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The reproductive cycle of *Patella candei gomesii* Drouët, 1858 (Mollusca: Patellogastropoda), an Azorean endemic subspecies

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Summary

Patella candei gomesii is morphologically plastic comprising two ecomorphs. The "smooth limpet" is characteristic of the eulittoral zone, whereas the "fly limpet" is mainly found higher in the shore, on the splash zone of exposed areas. Their reproductive strategies are poorly understood. This study investigated the reproductive cycles of the ecomorphs using histological techniques. The annual cycles were found to be similar. Both sexes exhibited synchronous patterns and were mature most of the year. In April, significant increases were observed in the relative volume occupied by previtellogenic and vitellogenic cells in females, and by spermatogonia, spermatids and spermatocytes in males. The maximum values for mature oocytes and spermatozoa were observed in July. It is concluded that the breeding season of *P. candei gomesii* lasts the whole year peaking in the summer (when reproductive condition is highest and the main spawning event must occur). The implications of these findings for the taxonomy and conservation of the subspecies are further discussed.

Key words: *Patella candei gomesii*, gametogenesis, Azores, reproductive cycle

Introduction

The limpet *Patella candei gomesii* Drouët 1858 belongs in the *Patella candei* complex, which is exclusive to Macaro-nesia (Azores, Madeira, Canary Islands, Selvagens and Cabo Verde). The subspecies *P. candei gomesii* is endemic to the Azores (Titselaar, 1998; Weber and Hawkins, 2002), occurring subtidally and in mid and high shores, especially on boulders (Côrte-Real et al., 1996; Hawkins, et al. 2000). Within the Azorean archipelago, this subspecies is morphologically plastic occurring as two habitat morphs: the "smooth limpet" and the "fly limpet". The former is characteristic of the eulittoral area, whereas the latter is mainly found higher on the shore, especially the splash

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zone in exposed areas (Hawkins et al., 1990). Plasticity has been described in other patellid species and linked to environmental variation (Lewis and Bowman, 1975).

Ridgway et al. (1998) emphasized the importance of knowledge on the biogeography of patellid species to understand the evolutionary trends in the NE Atlantic. The determination of the reproductive cycles of *P. candei gomesii*'s ecomorphs should provide information to clarify pending issues on the subspecies' taxonomical status. According to Martins et al. (1987), the gonochoristic *P. candei gomesii* spawns throughout the year, without synchronized resting periods. However, important information might have been left out since the two morphs were not addressed separately in the study. This is relevant not only because different reproductive cycles have been reported for separate conspecific populations of molluscs (Sutherland, 1970; Geller, 1990), but also because similar reproductive cycles have been traced in populations under different environmental conditions (Henninger and Hodgson, 2001).

Ultimately, knowing about the reproductive cycles of *P. candei gomesii* is important for the conservation of this subspecies. Due to its endemic character, there are no populations outside the Azores archipelago to act as reservoirs for recruitment during recovery, which, together with larval loss, makes the subspecies vulnerable (Hawkins et al., 2000). These authors suggested that there is a risk of complete extinction, taking into account its endemic nature and the existence of over-exploitation. The enormous impact on rocky shore communities of direct exploitation of resources is well known as targeted species show declining populations (Thompson et al., 2002). In São Miguel, *P. candei gomesii* is commercially exploited (Martins et al., 1987; Hawkins et al., 2000). Água d'Alto beach has been repeatedly harvested for limpets; these were conspicuously absent in 1992 (Morton et al., 1998), but did recover in 1994 when protection from indiscriminate collecting was enforced by the Azorean Government (Morton et al., 1998). Information regarding the reproductive cycles of this subspecies is crucial for an adequate management of limpet harvests.

Several studies have been undertaken concerning the reproductive cycles of *Patella* species (Orton et al., 1956; Orton and Southward, 1961; Blackmore, 1969; Lewis and Bowman, 1975; Bowman and Lewis, 1986). Most are based on the macroscopic index described by Orton et al. (1956). The present work reports on a preliminary investigation of the reproductive seasonality of the two ecological morphotypes of *P. candei gomesii* in São Miguel (Azores). A quantitative assessment of gametogenesis in *P. candei gomesii* was carried out applying histological methods.

Materials and Methods

Study area

Limpets were collected from a boulder/cobble beach at Água d'Alto (São Miguel, Azores, Portugal). Specimens of the two ecomorphs were sampled in characteristic habitats, separated horizontally by 100–150 m: "fly" specimens from a highly exposed area, in the supralittoral zone; "smooth" specimens from rolled stones and cobble, in the intertidal zone. Harvesting took place at four sampling periods: October 2002 (autumn); January 2003 (winter); April 2003 (spring) and July 2003 (summer).

