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Contrasting biological traits of *Clavelina lepadiformis* (Ascidiacea) populations from inside and outside harbours in the western Mediterranean

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ABSTRACT: *Clavelina lepadiformis* is a colonial ascidian found in harbour and rocky littoral habitats. The populations of these 2 habitat types in the Western Mediterranean showed marked genetic differences in mtDNA sequence data in previous works. However, no morphological differences between the forms inhabiting the 2 habitat types have been found. Here, we compared the biology and accumulation of heavy metals in populations of both habitats in NE of Spain. Abundance and seasonal cycles showed contrasting trends in the 2 habitat types: harbour populations reached densities of ca. 3900 zooids m^{-2} and active colonies were found all year round. Abundance in the rocky littoral environment was 1 order of magnitude lower and showed a clear seasonal pattern, with the disappearance of zooids during summer (aestivation). Reproductive cycles also differed, as larvae were present in the harbour population from November through June, with several sexual cycles during this period. In contrast, in the open littoral, larval occurrence was restricted to 2 to 3 mo during winter-spring, with only 1 gonadal cycle yr^{-1}. The zooids and larvae of the harbour were significantly larger. However, neither the total reproductive effort, tunic production (in weight ratios) nor fecundity significantly differed between these habitats. The harbour population accumulated significantly more Cu and Pb, and heavy metal concentrations showed a seasonal cycle with minima in summer. On the other hand, both populations accumulated a similar amount of V, a metal involved in ascidian metabolism. The production of secondary metabolites and the toxicity of polar extracts were higher in the open littoral form. These results, however, did not correlate with the outcome of palatability tests carried out with specialist and generalist predators, in which no preference was observed. Experimental juvenile transplantation between habitats showed that newly settled individuals from the rocky littoral habitat can survive in both environments (with survival rates of 30 to 50% during the first 4 wk), while those from harbours show low survivorship when moved out (ca. 5% survival after 4 wk). We conclude that there was a marked ecophenotypic variation between populations of both habitat types.

KEY WORDS: Ascidians · Harbours · Ecophenotypic variation · Genetic divergence · Sub-lethal pollution · Reproductive investment · Biological cycles

INTRODUCTION

The study of benthic assemblages in harbour environments and in other confined, marginal marine habitats such as estuaries and lagoons has potential interest both from a basic and applied point of view. Given the closed nature of these habitats, the populations are isolated from adjacent littoral communities (Boisselier-Dubayle & Gofas 1999). In species that thrive in both habitats and that feature benthic development or

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development via larvae with restricted dispersal (anchiplanic larvae, Levin & Bridges 1995), this isolation can constitute an effective genetic barrier. Although few studies have been devoted to this topic, there are several reports of genetically distinct varieties of invertebrates that inhabit marginal marine habitats and open littoral habitats along the same coast (Dalby 1997, Boisselier-Dubayle & Gofas 1999). Intense ship traffic results in the establishment in harbours of species which are either rare or absent from adjacent outer communities (Monniot et al. 1985). In this sense, marinas and harbours make a large contribution to the introduction and invasion of exotic fauna and flora (Zibrowius 1991), and ascidians in particular (Lambert & Lambert 1998).

In addition, harbours are frequently polluted by heavy metals, hydrocarbons and other organic pollutants (Fichet et al. 1998, Commendatore et al. 2000), and show a moderate to high degree of eutrophication (Papadopoulou et al. 1998, Dhainaut et al. 2000). Harbours are also shallow and closed, which results in greater fluctuations in the physical parameters than in open sea. Organisms that inhabit these environments adapt to these characteristics; therefore, they are suitable models to study the effect of pollution on their biology. In addition, these organisms are also appropriate for bioremediation studies, as many filter feeding invertebrates accumulate and immobilise pollutants from water or sediments (Chassard-Bouchaud 1985, Patel et al. 1985, Verdenal et al. 1990, Monniot et al. 1993).

Ascidians are benthic filter feeders that are particularly abundant in harbour environments (Monniot et al. 1985, Lambert & Lambert 1998). They are considered as indicators of water quality and they can accumulate toxicants like heavy metals or hydrocarbons in their tissues (Monniot et al. 1993, 1994). In addition, they have lecitotrophic, short-lived larvae which confers to them low dispersal capabilities, and especially so for colonial species (Svane & Young 1989). They have therefore a potentially high susceptibility to confinement effects. Our study focuses on the colonial ascidian *Clavelina lepadiformis* (Müller, 1776), an Atlanto-Mediterranean species that is abundant along rocky littorals at shallow depths (Thompson 1934, Berrill 1950, Millar 1966, Turon 1987, Koukouras et al. 1995). The colonies consist of groups of zooids interconnected by stolons. After an annual release of larvae, the colonies feature a dormant (inactive) period during which zooids disappear and the colonies survive in the form of stolons with budding chambers (Berrill 1951). The inactive period occurs during winter (hibernation) in Atlantic populations and during summer (aestivation) in Mediterranean populations (Mukai 1977). *C. lepadiformis* lives in open littoral habitats and in estuaries, fjords and harbours. A recent study of populations of this species in several harbours and open littoral habitats in the northwestern Mediterranean has revealed a noticeable genetic differentiation between the 2 types of habitat (ca. 5% in cytochrome oxidase 1 mtDNA sequence data, Tarjuelo et al. 2001).

Clavelina lepadiformis, therefore, is a suitable taxon for the study of adaptation to confined and polluted habitats such as harbours. We will refer to the populations inside and outside harbours as the inner and outer populations, respectively. Our goals were: (1) to compare the biology of the 2 forms of *C. lepadiformis* in terms of biological cycles, reproductive investment and defence; (2) to analyse the accumulation of heavy metals in tissues; and (3) to ascertain the ability of juveniles to survive in each other's habitat by way of transplant experiments.

MATERIALS AND METHODS

Study sites. The inner population was studied in Blanes harbour (NE of Spain, 41°40.4′N, 2°48.2′E). This is a relatively clean marina, which allows the study of the sublethal effects of pollutants. The outer populations studied were those along the rocky coastline of the same locality (Blanes) and in nearby Tossa de Mar (41°43.2′N, 2°56.4′E).

