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Reproductive and tissue plasticity in *Arca noae* (Bivalvia: Arcidae)

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Abstract
The reproductive strategy of an unexploited population of *Arca noae* from the salt-water Bizerte Lagoon (Tunisia, western Mediterranean), including its tissue plasticity, was studied. In total 309 individuals, collected monthly from October 2013 to September 2014, were examined; 142 were females, 42 were males and five were hermaphrodites. They were used in histological and immunohistochemical (stem marker: Pou5f1; proliferation marker: proliferating cell nuclear antigen (PCNA)) analyses of gonads and adjacent tissues (N = 189) as well as to compute the monthly condition index (N = 120). Water salinity, temperature and chlorophyll *a* concentration were recorded. Ripe ovaries were observed in two discrete periods, October–November and April–August. Both gonad ripenings were followed by spawning periods, November–April and July–September. The mature oocyte density showed that the first spawning period was less vigorous than the second one. These data also indicated that *A. noae* is a multiple spawner. Five cases of protandric hermaphroditism occurred from December to April. Gonad tissue was strictly associated and intermingled with the digestive gland and mantle muscle fibres. Seasonal variations were observed in the relative proportions of digestive gland and gonads: the former predominated when the latter regressed (March) and vice versa (peak in June). Seasonal transitions from germinal to somatic tissue and vice versa were hypothesised to occur through transdifferentiation mechanisms based on the activity of stem and proliferating cells. The condition index roughly increased along with gonad ripening and decreased during the spawning periods, although it did not run parallel to gonad evolution, because it also depended on chlorophyll *a* concentration, a proxy for phytoplankton density. The condition index was significantly correlated, by multiple regression, to both mature oocyte density and chlorophyll *a* concentration. *Arca noae* appears to have evolved a flexible reproductive strategy that makes it capable of exploiting diverse environmental conditions, which also involves tissue transdifferentiation.

Keywords: *Mollusca, reproduction, body condition, stem cells, Mediterranean Sea*

Introduction

The Noah's ark, *Arca noae* Linnaeus, 1758 (Bivalvia: Arcidae), is a commercial, edible bivalve widespread in the whole Mediterranean Sea and the eastern Atlantic Ocean from Portugal to Angola (Gofas 2008). Hence, it is a warm-affinity mollusc. It lives attached by a solid byssus on rocky grounds or other solid substrate (e.g. dead mollusc shells), from the low-tide level to about 120 m depth (Hrs-Brenko & Legac 1996). In the Mediterranean, it becomes sexually mature at an early age, i.e. 2 years, when slightly larger than 15 mm and 20 mm in males and females, respectively (compare data in Bello & Paparella 2001; Peharda et al. 2006), and reaches 12 cm in length and 25 years of age (Puljas et al. 2015). It takes 3 to 7 years to grow to about 50 mm length (Peharda et al. 2002, 2003), which corresponds to the minimum commercial size. It is the most important edible arcid species in the Adriatic Sea and is commercially exploited in Croatia, Italy and Slovenia (Valli & Parovel 1981; Peharda et al. 2006) where it attains high market quotations (Poutiers 1987).

The reproductive biology of *A. noae* has been investigated only in the Adriatic Sea. Studies on its life traits have been carried out by Bello and Paparella (2001), Peharda et al. (2002, 2003, 2006,

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2009), Bello et al. (2013), Župan et al. (2014) and Puljas et al. (2015).

No fishery management has ever been implemented for *A. noae*, although several papers have suggested that its Adriatic populations are overfished and require a suitable conservation policy (Bello & Paparella 2001; Peharda et al. 2002, 2003, 2006, 2009; Župan et al. 2012; Bello et al. 2013). The overexploitation of *A. noae* populations alters their structure both size- and sex-wise, since females, which are larger than males, are proportionally harvested more than males. This hinders the ascertainment of actual natural biological parameters, including sex ratio by size/age class and size/age at sex change (Bello et al. 2013).

The presence of *A. noae* has been occasionally recorded in Tunisia: in the Gulf of Gabes (Risso 1978; Darmoul et al. 1980; Ben Mustapha et al. 1999; Enzenross & Enzenross 2001), the North lagoon of Tunis (Saubade & Risso 1983), the Bizerte Lagoon, Tabarka (Enzenross & Enzenross 2001) and the Gulf of Tunis (Zouari 1985; Ayari & Afli 2008). Due to their negligible commercial exploitation, none of these (sub) populations has ever been studied and no information is available on their demographic structure and reproductive biology.

Since the reproductive cycle of *A. noae* was already satisfactorily described by Valli and Parovel (1981) and Peharda et al. (2006), the present study aimed mainly at knowing the reproductive strategy of this species in the Bizerte Lagoon, including periods and pattern of reproduction. This information is necessary for establishing a knowledge base (Gosling 2003) for an unexploited (sub)population in view of the management of stocks that will be likely exploited in the near future. Moreover, the information obtained from the study of unexploited stocks provides an insight into the natural biological parameters of a species, which in turn are an essential reference tool for the assessment and management of overexploited stocks from other geographical areas.

In addition to gaining this set of information, the histological analyses of *A. noae* gonads provided insights into the structural plasticity of this marine invertebrate, which displayed an unreported capability of modifying the structure and volume of visceral organs on a seasonal basis, most likely through a transdifferentiation mechanism also observed in other marine and terrestrial invertebrates but never reported in any bivalve.

