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Reproductive investment of the pen shell *Pinna nobilis* (Bivalvia, Pinnidae) Linnaeus, 1758 in Cabrera National Park, Spain

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Abstract

The spawning period can be a period of particular vulnerability for a species and in hermaphroditic species such as the long-lived pen shell *Pinna nobilis*, different costs could be associated with female, male or hermaphrodite stages. Here gonad development in a population with few anthropogenic pressures is evaluated and the timing and succession of developmental stages, the sex ratio, and condition of the animals described. Resource mobilisation and associated costs are assessed through the relationship between stable isotopes and condition indices with reproductive parameters, and the role of the adductor muscle as a storage organ used in gonad maturation is evaluated. In our study area only one spawning season is observed, starting in May and peaking in June-July. The onset of spawning coincided with water temperatures of 20°C. Condition indices drop during the spawning period and recover afterwards to reach pre-spawning values in November. Stable isotope signals are negatively related to the gonad condition index, while the C:N ratio showed a positive correlation with the same index. Additionally, the muscle condition index sharply decreased after the start of the spawning season, which suggests that *P. nobilis* uses the reserves stored in the adductor muscle for reproduction, as recorded in other Pinnidae species. Reproductive indices and stable isotopes ratios indicate 'capital breeding' as reproductive strategy of the pen shell. Decreased muscle force could mean a higher vulnerability during the summer period, coinciding with the peak in recreational activities involving poaching and boat anchoring.

Keywords: Gonad Index, histology, histochemistry, *Pinna nobilis*, reproduction cost, reproductive cycle, stable isotopes, western Mediterranean.

Introduction

The pen shell *Pinna nobilis* (Linnaeus, 1758), endemic to the Mediterranean Sea, is one of the largest bivalve species and reaches a total anterio-posterior length of up to 1.2 m (Zavodnik *et al.,* 1991). The pen shell is subject to strict protection as an endangered species under the European Council Directive 92/43/EEC (EEC, 1992) as population numbers of the bivalve *P. nobilis* are currently in decline (Centoducati *et al.,* 2007). This decline has been related to both an increase in anthropogenic impacts on coastal areas resulting from increased human population growth, and incidental damages by trawling and anchoring as well as collection by divers (Zavodnik *et al.,* 1991; Richardson *et al.,* 2004; Katsanevakis, 2007; Hendriks *et al.*, 2013; Deudero *et al.,* 2015; Vázquez-Luis *et al.,* 2015).

P. nobilis can achieve lifespans in excess of 20 years (Butler *et al.,* 1993; Richardson *et al.,* 2004; Galinou-Mitsoudi *et al.,* 2006; García-March & Márquez-Aliaga, 2007), but the full life cycle of the pen shell is still understudied, e.g. mortality during the larval phase has not been studied to date, while for juveniles there is very little information available (Katsanevakis, 2007). The pelagic larval phase of the pen shell is described as approximately 5-10 days (Butler *et al.,* 1993), while recruitment is highly variable (Katsanevakis, 2007). Spawning is expected in the late summer or early fall (De Gaulejac, 1993). In Mallorca (Balearic Islands) this has been further limited to a peak period between the last week of August and the first week of September (Cabanellas-Reboredo *et al*., 2009a). Due to the uncertain length of its pelagic lifespan, and lack of specific information about

occurrence and duration of the spawning period, little is known about the actual dispersal capacity of the species.

However, some basic information about sexual reproduction of the species is available; *Pinna nobilis* is hermaphroditic (De Gaulejac, 1995) in contrast with other species of the Pinnidae family like *Atrina maura*, which is a stable gonochorist with only occasionally some hermaphroditic individuals (Butler, 1987; Shimao *et al*., 1987; Yongqiang & Xiang, 1988; Ceballos-Vázquez *et al.,* 2000; Soria *et al.,* 2002; Angel-Pérez *et al.,* 2007). *Pinna nobilis* exhibits a very particular hermaphroditism, successive and asynchronous in maturation, in which a succession of alternate spawning and fast gametogenesis occurs without interruptions during the spawning period. Gonochoric individuals are an exception in the pen shell (De Gaulejac, 1995).

Due to the costs associated to egg and sperm production (Le Pennec *et al.,* 1991), the build-up of gonads and the subsequent spawning period can be a period of particular vulnerability for a species. In bivalves, sex changes in individuals are common and can be influenced by many factors, among which the condition of the animal and environmental conditions. Different costs are associated with female, male or hermaphrodite stages (Galtsoff, 1964), and determining the ratio of sexes and the exact period in which gonad build-up takes places can help pinpoint a sensitive phase in the life cycle of an endangered species. Histological analyses of gonadal tissue of individuals collected at regular intervals provide the most reliable method for determining seasonal gonadal changes in bivalves (Camacho-Mondragón *et al.,* 2012). With this method the timing of spawning, the percentage of spawning individuals in a natural population and the sex ratio can also be evaluated, which is useful to predict the occurrence of seed settlement and to establish fishery management measures (Camacho-Mondragón *et al*., 2012).

Strategies in allocation of obtained energy resources to reproduction range from strict 'income breeding' (females directing energy to gametes from resources concomitant to reproduction) to strict 'capital breeding' (females storing nutrients prior to reproduction; Pélisson *et al.,* 2013). In bivalves, timing and duration of reproduction and spawning might be determined by exogenous (environmental: temperature, particulate organic food availability) and endogenous (genetic and physiological) factors (Sastry, 1979). Reproduction in bivalves represents an energetic cost and discerning whether energy is fuelled to gametes via stored resources and further remobilisation (endogenous factors) or is being channelled from food availability (exogenous factors) is crucial for the conservation of vulnerable species such as the pen shell. Stable isotopes ratios of C and N, as ecological tracers, might provide information on these processes and have been used successfully to understand the dynamics of marine ecosystems (Deudero *et al*., 2004, 2011, 2014; Box *et al.,* 2010), providing information on tissue fractionation and species´ diets (Blanco *et al.,* 2009).