Morphometric analysis

Quantitative measurements of shell length (SL, greatest distance between the anterior and posterior ends of the shell), shell width (SW, greatest distance between margins perpendicular to the anterior/posterior axis) and shell height (SH, greatest vertical distance from the apex to the base of the shell) were performed to the nearest 0.05 mm using vernier callipers. In addition, total fresh weight (FW) of each individual and shell dry weight (SDW) were measured using a precision scale (0.0001 g). The shell length/shell height ratio was calculated.

Gonadal maturation state

For each sampling period, 16 specimens (eight males and eight females) were sampled for histological analysis in both habitats. The fresh weight was obtained before excision of the gonad for histological purposes. Testes and ovaries were fixed in 10% formalin and embedded in paraffin. Serial sections, 7 µm thick, were stained with Mayer's haemalum and eosin (Martoja and Martoja-Pierson, 1970). The relative volumetric density of gametes was estimated using the M168 Weibel Multipurpose Test System (Weibel, 1979).

Four stages of spermatogenesis were identified based on the classification of Griffond et al. (1991): (1) spermatogonia — medium-sized cells rectangular in shape viewed by light microscopy, with a large nucleus in relation to the quantity of cytoplasm, always located near the acinus epithelium; (2) spermatocytes — smaller than spermatogonia with basophilic cytoplasm; (3) spermatids — smaller than spermatocytes, spheroid in shape and slightly more basophilic than spermatocytes; (4) spermatozoa — with strong basophilic head and esosinophilic tail. With light microscopy, no differentiation was made between spermatocytes II and I.

Following Hill and Bowen (1976), three stages of development were distinguished during oogenesis: (1) previtellogenic oocytes (PV), small, rounded and with strong basophilic cytoplasm; (2) vitellogenic oocytes (V), larger than the previous ones, irregular in shape, sometimes with multiple visible nucleoles, cytoplasm with slight granulations and lightly basophilic; (3) maturing oocytes (M), larger than the vitellogenic oocytes, round in shape with eosinophilic and granular cytoplasm.

Statistical analysis

In order to identify the gonadal maturation state, scores for volumetric density were summed for each specimen and converted to percentages.

Data were analysed with Statistica V.5.1 (StatSoft). The mean and standard error were calculated. Relative volumetric density of each oogenesis and spermatogenesis stage was compared using a two-way ANOVA (for spermatogonia arcsin transformation was performed to comply with requirements for ANOVA) or using the Kruskal–Wallis test (for previttelogenic cells, as data did not comply with ANOVA assumptions). Multivariate analyses were undertaken to assess for seasonal patterns.

Results

This study focused on investigating the reproductive cycles of both morphs. The two sexes were analysed. The results for oogenesis and spermatogenesis are presented separately, in sequence, so as to facilitate comparisons between morphs.

Oogenesis

Generally, the two morphotypes followed the same pattern. The differences due to morphotype concerning the three oogenic stages were not statistically significant (Table 1; Fig. 1A and C). The relative volumetric densities of previttelogenic oocytes were relatively low in October, January and July (fly: 8.6%, 4.2% and 2.2%, respectively; smooth: 4.8%, 6.6% and 3.8%, respectively), with a small peak in April, particularly for the smooth morphotype (21.41%). Medians obtained for April were significantly different from those for the other periods (Kruskal–Wallis, H = 32.7844, p<0.001, Table 1). The percentage volume of vitellogenic cells was low during October, January and July (less than 15%), increasing significantly in April (24.50% fly; 30.71% smooth; ANOVA, p<0.001, Table 1). Maturing oocytes comprised most of the gonadal volume throughout the entire sampling