Materials and methods

Specimens of *Arca noae* were collected in the Bizerte Lagoon, Tunisia, western Mediterranean Sea, close to the Sicilian–Tunisian sill. This is a salt-water coastal lagoon 150 km^2 wide, with a maximum depth of 12 m (Souissi 1981). It is connected to the Mediterranean Sea by a large, 8-km-long channel, which allows a considerable water exchange with the sea. Freshwater inputs to the lagoon derive from the Ichkeul Lake, through a 5-km-long channel, and from several *wadis* (seasonal streams) as well as from rainfall. Although the Bizerte Lagoon is affected by agricultural runoff, the sampling station for *A. noae*, in its southern

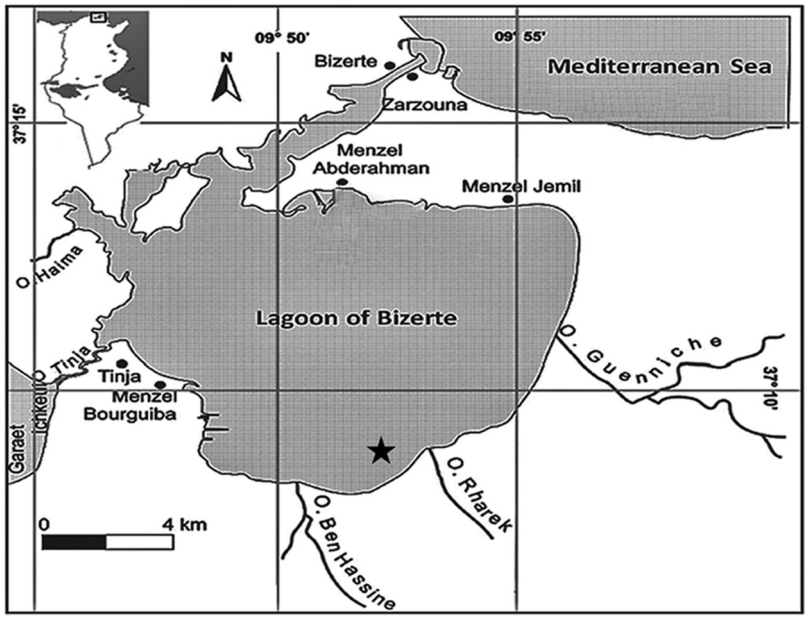

Figure 1. Map of the Bizerte Lagoon (North Tunisia). Black star: sampling site.

Table I. Number (N) and size range of *Arca noae* specimens from the Bizerte Lagoon.

Month	Specimens collected (N)	Size range (mm)
October	20	59.4–70.6
November	20	52.9–73.8
December	20	47.0–62.2
January	20	53.2–70.8
February	24	28.4–62.1
March	25	16.4–65.0
April	25	46.2–74.6
May	25	58.5–75.5
June	25	43.5–64.0
July	26	39.9–58.9
August	41	23.7–69.9
September	40	44.5–68.6

part (coordinates: 37°08'36"N, 9°52'20"E), was far away from urban and industrial sources of pollution (Figure 1).

A total of 309 specimens were hand-collected by scuba diving at about 3 m depth from October 2013 to September 2014, on the $15^{th} \pm 1$ day of each month (Table I), and carried to the laboratory in a cool box. In the laboratory, the shell length (*SL*) of all specimens was measured with a digital calliper and rounded down to the nearest 0.1 mm. The gonads from 10 to 31 sexually mature individuals per month were used for histological and immunohistochemical analyses (in total 189 specimens; *SL* range = 16.4 to 75.5 mm, all of them > 12 mm, size at first maturity according to Peharda et al. (2006)). The gonads were fixed in 10% buffered formalin, dehydrated in ethanol and embedded in paraffin wax; 5-µm-thick sections were obtained by means of a microtome and stained with haematoxylin–eosin. In order to detect the spawning period of the species in the sampling area, the occurrence of mature specimens – i.e. specimens with ripe or partially spawned gonads (cf. Walker & Power 2004; Peharda et al. 2006) – was recorded.

The identification of stem cells was carried out through the immunohistochemical identification of Pou5f1, a transcription factor involved in the maintenance and self-renewal of undifferentiated and pluripotent cells (Sánchez-Sánchez et al. 2010; Schulz et al. 2010; Lacerda et al. 2014).

The identification of proliferating germ cells was performed through the immunohistochemical localisation of proliferating cell nuclear antigen (PCNA), a polymerase delta accessory protein that is synthesised in late G1 and S phases of the cell cycle and is, therefore, used as a nuclear marker of proliferation.

The immunohistochemical detection of Pou5f1 and PCNA was performed using the same protocol, with the exception of an antigen retrieval procedure that was applied only to Pou5f1 immunostaining. This procedure was performed by boiling testis sections in citrate buffer (0.01 M, pH 6.0; 4 × 5 min cycles) in a microwave oven on high power (750 watts). Endogenous peroxidase was inhibited by treating sections for 10 min with 3% hydrogen peroxide (H_2O_2) and then rinsing them with distilled water and phosphate-buffered saline (PBS, 0.01 M, pH 7.4, containing 0.15 M sodium chloride [NaCl]). Subsequently, sections were incubated for 30 min in normal horse serum (NHS; Vector, Burlingame, CA), to block non-specific binding sites for immunoglobulins, and then incubated overnight in a moist chamber at 4°C with rabbit polyclonal antibodies raised against synthetic peptide of Pou5f1 (Abnova, Taipei, Taiwan) and monoclonal antibodies to PCNA (Santa Cruz Biotechnology Inc., Dallas, Texas). Anti-Pou5f1 and anti-PCNA antibodies were diluted 1:500 and 1:100, respectively, in PBS containing 0.1% bovine serum albumin (BSA; Sigma–Aldrich, Milan, Italy). After rinsing for 10 min in PBS, immunohistochemical visualisation was obtained using the Vectastain Universal Elite Kit (Vector, Burlingame, CA). This method uses the avidin–biotin–peroxidase complex (ABC) procedure. Peroxidase activity was visualised by incubating for 10 min with a Vector DAB Peroxidase Substrate Kit (Vector, Burlingame, CA), which produces a brown precipitate. To confirm the specificity of the immunoreaction, a control-staining procedure was carried out by replacement of the primary antibody with NHS and PBS.