Pen shells exposed to anthropogenic pressures in the coastal waters around the Balearic Islands show differences in isotopic signals in their tissues as well as a marked seasonal variation, indicating a rapid isotopic response to environmental changes (Cabanellas-Reboredo *et al.,* 2009b; Alomar *et al.,* 2015). Values of $\delta^{15}N$ are used quantitatively to assess trophic level, whereas $\delta^{13}C$ is generally applied to indicate the relative contribution of different potential primary sources in a food web to the diet, and to distinguish inshore versus offshore food sources (Hobson *et al.,* 1995; Lepoint *et al.,* 2000). In addition, C:N ratios are good indicators of lipid content as samples containing more lipids have higher values (Tieszen *et al.,* 1983). Lipids are lower in ^{13}C compared to whole body or protein values, and protein-rich tissues such as muscles exhibit higher $\delta^{13}C$ values relative to those of lipid-rich tissues (DeNiro & Espstein, 1978; Tieszen *et al.,* 1983; Pinnegar & Polunin, 1999). Variability in $\delta^{15}N$ can reflect the relative abundance of different amino acids in tissues (Pinnegar & Polunin, 1999; Schmidt *et al.,* 2004). In general gonad tissues are enriched in lipids; therefore, seasonal shifts in ^{13}C of the pen shell will be indicative of translocation of energy storage towards gametes ('capital breeding') coupled with the reproductive cycle and gonadal development.

In this study gonad maturation of the pen shell (*P. nobilis*) in Cabrera National Park was evaluated, to 1) identify the spawning period, after which pelagic larvae disperse to recruit into new populations, 2) assess resource mobilisation and associated costs through the relationship between stable isotopes and condition indices with reproductive parameters, and 3) assess the role of the adductor muscle as storage organ used in gonad maturation.

Methods

Study area

Potential threats to pen shell populations are reduced in Marine Protected Areas (MPAs), which are essential tools for biodiversity conservation (Coll *et al.,* 2012). The MPA of Cabrera National Park is located 9 km southeast of Mallorca in the Balearic Archipelago, western Mediterranean Sea. It comprises 19 islands, from small islets to the 38.6 km perimeter Cabrera Island. The MPA was established in 1991 with an area of 100.21 km^2, of which 87.03 km^2 are maritime. A differential zonation guarantees full protection at no-take zones and pen shell populations in these zones where no anchoring damage is inflicted are more mature and more numerous compared to populations at the main island of Mallorca (Hendriks *et al.,* 2012a; Vázquez-Luis *et al.,* 2014a; Deudero *et al.,* 2015). Since most confounding factors present in intensively used coastal regions are excluded in this area, the National Park has been the site of choice for studies addressing ecological responses of

Pinna nobilis and evaluating the effect of anthropogenic impacts, with Cabrera MPA as a control zone (Hendriks *et al.,* 2012a, b; Vazquez-Luis *et al.,* 2014a, b; Alomar *et al*., 2015; Deudero *et al.,* 2015).

The study location, Cala Gandulf, is a small bay opening to the NW situated at the major island of the archipelago, Cabrera Island, and has been closed to visitors, except scientists, since 1993. The site has luscious *Posidonia oceanica* seagrass meadows, the main habitat for *P. nobilis*, which cover the seafloor of the whole embayment, ranging from 1 to 43 m depth (Marbà *et al.,* 2002).

Sampling

Sampling of *P. nobilis* specimen was carried out with governmental authorisation (dated 11 July 2011, reference number RE127). Tissue and gonad samples were collected every month over a depth range of 5-12 m by scuba divers.

Table 1. Description of developmental gonad stages for *P. nobilis* males and females.

	Females	**Males**
Undifferentiated (immature-resting) (1)	Acini very small with vesicular follicle cells closing the cavity. Some germinal cells can be observed. Not possible to determine sex in most of the samples (Fig. 1A). Externally gonads are not visible. The collapsed acinis are surrounded by vesicular connective tissue with some brown cells, which are very abundant in the case of resting gonads (Fig. 1 H).	Collapsed acini filled with enlarged follicular cells closing the acini cavity, and sex cannot be determined but, in the reproductive period, a few follicles with low spermatogenic activity permit the distinction of some immature male specimens.
Early developing (2)	Acini enlarged and irregular in shape with visible follicle cells at the beginning of the reproductive period. Previtellogenic oocytes attached to the walls of the acini. Eosinophilic granular haemocytes are observed inside follicular cavity as spawning season progresses. Decreasing vesicular connective tissue with some brown cells (Fig. 1B).	Acini enlarged and irregular in shape with visible follicle cells seen at the beginning of the reproductive period. Spermatogonia and spermatocytes observed attached to the acini wall. Vesicular connective tissue abundant, with some brown cells. Granular haemocytes observed inside acini as spawning season advances (Fig. 1C).
Late developing (3)	Acini very enlarged and dilated with pear-shaped vitellogenic oocytes attached to wall between smaller vesicular follicle cells. Scarce connective tissue with some brown cells. Abundant eosinophilic granular haemocytes inside the acini lumen (Fig. 1D).	Acini larger and filled with all sexual cell categories including spermatozoa but not all the acini contain spermatozoa in the lumen. Spermatogenic activity exhibits centripetal evolution. Scarce connective tissue with some brown cells. Abundant eosinophilic granular haemocytes inside the acini lumen
Spawning (4)	Ripe polygonal or rounded oocytes observed free in acini lumen whilst some oocytes remain attached to the wall. Acini very distended and enlarged. Considerable reduction of vesicular connective tissue, with some brown cells. Eosinophilic granular haemocytes seen inside the acini (Fig. 1E).	Acini larger, very distended, full of spermatozoa with the tails orientated towards the lumen. Spermatogenic activity very intense in acini walls. Scarce connective tissue between acini with some brown cells observed. Eosinophilic granular haemocytes present in the acini (Fig. 1F).
Regressing (5)	Some residual vitellogenic oocytes, free in the lumen, seen inside the acini with abundant eosinophilic granular haemocytes. Abundant cellular debris observed. Acini collapsed as a result of oocyte release. Connective tissue proliferates and is filled with brown cells. At the resting stage, collapsed acini filled with enlarged follicular cells closing the acini cavity, and sex cannot be determined (Fig. 1G).	Some residual spermatozoa observed in the lumen of the collapsed acini. No evidence of active gametogenesis. Eosinophilic granular haemocytes proliferate during this regressing stage. Connective tissue proliferates and is filled with brown cells.