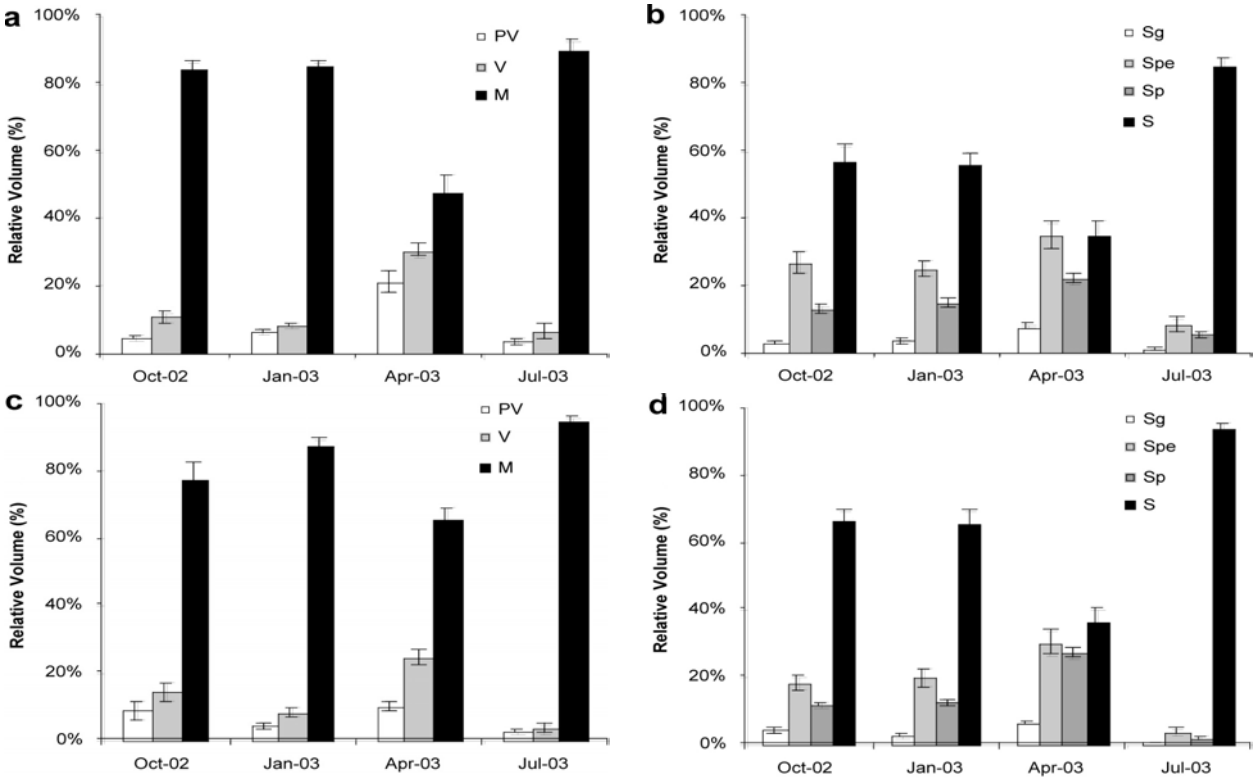

Fig. 1. Mean values and standard errors of the previtellogenic (PV), vitellogenic (V) and maturing oocytes (M) for "smooth" females (A) and "fly" females (C). Mean values and standard errors of the spermatogonia (Sg), spermatocyte (spe), spermatid (sp) and spermatozoa (S) for "smooth" (B) and "fly" males (D).

Table 1. Two-way ANOVA results for each gametogenic stage

Source of Variation	df	SS	MS	F	p	
Previtellogenic [a]						
Season	-	-	-	32.7844	0.0000	***
Morphotype	-	-	-	1.9127	0.1667	N.S.
Vitellogenic						
Season	3	0.47628	0.15876	51.5395	0.0000	***
Morphotype	1	0.00418	0.00418	1.3571	0.2490	N.S.
Season x Morphotype	3	0.02090	0.00697	2.2616	0.0912	N.S.
Residual	56	0.17250	0.00308			
Total	63	0.67386	0.01070			
Mature						
Season	3	2.97347	0.99116	45.1412	0.0000	***
Morphotype	1	0.08458	0.08458	3.8520	0.0547	N.S.
Season x Morphotype	3	0.22182	0.07394	3.3675	0.0248	*
Residual	56	1.22958	0.02196			
Total	63	4.50945	0.07158			
Spermatogonia [b]						
Season	3	0.60638	0.20213	33.3770	0.0000	***
Morphotype	1	0.04495	0.04495	7.4225	0.0087	**
Season x Morphotype	3	0.00870	0.00290	0.4791	0.6981	N.S.
Residual	54	0.32702	0.00606			
Total	61	0.98705	0.01618			
Spermatocyte						
Season	3	0.57416	0.19139	35.2532	0.0000	***
Morphotype	1	0.04198	0.04198	7.7331	0.0074	**
Season x Morphotype	3	0.00807	0.00269	0.4952	0.6871	N.S.
Residual	54	0.29316	0.00543			
Total	61	0.91737	0.01504			
Spermatid						
Season	3	0.20365	0.06788	73.2993	0.0000	***
Morphotype	1	0.00242	0.00242	2.6094	0.1121	N.S.
Season x Morphotype	3	0.00584	0.00195	2.1024	0.1107	N.S.
Residual	54	0.05001	0.00093			
Total	61	0.26192	0.00429			
Spermatozoa						
Season	3	1.82066	0.60689	68.6040	0.0000	***
Morphotype	1	0.06610	0.06610	7.4720	0.0085	**
Season x Morphotype	3	0.01573	0.00524	0.5929	0.6224	N.S.
Residual	54	0.47770	0.00885			
Total	61	2.38019	0.03902			