To estimate the mean monthly density of mature oocytes (MOD), a representative histological slide per each female specimen was examined and all of the oocytes larger than 50 µm occurring in five different microscope fields (field surface = 0.244 mm^2) were counted. The mean density (oocytes/mm^2) of each month was compared with that of the contiguous months by the Student's *t*-test.

In addition to the microscopic examination of gonads, the analysis of "body condition" – an indicator of the individual overall body mass, which depends on both trophic state of soma and gonad state – was carried out. Ten specimens per month were chosen at random and sexed macroscopically by the colour of their gonads: whitish in males, orange to purple-red in females (Figure 2).

Their soft tissues were carefully removed from the shell and dried in the oven at 60°C for 48 h; the shells were scraped to remove all epibiotic material and dried in an oven at 60°C for 48 h. Soon after, the mass of both dried soft tissues (*BW*) and dried shells (*SW*) were measured with a digital scale to the nearest 0.01 g. Incidentally, in this comparatively irregularly shaped mollusc, shell mass was found to

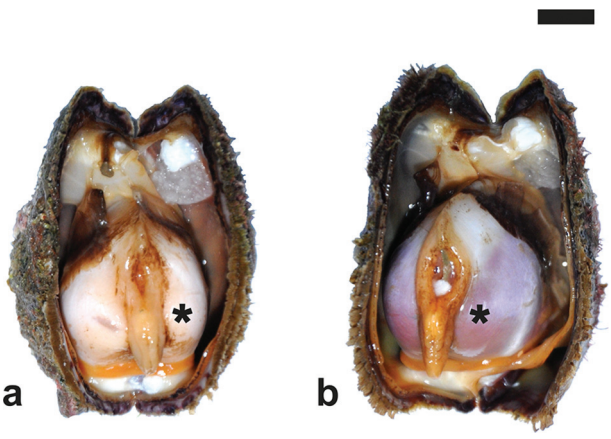

Figure 2. Fully ripe specimens of *Arca noae* collected in late July from the Bizerte Lagoon. (a) Male. (b) Female. Asterisk, right gonad. Scale bar = 1 cm.

be a better descriptor of the animal's overall size than shell length. To evaluate the year-round evolution of "body condition", the monthly relationship between *BW* and *SW* was analysed; since no statistically significant differences were found in any month between the male and female *BW*/*SW* ratio, the raw data for the two sexes were pooled. The monthly individual *BW* and *SW* values were log-transformed (natural logarithms) and fitted to predictive linear regression equations (model I of Sokal & Rohlf 2012) of the type $\ln BW = a + b \ln SW$. The set of 12 regression equations so obtained was tested by analysis of covariance (ANCOVA). In order to avoid the effects of body size (see Results), the monthly adjusted means of log-transformed soft tissue dry masses, $\ln BW_{adj}$, were computed by ANCOVA as suggested by Trippel and Hubert (1990). Pairs of $\ln BW_{adj}$ values of consecutive months were compared by the Tukey–Kramer multiple-comparison test (Sokal & Rohlf 2012).

The size class frequency distributions of males and females were compared by the non-parametric Mann–Whitney *U*-test (Sokal & Rohlf 2012).

The mollusc sampling was complemented by temperature (°C) and salinity (S‰) records at 1 m depth using a WTW-197i multimeter, as well as by surface water collection for the determination in the laboratory of chlorophyll *a* concentration (Chl *a* [mg l^{-1}]). Chlorophyll *a* was extracted using Whattman GF/F filters with 90% methanol, and its concentrations were determined spectrophotometrically at 665 and 750 nm (Aminot & Chaussepied 1983). The seasonal variation of Chl *a* was monitored during this study as a proxy for the primary production, and hence food availability, for bivalves.

Correlations among these parameters, as well as between them and body condition and between the latter and mature oocyte density, were examined by both single and multiple regressions, in order to understand how the environmental situation affects oocyte maturation and animal condition.

Results

Gonad structure

The *Arca noae* gonad tissue was found to be strictly associated and mixed with the digestive gland and with mantle muscle fibres (Figure 3(a,b)) (see the section G*onad/digestive gland tissue plasticity*).

Both ovaries and testes were arranged in acini (follicles), in which germ cells at different stages of development were observed (Figure 3(a,b)). Gonad acini conveyed their products in small gonadal ducts (250–370 µm in diameter) lined with a simple columnar ciliated epithelium with scattered secretory cells containing acidophilic granules (Figure 3(c)). Small gonadal ducts merged into a large main gonadal duct (700–800 µm in diameter) lined with a simple columnar ciliated epithelium containing both cells with acidophilic secretory granules and cells with large vacuoles (Figure 3(d), inset). Gonadal ducts containing gametes were observed in few specimens (Figure 3(c,d)).

Reproduction seasonality

Out of the 189 histologically analysed gonads, 142 were ovaries, 42 testes and five hermaphroditic. Incidentally, the Mann–Whitney *U*-test showed that the females in the sample were significantly larger than the males ($P < 0.05$).