The temperature inside the marina did not vary more than 0.2 to 0.3°C with respect to the adjacent outer habitats during the study period. Only on 1 sampling date was a difference of 0.5°C observed. Other environmental parameters of the harbour and the adjacent habitats outside it have been studied by Cebrian (2001). Her findings can be summarised as follows: irradiance was slightly higher inside the harbour than at the outside (371 ± 26.04 µE m^{-2} s^{-2} and 439 ± 31.4 µE m^{-2} s^{-2}, mean ± SE, respectively). Particulate organic carbon in the water varied with season but not with habitat (from 1300 to 2100 µg C l^{-1} at both sites). Sedimentation rates had maximal values of 58.16 g m^{-2} d^{-1} outside the harbour and 18.8 g m^{-2} d^{-1} inside it. Total organic matter in the sediment varied seasonally with a maximum of 2.5 g m^{-2} inside the harbour and 11 g m^{-2} outside it; although it was significantly higher in the harbour than outside it in the summer months. Pb concentration in the water column was higher inside than outside the harbour (8.0 ± 2.2 and 3.3 ± 0.9 µg l^{-1}, respectively), while hydrocarbon levels in the water column were undetectable at both habitats. Cu concentration reached 98 and 6 ppm, respectively, in sediments within the harbour and outside it (Pinedo 1998). Presence of heavy metals in the water column and the sediment, organic matter in the sediment and resuspension rates (as measured by sedimentation rates) emerged therefore as the main differential factors that may influence benthic assemblages at both habitats.

Morphology, abundance and reproductive cycles. Specimens from both types of habitat were collected, anaesthetised and formalin-fixed for morphological observation. The abundances were quantified by monitoring fixed points at the harbour and at the outer habitat. Monitoring started at Tossa de Mar in March 1998 and in the harbour of Blanes in September 1999, and ended in September 2001. Plots on vertical walls at a depth of between 3.5 and 5 m in both localities were randomly selected and marked with nails. Thirty-two plots were surveyed per locality, each covering 0.075 m^2. They were photographed monthly with an underwater camera with a close-up lens. Zooids were then counted in each picture. We found the estimate of zooid abundance more reliable than that of the number of colonies, since in the harbour, colonies carpeted the wall almost continuously during certain months, making between-colony boundaries difficult to define.

Reproductive cycles were followed only in the Blanes harbour, as those from the population of Tossa de Mar have been established from the work by Tarjuelo (2001) at this locality from 1997 to 1999. A minimum of 10 colonies was collected monthly and preserved from October 1999 to December 2000. To assess their reproductive state, 10 zooids of each colony were examined under the stereomicroscope and the presence or absence of gonads and incubating embryos was recorded. Zooids lacking all these features were scored as immature. From these data, we obtained the percentage of immature zooids and the percentage with gonads and embryos (not mutually exclusive) per month.

Investment in reproduction and tunic production. To measure reproductive investment and tunic production, we compared the populations of Blanes harbour and Tossa de Mar sampled. At least 10 mature colonies with testes, ova and larvae were collected at each habitat during the reproductive season and, of these, 5 zooids per colony were dissected. The number of mature larvae and developing embryos in the brood pouch and ova in the ovary were counted and summed up. The per zooid fecundity of each colony was estimated as the average of these sums. This procedure may underestimate the true number of larvae produced per zooid, as some could have been released at the time of observation. However, not all ova in the ovaries may become mature larvae, as resorption may occur, especially at the end of the reproductive season. The zooids of this species do not appear to be able to produce several batches of larvae, rather, they mature, release larvae and then degenerate (Berrill 1951). Even if our data might give somewhat biased estimates of the true fecundity, they are still valid for comparative purposes. The number of embryos and larvae (excluding ova) was also compared between populations.

Ten further zooids per colony were then dissected under the stereomicroscope, the tunic, zooid tissues, mature larvae and testes were separated. This material was pooled (some components were too small to be weighed separately for each zooid), freeze-dried and weighed. For each colony, we obtained a mean zooid, tunic and testes weight per zooid, and an average weight of mature larvae. A measure of reproductive investment that includes both male and female investments was estimated by multiplying the mean larval weight by the per zooid fecundity of the colony and adding the mean testes weight, and then dividing this sum by the average zooid weight. In ascidians, investment calculated as weight ratios correlate well with investment in caloric ratios (Tarjuelo 2001). Thus, the former can be used as reliable estimates of energy investment.

Accumulation of heavy metals. The amount of heavy metals in tissues was monitored monthly in the population of Blanes harbour from October 1999 to December 2000. Samples from the outer population at Blanes were collected in April and December 2000 for comparison. The rationale was to compare the accumulation of heavy metals inside and outside the harbour at the beginning of the seasonal cycle of the outer population after aestivation (December) and at its maximum development (April).

Five colonies were collected and freeze-dried monthly. The zooids were then carefully cleaned of foreign material (algae, sediment) and were ground in a glass mortar. Approximately 0.1 g of each colony was subjected to digestion in Teflon reactors to which 3 ml of Merck suprapure nitric acid (65%) and 1 ml of H_2O_2 were added. The reactors were then digested at 95°C for 20 h. Afterwards the contents were transferred to previously weighed vials. Six ml of Milli-Q water was first added to the reactors and then emptied in the corresponding vials, which were weighed again. The attack solutions in the vials were then diluted 1 to 20 with HNO_3 (1%) and 10 ppb of Rh was added as an internal standard. Solutions were measured against a calibration prepared using 1 blank and 4 increasing concentrations of commercial standards of every element. Appropriate concentrations of standards were selected according to the element. Cu, Pb, V, Cd and Hg were studied, and Cl was also analysed to estimate possible interference with V. Standards and samples were analysed in an inductively coupled plasma mass spectrometer (Perkin Elmer Elan 6000) under standard conditions. Results were expressed as ppm of metal with respect to sample tissue (dry weight).