[a]Kruskal–Wallis test was used.
[b]Data transformed to arcsine.
N.S., non significant; *Significant at p <0.05; **Significant at p <0.01; ***Significant at p < 0.001.

periods. Maximum values (around 80%) were found for October, January and July. For mature cells, the interaction between the factors Season and Morphotype was significant (ANOVA, p<0.05, Table 1), meaning that the differences observed in different seasons are not independent of morphotype, even though differences among morphotypes are not significant.

Cluster analysis (Fig. 2A) showed a clear seasonal trend with three main groups. Most April samples cluster together in a separate group (group A, Fig. 2A), with the rest of the samples comprising a major group (group B). Within this group, two subgroups are formed: group B1, mainly July samples; and group B2, most samples from October and January. This seasonal trend is also shown by the Multidimensional Scaling diagram, Fig. 2B). April and July samples were separated and October and January samples were distributed in the middle. It is relevant that specimens from the ecomorphs have grouped together in terms of the relative volume of each cell type, since it indicates that both follow similar reproductive cycles.

Spermatogenesis

The general pattern was very similar in both morphotypes (Fig. 1B and D). The percentage volume of spermatogonia was lower in October and January, increasing to a maximum in April and decreasing to the lowest values in July. This trend was also observable for spermatocytes and spermatids. The differences found between seasons were statistically significant