FEMALES (N = 142; *SL* range: 23.7–75.5 mm; mean *SL* = 56.6):

Specimens with mature oocytes, inside either ripe or partially spent ovaries, were found throughout the year (Figure 4).

Ripe ovaries showed densely packed mature oocytes, polygonal in shape, which enclosed a large euchromatic nucleus with a single eccentric nucleolus (Figure 5(a)). Oocytes at this stage incorporated plenty of acidophilic yolk granules, mainly accumulated at their lumen-facing pole (inset of Figure 5 (a)). Females with ripe ovaries were collected from October to November and from April to August.

Partially spawned ovaries (Figure 5(b)) showed mature oocytes with the same morphological aspect as those observed in ripe females; however, these ovaries had released part of their oocytes and hence their acini were partially empty. This condition was

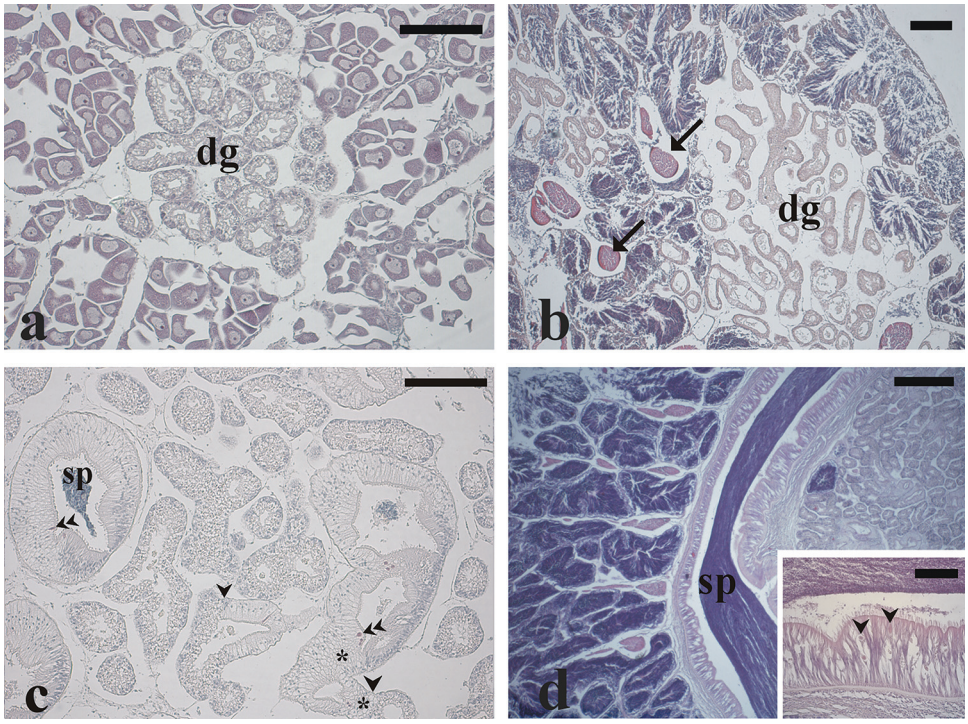

Figure 3. Microphotographs of *Arca noae* visceral mass sections showing gonad tissue associated and mixed with digestive gland and with muscle fibers. (a) *Arca noae* specimen collected in August showing partially spawned ovarian acini and digestive gland. (b) *Arca noae* specimen showing partially spawned testicular acini and digestive gland. (c) Small sperm ducts containing spermatozoa along with digestive gland in an *Arca noae* specimen collected in June. Note the degeneration of the digestive gland adenomeres concomitant with the proliferation of gonad duct epithelial cells. (d) Main sperm duct containing large amount of sperm between testis (left) and digestive gland (right) tissues. The inset shows the ciliated columnar epithelium of the sperm duct. Haematoxylin–eosin staining. Arrowhead, transition between digestive gland and gonadal duct; double arrowhead, secretory granules; arrow, muscle tissue; asterisk, proliferating gonadal duct epithelial cells, dg, digestive gland; sp, spermatozoa. Scale bars: a, b = 200 μm; c = 50 μm; d = 600 μm; inset of d = 50 μm.

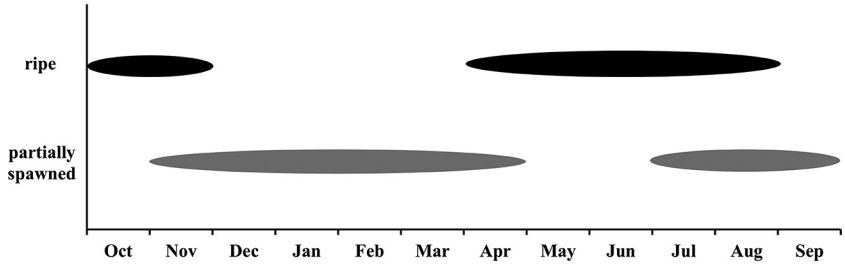

Figure 4. Months in which *Arca noae* specimens from the Bizerte Lagoon showed either ripe or partially spawned ovaries.

considered evidence of an effective spawning activity. Females in this condition were found in two discrete periods: from November to April and from July to September.

MALES (N = 42; *SL* range: 16.4–72.0 mm; mean *SL* = 53.0):

The histological examination of testes showed that all males collected in March (N = 4) had polygonal-shaped ripe acini and seemingly had not started to spawn yet (Figure 6(a)). Spermatogonia in active proliferation, as well as spermatocytes and spermatids, were assembled in different layers at the periphery of the acini, the lumina of which were packed with flagellated spermatozoa. The nuclei of both spermatogonia and spermatocytes immunoreacted with anti-PCNA antibodies (Figure 6(b)). Testes from all other months (N = 38) showed ripe as well as partially emptied acini, the latter state being indicative of ongoing spawning activity (Figure 6(c)).