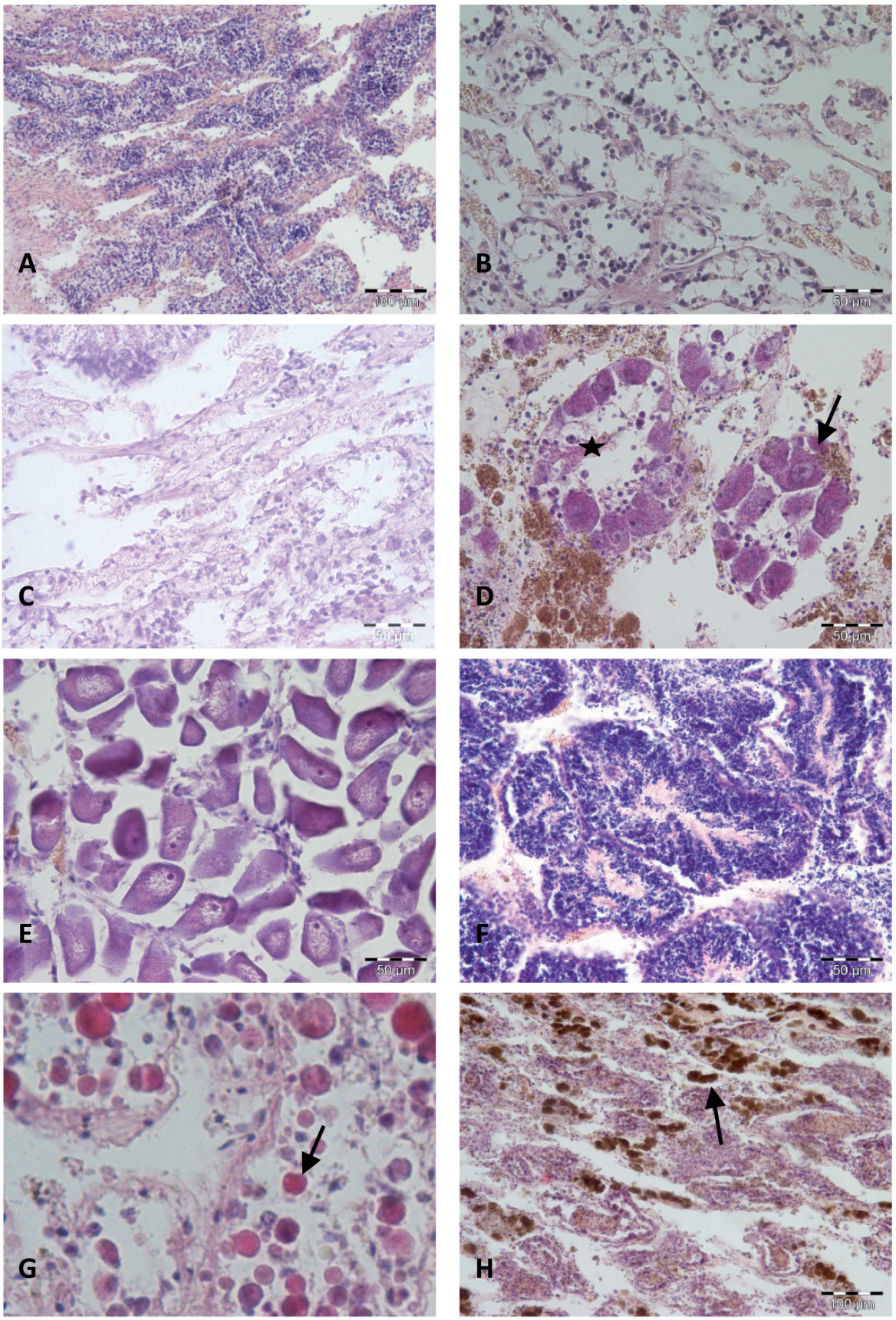

Fig. 1: Different stages of gonad development in *P. nobilis*: A) Undifferentiated, B) early developing female, C) early developing male, D) late developing female [note the presence of vitellogenic oocytes attached to the acini wall (arrow), and the many eosinophilic granulocytes in the acini lumen (asterisk)], E) spawning female, F) spawning male, G) regressing [eosinophilic granulocytes can be seen (arrow)), and H) resting (note the presence of abundant brown cells (arrow)).

The pen shell population was sampled for a full year (April 2011- March 2012) during which approximately 10 (9-12) individuals were collected every month. Individuals were measured (shell length and width) and visceral mass was dissected immediately from the shells at the sample site and weighed (edible or flesh weight), while muscle tissue was weighed separately and removed for further isotope analysis. Tissue samples were preserved in 10% phosphate-buffered formalin. In total, 120 individuals ranging between 0.13 and 0.69 m shell length and 0.06 to 0.28 m shell width were extracted. This number represents a very low percentage of the pen shell population in the MPA and this number should not have affected the natural population (Vázquez-Luis *et al.,* 2014a; Deudero *et al.,* 2015).

Seasonal trends in gonad development

Reproductive development was determined for 120 pen shells based on microscopic observations of the gonad. A macroscopic classification of development was not possible due to the absence of colour differences in gonads between sexes; all gonads have an orange-white colour throughout the year. Therefore, a transverse section of the visceral mass was made, caudal to the digestive gland, to observe the gonad tissue. Samples were dehydrated in alcohol and embedded in paraffin wax, sectioned in 3-4 µm slices, and stained with Mayer's haematoxylin and eosin for routine light microscopic examination.

The following scale of gonad maturation (based on histological observations, Table 1) was established: undifferentiated (immature-resting) (1), early developing (2), late developing (3), spawning (4), and a regressing phase (5). Female reproductive phases were determined based on the most advanced oocytes, and the occurrence of free post-vitellogenic oocytes at the lumen of the follicle. Male gonad stages were based on male germ cell development and the presence of spermatozoa in the lumen of the follicle. The presence of so-called brown cells (Cheng, 1981), which are involved in phagocytosis processes (Adema *et al.,* 1991), was used as an additional criterion.

The reproductive cycle was evaluated for all sampled individuals. Hermaphrodites were considered as one individual and classified according to the most advanced developing stage. The spawning season is defined as the time period containing individuals in the spawning phase (stage 4 in both sexes).