Toxicity and palatability. We compared the toxicity of tissue extracts of the inner and outer populations of Blanes by the Microtox method (Ribó & Kaiser 1987) based on the reduction of the bioluminescence of the

marine bacterium *Photobacterium phosphoreum*. This method has proven to be precise and repeatable, and it correlates well with other common toxicity tests (Becerro et al. 1995). It can be used, therefore, for general toxicity estimation. Ten replicates (colonies) were used for each population. Colonies were collected on the same day in winter in the Blanes harbour and the littoral of the same locality. The zooids of each colony were cleaned under the stereomicroscope, freeze-dried and ground in a mortar. We used dichloromethane: methanol (2:1) for the extraction of secondary metabolites. Three extractions (1 h each) with 10 ml of dichloromethane:methanol were separately performed on the 20 colonies included in the study. Extraction vials were periodically placed in an ultrasonic bath. The extracts were then pooled, the solvent evaporated and the crude extract weighed. Assays were made by resuspending the extracts (in an ultrasonic bath) in artificial seawater. The Microtox standardised test uses 4 increasing concentrations of the sample solution (Ribó & Kaiser 1982). In our case, the concentrations of extract (relative to dry sample weight) tested were 250, 500, 1000 and 2000 ppm. With the values of reduction of bioluminescence, the Microtox device calculates a regression line and the EC_{50}, the effective concentration at which bioluminescence is reduced to one half. Both the percentage yield of crude extract and the EC_{50} were compared for both populations.

As the Microtox assay indicated differences in secondary chemistry, we tested whether the 2 forms were equally defended against predation by using several predators. We tested artificial food pellets with extracts of the ascidians in the field with the damselfish *Chromis chromis* (Linnaeus, 1758), the commonest Pomacentridae in this area. This species inhabits both habitat types, it is easily amenable to field palatability assays, and the pellet size is very adequate for it (Tarjuelo 2001). We extracted zooids (mixing together not less than 5 colonies) of the 2 forms from the Blanes locality as described above, and added an appropriate volume of solvent with extract (to keep natural concentrations) to 0.3 g of food pellets (Sera Granumarin®). When dry, the pellets are small, 1 mm long, rice-like grains but they become more spherical after absorbing liquids. We added the solvent to small vials with the 0.3 g of pellets and let the solvent evaporate so that the pellets absorbed the extract. The pellets were then removed and a few drops of solvent were added to clean the vials' walls and released again, drop by drop, over the pellets. This was done to ensure that no extract was left behind. Control pellets were prepared with solvent only.

For the field assay, pellets were taken underwater in 100 ml syringes. Fish were first fed with control pellets to accustom them to feed from the syringes. After a few minutes, damselfish used to cluster around the diver and the test began. This diver released a control pellet, then a pellet from the first treatment, then a second control, then a pellet from the second treatment. The syringe was aimed in different directions each time to ensure that different fish encounter the pellets. Another diver recorded whether the pellet was consumed or not. One pellet was scored as consumed if it was eaten immediately or after a few (less than 5) trials by the same or different fish. It was scored as rejected if it was mouthed and rejected 5 or more times. Pellets not mouthed were not included in the analysis as fish probably did not see them. The number of valid observations varied between 45 and 61 per treatment. The results were arranged in a 3-way contingency table with variables population (inner vs outer), treatment (control and treated food) and consumption (eaten or rejected). Data were number of pellets under the categories of these variables.

We also tested raw ascidian material against a specialist and a generalist predator. For the former, we selected the flatworm *Prostheceraeus moseleyi* Lang, 1884, a species that commonly feeds on *Clavelina lepadiformis* colonies in outer habitats. It can be noted that in this ascidian the incorporation of prey secondary metabolites by a flatworm predator was described for the first time (Kubanek et al. 1995, for *P. vittatus*). It seems likely that *P. moseleyi* is similarly able to use the ascidian metabolites for its own defence. This flatworm was not detected inside marinas. The worms were collected and placed in 6-well plates filled with filtered sea-water. After 1 d of establishment, 1 live zooid from the inner population and 1 from the outer (both from Blanes) was added to each well. After 12 h, we recorded which zooid, if any, had been consumed. We noted any difference between the zooids (such as wrinkles, size, form of stolons) to be able to tell which one has been consumed. Twenty-four replicate tests were performed.

As a generalist predator we selected the hermit crab *Calcinus ornatus* (Roux, 1830), which is present in both habitats. We used the same procedure as with the flatworm test. Twenty-four replicates were made, and consumption after 1 h was recorded. Note that the lengths of the observation times for this and the flatworm experiment was established after preliminary trials.

Juvenile mortality. To evaluate the possibility of survival of the 2 forms in the other's habitat, we cross-transplanted juveniles. In April 2000, mature inner and outer colonies from Blanes were collected. The brooding chamber of the zooids was then dissected in the laboratory, and the mature larvae that began swimming spontaneously were pipetted out and placed in 90 mm plastic Petri dishes filled with filtered sea water.

Larvae from about 12 colonies from each habitat were pooled and distributed in the Petri dishes. Twenty dishes were 'seeded' with 20 to 30 larvae each, 10 dishes for each population. The dishes were kept for 10 d at a constant temperature (13°C, the water temperature at sampling time) and under natural light/dark regimes. The water was changed every 3 d. After 10 d, the number of larvae successfully settled and metamorphosed (as judged by the open siphons) was recorded and their position mapped onto the dish lids. Of the 10 dishes with larvae from the inner habitat, 5 were submerged in the harbour and 5 along the rocky littoral. The same was done with the larvae plates from the outer habitat. The dishes were fixed underwater with elastic bands attached to nails driven into rocks. Care was taken in interspersing the treatments and in placing the dishes at the same depth and community inhabited by adult populations. Dishes were left undisturbed for 4 wk, after which they were taken to the laboratory and the number of surviving juveniles was recorded. The mappings on the lids allowed us to distinguish the experimental settlers from occasional natural settlements (a fact that occurred only in some dishes in the harbour). The survival of juveniles (each dish was considered a replicate) as a function of the origin and the place of transplantation was recorded and analysed.

Statistical analyses. For among-habitat comparisons of variables related to investment in reproduction and tunic production, as well as extract yield, toxicity and survival of transplanted juveniles, *t*-tests were used. A nested model ANOVA, with colony nested under the factor habitat, was used to compare fecundity among habitats. A 2-way ANOVA with habitat and month as main factors was used to ascertain differences in accumulation of heavy metals. Assumptions of normality and homoscedasticity were tested by Kolmogorov-Smirnov and Bartlett tests, respectively, before applying parametric analyses. For variables related to zooid and tunic weight, as well as for heavy metal concentrations, log-transformation was applied to satisfy these assumptions. Survival rates were arc-sine transformed prior to analysis.

