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RESEARCH ARTICLE

Secondary metabolite and inorganic contents in *Cystodytes* sp. (Ascidiacea): temporal patterns and association with reproduction and growth

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Abstract Temporal variation is one of the least known components of defence production in marine organisms. Here we examined whether there is a predictable temporal pattern in the production of chemical and structural defences by a marine invertebrate. To assess the seasonal variation in chemical defence, we measured ascididemin, the main pyridoacridine alkaloid in the blue Mediterranean morph of the ascidian *Cystodytes* sp. Structural defence variability was assessed on the basis of colony ash content, as it contains mainly spicules. Ascididemin concentration and the colony ash content displayed an annual cycle, reaching a minimum in spring and peaking in summer. Cross-correlation analyses with existing data on growth and reproduction suggested a significant trade-off between investment in reproduction and the other biological parameters considered (growth, inorganic content and ascididemin concentration). Our results suggest that optimization of resource allocation, probably influenced by biotic interactions and physical factors, shaped the temporal trends observed in secondary metabolite concentration and inorganic content.

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Introduction

Defence mechanisms are commonplace among sessile invertebrates, especially those that require exposed surfaces to acquire resources, such as many filter feeders. Defences can be structural, associational, nutritional or chemical (McClintock and Baker 2001). Although the estimation of costs is difficult, it is generally agreed that production, maintenance, transport and storage of defences have associated costs that can be demonstrated in terms of trade-offs with other biological functions (Karban and Myers 1989; Skogsmyr and Fagerström 1992). Thus, it is predicted that natural selection will 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 defence theory (ODT) postulates that the allocation of resources to chemical and structural defences will be optimized with respect to the requirements of an organism (e.g. risk of attack) and its energy budget (growth, somatic maintenance, regeneration and reproduction; Rhoades 1979; Fagerström et al. 1987). Under the ODT, predictable intraspecific variation is expected both between individuals (because of environmental conditions) and within individuals, caused by distinct pressures on different parts of an organism (particularly so in colonial forms), life-history stages (e.g. between larvae and

adults) and biological cycles (e.g. reproduction and growth).

In marine organisms, intraspecific patterns of variation have been reported within-organisms (e.g. Paul and Van Alstyne 1988; Harvell and Fenical 1989; Van Alstyne et al. 1994; Turon et al. 1996; Becerro et al. 1998; Schupp et al. 1999; Puglisi et al. 2002), and related to geography or ecological conditions (e.g. Harvell et al. 1993; Maida et al. 1993; Valls et al. 1993; Yates and Peckol 1993; Becerro et al. 1995; Dube et al. 2002). Although the observations of patterns or lack of patterns are the fundamental starting-blocks for ecological studies (Underwood et al. 2000), temporal variation is one of the least known components of defence production in marine organisms (reviewed in Paul et al. 2006). Moreover, most studies had focused on plant species (e.g. Hay and Steinberg 1992; Steinberg and van Altena 1992; Yates and Peckol 1993; Steinberg 1995; Pavia et al. 1999) and, to our knowledge, only Page et al. (2005) and López-Legentil et al. (2006a) had quantified bioactive metabolites over time in marine invertebrates. Our study is the first to relate the temporal production of a secondary metabolite to other biological parameters such as reproduction and growth.

The ascidian genus *Cystodytes* is widely distributed around the world and shows a variety of colour and spicular composition (López-Legentil and Turon 2005). In the Mediterranean, López-Legentil et al. (2005a) described two chemotypes which, on the basis of genetic and reproductive studies, were found to correspond to sibling species (López-Legentil et al. 2005b; López-Legentil and Turon 2006). The first chemotype was characterized by the presence of C_9-unsubstituted pyridoacridines such as ascididemin (Kobayashi et al. 1988) and 11-hydroxyascididemin (Schmitz et al. 1991), and was found in blue and green morphs, while the second (found in purple morphs) contained sulphur-containing pyridoacridines.