HERMAPHRODITES (N = 5; *SL* range: 47.2–56.4 mm; mean *SL* = 52.0):

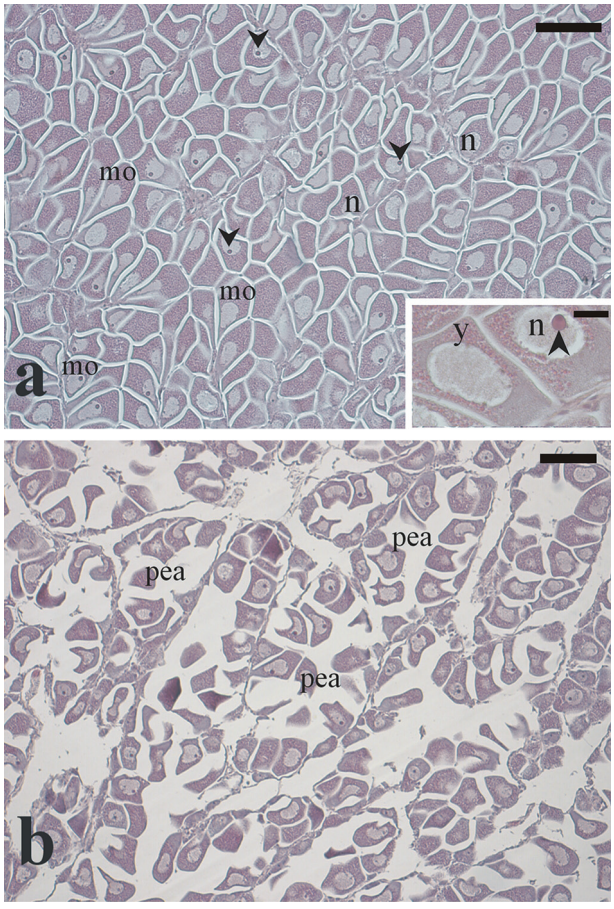

Figure 5. Microphotographs showing ripe and partially spawned *Arca noae* ovaries. (a) Ripe ovary showing large vitellogenic oocytes. (b) Partially spawned ovary showing mature oocytes and empty spaces, signs of oocyte release. Haematoxylin–eosin staining. Arrowhead, nucleolus; mo, mature oocyte; n, nucleus; pea, partially empty acini; y, yolk granules. Scale bars: a = 100 μm; inset of a = 20 μm; b = 100 μm.

The co-occurrence of female and male germ cells in one gonad, i.e. hermaphroditism, was observed in five specimens (Figure 7). They were collected in December (*SL* = 47.5 mm), February (*SL* = 55.3 mm), March (*SL* = 47.2 mm) and April (*SL* = 53.6 and 56.4 mm). All the hermaphroditic gonads were purple red-coloured and could be set apart from normal ovaries only through histological examination.

The February specimen's acini contained previtellogenic oocytes, still attached to the acini wall, along with few luminal ripe oocytes. Clusters of spermatozoa were visible within the acini lumen intermixed with mature oocytes (Figure 7). The hermaphroditic gonads from the following months were at a further advanced maturation phase, showing acini either full of ripe oocytes or partially empty (i.e. with a lower oocyte density). As for the male component, there were only