The time course of the abundances in the fixed plots at both habitats was compared by a repeated measures analysis of variance. As the data did not comply with the assumptions of this test, even after transformation (Von Ende 1991), we used the randomisation technique described in Manly (1997) and implemented in a TurboPascal routine (available from the authors). This procedure consisted of a 2 stage permutation of the data: first, plots were randomly reassigned to the 2 localities and then readings for each plot were randomly rearranged among observation times. With this 2-level randomisation, the overall sum of squares, as well as the total between- and within-plot sums of squares, remains unchanged (Manly 1997). The significance of the *F*-ratios associated with each factor and their interaction was determined by comparing them with the distribution of *F*-ratios obtained by 999 randomisations of the data set plus the observed one.

The contingency table from the fish predation test with pellets was analysed with log-linear models. By leaving out of the model the appropriate variables and their interaction, we tested the significance of the variables considered. The likelihood of the outcomes of the specialist and generalist predator choices was estimated by comparison with a binomial distribution in which $p = q = 0.5$. All analyses were performed with Systat v 9 and Sigmastat v 1.

RESULTS

Morphology, abundance and reproductive cycles

Specimens of both habitats conform to the description of the species (e.g. Berrill 1950) in all respects. The larvae are also typical, with an anterior outgrowth that bears 3 simple, coniform papillae and a sensory vesicle with otolith and ocellus (see Turon 1988a, 1991). The zooids of the inner population were on average slightly larger than those from the outer one, although this was difficult to quantify on the collected material due to the high contractibility of zooids (even anaesthetised). We therefore used zooid weight for statistical comparison (see below). The number of rows of stigmata in the branchial sac was the same in both populations (up to 17 rows in mature zooids). No morphological character of the colonies, zooids or larvae was detected that allowed a distinction between the 2 forms.

From March 1998 to September 2001, strong seasonality was observed in *Clavelina lepadiformis* in fixed plots at Tossa de Mar, with active colonies present in winter and then disappearing (aestivation) during the period of highest temperatures, lasting from 4 to 7 mo (Fig. 1). Moreover, strong inter-annual differences in abundance were detected, with maximal densities in 1998 (up to a mean value of ca. 700 zooids m^{-2}) and lower densities in the following years. These 'bloom' dynamics in this population have been observed in the area with peak episodes every 4 to 7 yr (X.T. pers. obs.). Among-plot differences were also high, the coefficient of variation among them being 171.7 ± 19.0% (mean ± SE of the coefficient of variation found each month). The abrupt decrease in December 2000 was due to an accumulation of sediments that buried part of the walls of study, although the population partly recovered in the following months.

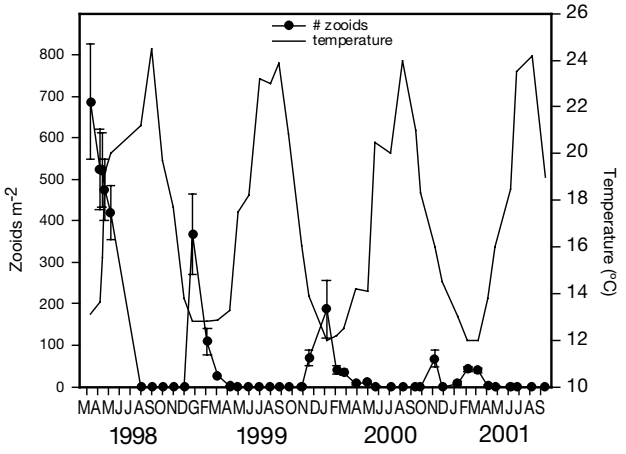

Fig. 1. *Clavelina lepadiformis.* Monthly time course of abundance, and water temperature at Tossa de Mar during the study period. Bars are standard errors

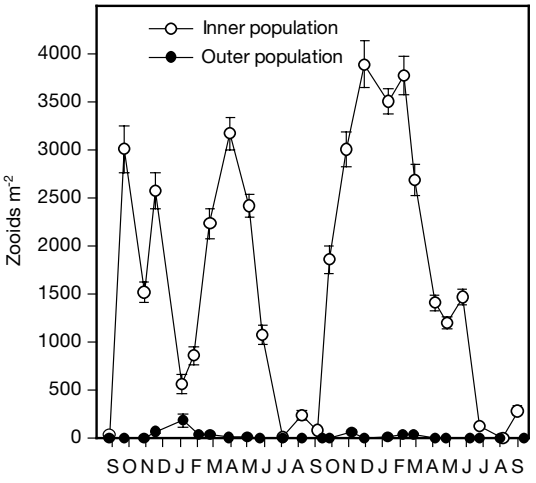

Fig. 2. *Clavelina lepadiformis.* Comparison of monthly abundances of an inner (Blanes harbour) and an outer (Tossa de Mar) population from September 1999 to September 2001. Bars are standard errors

From September 1999 onwards we also obtained data from Blanes harbour (Fig. 2). Zooid densities in the harbour were about 1 order of magnitude higher than in the outer population of Tossa during this period. The former peaked at ca. 3900 zooids m^{-2} (December 2000). Among-plot variability was lower than in the outer population (coefficient of variation 74.5 ± 13.1%, mean ± SE of the months studied), indicating a spatially more homogeneous population. In addition, the population did not disappear in summer and, although densities were very low in July 2000 and August 2001 (mean of 9 and 6 zooids m^{-2}, respectively), active zooids were found all year round. Densities varied greatly between observation times, with 3 peaks in October, December and April in the 1999/2000 season, and a peak during winter of 2000/2001, when densities were above 3500 zooids m^{-2} from December through February. The repeated measures analysis of variance (randomisation procedure) showed that the factors locality (F-ratio: 694.54; df: 1, 66), time (F-ratio: 95.26; df: 24, 1584), as well as their interaction (F-ratio: 84.64; df: 24, 1584), were highly significant (p = 0.001 of the randomised series in all cases), indicating a different time course of abundances at both habitats.

In the inner population of Blanes harbour, the zooids presented gonads and embryos from November 1999 until June 2000 (Fig. 3), although incubated larvae were mature (as determined by the development of both pigment spots and the anterior ampulla) from December onwards. In January, reproductive activity peaked, with 99% of zooids with gonads and 72% with mature larvae. A second peak of zooids with mature larvae (54%) was found in April. In contrast, from July to November, most zooids were immature or with testes only. Gonads were completely absent only in July, when densities were lowest. At this time, the population was composed of immature, isolated zooids that did not form colonies. The results indicate that in the inner population several batches of larvae can be released during the reproductive period. This results in a population composed of several cohorts, as shown by the common finding of colonies in various stages of development during winter-spring.