Seasonality is particularly likely in temperate seas where strong periodicities affect most of the biological parameters of organisms (Sebens 1986; Coma et al. 2000). Temperature and food availability are often reported as determinants of the cycles that are described. In Mediterranean littoral ecosystems, however, annual fluctuations of seston do not affect the abundance of pico- and nanoplankton, which accounts for most of the diet of ascidians (Seiderer and Newell 1988; Ribes et al. 1998; Coma and Ribes 2003). A clear-cut seasonal reproductive pattern was observed in the Mediterranean blue morph of *Cystodytes*, in which larval release occurred in June, followed by a period of active growth in late summer (López-Legentil et al. 2005b). Both the reproductive and growth patterns were significantly correlated with sea temperature fluctuations and displayed a temporal lag that suggested the partitioning of resources to either reproduction or growth (López-Legentil et al. 2005b).

Here we assessed temporal variability in the production of defences in the blue Mediterranean morph of *Cystodytes*. To study the variation in this production we quantified ascididemin, the main alkaloid in this morph (López-Legentil et al. 2005a). Ascididemin showed anti-predatory properties against both puffer and damselfish, but not against sea urchins (López-Legentil et al. 2006b). We also quantified the colony ash content as an estimation of investment in spicules and structural material in the ascidian. Indeed, several studies have suggested a defensive role for both spicules and structural material in many benthic organisms (e.g. Harvell et al. 1988; Uriz et al. 1996; O'Neal and Pawlik 2002; Burns and Ilan 2003). Although spicules of *Cystodytes* spp. failed to deter predators, they may contribute to the overall defensive strategy of the ascidian by protecting the zooids (López-Legentil et al. 2006b) and complementing the effectiveness of the remaining structural materials and chemical defences (Van Alstyne et al. 1994; Uriz et al. 1996; Tarjuelo et al. 2002). In the Mediterranean, *Cystodytes* colonies generally lack epibionts and present scarce signs of predation, although occasional sea urchin and fish bites have been observed (López-Legentil 2005). The main goal of this study is to determine whether a temporal pattern in chemical and structural defence production exists and if so, to relate it to other biological parameters such as investment in reproduction and growth.

Materials and methods

Sampling and storage

We monitored a population of the blue morph of *Cystodytes* in Palamós (NE Spain; 41°50.4′N 3°07.6′E). Two annual cycles were studied: one from February 2003 to February 2004, coinciding with a study on the growth rates and reproductive cycles in the same population (López-Legentil et al. 2005b), and a second one from July 2004 to July 2005 to verify that the pattern was repeatable. During the study periods, five different colonies per month (separated by at least 2 m) were randomly collected by scuba diving. Dissecting zooids from the tunic or into their respective components was not feasible because each zooid is encased in a capsule formed by layers of overlapping spicules and measures less than 1 mm when

contracted. Therefore, whole colonies were frozen alive, freeze-dried within a month and kept at −30°C until analysed (which was less than 5 months in all cases). This protocol was chosen after performing two preliminary tests to check the effects of storage procedures in the amount of ascididemin recovered. In the first one, five colonies were divided into halves and freeze-dried within hours. One half was extracted and analysed immediately, while the remaining half was kept at −30°C and analysed after 10 months. No significant changes in ascididemin concentration were observed (Wilcoxon signed rank test, $P = 0.313$). In the second experiment, again five colonies were divided into two halves but this time, one half was freeze-dried immediately and kept at −30°C while the second was kept frozen but not freeze-dried. After 5 months, the frozen pieces were freeze-dried and both halves were extracted and analysed. Again, yields of ascididemin did not differ significantly (paired sample *t*-test, $P = 0.179$).

Chemical extraction procedure

We followed the optimized protocol for extraction and identification of pyridoacridine alkaloids from *Cystodytes* spp. described by Bontemps (1996). Approximately 250 mg of each freeze-dried colony was weighed and extracted three times in a 1:1 (v : v) mixture of dichloromethane and methanol and passed through a 20 µm PTFE filter in a vacuum chamber. The resulting solution was then dried by vacuum rotary evaporation. Each crude extract was then redissolved in methanol. The final volume was adjusted to 10 ml and an aliquot of 1 ml was passed through a 13 mm, 0.20 µm PTFE syringe filter before HPLC injection. The injection volume was fixed at 10 µl.

