marins. Très peu de marques de prédation ont été observées chez les ascidies. Ces animaux possèdent des défenses aussi bien physiques (spicules, consistance plus ou moins rigide de la tunique) que chimiques. L'utilisation de métabolites secondaires, la concentration et la forme des spicules, les concentrations élevées de Vanadium et un pH souvent inférieur à 2 ont été proposés comme défenses potentiellement utilisables par les ascidies pour diminuer l'impact de la prédation.

L'objectif de ce chapitre est de déterminer si les métabolites secondaires bioactifs, les spicules calcaires et l'acidité de la tunique (pH < 1) de *Cystodytes* agissent comme mécanismes défensifs contre la prédation et, si c'est le cas, s'ils agissent de manière indépendante ou en se combinant les uns avec les autres pour en augmenter l'effet. Pour faire ceci, nous avons étudié 3 morphotypes de *Cystodytes* : un violet et un bleu de la Méditerranée Occidentale et un violet de Guam (USA ; Pacifique Occidental) ; les extraits bruts ont été préparés et les spicules typiques de chacun isolés. L'ascididemine, alcaloïde majoritaire de la forme bleue et disponible en quantité au laboratoire, a été utilisée comme exemple de métabolite secondaire pouvant agir de façon indépendante ou en combinaison avec d'autres mécanismes. Le rôle éventuel de l'acidité de la tunique a été étudié en ajoutant de l'acide sulfurique aux différents traitements et en considérant les changements de pH en fonction du temps. La toxicité des extraits bruts et de l'ascididemine a été évaluée avec la méthode Microtox. Les tests d'anti-prédation ont été effectués à Guam (USA) avec des poissons appartenant aux espèces *Abudefduf vaigiensis* et *A. sexfasciatus in situ* et avec *Canthigaster solandri* et l'oursin *Diadema savigny* en aquarium.

Tous les extraits bruts et l'ascididemine sont toxiques et réduisent significativement la prédation par les poissons mais pas par les oursins. Par contre, l'acidité toute seule ou combinée avec les spicules ou l'ascididemine n'a, dans aucun des cas testés, empêché la prédation. Si on ajoute ces résultats à ceux existant dans la littérature, il semblerait que les différents mécanismes défensifs pouvant être utilisés par *Cystodytes*, ont été évolutivement sélectionnés pour agir seuls ou en se combinant avec d'autres mécanismes pour diminuer l'impact d'un nombre plus élevé de prédateurs potentiels. Il est donc important, dans ce type d'étude sur l'évolution de l'écologie chimique des invertébrés marins, de prendre en compte tous les mécanismes de défense théoriquement utilisables par un organisme.

COMMENT SE FAIT LA RELATION ENTRE MORPHOTYPES, CHEMOTYPES ET GENOTYPES ?

La variabilité intra-spécifique est un phénomène commun aux invertébrés marins. La taille, la couleur, la texture, la forme générale, la chimie et autres caractéristiques peuvent varier de façon assez radicale d'un organisme à un autre. Cette variabilité représente aussi un problème lorsqu'il faut déterminer le statut taxonomique d'une espèce. Les espèces largement répandues présentent, normalement, des variations morphologiques généralement attribuées à leur répartition géographique ou/et bathymétrique. D'un autre côté l'utilisation de nouveaux outils moléculaires permet d'établir le statut taxonomique d'espèces présentant une certaine variabilité morphologique. Bien que dans certain cas il existe une véritable variation intra-spécifique, la majorité des études ont mené à la description de nouvelles espèces cryptiques ou voisines. La détermination d'espèces n'est pas importante uniquement pour les études taxonomiques. Elle comporte aussi des implications évidentes dans le domaine de la biologie appliquée, la gestion de la biodiversité, le suivi d'espèces potentiellement envahissantes ou la détermination des organismes producteurs de substances à haute valeur ajoutée. *Cystodytes dellechiajei* est une ascidie coloniale, supposée cosmopolite et qui présente plusieurs morphotypes. Les morphotypes différent entre eux par la couleur, la composition spiculaire et la composition en métabolites secondaires (voir chapitre 2). C'est pour cela qu'il existe une certaine controverse pour savoir si *C. dellechiajei* représente une seule ou plusieurs espèces en Méditerranée.

