Journal of the Marine Biological Association of the United Kingdom

cambridge.org/mbi

Original Article

Cite this article: Sousa R, Henriques P, Vasconcelos J, Faria G, Riera R, Pinto AR, Delgado J, Hawkins SJ (2019). First observations of hermaphroditism in the patellid limpet *Patella piperata* Gould, 1846. *Journal of the Marine Biological Association of the United Kingdom* 1–6. https://doi.org/10.1017/S0025315419000559

Received: 12 December 2018
Revised: 15 May 2019
Accepted: 20 May 2019

Key words:
Atlantic Ocean; intertidal; Madeira; Mollusca; Patellogastropoda; sex change

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First observations of hermaphroditism in the patellid limpet *Patella piperata* Gould, 1846

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Abstract

Hermaphroditism is thought to be an advantageous strategy common in marine molluscs that exhibit simultaneous, sequential or alternating hermaphroditism. Several species of patellid limpets have previously been shown to be protandrous hermaphrodites. The present study aimed to confirm whether this phenomenon occurs in *Patella piperata*. Transitional forms of simultaneous protandrous hermaphroditism were found in intermediate size classes of *P. piperata*, in Madeira (North-eastern Atlantic). Sequential hermaphroditism was confirmed after histological analysis. The overall sex-ratio was biased towards females but approached similar proportions in the larger size classes. Analysis of size at sex change showed that at a shell length of 36 mm 50% of the population probably have changed sex. The results reported confirm the occurrence of sequential hermaphroditism. These findings are of utmost importance to the understanding of the reproductive biology of this species with direct effect on management and conservation of this traditionally harvested limpet.

Introduction

Patellid limpets are among the most successful marine gastropods inhabiting rocky shores (Branch, 1981) from the supratidal to the subtidal zones and as such, are subject to the most variable and unpredictable environmental conditions in nature (Harley *et al.*, 2009). The sharp environmental gradients of wave action, temperature variation and desiccation can prompt significant changes to the overall morpho-physiological characteristics of patellid limpets (Davies, 1969). For instance, the reproductive biology and phenology of limpets are influenced by factors such as temperature and storminess (Branch, 1974; Bowman & Lewis, 1986), latitude and temperature (Lewis, 1986), climate change (Moore *et al.*, 2011), parasitism (Firth *et al.*, 2017) and anthropogenic pressures such as harvesting, pollution and habitat loss (Boukhicha *et al.*, 2013; Henriques *et al.*, 2017).

Hermaphroditism is regarded as an evolutionarily advantageous strategy supposed to increase the likelihood of successful fertilization for sedentary broadcast spawning species such as limpets, especially when occurring in low population densities (Branch, 1981; Guallart *et al.*, 2013). Hermaphroditism may be simultaneous, with one individual releasing both types of gametes during the same season, or sequential, with one individual functioning first as one sex and then changing to another later in life (Orton, 1928; Branch, 1974; Ghiselin, 1987). Sequential hermaphroditism is designated as protandry when an individual is first a male and then changes to female (Branch, 1981). Protogyny is when an individual first matures as female and then changes to male. In some limpet species the sex change can be reversed (Le Quesne & Hawkins, 2006; Rivera-Ingraham *et al.*, 2011), whereby males that became females can become males again. This could be linked to their reproductive fitness, since small females produce fewer oocytes and they would have a greater reproductive fitness as males (Guallart *et al.*, 2013). When sex change occurs repeatedly it is known as alternating sexuality (Hoagland, 1984). Sex can also be determined by environmental circumstances where one individual can influence the sex of another (Heller, 1993). Sex change in limpets has been shown to be density dependent by experimental manipulation in *Lottia gigantea* Gray in G. B. Sowerby I, 1834 (Fenberg & Roy, 2008) and in *Patella vulgata* Linnaeus, 1758 (Borges *et al.*, 2015). Earlier onset of switching to females has been shown in heavily exploited populations of *Patella aspera* Röding, 1798 in the Macaronesian Islands (Martins *et al.*, 2017). In some species, sex change is triggered by specific environmental cues, in other species, sex change seems to be determined genetically, occurring mainly after the first reproductive season (Wright, 1989; Fretter *et al.*, 1998).