HPLC analysis and quantification

The HPLC elution conditions consisted of eluents A (water-methanol-acetic acid, 9:1:0.1 v : v : v) and B (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 × 15 cm) analytical column. Analyses were performed using a Waters Alliance 2695 Separations Module with a Waters 996 photodiode array detector.

The method for ascididemin purification and identification was optimized by López-Legentil et al. (2005a). Ascididemin was detected at 380 nm and the peak 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 at the 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.

Quantification of ash content

After chemical extraction, the remaining colony pieces from February 2003 to February 2004 were oven-dried to a constant weight (60°C for at least 24 h), placed in aluminium cups, weighed and burned in a furnace oven at 500°C for 12 h. The resulting ash was kept in a dry atmosphere until they were reweighed. The ash was then observed under a stereomicroscope and all foreign material, such as small shells and worm tubes, was carefully removed and its weight was subtracted from the ash weight.

We used maturity index and growth rate data from López-Legentil et al. (2005b) to investigate how those biological parameters correlate with the concentration of ascididemin and inorganic content quantified in our study. To do so, we performed cross-correlation analyses using Systat Version 9.0 package.

Results

Ascididemin quantification

All the samples studied showed low-chemical diversity, with one major peak (retention time: 9.3 min), which was identified as ascididemin. Some minor peaks, including 11-hydroxyascididemin (Schmitz et al. 1991), were also detected; however, because they had a negligible contribution to the total area of pyridoacridines, they were not quantified.

Changes in the concentration of ascididemin (mean ± SE) are shown in Fig. 1a as the percentage of the compound relative to total organic matter (the data from the samples collected between July 2004 and July 2005 are superimposed). The percentage of ascididemin relative to total organic matter fluctuated seasonally, ranging from 0.18 to 0.62%. In 2003, the lowest value was found in April, while the highest appeared in August. Examination of the samples collected between July 2004 and July 2005 (Fig. 1a) yielded the same general pattern of ascididemin production as in the corresponding months of 2003. However, there was a lag of 1 month in the minimum value (April in 2003 and May in 2005), and this value was not as low as in 2003.

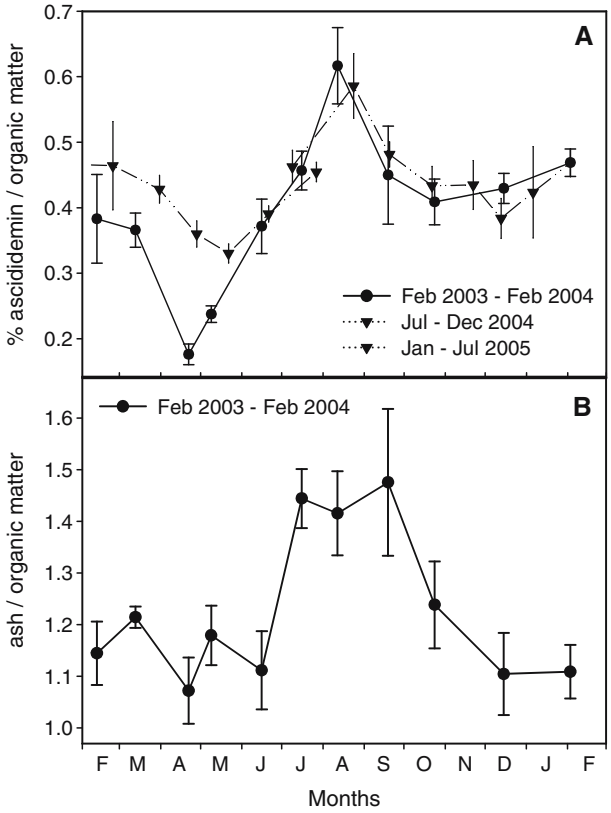

Fig. 1 **a** Time course of the percentage of ascididemin relative to total organic matter from February 2003 to February 2004 (*solid line*) and during the second monitoring from July 2004 to July 2005 (*dotted line*). **b** The ratio of ash per total organic matter obtained for the monitored colonies from February 2003 to February 2004. *Bars* indicate standard errors (*SE*)