Reproductive activity was estimated by calculating a Gonad Index (GI), a numerical grading system based on developmental stages observed during each monthly sampling (Soria *et al.,* 2002). Unlike the more detailed categories (5) of the scale of gonad maturation described above, here three categories were established on the basis of gonad development: 1 (not developing) = immature-indifferent + regressing-resting, 2 (developing) = early developing + late developing, and 3 = spawning. The GI was estimated by multiplying the number of individuals belonging to each category by the category score (1 to 3 points), from which average values and standard errors were calculated.

Additionally, lipids, carbohydrates, and proteins were determined with histochemical reactions on unstained sections embedded in paraffin (Table 2). The histochemical composition of oocyte granules allows distinction of the onset of yolk formation (vitellogenesis). For each histochemical reaction, sections from different specimens were stained simultaneously to reduce variability in stain intensities due to different staining runs. Presence/absence of these compounds was indicated in the results.

Cost of maturation

To determine if remobilisation of resources takes place during gonad maturation, the Muscle Condition Index (MCI) and an adapted Gonadosomatic Index (GSI) were calculated with tissue previously stored in formalin. We applied a modification of the GSI because *P. nobilis* presents a diffuse gonad (the gonad infiltrates the digestive gland forming the visceral mass) and the more conventional GSI cannot be calculated. We also calculate a Condition Index (CI), according Camacho-Mondragon *et al.* (2012), where the weight of the shell is taken into ac-

Table 2. Histochemical methods used to evaluate lipids, carbohydrates, and proteins.

Evaluation	Method	Literature
Glycogen, neutral mucosubstances and/or glycoproteins	Periodic acid−Schiff (PAS)	Mc Manus, 1948
Acid glycoconjugates	Alcian Blue (AB) (pH 2.5)	Lev & Spicer, 1959
Proteins in general	Hg Bromophenol Blue	Chapman, 1971
Lipids in general (unsatured)	Sudan Black B	McManus, 1945

References cited in the table from the monographs by Pearse (1985) and Bancroft & Stevens (1990).

count in the total weight of the organism. The indices are calculated as:

MCI = muscle weight/ weight of total soft tissue x 100

GSI = weight of visceral mass (including gonad)/weight of total soft tissue x 100

CI = fresh weight/total weight x 100

Additionally, the qualitative composition of the muscle tissue was determined by histochemical reactions, specifying the neutral lipids and carbohydrates (neutral/acid) as well as proteins. In total 106 samples were analysed, as some muscle samples were unrecoverable during processing.

Relationships between stable isotopes and reproductive parameters

Stable isotope ratios of $\delta^{13}C$, $\delta^{15}N$ and the ratio C:N were evaluated in all 120 muscle samples. Samples were rinsed with distilled water and dried at 60ºC for 24 h and then ground to a fine powder using a mortar and pestle. For each sample, 2.0 ± 0.1 mg of dried powdered sample was placed into a tin cup and combusted for ^{13}C and ^{15}N isotopic analysis with a continuous flow mass spectrometer (Thermo Finnegan Delta x-plus). Vienne Pee Dee Belemnite (VPDB) standards for carbon and atmospheric nitrogen for nitrogen were used. In order to calibrate the system and to compensate for drift over time, one sample