Table 1. *Clavelina lepadiformis.* Variables measured in zooids from the 2 habitats. Mean ± SE values are given. The value of the t-statistic, degrees of freedom and associated p-values are indicated

Variable	Inner habitat	Outer habitat	t (df)	p
Zooid + tunic weight	2.416 ± 0.249 mg	0.641 ± 0.166 mg	6.30 (28)[a]	<0.0001
Zooid weight	1.198 ± 0.131 mg	0.319 ± 0.072 mg	6.66 (28)[a]	<0.0001
Investment in tunic	1.121 ± 0.173	0.827 ± 0.106	1.52 (28)[a]	0.139
Weight of a larva	$3.86 \pm 4.1 \times 10^{-3}$ mg	$2.31 \pm 4.3 \times 10^{-3}$ mg	2.50 (18)[a]	0.022
Investment in reproduction	0.438 ± 0.05	0.424 ± 0.092	0.41 (18)	0.889

[a]Logarithmic transformation applied

Investment in reproduction and tunic production

Zooid weight of the inner population was higher in terms of both total weight and zooid weight without tunic. However, the relative investment in structural material (tunic) did not significantly differ between the 2 populations compared (Table 1, Fig. 4).

Fig. 5 compares reproductive parameters of the outer population with those of the inner population in April. The weight of the mature larvae was significantly higher in the inner than the outer population (Table 1). However, due to the higher weight of zooids in the former, the relative investment in reproduction did not vary between the 2 forms (Table 1).

The results of the nested model ANOVA on fecundity estimates (Table 2) showed that fecundity was not different between populations, but that there were significant differences between colonies of the same locality. The same results (Table 2) were obtained including the ova in the ovary in the fecundity estimate (fecundity = 66.93 ± 17.6 in the outer and 62.87 ± 6.73 in the inner population) or considering only embryos in the brooding chamber (59.80 ± 10.44 in the outer and 55.05 ± 6.16 in the inner population).

Table 2. *Clavelina lepadiformis.* Nested analyses of variance on the fecundity of zooids at both habitats. SS: sum of squares; MS: mean square

Source	SS	df	MS	*F*-ratio	p
Embryos + ova					
Habitat	925.8	1	925.8	1.04	0.314
Colony (habitat)	41498.1	17	2441.1	2.75	0.005
Error	32854.7	37	888.0		
Only embryos					
Habitat	184.1	1	184.1	1.15	0.291
Colony (habitat)	30178.8	17	1775.2	11.06	<0.001
Error	5938.5	37	160.5		

Accumulation of heavy metals

The analysis of Cu, Pb, V, Hg and Cd in the tissues of the inner population in Blanes harbour revealed that only the first 3 elements were present in detectable amounts, Cu being by far the most abundant metal.

The lowest values for Cu were obtained from April through July, and the maxima were observed from October to December in both years (Fig. 6). Overall, the mean values ranged from 54.51 ppm (July) to 248.15 ppm in December 2000. As regards Pb, no clear pattern was observed, but again the minimal mean values were observed in July (8.78 ppm). Maxima were observed in April and in December 1999 and 2000 (mean values above 40 ppm). V accumulation was low from May to September, with a minimum in May (1.82 ppm), while the maximal values were observed in January and December of 1999 and 2000 (mean values above 25 ppm). It appears, therefore, that heavy metal accumulation is highest for the 3 metals in winter, and lowest in spring-summer.

Comparisons of accumulations of Cu, Pb and V between the inner and outer populations of Blanes in April and December 2000 showed contrasting pat-

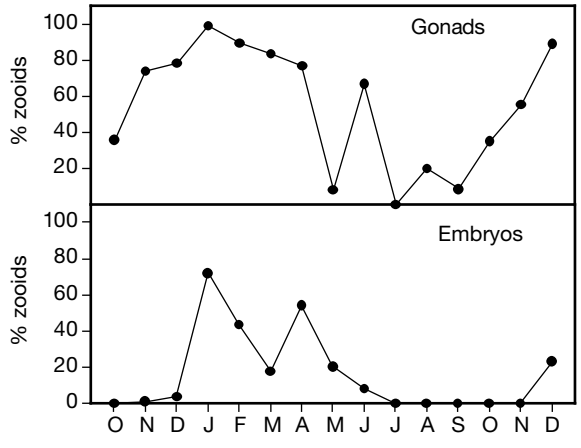

Fig. 3. *Clavelina lepadiformis.* Percent zooids of the Blanes harbour population with gonads and incubating embryos or larvae from October 1999 to December 2000

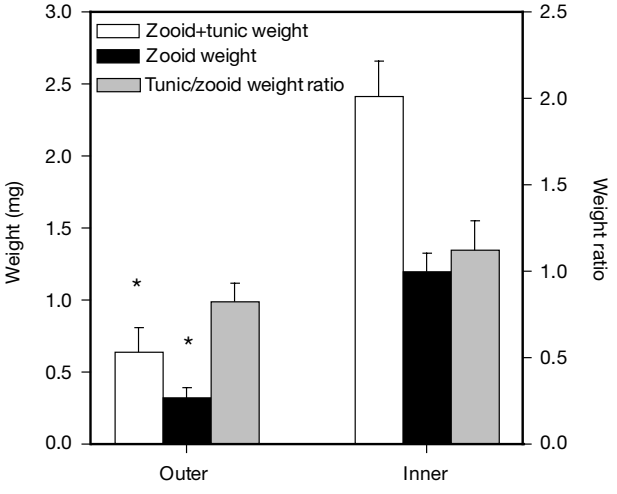

Fig. 4. *Clavelina lepadiformis.* Comparison of the zooid weights (with and without tunic) and the production of structural material (tunic) in an inner (Blanes harbour) and an outer (Tossa de Mar) population. *Variables for which statistical differences between habitats have been found. Bars are standard errors