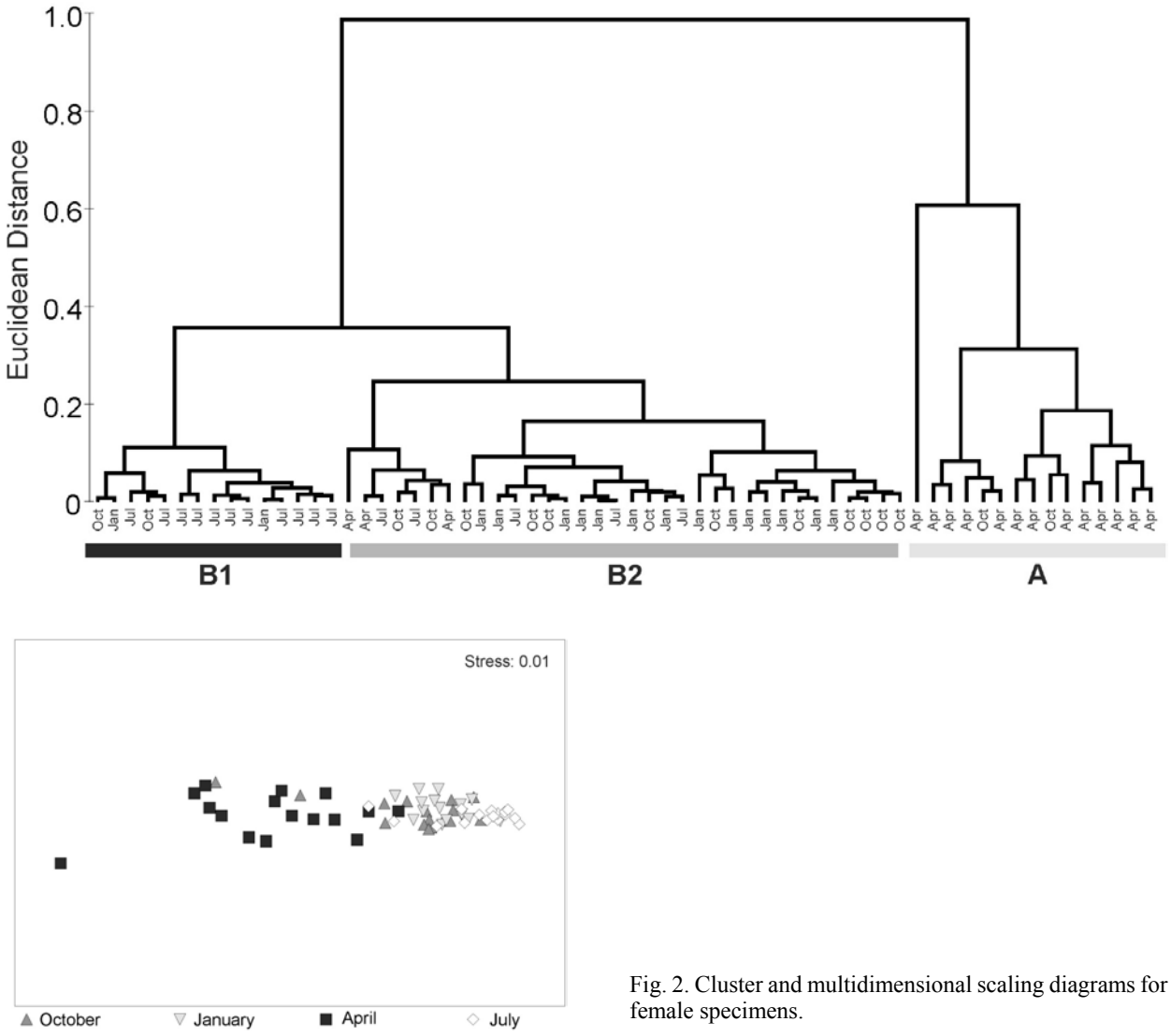

Fig. 2. Cluster and multidimensional scaling diagrams for female specimens.

(ANOVA, $p<0.001$, Table 1), with three homogeneous groups (Tukey HSD test, $p<0.05$), July < October and January < April. In October and January, spermatozoa comprised approximately 60% of the gonadal volume, decreasing to about 35% in April, and increasing to over 85% in July. There were three homogeneous groups, April < October and January < July (Tukey HSD test, $p<0.05$), reflecting statistically significant differences between seasons (ANOVA, $p<0.001$).

Results from multivariate analysis supported a clear seasonal trend. They did not, however, separate the morphotypes, suggesting that males from both ecomorphs follow similar reproductive cycles. Cluster analysis (Fig. 3A) showed three different groups. The majority of April samples clustered together in a separate group (group A′, Fig. 3A) with the remaining samples forming two subgroups in a larger group: group B1′, mainly July samples; group B2′, most samples from October and January. Multidimensional Scaling also showed the same seasonal pattern (Fig. 3B). April and July samples were clearly separated, the latter presenting a higher similarity to October and January samples.

In contrast to oogenesis, differences between morphotypes were statistically significant for spermatogonia, spermatocyte and spermatozoa (ANOVA, $p<0.01$, Table 1). The interaction of the two sources of variation was, however, not statistically significant (Season×Morphotype, Table 1), showing that differences between seasons were independent of the differences among morphotypes.

Morphometric parameters

The morphometric parameters had similar values across the time scale (Fig. 4). The two ecomorphs did

