

## Reproductive biology of *Oxychilus (Atlantoxychilus) spectabilis* (Milne-Edwards, 1885) (Gastropoda: Pulmonata): a gametogenic approach

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The taxonomic status and anatomy of *Oxychilus (Atlantoxychilus) spectabilis* (Milne-Edwards, 1885), an endemic land snail from Santa Maria Island, Azores, has been subject of detailed study, yet information about its life history is wanting. This study describes the reproductive cycle of *O. (A.) spectabilis* and assesses the validity of three morphometric shell parameters as maturation diagnostic characters. Our results indicate that individuals are reproductively more active from May to November. However, the availability of spermatozoa throughout the year and the residual values of mature oocytes during the remaining months seem to provide minimum conditions for reproduction all year round. The snail has a functional protandric tendency and gonadal maturation is initially triggered by photophase and after regulated by temperature. The positive correlation between gonadal maturation and morphometric shell characters indicate that these parameters might be a useful tool for the diagnosis of snail's maturation.

Key words: Azores, gametogenesis, land snails, reproduction

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### INTRODUCTION

In the Azorean Islands, the oxychilid land snails are the most speciose pulmonate group, representing approximately one third of their endemic molluscan species (Riedel 1997; Martins 1999, 2011) and aspects of the life history of various species have been investigated (Rodrigues et al. 1998; Cunha 1999; Cunha et al. 2001; Ferreira et al. 2011). Santa Maria is the oldest island (about 8 Mys) of the archipelago and despite of the small area of only 97 km<sup>2</sup> it comprises 31% of

Azorean endemism (Cunha et al. 2010). Therefore, the knowledge of the reproductive cycle of these endemic species is important, namely for conservation purposes.

Several studies were performed in an attempt to correlate the maturation of the reproductive system with the growth of the animal. According to Cuezco (1993), the gonadal development depends on the season, age and size of the animal. Rodrigues et al. (1998) for *Oxychilus atlanticus* (Morelet & Drouët, 1857) reported a positive correlation between the maturation degrees of

reproductive system and maximum diameter of the shell. In addition, Rodrigues & Medeiros (2005) for *Leptaxis caldeirarum* (Morelet & Drouët, 1857) pointed out that the degree of maturity is related not only with maximum diameter but also with total height of the shell, whereas Ferreira et al. (2011) reported as well a correlation between number of whorls and sexual maturity, for *Oxychilus brincki* Riedel, 1964.

Although the systematics and the anatomy of *Oxychilus spectabilis* has been abundantly dealt with (see Milne-Edwards 1885; Riedel 1964; Hausdorf 1993 but also Bank et al. 2002; Martins 2005, 2011), nothing is known about the life cycle of this species, in particular with reference to its reproductive cycle. Thus, the aim of this work is to study the reproductive cycle of *O. spectabilis* and to assess the validity of three shell morphometric parameters, namely, maximum diameter, total height and number of whorls, as being diagnostic for reproductive maturation, in order to minimize the ecological impact of future studies and to support conservation measures.

## MATERIAL AND METHODS

### THE STUDY AREA

The climate in Azores is considered as marine temperate, with low thermal amplitude, high precipitation, high air humidity and persistent wind, as well as by a marked contrast between a dry season and a colder and wet season. Estimates from the monthly precipitation measurements show that about 75 % of the annual precipitation occurs among October and March (DROTRH-INAG 2001). The photophase range from around 9 hours in December and January, increasing after that, and reaching the maximum day length (14h30) in June and July, followed afterwards by a decrease at the same rate (Beck 1968).

The study was carried out in Santa Maria island, at Chã do João Tomé (Latitude 38° 32' 098''N; Longitude 28° 20' 216''W; altitude 192 m). The sampling site is a forest of *Platanus orientalis*, *Cryptomeria japonica* and *Pittosporum undulatum*, with the soil covered with abundant leaf-litter and some gramineae and *Tradescantia fluminensis* in the understorey. Considering the data from the closest meteorological station at the

airport (S. Pedro), monthly average precipitation during the sampling period is 98.1 mm, ranging from 5.9 mm in August to 256.7 mm in March. Monthly average air temperature, measured in the same station, is 18.1 °C, ranging from 14.4 °C in February to 23.3 °C in August. At each sampling period, soil pH and soil temperature were measured with a Hanna instruments (model HI 99121). During the sampling period the soil pH ranged between 4.16 and 6.12 and soil temperature varied similarly with the profile exhibited by the average air temperature, with the lower values (13.8 °C and 14.8 °C) occurring also between January and March, and the highest (19 °C) in July.