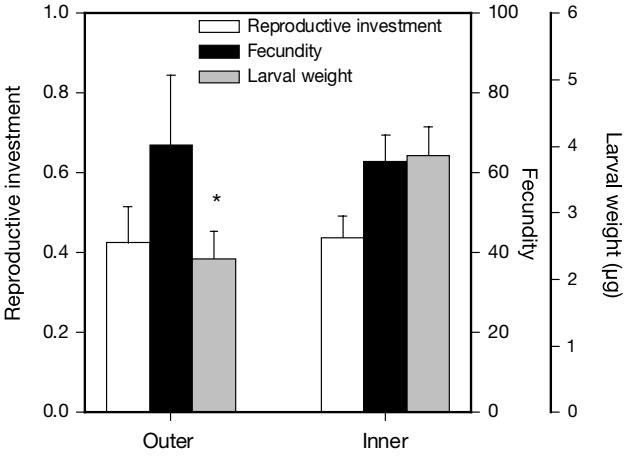

Fig. 5. *Clavelina lepadiformis.* Comparison of the reproductive investment (in weight ratio), fecundity and larval weight in an inner (Blanes harbour) and an outer (Tossa de Mar) population. *Variables for which statistical differences between habitats have been found. Bars are standard errors

terns (Fig. 7). In the first 2 metals, there was a much higher concentration in the inner population. The Cu values increased in both habitats in December, and the differences between the 2 populations were also greater in December. Thus, for Cu there was between 5 (April) and 8 (December) times more Cu in the inner population. A 2-way ANOVA showed a significant effect of the factors habitat and month, while the interaction was not significant (Table 3). For Pb the difference was 5-fold in April and 10-fold in December. The corresponding 2-way ANOVA also showed significant effects of habitat and month, and a non-significant interaction (Table 3). On the other hand, the dynamics for V clearly differed, with similar values at both locations in April and slightly higher values in the inner population in December. A 2-way ANOVA revealed that neither the effect of habitat type, month nor interaction of the 2 was significant (Table 3).

Table 3. *Clavelina lepadiformis.* Analyses of variance on the concentrations of heavy metals found in *C. lepadiformis* tissues on 2 sampling dates. Values were log-transformed prior to analysis. SS: sum of squares; MS: mean square

Source	SS	df	MS	*F*-ratio	p
Cu					
Habitat	3.043	1	3.043	259.38	<0.0001
Month	1.075	1	1.075	91.62	<0.0001
Habitat × Month	0.018	1	0.018	1.51	0.237
Error	0.188	16	0.012		
Pb					
Habitat	3.51	1	3.51	267.35	<0.0001
Month	0.137	1	0.137	10.40	0.005
Habitat × Month	0.019	1	0.019	1.50	0.234
Error	0.210	16	0.013		
V					
Habitat	0.043	1	0.043	3.81	0.069
Month	0.047	1	0.047	4.13	0.059
Habitat × Month	0.045	1	0.045	3.92	0.065
Error	0.182	16	0.018		

Toxicity and palatability

Two variables were analysed in the toxicity study, the percentage of crude extract and the EC_{50} values. For the former (Fig. 8), the inner colonies yielded significantly less (*t*-test, $p < 0.01$) extract than outer ones (4.09 ± 0.76% and 6.58 ± 0.96%, mean ± SE, respectively). For EC_{50} (Fig. 8), the values were significantly (*t*-test, $p < 0.01$) higher (i.e. the extracts were less toxic) inside the harbour than outside (491.4 ± 48.2 ppm and 322.1 ± 42.1 ppm, respectively).

In the assay with treated artificial food and the damselfish *Chromis chromis*, 52.3% of pellets treated

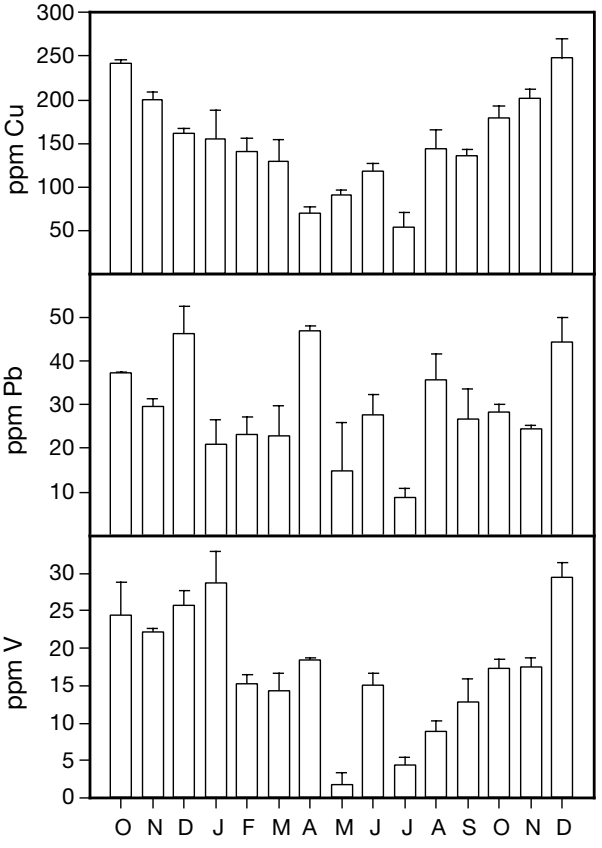

Fig. 6. *Clavelina lepadiformis.* Concentration of heavy metals in the ascidian tissues at the Blanes harbour from October 1999 to December 2000. Bars are standard errors

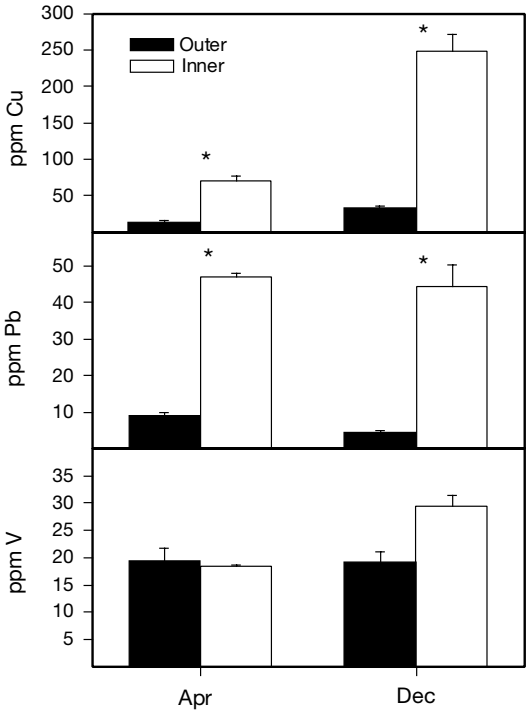

Fig. 7. *Clavelina lepadiformis.* Comparison of heavy metal concentrations in the inner and outer populations at the locality of Blanes in April and December 2000. *Significant differences. Bars are standard errors

with extracts from the inner population and 50% of those with extracts from the outer population were rejected by fish, while only 18 and 19.1%, respectively, of the control pellets were rejected in each test. The log-linear analyses showed that there was a significant deterrency of both treatments (i.e. populations) with respect to their controls (likelihood ratio chi-square = 20.12, df = 4, $p < 0.001$), while treatments did not differ among them (likelihood ratio chi-square = 4.81, df = 4, $p = 0.308$).