Hermaphroditism has been described in several families of limpets, for example Nacellidae (Mau *et al.*, 2017), Siphonariidae (Pal & Hodgson, 2005) and Patellidae (Orton, 1928). This phenomenon is prevalent in some genera of the Patellidae, *Cymbula* (Branch, 1981), *Scutellastra* (Branch, 1974; Lindberg, 2007), *Helcion* (Henninger & Hodgson, 2001), and especially in the genus *Patella*. *Patella vulgata* (Orton *et al.*, 1956; Le Quesne & Hawkins, 2006), *Patella ulyssiponensis* Gmelin, 1791 (Thompson, 1979), *Patella caerulea* Linnaeus, 1758 (Montalenti, 1958), *Patella aspera* (Martins *et al.*, 2017), *Patella ferruginea* Gmelin, 1791 (Espinosa *et al.*, 2009) and *Patella rustica* Linnaeus, 1758 (Prusina *et al.*, 2014) are known to be protandrous hermaphrodites (Dodd, 1956; Le Quesne & Hawkins, 2006; Rivera-Ingraham *et al.*, 2011; Martins *et al.*, 2017). Simultaneous hermaphroditism has been reported for the Azorean endemic limpet *Patella candei gomesii* (Cunha *et al.*, 2007). Protandry does not seem to occur in populations of *Patella depressa* Pennant, 1777 in southern England (Orton & Southward, 1961; Guerra & Gaudêncio, 1986), however, more recently some indications of protandry in populations from Portugal have been reported (Borges *et al.*, 2015).

Hermaphroditism in patellid limpets is an important trait that needs to be taken into consideration in the conservation and management of heavily exploited populations (Espinosa *et al.*, 2006). It is known that size-selective harvesting negatively impacts the reproductive output threatening species survival (Fenberg & Roy, 2008). In fact, larger specimens are more visible and valuable thus more prone to being harvested and at the same time contribute the most to the reproductive effort, since in these species the reproductive output is directly related to size, with larger individuals with larger gonads producing more gametes (Creese, 1980). Removal of these individuals will therefore diminish the reproductive success of exploited populations (Espinosa *et al.*, 2009; Henriques *et al.*, 2017). In hermaphroditic species, removal of larger individuals might also cause alterations in population dynamics, since the absence of the predominant sex at larger size is considered one of the cues that triggers sex change to occur earlier at smaller sizes (Rivera-Ingraham *et al.*, 2011).

Patella piperata Gould, 1846 is one of the 15 worldwide recognized species of the genus *Patella* Linnaeus, 1758 (WoRMS, 2018). This species is endemic to the Macaronesian archipelagos of Madeira, Canaries and Cape Verde (Christiaens, 1973) and occurs together with *P. aspera* and *Patella candei* d'Orbigny, 1840 in the Madeira archipelago (Sousa *et al.*, 2017). It is closely related to *P. rustica*, a sister species occurring on continental Atlantic coasts and throughout the Mediterranean (Côrte-Real *et al.*, 1996; Sá-Pinto *et al.*, 2005). This important microphagous grazer controls algal biomass of the intertidal rocky shores (Bergasa *et al.*, 2007) and is collected for human consumption by locals throughout the Canaries (Moro & Herrera, 2000; Bergasa *et al.*, 2007) and occasionally in Madeira. A potentially hermaphroditic individual of *P. piperata* was macroscopically identified, while studying this species' reproductive biology. This find prompted the need to confirm whether hermaphroditism occurs in this species similar to its protandrous sister clade *P. rustica* (Prusina *et al.*, 2014). Our specific objectives were: (i) to report the histological validation of sex change in *P. piperata* and (ii) to determine the size at which sex change occurs.

Materials and methods

Monthly samples of *P. piperata* were collected from the coast of Madeira, North-eastern Atlantic (32°00′–33°30′N 15°30′–18°00′W), at two different sites (São Vicente and Santa Cruz), between 2017 and 2018, as part of a study focussing on growth and reproduction of this species. Specimens were randomly collected from the supra-tidal rocky shore, removing all the specimens found without considering their size, for a period of 30 min. Shell length (L) was measured using a Vernier calliper to the nearest 0.01 mm, and total wet weight (W) to the nearest 0.01 g was measured using an electronic balance. Specimens were dissected for macroscopic observation of the gonads and sexed according to Orton *et al.* (1956) considering gonad pigmentation, with males exhibiting pale white to pink gonads and females brown to red gonads.

Gonads were removed, damp-dried, weighed to 0.01 g accuracy and preserved in a 10% buffered formaldehyde solution. All measurements were taken from fresh samples.