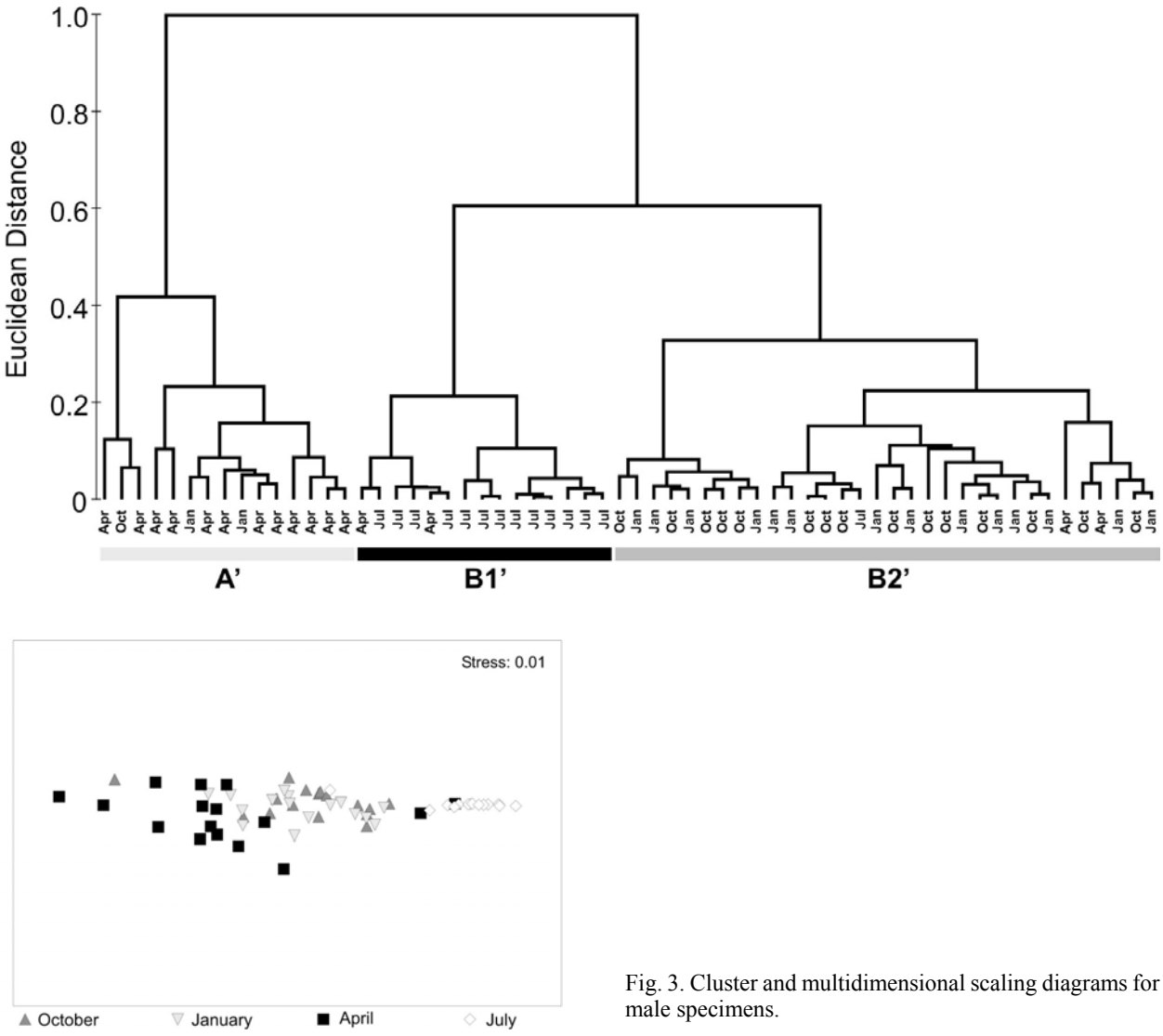

Fig. 3. Cluster and multidimensional scaling diagrams for male specimens.

differ in the morphometric variables studied, but the pattern was similar among seasons. The morphometric parameters were concordant with unpublished data concerning larger samples (total of 117 fly limpets and 132 smooth limpets) for the same sites and sampling periods.

Discussion

The main goals of this investigation were the definition and comparison of the reproductive cycles of the two ecomorphs of *P. candei gomesii*. The results revealed that the morphs' annual reproductive patterns are similar. This is in contrast to what has been described for *Patella depressa* from exposed and sheltered sites in the centre of Portugal (Cabo Raso, Cascais). Morais et al. (2003) found that, in the summer, gametogenesis in limpets from the exposed site was more advanced (mainly stage III and stage IV) than that in limpets from the sheltered site (stage III), according to the maturation scale of Orton et al. (1961). The similar reproductive cycles of the two ecomorphs described in this study may reflect phylogenetic preservation of reproductive patterns, as referred to by Henninger and Hodgson (2001).

This paper presents findings that challenge the thesis advanced elsewhere of an absence of annual resting periods in this limpet (Martins et al., 1987) by describing quantitative and qualitative seasonal changes in the gonads of *P. candei gomesii*. Even though females were mature most of the year, the relative volume occupied by previtellogenic and vitellogenic oocytes over mature cells increased significantly in April, but decrease to minimum levels in July, when maximum number of mature cells was registered. The production of new oocytes is the most likely