L'objectif de ce travail est d'étudier, du point de vue génétique et morphologique, un échantillon représentatif d'exemplaires de ce genre récoltés dans diverses localités de la Méditerranée Occidentale et qui montrent un ensemble de variétés chromatiques. Nous voulons déterminer si les différences de couleurs et de composition spiculaire qui ont été traditionnellement utilisées pour identifier les espèces du genre *Cystodytes* coïncident avec l'information chimique et génétique.

Nous avons séquencé un fragment de 617pb du gêne mitochondrial Cytochrome Oxidase *c*, sous unité I (COI) de 46 exemplaires provenant de différentes localités étudiées. Avec les séquences obtenues nous avons réalisé des cladogrammes en appliquant divers critères (distance, maximum de parcimonie, maximum de vraisemblance). Parallèlement plusieurs caractères morphologiques comme la couleur, la forme des zooïdes et la composition spiculaire (en microscopie

électronique à balayage) ont été étudiés sur ces mêmes échantillons. Ces résultats ont été comparés à ceux obtenus en chimie (cf. chapitres précédents).

Au total nous avons recensé 15 couleurs différentes et 4 types de spicules : discoïdales, sphériques, en forme d'étoile et discoïdales avec le bord dentelé. Les divers arbres évolutifs sont assez ressemblants et nous permettent de différencier 6 clades qui ne correspondent pas avec les types spiculaires et seulement partiellement avec les couleurs. Cependant, quelques-uns des clades trouvés correspondent aux chemotypes décrits dans le chapitre 2.

Bien que les différentes couleurs et contenus spiculaires semblent indiquer la présence de plusieurs espèces, nous concluons, en nous basant sur les études génétiques et chimiques, que les différences morphologiques observées ne sont pas assez consistantes pour subdiviser le genre *Cystodytes* de la Méditerranée en plusieurs espèces. Il est donc nécessaire de réaliser des études complémentaires, notamment en génétique des populations et sur les cycles biologiques afin de confirmer si nous nous trouvons en présence d'une seule espèce. Finalement, nous soulignons l'importance de la génétique et de la chimie lorsqu'il s'agit d'établir le statut taxonomique d'espèces morphologiquement variables.

GENETIQUE DE POPULATIONS, PHYLOGÉOGRAPHIE ET SPECIATION

Bien que les études sur les subdivisions au niveau des populations et des phénomènes de spéciation soient cruciales pour notre connaissance et pour la gestion de la biodiversité, le concept même d'espèce est, actuellement, remis en cause. Dans le chapitre précédent nous avons conclu que les caractères morphologiques étudiés n'étaient pas suffisants pour subdiviser le genre *Cystodytes* en plusieurs espèces en Méditerranée. En réalité, jusqu'ici, tous les morphotypes observés en Méditerranée Occidentale ont été attribués à l'espèce cosmopolite *Cystodytes dellechiajei*, la différence la plus fréquemment observée entre ces morphotypes étant la couleur. Templeton (2001) a démontré que des études phylogéographiques rigoureuses sur des donnés génétiques pouvaient apporter une information clé sur l'interphase évolutive inter/intra espèce dans le concept de la cohésion d'espèces. Bien que les espèces largement réparties présentent habituellement une certaine variabilité génétique, les différentes populations

appartenant à une même espèce doivent maintenir une certaine cohésion génétique dans toute leur zone de distribution.

Nous avons considéré sept populations représentatives des principaux morphotypes et de la surface géographique de distribution du genre *Cystodytes* de Méditerranée Occidentale afin de déterminer s'il existait des subdivisions des populations et, dans le cas où elles existent, si elles peuvent être mises en relation au point de vue phylogéographique (en utilisant l'analyse des clades joints, NCA). Nous avons utilisé 67 séquences partielles du gêne mitochondrial COI. La variabilité génétique rencontrée a été séparée en plusieurs composants en fonction de l'origine géographique et de la couleur des morphotypes. À partir de cette répartition, nous avons réalisé diverses analyses de structure génétique : F_{ST}, analyse de la variation moléculaire (AMOVA) et multidimensionnelle (MDS). De cette façon, nous avons éclairci la relation entre les différents morphotypes observée en Méditerranée et la variabilité génétique trouvée.