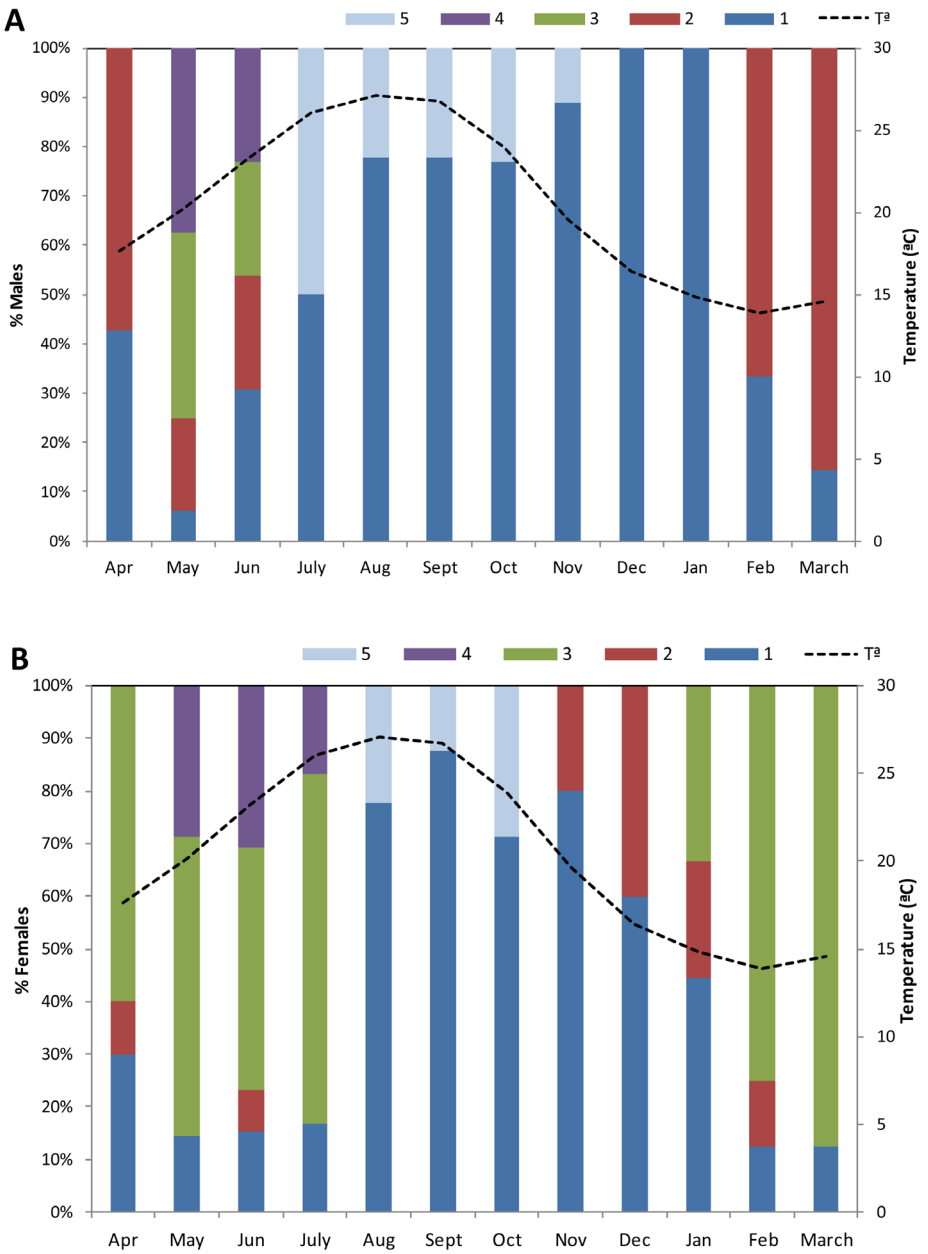

Fig. 2: Gonad maturation (phase) vs month of *Pinna nobilis* for A) males and B) females with indifferent individuals. 1. (blue) indifferent, 2. (red) early developing, 3. (green) late developing, 4. (purple) spawning, 5. (cyan) resting stage. The black dotted line denotes the evolution of temperature (°C) at the site during the sampling period.

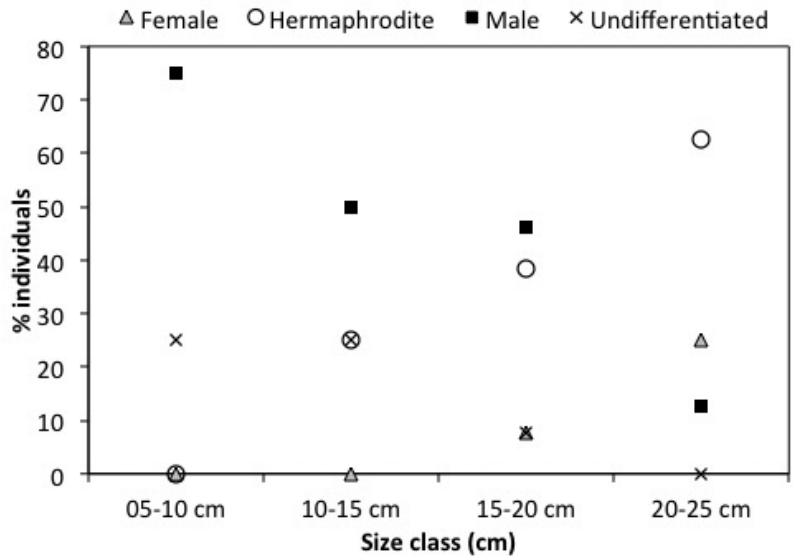

Fig. 3: Percentage of individuals with undifferentiated gonad development during the spawning period (May-July), or exclusively male or female gonad development or both (hermaphrodites) for size classes examined.

of an internal reference material, Bovine Liver Standard was analysed every eight samples (BLS, 1577b, U.S Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA). Analytical precision of SIA based on the mean standard deviation of replicates of internal BLS was 0.09 ‰ for $\delta^{13}C$ and 0.07 ‰ for $\delta^{15}N$.

Stable isotope abundances were measured by comparison of the ratio of the most abundant isotopes ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the sample with the international isotope standards. Ratios were expressed in δ notation with units of parts per thousand (‰) according to the equation $\delta^{13}C$ or $\delta^{15}N$ = [(R sample /R reference) -1] x 1000, where R is the corresponding $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratio for $\delta^{13}C$ and $\delta^{15}N$, respectively. The C:N ratio was calculated by dividing the percentage of carbon by the percentage of nitrogen present in tissue samples and multiplying the number by 12/14, due to the different atomic weights of these elements.

Statistical analyses

To analyse the isotopic values $\delta^{13}C$, $\delta^{15}N$, and C:N in adductor muscle of *P. nobilis* according to sampling month, a permutational multivariate ANOVA was used with 'month' as fixed factor (PERMANOVA: Anderson *et al.,* 2008). Significant differences between months were tested with a pair-wise test. A non-parametric Spearman correlation test was conducted to explore the correlation between stable isotopes and the Gonadosomatic Index. Statistical analyses were performed with PRIMER v.6 and its add-on package PERMANOVA+ (Anderson *et al.,* 2008) and SPSS.

Results

Sex ratio

Of all the pen shells sampled 24 were males, 32 females and 29 were hermaphrodites while 52 could not be sexed. The sex ratio in the reproductive season is M:F:H = 1.12:1.00:1.77 (M = Male, F = Female, H = Hermaphrodite), while the average sex ratio over the whole year in all samples is M:F:H = 1.63:1.00:1.63.

Seasonal trends in gonad maturation

During the study period, the surface water temperature varied from 27.1 ºC (August 2011) to 13.9 ºC (February 2012). The annual reproductive cycle during this period is summarised in Figure 2. In April 2011, female pen shells are mostly in early or final developing gonad stages whereas males are beginning their differentiation. In May there are some individuals in the spawning phase (Fig. 1e, f) with many hermaphrodites. In the hermaphrodite gonads, the development of both sexes is not synchronous since one sex is always in a more advanced stage of development. Development of gonads is also size dependent, as the smallest individuals only develop male gonads during peak spawning while with increasing size, a higher percentage of the population develops first both characteristics (hermaphrodites) while only the largest samples individuals can be female only (Fig. 3). The highest proportion of spawning individuals is found in June (41%) and July (57%). In June, all mature individuals are hermaphrodites and alternating sexes are observed over the spawning period (Fig. 2). In July, the spawning continues and in August all individuals are in

Table 3. Average values for the Gonad Index (GI) ± Standard Error (SE) for males and females during each monthly sample. 1 = immature-indifferent + regressing-resting, 2 = early developing + late developing, 3 = spawning.