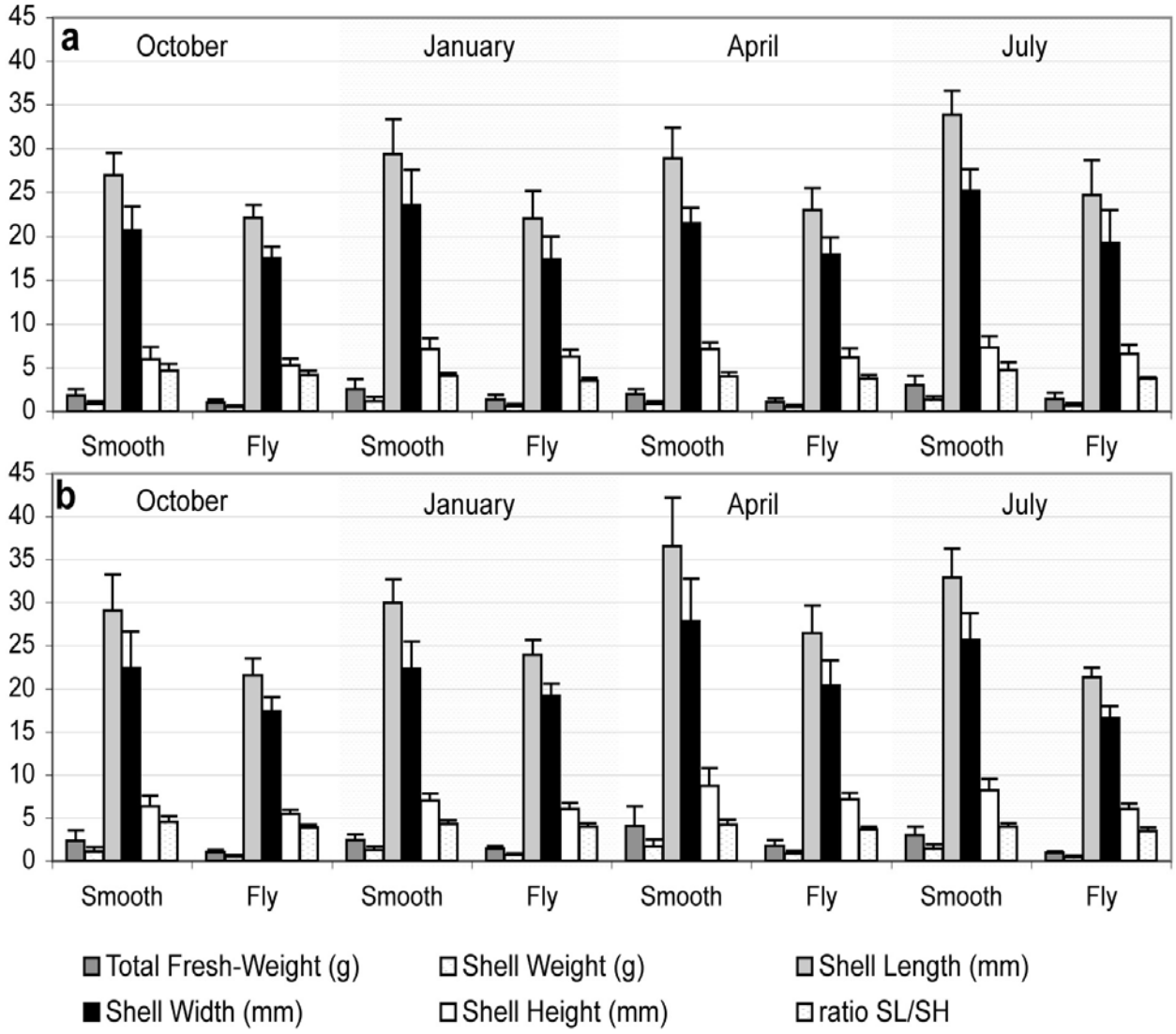

Fig. 4. Averages and standard errors of fresh weight of the animal (FWG) and weight of the shell (WS), length (SL), width (SW) and height (SH) for males (a) and females (b), morphs are discriminated.

explanation for this pattern, thus suggesting that *P. candei gomesii* females spawn mainly in the summer. Males present a similar annual pattern: the abundance of spermatogonia, spermatocytes and spermatids increases sharply in April, but decreases to minimum values in July, when maximum values of spermatozoa are observed. The annual cycle suggested by our data, featuring a major spawning event (but with an extended spawning period until January), is consistent with those of most patellid species, which have a marked annual cycle with a single spawning each year (Branch, 1981).

The seasonal changes in the reproduction of *P. candei gomesii* found in the present work are not unique since reproductive cycles have been described for other, especially European, *Patella* species. Summer spawning events have been reported for *Patella vulgata* in NE England (August: Bowman and Lewis, 1986) and in Portugal (September/October: Guerra and Gaudêncio, 1986). *P. depressa* has also been identified as a summer breeder in Britain (Orton and Southward, 1961) and in Portugal (early summer and early autumn: Guerra and Gaudêncio, 1986). The fact that in *P. candei gomesii* the levels of gametes remain high until January is not surprising, as *P. depressa* in the south of Portugal also presents fat and ripe gonads over the winter season (Guerra and Gaudêncio, 1986).