### SAMPLING AND MORPHOMETRICS

The sampling period, chosen according to the new moon phase, started in May 2009 and continued for a full year until May 2010, with bi-monthly periodicity. Specimens were handpicked, among leaves and under stones, during a time effort of one hour. According with the methods used by other authors in similar studies (Rodrigues & Medeiros 2005; Ávila et al. 2008), the individuals with the largest shell diameter were selected (May, n = 8; July, n = 6; September, n = 3; November, n = 3; January, n = 8; March, n = 13; May, n = 10). Prior to the preparation for histological studies, the shell of each specimen was measured for maximum diameter (MD), total height (TH) and number of whorls (NW). The measurement of the shell size was done using a vernier caliper and the number of whorls was counted, under the stereomicroscope, according to the methodology used by Martins (2005).

### GONADAL MATURATION STATE

Specimens were fixed in aqueous Bouin's solution for about 16h, dehydrated and embedded in paraffin wax. Serial sections of 5 µm thickness were obtained and stained with Mayer's haemalum and eosin (Martoja & Martoja-Pierson 1970). The relative volumetric density of the gametes was estimated using the M168 Weibel Multipurpose Test System (Weibel 1979). Three stages of development were distinguished during oogenesis (Fig. 1) (Hill & Bowen 1976; Cúrdia et al. 2005; Rodrigues & Medeiros, 2005): (1) previtellogenic

oocytes (PVO) - small rounded and with strong basophilous cytoplasm; (2) vitellogenic oocytes (VO) - larger than the earlier stage, more flattened and light basophilous and (3) maturing oocytes (MO) - rounded and larger than vitellogenic oocytes and presenting an eosinophilous and granular cytoplasm. This stage also includes the fully mature oocytes. Four development stages were identified during spermatogenesis (Fig. 1) (Griffond et al. 1991; Cúrdia et al. 2005; Rodrigues & Medeiros 2005): (1) spermatogonia (Sg) - sphaeroidal cell, small size and with a large nucleus in

relation to the small quantity of cytoplasm; (2) spermatocyte (Sc) - larger than earlier stage with a more abundant and eosinophilous cytoplasm; (3) spermatids (St) - in early phase spermatids have a small size with sphaeroidal shape and with the nucleus sphaeroidal shape, too, but showing frequently basophilous condensations at its poles; in a late phase, the whole cell stretches and elongates showing small tails and (4) spermatozoa (Sz) - with strong basophilous head and long eosinophilous tail.

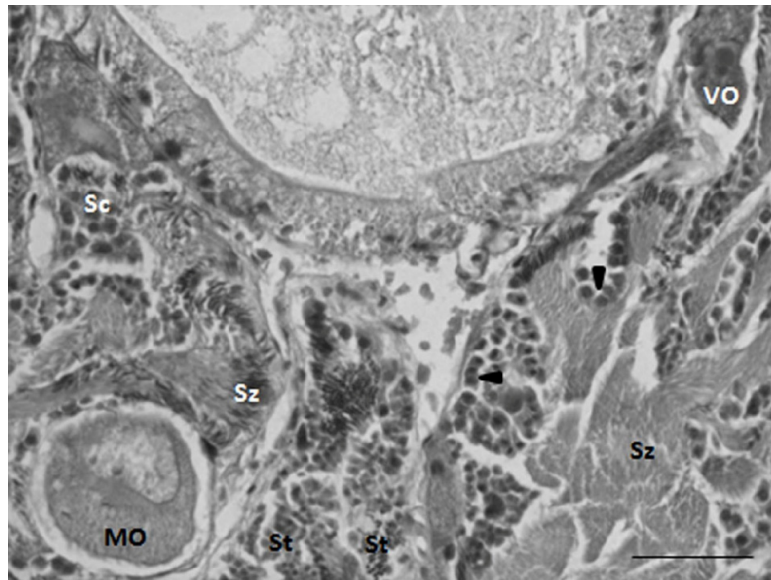


Fig. 1. Histological section of the gonad of the *Oxychilus (Atlantoxychilus) spectabilis*. Arrows, spermatogonia; Sc, spermatocytes; Sz, spermatozoa; VO, vitellogenic oocytes; MO, mature oocytes. Scale bar: 50  $\mu$ m.

#### A STATISTICAL ANALYSIS

Scores for volumetric density were summed for each specimen and converted to percentages, in order to reach the gonadal maturation state. Statistical tests were carried out with Excel and SPSS 18.0 for Windows (SPSS Inc. 2010) software package. Nonparametric tests were performed, given that data were not normally distributed and had heterogeneous variance. The variation of relative volumetric density of each gametogenic stage during the sampling period was analyzed with Kruskal-Wallis test, followed by

the Dunn's post hoc test. Spearman's correlation was determined among three shell variables (maximum diameter, total height and number of whorls) and the relative volumetric density of mature gametes (when considered together and separately).