Regarding the specialist predator test (*Prosthecerae us moseleyi*), after 12 h, 10 flatworms had consumed no zooid, 8 had consumed the zooid from the inner population, 5 the outer one and 1 had consumed both. Among those that consumed 1 zooid only, the proportion of the 2 forms consumed did not deviate significantly from 1:1 (binomial test, $p = 0.291$). In all cases, the flatworm sucked the inner tissues through its everted pharynx, leaving only tunic and faecal pellets. In the generalist predator assay (*Calcinus ornatus*), the results of the test were: no consumption, 1; both zooids consumed, 5; inner zooids consumed, 10; and outer zooids consumed, 8. The proportion of predators that chose one or the other forms did not deviate from the expectancy of 50% each (binomial test, $p = 0.407$).

Juvenile mortality

A total of 146 and 174 larvae from inner and outer populations, respectively, settled and metamorphosed successfully in the experimental Petri dishes during the 10 d laboratory incubation. The juveniles originating from the outer habitat did not feature significant differences in survival rate according to the habitat where they were transplanted (mean of 46.3% in the outer and 35.6 % in the inner habitat, *t*-test, $p = 0.421$), while differences were highly significant in favour of the autochthonous habitat in the juveniles originating from the harbour population (mean survival of 4.3% in the outer and 31.9% in the inner habitat, *t*-test, $p = 0.005$) (Fig. 9).

DISCUSSION

We detected large differences between the inner and outer populations for most of the biological parameters studied. However, no clear morphological distinction could be made, aside the zooids of the inner population being larger (which is not reflected by a higher number of stigmata rows). As size is dependent on food availability, no taxonomic significance is usually attached to this parameter in ascidians. No formal taxonomic distinction between these 2 forms can therefore be established at present, although it has been suggested that they may constitute an instance of cryptic speciation (Tarjuelo et al. 2001). In fact, there is more genetic differentiation among habitats on the same locality than among geographically separated populations from the same type of habitat (Tarjuelo et

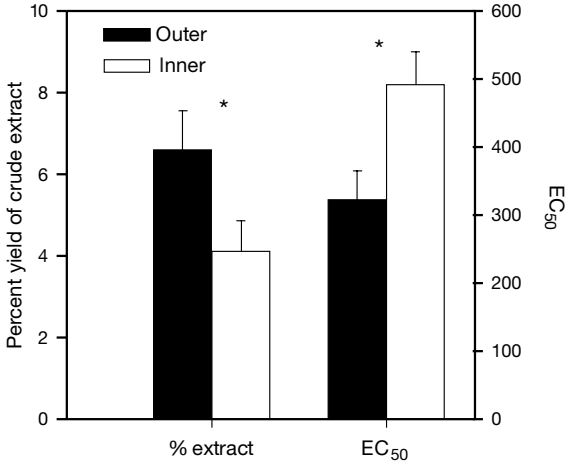

Fig. 8. *Clavelina lepadiformis.* Percent of crude extract and toxicity (in EC_{50} values) in the inner and outer populations at the locality of Blanes. *Significant differences. Bars are standard errors

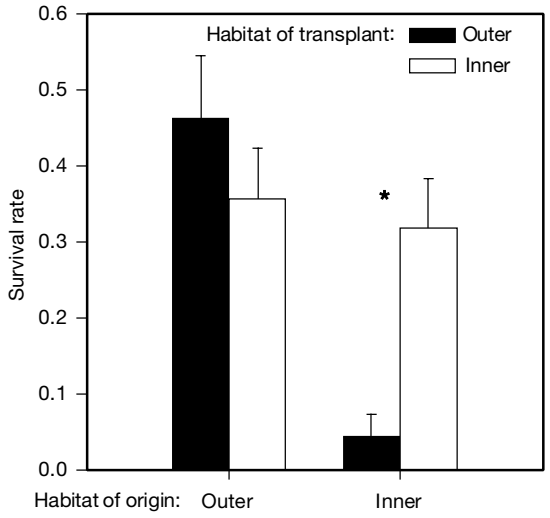

Fig. 9. *Clavelina lepadiformis.* Survival of juveniles of transplanted between the inner and outer populations of Blanes. *Significant differences. Bars are standard errors

al. 2001). We do not know whether the larvae of these forms can reach each other's habitats. In the ca. 100 specimens genetically studied in the Spanish littoral, a colony of the outer type was never found inside harbours, and only in 1 instance was 1 colony of the inner type found on the external side of the Blanes harbour breakwater (I.T. pers. obs.). Ascidian larvae are lecitotrophic and short-lived, especially those of colonial forms (Svane & Young 1989), so a confinement effect may explain a lack of gene flow between habitats.

Abundance cycles showed a distinct pattern between populations. The density values were ca. 1 order of magnitude higher in the inner population. The dynamics of the outer population studied were clearly seasonal, with peak abundance in winter and large differences in density from year to year. In addition, the outer population showed prolonged aestivation, lasting for several months. In contrast, in the inner population, abundance maxima were found from autumn to spring with strong changes in density from one month to the next, and minimal abundance was detected in summer. A reduced aestivation was evident, as active individuals were found all year round, although with minimum abundances in summer, when only scarce, immature zooids were detected. It cannot be said at present whether this form also produced dormant stolons, but at least some of the zooids were able to pass the unfavourable season in an active form. Aestivation and other resting periods in ascidians have been commonly linked to temperature (Turon 1992, Turon & Becerrro 1992), but in this case the reduced aestivation in the inner population did not correspond to any significant temperature difference among populations. It can be argued that we have made observations of the cycles at different years in the inner and outer population, but the pattern reported here has been observed for many years in the outer population and for 3 yr in the inner one (X.T. pers. obs.); therefore, it seems to be a constant feature. Differences in food availability at the 2 habitats seems a likely explanation for the pattern found.