The annual patterns were found to show considerable synchrony between sexes, in agreement with what has been described in other limpets (Branch, 1981). According to Yund (2000), free-spawning invertebrates with synchronous spawning increase the

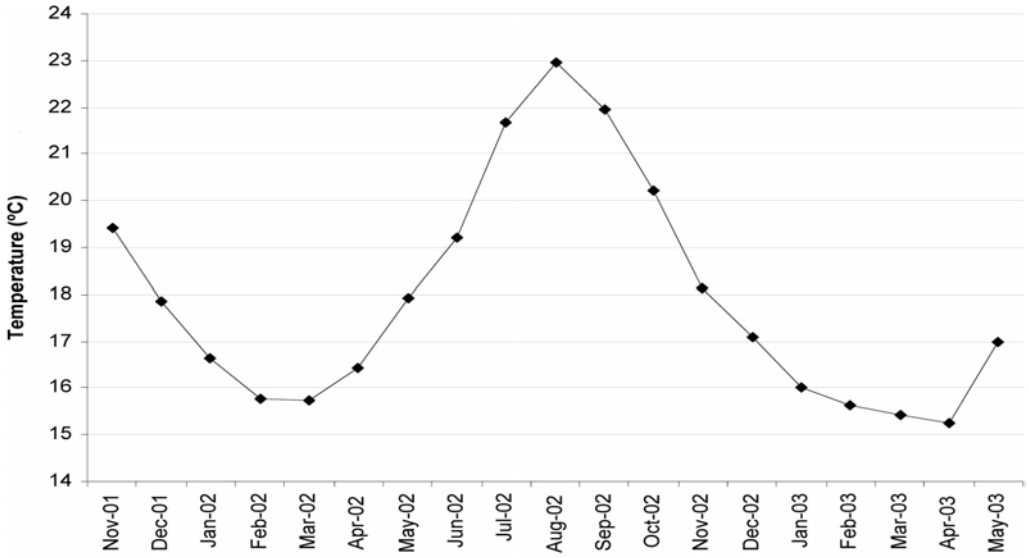

Fig. 5. Average sea surface temperatures for the period of November 2001 to May 2003 in the geographical area of São Miguel (37°–38°N; 26°–25°W). Data obtained from PODAAC–ESIP (http://podaac-esip.jpl.nasa.gov).

chances of fertilization by reducing the degree of sperm limitation. This is an issue of probable relevance to *Patella* species in the Azores, since decreases in population density due to human exploitation (Hawkins et al., 2000) are likely to bear a substantial influence on the availability of sperm.

The present research project did not address the factors that possibly trigger the annual changes. In the literature, temperature has been linked to the reproductive cycles of *Patella* species (Orton et al., 1956; Fretter and Graham, 1976). Species with planktotrophic larvae spawn just prior to the time when plankton is maximal (ensuring food for the larvae) at a time of rising or high sea surface temperatures (Branch, 1981). In fact, spawning in *P. depressa* seems to coincide with maximum air temperatures (Orton and Southward, 1961; Fretter and Graham, 1976). Such is the case for *P. candei gomesii*. Sea surface temperatures (PODAAC-ESIP; http://podaac-esip.jpl.nasa.gov) for São Miguel present a rather small variation ranging from 15 to 23°C (Fig. 5), with a clear seasonal pattern of higher temperatures during summer and early autumn (July–October) and lower temperatures during late winter and early/mid spring (February–April). This pattern agrees fairly well with our findings, since in April, when temperatures are low, limpets start to develop their gonads; and in July, when temperatures are high, they are fully developed for the postulated major spawning event. Even though temperatures gradually decrease in autumn and early winter, limpets remain capable of spawning until January when temperatures attain the lowest values.

Spawning may also be triggered by sea roughness (Orton et al., 1956; Fretter and Graham, 1976; Bowman and Lewis, 1977; Bowman and Lewis, 1986). In the Azores, sea roughness is higher in winter, arguing in favour of the extended period of spawning. This can be extremely important for species (or populations) that live high in the shore, like "fly limpets". In fact, *Notoacmea petterdi*, a limpet that lives on the extreme high shore, spawns when storms are frequent, suggesting that these are important for larvae to reach their settlement zones (Branch, 1981). Furthermore, in other gonochoric species such as *Patella aspera* and *P. depressa*, spawning is linked to wave action (Fretter and Graham, 1976).

This paper presents clear evidence on the reproductive cycles of the two ecomorphs of *P. candei gomesii*. Highlighting their similarity, the thesis that they are in fact two morphs of the same subspecies is strengthened.

The histological approach provided quantitative data and sharpened the analysis of the reproduction of *P. candei gomesii*, indicating that the period from April through July is crucial for conservation measures and for further investigations. Extending this approach to other limpets of the Macaronesia should provide interesting cues to understand their biogeography and to foresee adequate conservation strategies.

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