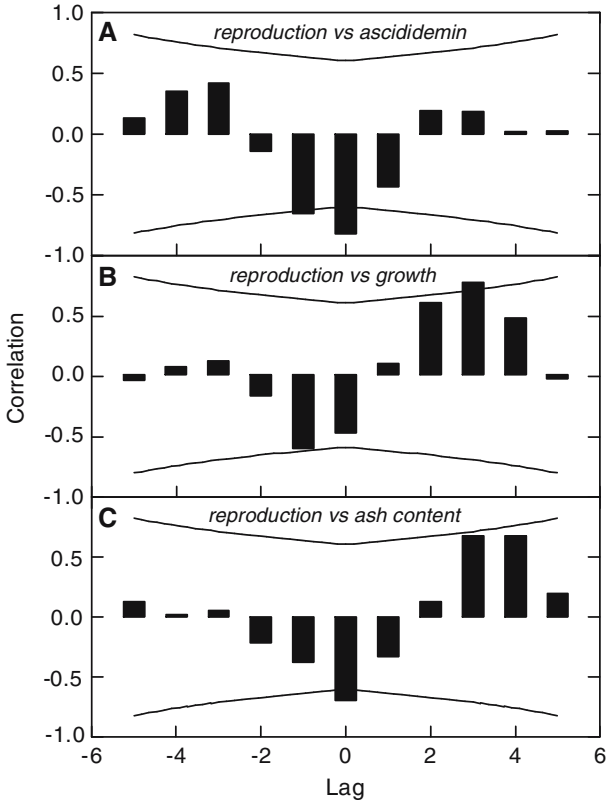

Fig. 2 Cross correlation analyses of the cycles of reproduction and: **a** ascididemin concentration, **b** growth rates and **c** ash content of the colonies. *Horizontal curved lines* represent 95% confidence bounds of the correlation values. Time lag is in months. Growth rates and reproduction data are from López-Legentil et al. (2005b)

Quantification of ash content

The time course of the variation in ash content during 2003 is shown in Fig. 1b as the ratio of ash per total organic matter of the ascidian. The percentage of ash relative to dry weight ranged from 38.89 to 67.05%. Although replicates from the same month varied greatly (see standard error bars), a seasonal trend similar to the one observed for ascididemin concentration during the same year was evident. The ash content increased and remained high from July to September 2003, decreasing again during wintertime.

The cross-correlation analyses (Fig. 2) showed that the cycle of reproduction featured a reverse trend with respect to those of the other functions. In particular, the lowest negative correlation values were found at time lag 0, indicating a trade-off with ascididemin concentration and with ash content. For growth rates, the relationship at time lag 0 was also negative, and the strongest positive correlation occurred at time lag 3, indicating that growth rates peak some months later than reproduction in this species.

Discussion

Ascididemin concentration displayed cyclic behaviour, which supports the hypothesis of annual periodicity in the production of bioactive substances, with a minimum in spring and a maximum in summer after the reproduction peak. A seasonal pattern was also observed for colony ash content, indicating that annual periodicity also occurs for the production of inorganic content, mainly spicules. The average percentage of ash content was within the range found for other colonial ascidians (Tarjuelo et al. 2002; McClintock et al. 2004). The large standard errors found in both the concentration of ascididemin and inorganic content showed that there was noticeable inter-individual variability, which is commonplace among sessile invertebrates (e.g. Van Alstyne et al. 1994; Turon et al. 1996). The ascididemin content cycles obtained in 2003 and 2004–2005

followed the same general pattern, but minimum values of this alkaloid were higher and occurred ~1 month later in the second cycle. The same month lag was observed in the reproductive cycle and the appearance of resting forms in 2005 (personal observation).