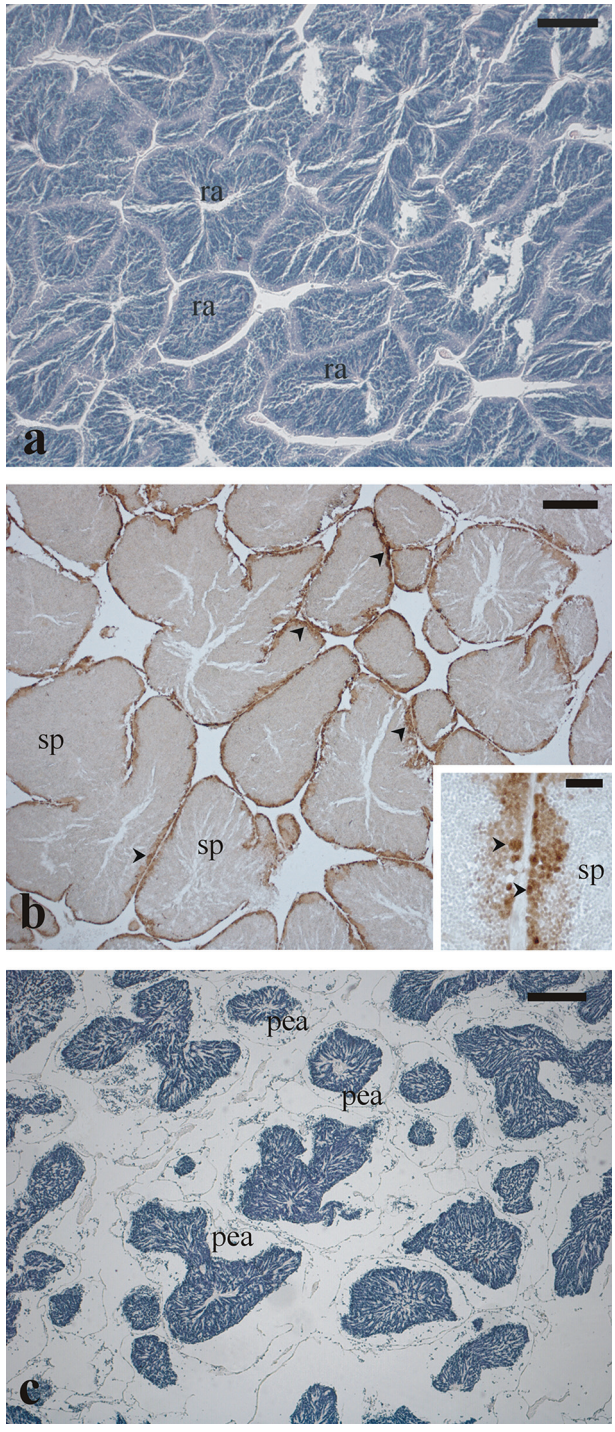

Figure 6. Microphotographs showing ripe and partially spawned *Arca noae* testes. (a) Ripe testis showing large acini filled with spermatozoa. Haematoxylin–eosin staining. (b) Section from a ripe testis immunostained with anti-PCNA antibodies showing dividing cells confined to the periphery of the acini (arrowheads). Inset: particulars of two acini from (b) showing the nucleus of dividing cells stained in brown. (c) Partially spawned testis showing partially empty acini. Haematoxylin–eosin staining. pea, partially empty acini; ra, ripe acini; sp, spermatozoa. Scale bars: a, b, c = 200 μm; inset of b = 20 μm.

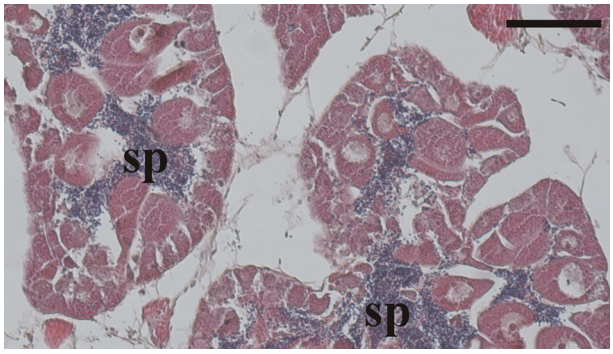

Figure 7. Microphotograph from an *Arca noae* hermaphrodite specimen sampled in February (shell length (*SL*) = 55.3 mm). Spermatozoa are visible within ovarian acini containing previtellogenic oocytes, still attached to the acini walls, along with few ripe oocytes in the lumina. Haematoxylin–eosin staining. sp, spermatozoa. Scale bar = 100 μm.

residual spermatozoa grouped in comparatively small clusters either inside the acini or among them.

This February specimen was at an earlier phase of the protandric sex-change than the March and April hermaphrodites, because its gonad male component was more conspicuous and the female component was at an earlier maturation stage than the others. In particular, the March specimen had seemingly already spawned a fraction of its oocytes and some of the remaining ones were degenerating (see below).

As for the scantiness of hermaphroditic individuals, the low overall percentage of hermaphrodites in the Bizerte Lagoon (2.1%) masks a much higher percentage. In fact, when only the male fraction of the only period when hermaphrodites occurred (December to April) is taken into consideration, the percentage of hermaphroditic specimens reaches 33.3 (calculation according to Bello et al. 2013).

Mature oocyte density monthly progression

From October to March, the MOD decreased from 178.9 to 55.7 oocytes/mm^2 (Figure 8), thus indicating that the ovary was steadily releasing its products. This is in agreement with the monthly progress of mature and partially spawned gonads: we found only mature individuals in October, mature and partially spawned individuals in November, and only partially spawned individuals from December to March (Figure 4), which trend is a robust indication that spawning occurred gradually, in fractionated batches from October to March. Hence, the oocyte density increased progressively through June (MOD = 267.4 oocytes/mm^2) because of the progressive ripening of the ovaries and the accumulation of mature oocytes. Afterwards, a second oocyte release phase ensued through September, which was faster and more massive than the previous one (MOD = 267.4 to 30.4 oocytes/mm^2; note the rapid drop in MOD from July to September in Figure 8).

This overall picture is corroborated by the histological examination of the ovaries. They were for the most part partially spawned from November to March, packed with mature oocytes from April to June, and partially spawned or spent in July–September after the massive summer spawning.

Gonad/digestive gland tissue plasticity

In the examined histological sections, the portions occupied by digestive and gonadal components showed seasonal variations; that is, digestive gland tissues predominated when the gonad regressed and vice versa. In March, when oocyte density was at a minimum and gonad acini were almost completely empty, the gonad tissue appeared to be degenerating with the disintegration of the gonad acini structure and the degeneration and reabsorption of residual oocytes and spermatozoa (Figure 9(a,b)). In June, when gonads were fully ripe and ready to spawn, and mature oocyte density peaked, a massive development of the gonadal duct system was observed concomitantly with the digestive tissue regression. This was shown, in histological sections, by the degeneration of adenomere cells, which were replaced by gonadal duct cells. In particular, in some males, clutches of spermatozoa were visible inside the lumen of well-structured gonadal ducts, as well as within degenerating digestive glands, which tissue

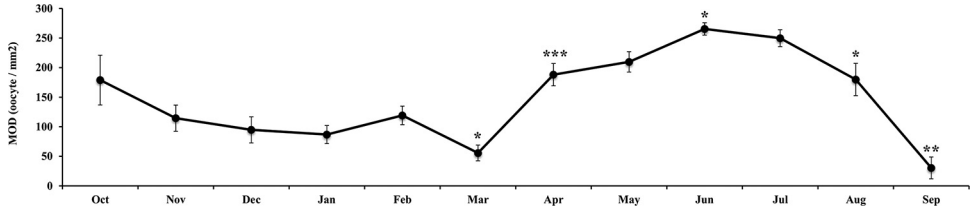

Figure 8. Monthly oocyte density (MOD) progression for female *Arca noae* from the Bizerte Lagoon. Significance levels: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