Toutes les analyses utilisées pour déterminer la structure génétique des populations indiquent clairement que les différences trouvées entre les morphotypes, en ce qui concerne la couleur, sont suffisamment importantes pour masquer n'importe quelle différence géographique lorsqu'on trouve des combinaisons de couleurs dans une même localité. La divergence génétique entre couleurs est significative lorsqu'on compare les formes marron et violette avec les autres, mais non entre les formes vertes, bleues et blanches. De plus, quand on élimine la variabilité due à la divergence entre couleurs, on trouve une variabilité significative entre localités géographiques. Les études phylogéographiques (NCA) suggèrent que la distribution actuelle des haplotypes étudiés est due à des phénomènes de fragmentation et d'agrandissement d'aire de distribution. En conclusion, et en tenant compte aussi des différences chimiques trouvées antérieurement, nos résultats indiquent qu'il existe diverses espèces de *Cystodytes* en Méditerranée Occidentale. Une révision taxonomique des espèces appartenant à ce genre est donc nécessaire, mais difficile, car elle ne pourra pas se baser uniquement sur les différences morphologiques (voir chapitre 5).

CYCLES BIOLOGIQUES ET TAUX DE CROISSANCE DE DEUX MORPHOTYPES DE *CYSTODYTES*

Chez les invertébrés marins coloniaux l'interaction entre génotype et environnement résulte souvent en des taux de croissance, fission, fusion, régénération et sénescence différents pour chacun des clones. Le peu d'études existantes sur les cycles biologiques d'ascidies coloniales incluant la croissance, la reproduction et la mortalité font souvent part de cycles très complexes. Les mers tempérées présentent une forte fluctuation selon les saisons et les paramètres environnementaux, qui ont une incidence sur les cycles de vie des ascidies. Les variations saisonnières sont souvent dues aux changements de température, mais d'autres facteurs comme, par exemple, la disponibilité de nutriments, devraient être pris en considération. Bien que les études génétiques et chimiques apportent des informations importantes sur les possibles procédés de spéciation d'une population ou d'ensembles de populations, l'étude des cycles biologiques continue à être nécessaire pour montrer l'existence d'un isolement reproductif qui serait en accord avec le concept biologique de cohésion d'espèce. Ce genre d'étude fournit alors, des données importantes sur la biologie et l'écologie des invertébrés benthiques.

Les objectifs de cette étude sont de comparer les cycles de reproduction, de croissance et de survie des deux formes de *Cystodytes* les plus abondantes en Méditerranée Occidentale (violet et bleu), pendant plus de 20 mois. Ces formes ont été identifiées, antérieurement, comme appartenant à l'espèce *Cystodytes dellechiajei*. Des corrélations entre ces paramètres biologiques et d'autres paramètres comme la taille des colonies, la température de l'eau et la présence ou l'absence de concurrents pour l'espace, ont été réalisés. Finalement, les divergences génétiques et chimiques trouvées durant les chapitres précédents et les différences biologiques mises en évidence durant ce chapitre afin de découvrir le degré d'isolement de ces morphotypes et son statut taxonomique, ont été corrélées.

Nous avons trouvé un modèle saisonnier des cycles de vie de ces deux morphotypes. Malgré ce modèle commun, nous avons mis en évidence des différences significatives entre les deux. Les pics saisonniers de reproduction et de croissance de la forme de couleur violette, ont lieu, approximativement, deux mois plus tard que pour celles de la forme bleue. Pour chacun, le pic de reproduction est décalé du pic de croissance montrant que les ressources énergétiques disponibles sont consacrées soit à la reproduction soit à la croissance. La croissance des deux

morphotypes est maximale une fois les larves relâchées. Nous n'avons pas trouvé de différences significatives du taux de mortalité entre les deux formes, ni de la croissance des colonies suivant qu'elles soient en contact ou non avec l'espèce toxique *Crambe crambe* (Porifera). D'autre part, il existe une corrélation négative entre la mortalité et la taille des colonies bleues, alors que ce n'est pas le cas pour les violettes. Les différences trouvées dans les cycles de reproduction de ces deux formes de *Cystodytes* coïncident avec les divergences chimiques et génétiques trouvées antérieurement. Nous concluons, donc, que ces deux morphotypes sont, en fait, des espèces différentes.