Histological analysis was performed on dissected gonads in five stages: (I) fixation; (II) dehydration (alcohol with different concentrations: 70, 90 and 95%); (III) embedding in resin; (IV) preparation of trimming blocks and (V) sectioning. A portion of about 1 cm^3 from the middle of each gonad was embedded in Technovit 7100 resin, following standard protocols. Histological sections (3–4 μm thickness) were cut and stained with methylene blue solution. The slides of each specimen were sealed with a drop of neo-mount resin, and image acquisition using the visual image analysis system composed of the Leica DMLB microscope coupled to the Leica EC 3 camera and the software LAS 4.5. (Vasconcelos, 2017).

An analysis of variance (ANOVA) was employed to compare limpet size among males, females and hermaphrodites. The size cumulative frequency was analysed using the non-parametric Kolmogorov–Smirnov test and a Pearson χ^2 was employed to compare the sexual proportion by size classes between areas. All statistical analyses were performed using SPSS v.24.0 (IBM Corp., Armonk, NY). For all tests, statistical significance was accepted when $P < 0.05$.

The size at sex-change (L_{50}) corresponds to the size at which 50% of mature individuals are the second sex (male for protogynous, female for protandrous) and was determined using a logistic regression (Allsop & West, 2003).

Results

A total of 1164 specimens of *P. piperata* were sampled between 2017 and 2018. Among the sampled individuals, 53 were macroscopically identified as potential hermaphrodites (Figure 1), representing 5% of the total sample. 65% were females and 30% were males. Hermaphrodites were only found from March to June.

The shell length of the hermaphrodites varied between 21.72 and 37.81 mm ($\bar{x} =$ 31.54 ± 4.98 mm), female shell length varied between 11.17 and 37.34 mm ($\bar{x} =$ 25.11 ± 4.56 mm) and male shell length varied between 11.65 and 37.52 mm ($\bar{x} =$ 22.99 ± 5.34 mm). Differences in mean shell length among sexes were statistically significant ($F = 94.067$, $P < 0.001$).

The overall sex ratio was 2.16:1 (female:male) favouring females. The distribution of the cumulative frequency of males, females and hermaphrodites per size is represented in Figure 2. The observed differences were statistically significant ($Z = -4.037$, $P < 0.001$).

The Pearson's χ^2 showed that sexual proportion between size classes did not differ between sampling sites ($\chi^2 = 2.006$, $P = 0.157$) and that females were predominant between 17.00 and 35.00 mm ($\chi^2 = 33.999$, $P = 0.005$), indicating that the size structure of the populations was similar between localities and that males were more abundant between 11.00 and 16.00 mm and females between 17.00 and 37.00 mm. The size at sex change (L_{50}) was estimated at a shell length of 36.01 mm, and at 29.22 mm 25% of limpets had started to change sex.

https://doi.org/10.1017/S0025315419000559

Fig. 1. Hermaphrodite gonad of *Patella piperata*, where the ovary is represented by the central dark area and the testis is represented by the lighter areas.

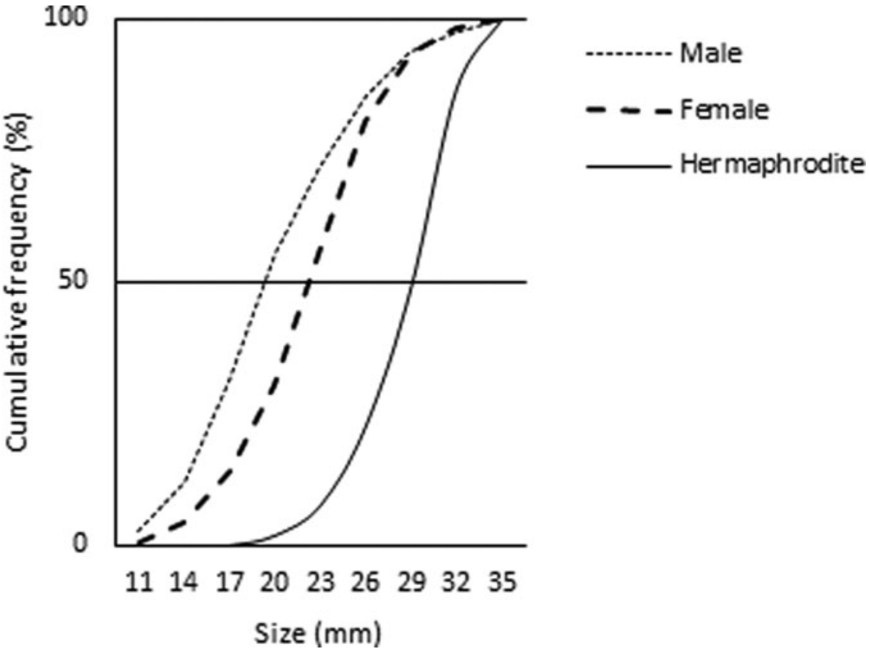

Fig. 2. Cumulative size frequency of males, females and hermaphrodites of *Patella piperata*.