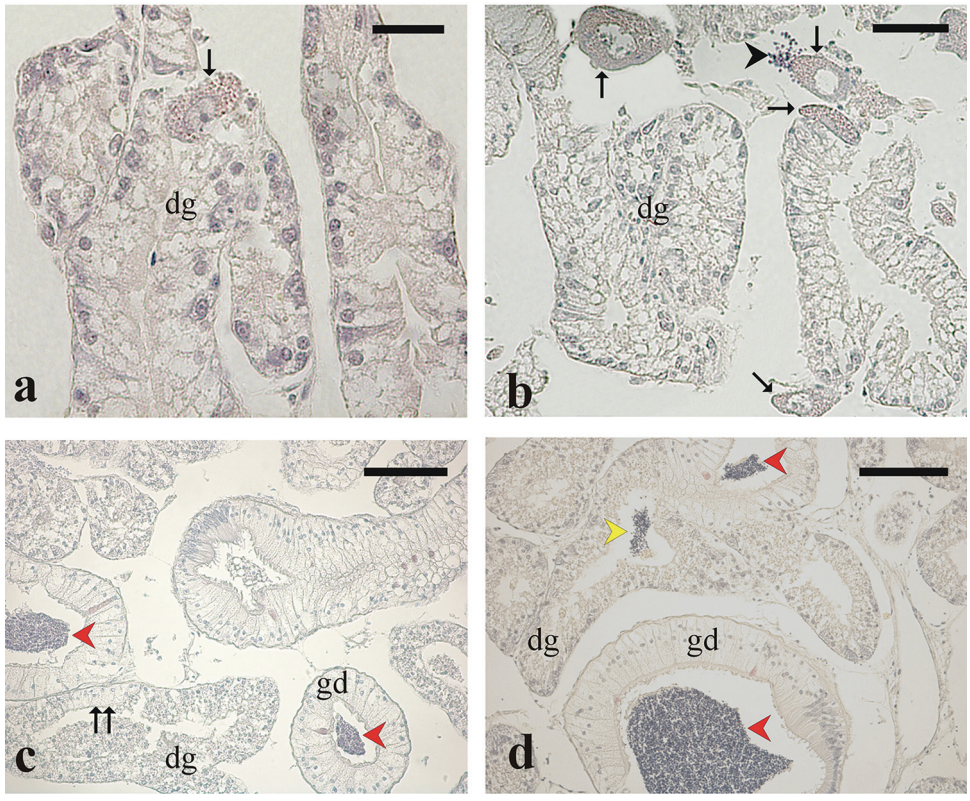

Figure 9. Micrographs from *Arca noae* gonad/digestive gland sections. (a) and (b) show the development of the digestive gland concomitant with the degeneration of the gonad in a female and a hermaphrodite specimen, respectively. In (a) note the presence of a reabsorbing vitellogenic oocyte inside a digestive gland adenomere. In (b) a few degenerating vitellogenic oocytes (arrows) and a cluster of spermatozoa (black arrowhead) are visible within the digestive gland tissue. (c) and (d) show degenerating digestive gland adenomeres along with developing gonadal ducts. Spermatozoa are visible within gonad ducts and in the lumen of a digestive gland adenomere. Haematoxylin–eosin staining. Arrow: degenerating oocyte; red arrowhead, spermatozoa within gonadal duct; yellow arrowhead, spermatozoa within the lumen of a digestive gland adenomere (please, refer to the online version for the explanation of coloured symbols). Double arrow indicates a digestive gland adenomere in which lining epithelial cells, morphologically similar to gonadal duct cells, are developing. dg, digestive gland, gd, gonadal duct. Scale bars: a = 25 μm; b, d = 50 μm; c = 100 μm.

was seemingly being remoulded into gonadal ducts (Figure 9(c,d)).

The immunohistochemical staining with antibodies against the stemness marker Pou5f1 labelled single small cells (diameter 6.1 ± 1.4 μm) scattered in the interstitial tissue as well as at the periphery of ovarian acini and around adenomeres of digestive glands (Figure 10(a)). The development of the gonadal duct system was supported by cells arranged within the glandular tissue that actively proliferated and gave rise to the epithelium lining the gonadal ducts (Figure 10(b,c)).

Environmental parameters

Monthly variations in water temperature (°C), salinity (S‰) and chlorophyll *a* concentration are shown in Figure 11. The highest water temperature (28°C) was recorded in July and August and the lowest (12°C) in January. Salinity varied from 33.4‰ in the winter (January), which is marked by rainfall and freshwater input from the Ichkeul Lake and several *wadis*, to 38.5‰ in the summer (July), when rainfall is fairly rare and water evaporation is very high (Figure 11(a)). The first notable Chl *a* peak was recorded in December (1.7 mg l^{-1}) and was likely due to the input of nutrient-rich fresh water after heavy rains. A second, much more marked peak occurred in May (2.5 mg l^{-1}) depending on spring phytoplankton blooms (Figure 11(b)). No statistically significant correlation was found between Chl *a* and either °C or S‰ (P = 0.76 and 0.99, respectively).