#### RESULTS AND DISCUSSION

Among the specimens of *O. (A.) spectabilis* under analysis (N = 51), maximum shell diameter

(Kruskal-Wallis test:  $H = 29.715$ ;  $df = 6$ ;  $p < 0.001$ ), total height of the shell ( $H = 16.620$ ;  $df = 6$ ;  $p < 0.05$ ) and number of whorls ( $H = 32.875$ ;  $df = 6$ ;  $p < 0.001$ ) showed significant differences during the sampling period. Post hoc Dunn's test indicated that in September and January the maximum shell diameter differed statistically from May 2010 ( $p < 0.05$ ) and the total height of the shell in January was also significantly different from May 2010 ( $p < 0.05$ ). The Dunn's post hoc test showed significant differences in the number of whorls between May 2009 and Janu-

ary, and in September, January and March this variable differed statistically from May 2010 ( $p < 0.05$ ). In general, the three morphometric parameters under analysis showed a very similar pattern of temporal growth, with the highest values always found in May 2010 and the lowest in September/January: maximum shell diameter ranged from 4.8 to 5.6 mm; total height of the shell ranged from 2.3 to 2.7 mm, and the number of whorls ranged from 4.5 to 5.3 mm (Fig. 2); however, the lowest values found in September could be due to the reduced size of the sample.

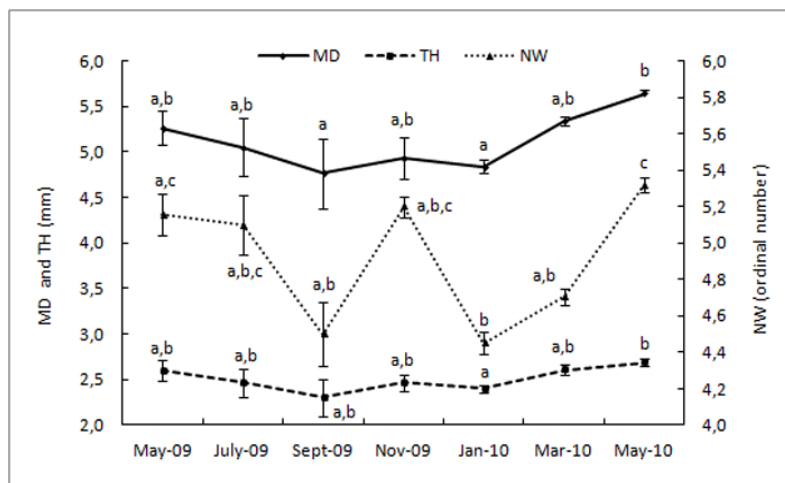


Fig. 2. Mean value  $\pm$  standard errors (in mm) of the maximum diameter of the shell (MD), total height of the shell (TH) and mean value  $\pm$  standard errors (in ordinal number) of the number of whorls (NW) during the sampling period. The mean values having the same letter do not differ significantly among months, Kruskal-Wallis (post hoc Dunn's test,  $p < 0.05$ ).

The relative volumetric density of early stages of spermatogenesis (spermatogonia and spermatocyte) and spermatozoa showed significant differences during the sampling period ( $H = 25.063$ ;  $df = 6$ ;  $p < 0.001$  and  $H = 25.487$ ;  $df = 6$ ;  $p < 0.001$ , respectively). The percentage of early stages of spermatogenesis in September differed statistically from January, and this month also differed statically from May 2010 ( $p < 0.05$ ). With regard to the gonadal volume occupied by spermatozoa, significant differences occurred between July and January as well as between January and May 2010 ( $p < 0.05$ ). The percentage of early stages of spermatogenesis reached the highest value (45.6 %) in January and the lowest in September (4.8

%), while the percentage of spermatozoa reached the highest value (72.7 %) in May 2010 and the lowest value (36.9 %) in January (Fig. 3a). Apart from January, the relative volumetric density of spermatozoa occupied more than 57 % of the gonad (Fig. 2a). The relative volumetric density of mature oocytes during the sampling period showed significant differences ( $H = 16.069$ ;  $df = 6$ ;  $p < 0.05$ ), between September and January ( $p < 0.05$ ). The percentage of mature oocytes reached the highest values in September and November (13.5 % and 6.37 %, respectively) and the lowest in January (1.1 %) (Fig. 3b).