Months	Males		Females	
	N	GI ± SE	N	GI ± SE
April	7	1.6 ± 0.2	10	1.7 ± 0.2
May	10	1.9 ± 0.1	7	2.1 ± 0.3
June	7	2.4 ± 0.4	4	2.5 ± 0.3
July	9	2.8 ± 0.2	6	1.8 ± 0.3
August	10	1 ± 0	10	1.0 ± 0
September	10	1.2 ± 0.2	9	1.0 ± 0
October	6	1.0 ± 0	9	1.0 ± 0
November	8	1.0 ± 0	11	1.0 ± 0
December	6	1.0 ± 0	10	1.3 ± 0.2
January	5	1.0 ± 0	9	1.4 ± 0.2
February	3	1.7 ± 0.3	8	1.9 ± 0.1
March	8	1.9 ± 0.1	8	1.9 ± 0.1

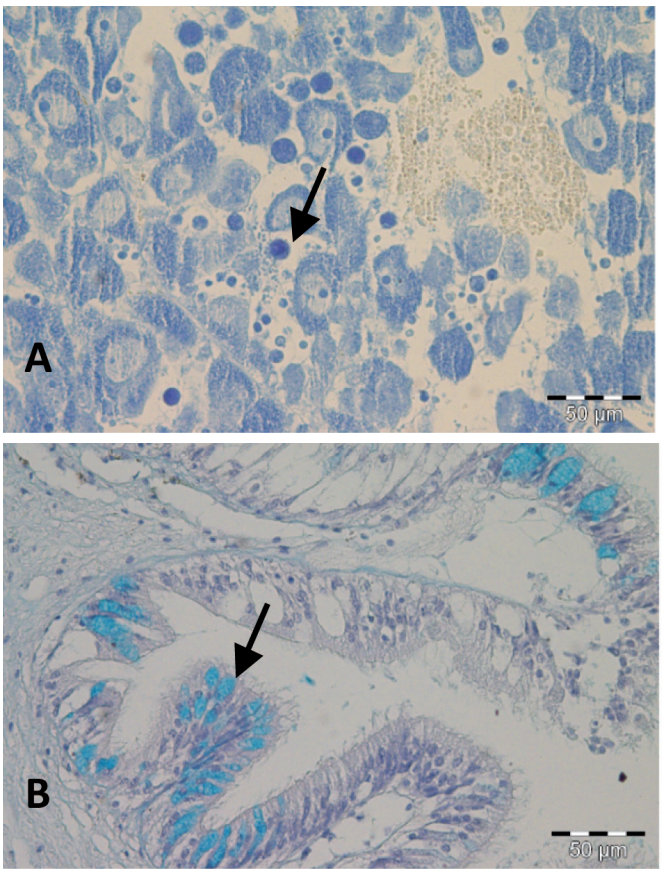

Fig. 4: Histochemical reactions in *P. nobilis*. A) Bromophenol blue. Note the intensity of the reaction inside vitellogenic oocytes and granulocytes (arrow). Brown cells are negative to the reaction (yellow coloration). B) PAS-Alcian blue. Goblet cells are deeply stained in blue (arrow).

the post-spawning phase (regressing or resting) (Fig. 1g, h, Fig. 2). Individuals remain in this phase during September until November. Until January, the majority of individuals are in the resting stage. Gonad differentiation begins in December, in only a small portion of the female population. During February and March many females are in the early (Fig. 1b) or late developing stages of gonad maturation while males are in the early developing stage (Fig. 1c, Fig. 2), confirmed by the higher score for females of the GI in these months (Table 3). Therefore, it can be stated that the spawning period begins in May and peaks in June-July. Only one spawning season is observed per year, ranging from late spring to summer. From our monthly observations of gonad maturation we can fit a linear regression of ripe individuals (%) over time (in Julian days) as % ripe = 200.42*ln (Julian day)-990.56. According to this model, the day we expect 100% of the adults to show mature gonads occurs on Julian day 209, or the 28th of July in our sampling year.

The temporal pattern of gonad development in sampled individuals is dependent on the size of the bivalve, with a lower threshold for size, as individuals smaller than 0.165 m shell length do not show any gonad development throughout the year.

Organic compounds in pen shell tissues

Spermatogenic cells are mainly protein, as well as yolk granules of vitellogenic oocytes (Fig. 4a). Haemocytes have the same histochemical composition as the yolk granules (Fig. 4a). Brown cells, typical of the connective tissue surrounding gonad acini, are negative to all the histochemical reactions (Fig 4a). Epithelia from the digestive tract and from the mantle display different intensity reactions not only between individuals but also between locations at the same individual. Muscular tissue, as well as the byssum, contains mainly protein. Goblet cells are rich in acid muco-substances (Fig. 4b), as well as the shell gland, whilst granular cells of the mantle are rich in protein.

Cost of reproduction

The reproductive peak in May is well-represented by the high GSI, which then sharply decreases from May to