ÉTUDE DU BILAN ÉNERGÉTIQUE : VARIATION TEMPORELLE DE LA PRODUCTION D 'ASCIDIDEMINE PAR RAPPORT À D 'AUTRES PARAMÈTRES BIOLOGIQUES

Les relations entre production de métabolites secondaires, présence de défenses physiques (comme les spicules) et paramètres biologiques d'une espèce comme son cycle reproducteur ou de croissance ont été très rarement étudiées. La théorie de la défense optimale établie que la répartition des ressources énergétiques pour la production des défenses physiques et chimiques doit être optimisée par rapport aux autres nécessités d'un organisme (croissance, maintenance somatique et réparation, et reproduction). Il devrait alors exister une variation temporelle de la production de ces défenses, dépendant aussi des conditions environnementales et de l'état physiologique de l'organisme qui les produit. Malgré l'importance de ce genre d'étude, il n'existe actuellement aucune recherche approfondie établissant la relation entre cette variabilité temporelle de la production de défenses (physiques et chimiques) et les autres paramètres biologiques d'une espèce.

L'objectif de ce chapitre est de mettre en évidence, expérimentalement cette variabilité temporelle de la production de défenses chez le morphotype bleu de *Cystodytes*. Pour étudier la variation de la production des défenses chimiques, nous avons quantifié l'ascididemine, pyridoacridine la plus abondante de cette forme. Les défenses physiques ont été estimées à partir de la variation quantitative des cendres, car celles-ci contiennent majoritairement les spicules et du matériel structurel inorganique.

Nous avons voulu savoir s'il existait une variation saisonnière de la quantité d'énergie investie pour la production de défenses physiques et chimiques et si nous pouvions la mettre en relation avec d'autres paramètres biologiques déjà étudiés comme la reproduction et la croissance. Pour répondre à ceci nous avons échantillonné, pendant un an, la même population du morphotype bleu étudiée au chapitre 7 afin de comparer les cycles obtenus.

Pour quantifier correctement l'ascididemine, il a fallu optimiser le protocole de conservation des échantillons et les conditions d'analyse en CLHP. Pour le stockage, on s'est aperçu que les échantillons conservés lyophilisés à température ambiante continuaient à évoluer et perdaient jusqu'à 30% d'ascididemine sur une durée de 10 mois. Par contre, nous n'avons pas observé d'évolution (diminution de la concentration en ascididemine) sur les échantillons maintenus congelés pendant cinq mois puis lyophilisés avant traitement. Nous nous sommes ensuite assurés que le processus de lyophilisation ne détériorait pas les échantillons. Ils ont donc été conservés lyophilisés au congélateur.

L'étude de la concentration d'ascididemine a montré des fluctuations annuelles, avec un minimum au printemps (juste avant que la période de reproduction ne commence) et un maximum en fin d'été et début d'automne. Pour le contenu en cendres, nous avons trouvé la même tendance. Ceci suggère qu'il existe aussi une périodicité annuelle pour la production du contenu inorganique des colonies, principalement des spicules.

Les résultats obtenus montrent que le cycle reproducteur conditionne fortement la quantité d'énergie disponible pour d'autres paramètres biologiques comme la production de métabolites secondaires, la croissance et l'augmentation du matériel inorganique (spicules). L'hypothèse selon laquelle la production de métabolites secondaires a un coût métabolique optimisé par rapport aux nécessités de l'organisme semble donc vérifiée (Fagerström et al. 1987).

En conclusion, l'approche pluridisciplinaire utilisée pour étudier l'ascidie coloniale *Cystodytes*, a permis de mieux connaître la biologie, l'écologie et la chimie de cet organisme. Elle a aussi permis de montrer l'importance de ce genre d'études afin d'obtenir une vision plus globale et sûrement plus proche de la réalité, de

l'évolution des invertébrés marins dans leur environnement. Malgré cela, beaucoup de questions restent encore sans réponse, comme la localisation cellulaire de l'ascididemine, la biosynthèse des pyridoacridines et le nombre total d'espèces du genre *Cystodytes* en Méditerranéenne. Ce qui est certain, c'est que plus on connaît un organisme, plus on se pose des nouvelles questions, qui vont susciter de nouvelles études avec l'utilisation de nouvelles techniques pour peu que l'on songe à apporter des réponses …