One month after larval release in June 2003, both the inorganic content (mainly spicules) of the colonies and the growth rates (López-Legentil et al. 2005b) increased rapidly for ~1 month. These two parameters may be linked, as during periods of rapid growth zooid budding increases, and spicules in this genus are formed surrounding the abdominal part of the zooids (Lambert 1979). Finally, ~2 months after the larval release and 1 month after maximal growth rate, the production of asciddemin, which appears to be the most efficient defence mechanism against predation (López-Legentil et al. 2006b), reached a maxima and remained high for some months. It can be noted that in June, when the maturity index is at its highest value (with presence of brooded larvae; López-Legentil et al. 2005b), growth rate and asciddemin production were already increasing. Colonial ascidian zooids are ovoviviparous (Svane and Young 1989). Therefore, the maximum investment in reproduction it's likely made during the gonad building phase, and not when fully formed larvae are already present. In the blue morph of *Cystodytes* sp., oocyte maturation occurred from March to May (López-Legentil et al. 2005b), coinciding with the period of low-asciddemin production.

Our results showed the existence of a temporal pattern in defence production and suggested a temporal trade-off between investment in reproduction and the other biological parameters considered (including defence production). This is in agreement with Tarjuelo and Turon (2004) who, after studying patterns of reproductive investment versus tunic production in 11 colonial ascidians, concluded that species with low-fecundity having large and complex larvae, such as *Cystodytes dellechiajei* (the name used in that study for the blue morph addressed here) invest the most energy in reproduction. The energetic value (in caloric content) of the reproductive material (testes and larvae) is in the same order or higher than that of the zooid itself [KJ (testes + larva)/KJ zooid is 1.14 ± 0.19, mean ± SE; Tarjuelo and 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 defence) was significantly reduced during periods of reproductive activity.

Larval release in itself could influence the cycles observed. As larvae have no spicules, its spawning will automatically increase the spicule concentration in the colony. Likewise, the observed increase in chemical defence concentration after spawning could be caused by the release of non-defended larvae. Asciddemin was present in both tunic and zooids (López-Legentil et al. 2005a), and larvae of *C. dellechiajei* were unpalatable to several predators (Tarjuelo et al. 2002). Therefore, it is likely that some amount of asciddemin is also present in the larvae for their defence. The presence of chemical defences in larvae has also been demonstrated for other ascidian species (Young and Bingham 1987; McClintock et al. 1991; Lindquist et al. 1992). The relative concentration of the compound in larvae with respect to that in tunic and zooids will determine whether their release implies an increase or a decrease in concentration in the remaining tissues. In any case, the larval weight is small compared to total colony weight: combining weight ratios of larvae versus zooids (0.95:1) with those of tunic versus zooids (21.36:1) (data from Tarjuelo and Turon 2004), larval weight accounts for a mere 4% of the total colony weight, so their release cannot explain the variations observed in the cycles of spicule and asciddemin production.

Biotic interactions and physical factors may also account for the temporal variation observed. For instance, summer is an unfavourable season for many sessile invertebrates in the Mediterranean due to the existence of a food shortage period (Boero et al. 1986; Turon and Becerro 1992; Coma and Ribes 2003). This unfavourable season may be reflected by a decrease in the production of secondary metabolites and structural material. Likely, the high concentration of asciddemin in early autumn could be related to the reactivation of growth of competing animal species and predators after summer. However, Coma and Ribes (2003) concluded that growth and reproduction in Mediterranean ascidians were little affected by food availability and only colonies that were not surrounded by known toxic species or/and good space competitors were collected for this study. Predation, on the other hand, was observed in the form of occasional bite marks during a 2-year photographic monitoring but no seasonal pattern of predation or mortality was found (López-Legentil et al. 2005b). It is noteworthy that a different chemotype of *Cystodytes* sp. (a purple morph, which is a sibling species of the blue morph studied here; López-Legentil et al. 2005b; López-Legentil and Turon 2006) showed no clear-cut temporal pattern in the production of four sulphur-containing pyridoacridines. In addition, minimum values in the concentration of these four pyridoacridines were recorded in late summer, after the reproductive period (López-Legentil et al. 2006a). The differences found in the temporal patterns of secondary metabolite production between these two

morphotypes match previously reported genetic, biological and chemical divergences (López-Legentil et al. 2005a, b, 2006a, b).

All in all, our results indicated that investment in reproduction significantly influences the temporal course of ascididemin concentration and inorganic content. This finding is in accordance with the hypothesis that defence production must be optimized with respect to other biological parameters (Fagerström et al. 1987), although environmentally modulated elements may also contribute to the fine-tuning of the seasonal pattern observed.

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