Reproductive cycles also differed. The outer population at Tossa de Mar, as described by Tarjuelo (2001) from 1997 to 1999, showed a restricted reproductive season. Colonies with immature zooids appeared from October to December according to the year, gonads appeared from January to February and mature larvae from February to March. After a period of about 2 mo incubation, larvae were released, the colonies disintegrated, and only juvenile, immature zooids not forming colonies could be seen for about 1 mo preceding the disappearance of the population in May/June. These observations agree with those of Turon (1988b) for the same population. In this outer population, therefore, a consistent feature was a single cycle of larval production, with the sequential appearance of testes, ovaries, embryos and larvae. In the inner population, however, mature larvae were present from December through June, with peaks in January and April. This indicates that larvae of this community are released throughout winter and spring. As zooids degenerate after sexual reproduction (Berrill 1951), the population is a mosaic of several cohorts. Colonies with large, mature zooids coexisted with those of small immature zooids during most of the year, an observation which was not made in the much more homogeneous outer population. The 2 maxima of mature larvae observed in January and April 2000 may indicate that there were 2 main generations during this year (lacking data for more years, we cannot determine whether this feature is constant). Colony degeneration after the winter peak could account for the low densities observed in January 2000 (Fig. 2), while the spring peak preceded the disappearance of most colonies and the arrival of juveniles that can survive the summer and rebuild the population from September/October onwards. A more detailed study of the different cohorts of colonies in the inner population would possibly require a sampling frequency higher than once per month.

With respect to investment in structural material (tunic), in spite of a significantly larger zooid weight of the inner population, the relative investment in tunic did not differ between forms. The same was true for fecundity and the relative investment in reproduction (in weight ratio). Larval weight was significantly higher in the inner population, which may be related to the overall larger zooids. Thus, the relative investment

in reproduction per zooid did not vary between populations, but the course of the reproductive cycles was different, as the reproductive period was longer and several cohorts overlapped in the inner population, a fact possibly attributable to differential food availability at the 2 habitats.

The accumulation of Cu presented a clear cycle with minima between April and July and maxima in autumn/winter. Cu is the main metal present in the Blanes harbour (Pinedo 1998, Cebrian 2001). Pb and V were present in ascidian tissues in smaller amounts and did not show clear cycles, although minimal values were again found in spring/summer. This behaviour may correspond to the dynamics of the metals in the harbour, but more likely it is the result of the dynamics of the ascidian, whose population in summer was composed mostly of juvenile colonies, which had had little time of exposure to metals. The inner population accumulated significantly more Cu and Pb (between 5 and 10 times more) and the differences were consistently higher in December than in April 2000. The between-habitat differences in metal concentration in tissues were much higher than those found in the corresponding water column (Cebrian 2001), which suggests that ascidians inside the harbour incorporate heavy metals by filtering resuspended sediments, which are much richer in metals than the surrounding water (Pinedo 1998).

The dynamics of V, on the other hand, clearly differed, as no significant differences could be found between the 2 habitats. In this respect, it should be noted that ascidians accumulate this metal in blood cells (Hawkins et al. 1983, Martoja et al. 1994). The biological function of V in ascidians is still disputed, although it may act against predation or as an antimicrobial agent (Rowley 1983, Martoja et al. 1994). It cannot be considered a pollutant that is passively incorporated into tissues but rather, it is actively incorporated and fulfils metabolic roles in this group. This may explain why no differences in the accumulation of this metal were detected between populations.

Clavelina lepadiformis produces highly active secondary metabolites (lepadines, Steffan 1991, Biard et al. 1996). We found that the inner population produced significantly fewer amounts of secondary metabolites and was also less toxic in a standard toxicity test. This may partly explain the higher growth of this population, if there is a cost for the production of chemical defences. An ecological interpretation of these differences is, however, difficult. We did find a similar deterrency level of extracts of both forms against a generalist fish predator. We did not find any significant preference either by a generalist or a specialist benthic predator assayed on live zooids, although there was a pattern of less consumption of the outer form, and we should acknowledge that small sample size limited the power of the test. On the other hand, the guild of potential predators is tremendously diverse, and any other predator may be more relevant for the ascidian population than the ones tested. Moreover, the distinct investment in chemical production may not be related to defence against predation, but to fouling avoidance or competition, the other functions usually assigned to bioactive secondary metabolites (Hay 1996). The same metabolites may even serve different functions (Becerro et al. 1997). Space competition is thought to be less crucial for stolonic invertebrates such as *C. lepadiformis* than for other types of growth morphologies (Jackson 1979); therefore, it might not be a relevant factor to explain differences in chemical production. On the other hand, a higher fouling pressure in the outer habitat, due to a more diverse larval pool, is likely. To unravel the factor or factors behind the differences in metabolite production in the 2 populations would require specific experimental work. In addition, larvae of the outer form are known to be palatable to several predators (Tarjuelo et al. 2002). It would therefore be informative to compare levels of larval defence among populations.

In our transplant experiment, juveniles originating from outer colonies survived in both habitats, but those from the inner habitat had a low survival rate in the outer habitat. Thus, even if confinement effects did not prevent the exchange of larvae totally, post-settlement mortality could explain, at least partially, the maintenance of the discontinuity between populations. Whether differential predation or any other physical or biological parameter can explain these differences in survival cannot be ascertained at present.

To summarise, the 2 forms of *Clavelina lepadiformis* have distinct biological traits. The inner population shows rapid growth, reproducing both asexually and sexually during most of the year, which results in a dense population that carpets submersed surfaces, with large abundance fluctuations from one month to the next, which are possibly linked to the succession of generations. The outer population shows a more restricted growth but less fluctuation between observation times. A single generation is produced per year and the juveniles of the year do not reproduce but enter aestivation. The production of secondary metabolites is higher in this form, which may reflect a higher investment in chemical defence. *C. lepadiformis* accumulates heavy metals, but pollutants in the harbour do not depress reproduction or growth in this population with respect to the outer ones. It cannot be said at present which threshold level of pollutants has to be reached to produce disruptive effects in the biology of this species.

Overall, the inner population had more opportunistic features (high growth rate, high density, long reproductive period and reduced aestivation), while that of the outer habitats showed more conservative traits (higher investment in secondary chemistry, low growth rates, dormancy during the adverse season). The effects of confinement and/or differential post-recruitment mortality may account for the persistence of the physical separation of these forms allowing divergent adaptation. Specific work is required to ascertain the genetic/environmental component of these adaptations and to definitely settle whether the 2 clades represent 2 ecotypes or 2 distinct species.

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