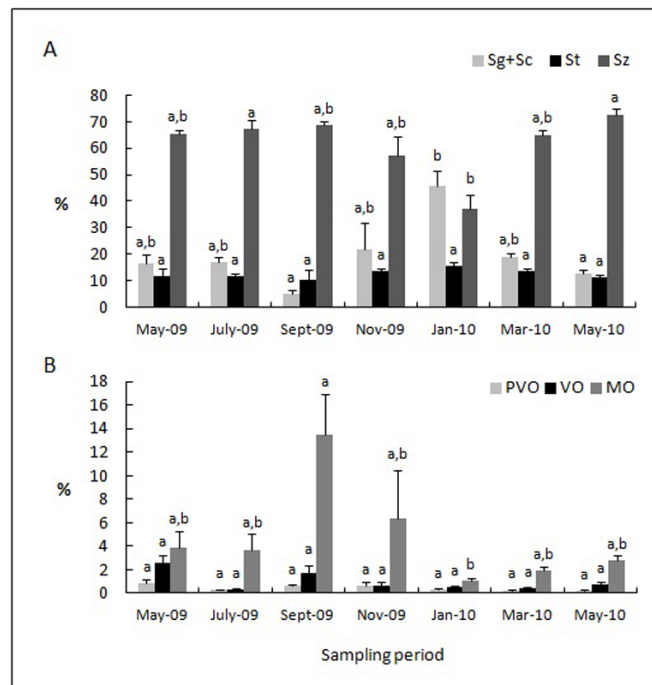


Fig. 3. Relative volumetric density (mean  $\pm$ SE) of the each gametogenic stages in *Oxychilus (Atlantoxychilus) spectabilis* from May 2009 to May 2010. A) Spermatogenesis: spermatogonia (Sg), spermatocytes (Sc), spermatids (St) and spermatozoa (Sz). B) Oogenesis: pre vitellogenic (PVO), vitellogenic (VO) and maturing oocytes (MO). Different lower cases above columns, within a stage of reproductive maturation between sampling dates, indicate significantly different values, Kruskal-Wallis (post hoc Dunn's test,  $p < 0.05$ ).

Our observations on gonadal maturation of *O. (A.) spectabilis* show that individuals are reproductively more active from May to November, when spermatozoa and mature oocytes are both present in higher relative volumetric density. Nevertheless, the availability of spermatozoa throughout the year (more than 36 %) and the residual values (< 2 %) of mature oocytes from December to April, appear to provide minimum condition for snail's reproduction throughout the year. Due to the short time of active life and low mobility, the availability of mature sperm during the whole year, also reported by our findings, would be of major advantage for snails allowing them to mate in each season, whenever they found a mate (Boato & Rasotto 1987). Our findings also demonstrate that this species has a functional protandric tendency, following the size advantage model proposed by Ghiselin (1969).

As reported by several authors (Rodrigues et al. 1998; Gómez 2001; Rodrigues & Medeiros 2005; Ferreira et al. 2011), individuals mature first as a male, given that sperm production is less energy demanding than eggs production, and once they reached a larger body size, female gametes initiate maturation. As described for other land snails, gamete maturation in *O. spectabilis* seems to be first triggered by photophase (see Segal 1960, for *Limax flavus* (Linnaeus, 1758); Bailey 1983, for *Helix aspersa* (Müller, 1774); Cunha et al. 2001, for *O. atlanticus*; Ferreira et al. 2011, for *O. brincki*) and then regulated by temperature (see Cunha et al. 2001; Ferreira et al. 2011). Our findings are in accordance with this hypothesis, since sperm production increased with day length and gonadal maturation, in particularly oogenesis, was regulated by temperature; in addition, several studies demonstrated that lengthy days promote

the development of male gametogenesis in slugs (Henderson & Pelluet 1960; Sokolove & Mccrone 1978; Mccrone & Sokolove 1979; Gomot & Gomot 1985; Gomot & Griffond 1987).

The morphometric variables of the shell (maximum diameter, total height and number of whorls) showed a positively significant correlation with the percentage of mature gametes (spermatozoa and maturing oocytes, considered together) ( $n = 51$ ,  $\rho_{MD} = 0.440$ ,  $p < 0.05$ ;  $\rho_{TH} = 0.353$ ,  $p < 0.05$  and  $\rho_{NW} = 0.414$ ,  $p < 0.05$ ). The percentage of spermatozoa showed a positive correlation with the three morphometric variables of the shell ( $n = 51$ ,  $\rho_{MD} = 0.531$ ,  $p < 0.001$ ;  $\rho_{TH} = 0.438$ ,  $p < 0.05$  and  $\rho_{NW} = 0.443$ ,  $p < 0.05$ ). By contrast, the percentage of mature oocytes did not show any significant correlation with the morphometric variables ( $n = 51$ ,  $p > 0.05$ ); this result could be due to the reduced number of mature oocytes per individual and their large cell size when compared with the volume occupied by male cells, since it was observed a very high standard error of the mean for the mature oocytes in contrast with that observed for the spermatozoa. Our data are in accordance to those reported for other terrestrial snails (Cuezzo 1993; Rodrigues et al. 1998; Rodrigues & Medeiros 2005; Ferreira et al. 2011). These findings could indicate that these morphometric variables might be a valuable tool for the diagnosis of snail's maturation, and therefore useful to minimize the ecological impact of future studies.

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