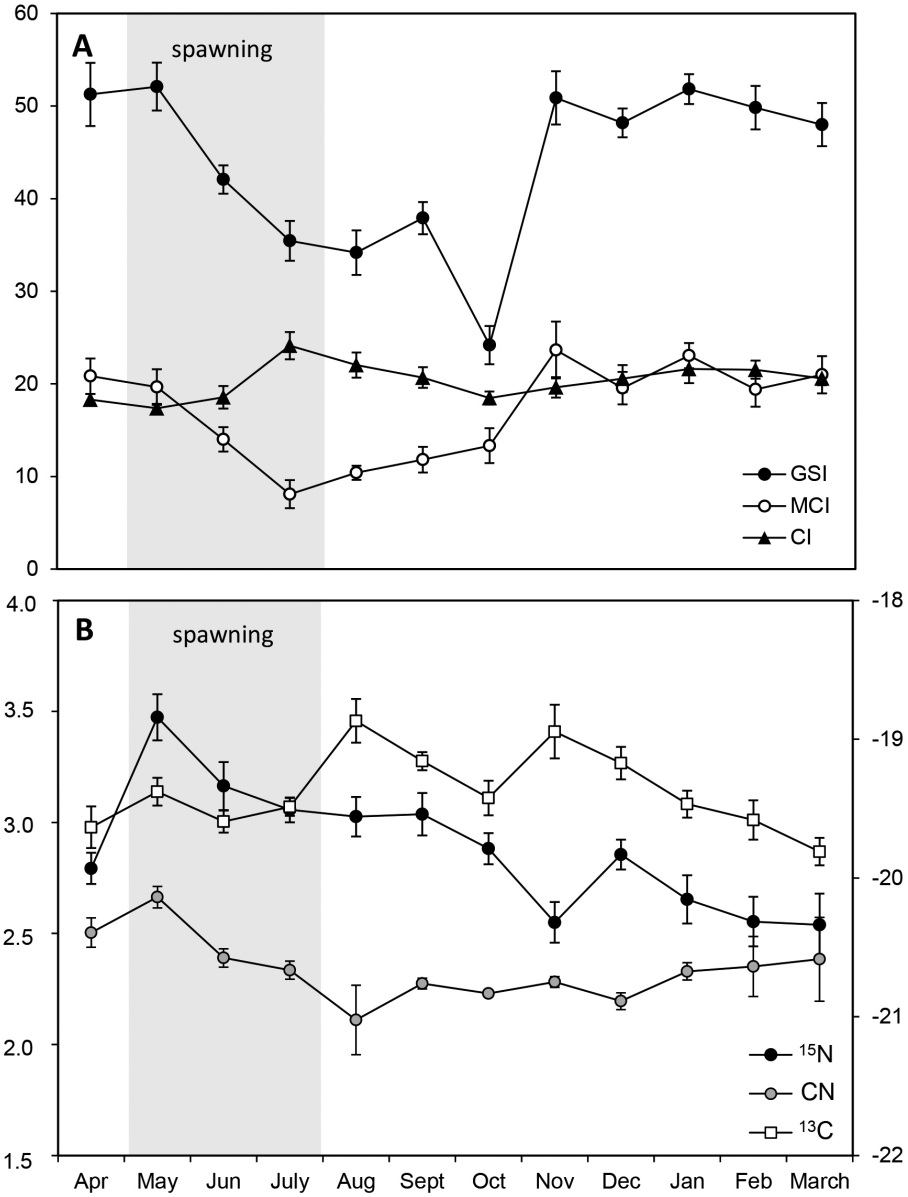

Fig. 5: A) Condition Index (CI, black triangles), development of the Gonadosomatic Index (GSI, black circles) and Muscle Condition Index (MCI, white circles) over time (month) with spawning period highlighted in grey. B) $\delta^{13}C$ (white squares), $d^{15}N$ (black circles), and C:N ratios (grey circles) over time (month). Error bars denote standard error. N = 109. Grey area denotes spawning period.

July, with the lowest value in October and only recovering, although abruptly, in November, just prior the beginning of gonad development (Fig. 5a). The MCI shows the same pattern on a smaller scale but only slowly recovers to pre-spawning values (Fig. 5a). The CI increases slightly during the period of reproduction (Fig. 5a) to decrease just before the end of the spawning peak.

Relationship between stable isotopes and reproductive parameters

Values of $\delta^{13}C$ in pen shell muscle tissue differ between months ($p < 0.001$; PERMANOVA), with the highest mean $\delta^{13}C$ values in August (-18.87 ± 0.16 ‰) and November (-18.95 ± 0.19 ‰). The lowest $\delta^{13}C$ values are obtained in April and March, -19.63 ± 0.15 ‰ and -19.81 ± 0.10 ‰, respectively (Fig. 5b).

P. nobilis $\delta^{15}N$ values show significant differences between months ($p < 0.001$; PERMANOVA) with a peak in May, mean maximum values of 3.47 ± 0.10 ‰, followed by decreasing nitrogen isotopic values reaching lowest $\delta^{15}N$ values in March (2.54 ± 0.14 ‰). This change in tendency of $\delta^{15}N$ values takes place in November, one month after a similar tendency change in $\delta^{13}C$ and GSI that takes place in October. In general, nitrogen isotopic signatures of adductor muscle samples during the spawn-

ing period are higher and less variable than during winter months (Nov-Feb, Fig. 5b).

The ratio between carbon and nitrogen in adductor muscle tissue (C:N ratio) shows significant differences between months ($p < 0.05$; PERMANOVA), and ranged from 2.11 ± 0.15 (August) to 2.66 ± 0.05 (May) (Fig. 5b).

GSI and $\delta^{13}C$ are negatively, although not significantly, correlated (Spearman, $\rho > 0.05$). GSI and $\delta^{15}N$ exhibit a significant negative correlation (Spearman, $\rho < 0.05$), while GSI is positively correlated with the C:N ratio (Spearman, $\rho < 0.05$).

Discussion

No histochemical differences were observed in goblet cell mucous along the different epithelia of *P. nobilis*, they only consist of acid mucosubstances. Mucous is an important layer protecting epithelia either from mechanical injuries or bacterial invasion (Petrinec *et al.,* 2005). Acid mucins probably lead to an increase in the viscosity, which is likely to be related to better protection (Suprasert *et al.,* 1987) and protects the intestinal epithelium against the degrading action of glycosidases (Carrassón *et al.,* 2006).

In *P. nobilis*, the brown cells surrounding gonad tissue are negative to all the tested histochemical reactions. These cells are very abundant in both sexes at the regressing –resting stage (stage 5) of gonad development inside the connective tissue. They contain translucent brown lysosomes (Zaroogian & Yevich, 1993), which are involved in phagocytosis (Hine, 1999). They appear to have a limited ability to degrade phagocytosed material, but may remove such matter by migrating across epithelial surfaces to the exterior of the animal (Hine, 1999). Their abundance in the connective tissue surrounding regressing gonads is in accordance with phagocytic resorption of residual cell debris after the spawning event. Oogenesis starts and ends before spermatogenesis as described in *A. seminuda* (Soria *et al.,* 2002). *Pinna nobilis* is a successive hermaphrodite with asynchronous development, as previously observed (De Gaulejac, 1995).

According to the histological analysis, and corroborated by the GSI values, only one single spawning period was observed for our population of pen shells in the bay of Cala Gandulf, Cabrera, at shallow depths (5-12 m). The duration of the spawning period is approximately three months (May-July) with peak intensity in July. This is not completely in agreement with published records of settlement peaks around the end of August (Cabanellas-Reboredo *et al.,* 2009a), since the larval period is thought to be short, but since these were observation in different years this discrepancy could be due to inter-annual variability as spawning in bivalves is usually triggered by temperature (e.g., Phillipart *et al.,* 2012). However direct observations of settlement peaks in the same year as this study (I. Hendriks pers. obs.) point at maximum settlement intensity in August, as observed previously by Cabanellas-Reboredo *et al.* (2009a). Therefore the duration of the larval phase in the Balearics is very likely to exceed the previous estimates. Observations of recruitment in the Bay of Palma (Cabanellas-Reboredo *et al.,* 2009a) have pointed to a second larval peak in January; however, this data shows that our particular shallow population cannot be the source of this second peak. It is possible that populations at greater depths are responsible for a second spawning period, as bivalve spawning triggers are related to water temperature and the thermocline around the island of Mallorca is approximately 20 m depth during the summer months and 60 m during the winter months (Reglero *et al.,* 2013; Olivar *et al.,* 2014).