The histological validation of the potential hermaphrodites showed that defined sections of ovary and testis occurred simultaneously in specimens of *P. piperata* between March and June, suggesting that these individuals represent a transitional stage of sequential sex-change, probably occurring in the late stages of the spawning season (Figure 3). Testicular tissue, with some distinguishable seminiferous tubules but without sperm was observed, implying that male structures during transition aren't functional. Ovarian tissue with vitellogenic oocytes and some atresia was also observed, suggesting post-spawning degeneration of female structures (Figure 3A, B).

Discussion

Hermaphroditism is thought to represent an advantageous strategy in increasing the reproductive success of organisms, based on the assumption that an individual changing sex will increase its reproductive efficiency (Charnov, 1979; Munday *et al*., 2006). When male and female reproductive values are closely related to size or age, natural selection will favour genes coding for sex change (Warner, 1988). The direction of sex change is thought to be determined by the relative fitness returns for the two sexes over the course of the limpet's lifetime and not by size (Allsop & West, 2003; Guallart *et al*., 2013). If males have lower

https://doi.org/10.1017/S0025315419000559

reproductive success than females in smaller sizes and greater in larger sizes, sex change will probably be protogynous to maximize the reproductive success of males later on life when their fitness accelerates above that of the females (Guallart *et al.*, 2013). The inverse holds for protandrous sex change (Cunha *et al.*, 2007). This sex changing allocation mechanism ensures that individuals have the optimal sex for the predominant circumstances, thus maximizing the organism's genetic contribution to the next generation (Warner *et al.*, 1996).

Sequential hermaphroditism assumes the existence of sexual dimorphism in size classes (Orton, 1919; Branch, 1974; Thompson, 1979; Creese *et al.*, 1990), with the occurrence of a small number of simultaneous hermaphrodites in intermediate size classes (Bacci, 1947; Branch, 1974; Creese *et al.*, 1990). In fact, direct observations have confirmed this pattern for the protandrous hermaphrodite *P. vulgata* (Le Quesne & Hawkins, 2006). Two forms of simultaneous hermaphroditism can occur, the very rare 'mosaic' form, in which patches of male and female gametes appear interspersed, usually considered aberrations since they mostly occur in gonochoristic species (Dodd, 1956) and the 'transitional' form, in which defined sections of male and female gamete have been observed to occur simultaneously and is thought to represent a gonad in a transitional state of sex change (Branch, 1974). Both these forms have been observed in the order Patellogastropoda (Orton, 1928; Branch, 1974), the mosaic form in *P. vulgata* and the transitional form in *P. caerulea* and *Cymbula oculus* (Born, 1778) (Dodd, 1956; Branch, 1974).

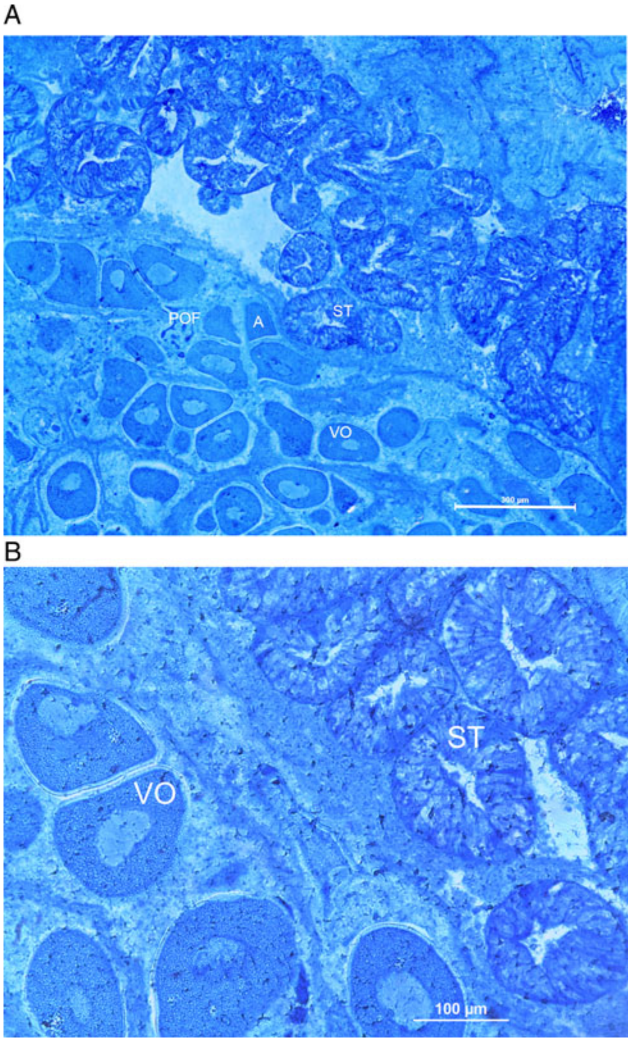

Fig. 3. Histological section of a hermaphrodite specimen of *Patella piperata* gonads. (A) General view highlighting the coexistence of spermatogenic and oogenic stages. ST, seminiferous tubules; VO, vitellogenic oocytes; POF, post-ovulatory follicles; A, atresia. (B) View detailing the spermatogenic and oogenic stages. ST, seminiferous tubules; VO, vitellogenic.

The results reported herein are the first observations of a transitional form of sequential hermaphroditism in *P. piperata*. This species size distribution ranges from 3 to 46 mm shell length (R. Sousa, personal communication) and the hermaphroditic individuals occurred in the size classes ranging from 20 to 38 mm representing ~5% of the examined individuals. The size at which sex change is estimated to occur was 36.08 mm. This corresponds to the size class at which 50% of the individuals in this population are of the second sex. Nonetheless, sex change in *P. piperata* starts to occur earlier, with 25% of limpets having changed sex at 29.22 mm shell length and the smallest hermaphrodite found had 21.72 mm shell length. The sex ratio was biased towards females in most of the sampled size range, with males being predominant in the smaller size classes and females in the larger size classes, corresponding to the size at which hermaphrodites become more common. The skewed sex ratio to females is uncommon in protandrous species where small males are often numerically dominant (Allsop & West, 2004). This could be a result of incomplete sampling with smaller recently matured males occurring lower on the shore or in cryptic habitats.

In gastropods, sex change is also thought to be determined genetically and to occur mainly after the first reproductive period (Fretter *et al.*, 1998). In some species, sex change is triggered by specific environmental signals and this may be the case of *P. piperata*. However, the environmental cues that trigger sex change in this species are yet to be determined.

Hoagland (1978) stated that in protandrous gastropods the presence of females or other larger conspecifics delays or inhibits sex change in males, with abundance acting as a trigger for sex change. As such, sex change in *P. piperata* in Madeira may be density dependent, where a greater overall abundance of one sex may prompt a sex change to balance the sex ratio of these populations, as reported for *P. aspera* (Martins *et al.*, 2017), *P. vulgata* (Borges *et al.*, 2015) and *P. ferruginea* (Espinosa *et al.*, 2009; Rivera-Ingraham *et al.*, 2011).

The occurrence of 5% of simultaneous hermaphroditic individuals in the sampled populations in the size classes between 20–38 mm, warrants further studies to better understand the impact of this phenomenon on the reproductive biology and population dynamics of *P. piperata* in Madeira namely, whether the mature gametes produced in hermaphrodites are functional or not and provide evidence of the direction of sex change. Another aspect that requires additional research is the environmental cues that control this phenomenon in this species.

Acknowledgements. The authors are grateful to the Fisheries Research Service (DSI) from the Regional Directorate of Fisheries of the Autonomous Region of Madeira. We also acknowledge Jorge Lucas and Filipe Andrade for their help during this work, namely in biological sampling and harvesting field surveys.

Financial support. The first author (RS) was supported by a grant from ARDITI OOM/2016/010 (M1420-01-0145-FEDER-000001-Observatório Oceânico da Madeira-OOM) and the third author (JV) by a grant from Fundação para a Ciência e a Tecnologia – FCT (SFRH/BSAB/143056/2018) and UID/MAR/04292/2019 granted to MARE. The present study has also been supported by the UE European Regional Development Fund in the framework of the Projects MARISCOMAC (MAC/2.3d/097), MACAROFOOD (MAC/2.3d/015) and the Regional Government of Madeira.

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