The onset of the spawning period for our population corresponds to a water temperature of 20°C, while during the spawning peak period the water temperature was recorded at 27°C. This value of 20°C is comparable to the temperature at the onset of spawning of *A. seminuda* and a close relative, *Pinna rugosa* (Soria *et al.,* 2002). Since the Mediterranean Sea is an area of rapid summer warming (Bethoux *et al.,* 1990), this might have consequences for the timing of spawning of the pen shell. Between 2002 and 2010 the mean maximum summer seawater temperature around the Balearic Islands (western Mediterranean) increased by 1.05 ± 0.37 °C relative to 1980-2000, while model projections forecast an increase in summer sea surface temperatures by 3.4 ± 1.3 °C in the Balearic Island by the end of this century (Marbà & Duarte, 2010).

The histochemical analysis presented confirms the observations of the ultrastructural study of De Gaulejac (1995). Yolk granules of *P. nobilis* vitellogenic oocytes are made up of lipid-proteinaceous yolk with glucidic elements. The granules observed in the haemocytes within the acini showed the same histochemical composition as the yolk granules, indicating that they are actively phagocytosing degenerating mature oocytes debris. Macrophagous haemocytes around degenerating oocytes of several bivalve species are responsible, in part, of their resorption (Dorange & Le Pennec, 1989). Resorption of degenerating oocytes is a commonly observed phenomenon in species whose gametogenesis is under control of natural environmental conditions (Pipe, 1987) and has been previously described in *P. nobilis* by auxiliary cells but not by haemocytes (De Gaulejac, 1995). The phenomenon of oocyte degeneration, or atresia, documented for many bivalves (Morse & Zardus, 1997), varies in intensity and may be a strategy to balance egg production with low nutrient availability or unfavourable environmental conditions (Le Pennec *et al.,* 1991, Morse & Zardus, 1997). In *P. nobilis*, macrophage haemocytes are involved in this process, as shown by the histochemical results presented in this study.

Judging from the MCI index and shifts in isotopes pen shells are fuelling energy from stored resources towards gametogenesis and can thus be classified as 'capital breeders'. As in other Pinnidae, the MCI sharply decreases after the start of the spawning season (Soria *et al.,* 2002; Angel-Perez *et al.,* 2007), suggesting that this species also uses the reserves stored in the adductor muscle for reproduction. In addition, nitrogen values in muscle tissues of *P. nobilis* decreased after the start of the spawning season, suggesting that this species allocates the nitrogen stored in muscles to the reproduction processes. As isotopic values in muscle, digestive glands, and gonads do not vary independently and variations reflect metabolic transfers between organs (Lorrain *et al.,* 2002), we assume that higher oscillations in the C:N ratio during the spawning period (2.66 ± 0.05 (May) to 2.11 ± 0.16 (Aug)) reflect the use of reserves during this period. As the CI rises slightly during the spawning period while the GSI clearly shows gonad resorption it is hypothesised that the increase in condition of the pen shells can only be attributed to an increased availability of food resources.

Physiological processes, together with tissue lipid content and food sources, can exert a profound effect on the isotopic signature of stable isotope tracers (Lorrain *et al.,* 2002). Thus, temporal variability in tissue lipid content often varies with degree of reproductive activity (Berthelin *et al.,* 2000; Ojea *et al.,* 2004) and may affect the carbon isotopic signature during the reproduction period. $\delta^{13}C$ values in the adductor muscle exhibit seasonal shifts that might be coupled with formation and transfer of lipids during gametogenesis (mobilisation of C from the adductor muscle to the visceral mass and gonads). These processes explain the low $\delta^{13}C$ values encountered during the spawning season. Instead, after the gametogenesis, the low $\delta^{13}C$ values recorded during the colder months could be linked with environmental conditions (Alomar *et al.,* 2015) since carbon isotope values reflect the source of food more than trophic levels; for instance, a terrestrial versus offshore source (Rau *et al.,* 1992; Hobson *et al.,* 1995). Moreover, exogenous factors such as sediment temperature (van der Geest *et al.,* 2014) might influence organic composition and subsequently modify $\delta^{13}C$. Indeed, increasing temperatures are related with the peak of $\delta^{13}C$ in August (Power *et al.,* 2003).

The lower threshold of 0.165 m shell length that limits gonad maturation is an indication only, as no smaller individuals were harvested or evaluated. This length would correspond to a shell width of approximately 0.07 m (formula in Hendriks *et al.,* 2012b). The smallest harvested individuals only had male gonads (75%), while hermaphrodite development (25% of sampled individuals) began in size classes of between 0.10-0.15 m shell width. Only the larger harvested individuals (8% for 0.15-0.20 m and 25% for 0.20-0.25 m shell width, Fig. 3) had exclusively female gonads. The average shell width in the harvesting area is 0.142 ± 0.047 m (Hendriks *et al.,* 2013), and harvested individuals were targeted specifically for their size and high probability of having mature gonads and not chosen to represent an average population. The size limits for gonad development, however, indicates that the local population consists largely of immature individuals and males with only a minor percentage of hermaphrodites and females. This will be even more important for populations around the main island of the Balearic archipelago as average population shell widths are invariably smaller there (i.e. between 0.081 m for Es Cargol, the site in Mallorca directly facing Cabrera and 0.127 m in Cala d´Or, at the east coast; Hendriks *et al.,* 2013).

Conclusions

In the shallow areas of the Cabrera National Parks the asynchronic hermaphrodite pen shell populations show a single three-month spawning season. The condition of pen shell adults clearly decreases during the spawning season with the mobilisation of resources from the adductor muscle to the gonad, indicating 'capital breeding'. Decreased muscle force could mean they are more vulnerable during this period, coinciding with summer and thus a peak in recreational activities involving anchoring and poaching along with environmental factors such as hydrodynamic forces, although this may not be the season of highest incidence in storms and wave height. Outside protected areas this could lead to increased mortality of reproductive adults, an already a limited part of the total population. The occurrence of the invasive macroalga *Lophocladia lallemandii* during this period could add further metabolic stress while high temperatures favour bacterial growth and a further increase in metabolic needs.

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