"Body condition"

The examination of monthly regressions of ln *BW* on ln *SW* showed that the correlation coefficient *b* was significantly different from 1 in most months, which

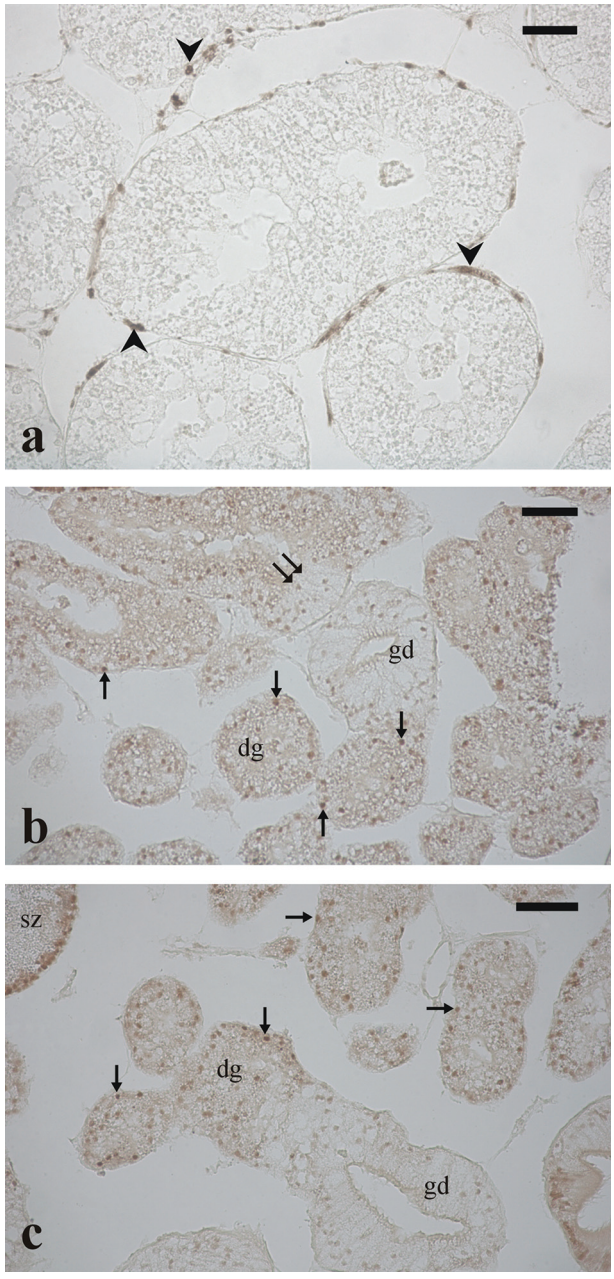

Figure 10. Micrographs from *Arca noae* testis/digestive gland sections immunostained with antibodies against the stemness marker Pou5f1 and with proliferating cell nuclear antigen (PCNA). In (a) the stem cells, scattered around digestive gland adenomeres, have brown-labelled nuclei. (b) and (c) show anti-PCNA positive (i.e. dividing) cells within digestive gland adenomeres, testicular acini and gonadal ducts. The immunostaining is stronger in the nucleus of digestive gland cells than in the epithelial cells lining the gonadal ducts. Arrowhead, stem cell; arrow, dividing cell. Double arrow indicates a digestive gland adenomere in which lining epithelial cells, morphologically similar to gonadal duct cells, are developing. dg, digestive gland, gd, gonadal duct. Scale bars: a = 25 μm; b, c = 50 μm.

was evidence of a significant size effect; that is, soft tissue mass was not proportional to shell mass throughout the *A. noae* size range. Hence, in order to avoid size bias, the computation of monthly adjusted means of log-transformed soft tissue dry masses, BW_{adj}, was carried out. Monthly BW_{adj} values generally increased as gonads ripened, and decreased during the spawning periods (Figure 12). However, such a relationship appeared to be modulated by other factors (see the following section). In particular, BW_{adj} remained fairly stable from October to February, despite the progressive reduction of mature oocyte density, and hence loss of gonadal mass. This seemingly depended on the fact that while the gonad tissue was being reduced, somatic mass was accreting anew. BW_{adj} started to increase in March, when Chl *a* increased thanks to spring phytoplankton blooms, and peaked in May, when oocyte density went on increasing and all specimens became mature, concomitantly with the highest Chl *a* (compare Figures 8 and 11(b)). The significant June drop depended as well on the concomitant considerable drop of phytoplankton density, in spite of the fact that oocyte density had reached its annual maximum. The subsequent decrease of BW_{adj}, from July to September, is related with the release of gametes.

Correlations between different parameters

The monthly mean mature oocyte density was found to be positively correlated to temperature and to salinity. Their regression equations are, respectively, MOD = −923.832 + 29.898 S‰ (r = 0.674; $P < 0.05$) and MOD = 1.962 + 7.357 °C (r = 0.577; $P < 0.05$). Moreover, the multiple regression of MOD on °C and S‰ for all months was not statistically significant (P = 0.064) but, when excluding the outlying September data from computation, the correlation of MOD to the two environmental factors became highly significant: MOD = 67.40 + 10.44 °C − 3.12 S ‰ (R^2 = 0.713; $P < 0.01$). Together °C and S‰ explained 71.3% of the MOD variance.

"Body condition", BW_{adj}, was found to be significantly correlated with the mature oocyte density (regression equation: $BW_{adj} = 1.292 + 3.120\text{x}10^{-3}$ MOD; r = 0.675; $P < 0.05$) and, to some degree although not significantly, with the chlorophyll *a* concentration (P = 0.07). The multiple regression examination showed that BW_{adj} is best correlated to both mature oocyte density and Chl *a*, a proxy for phytoplankton density (regression equation: $BW_{adj} = 1.146 + 2.781\text{x}10^{-3}$ MOD + 0.221 Chl *a;*

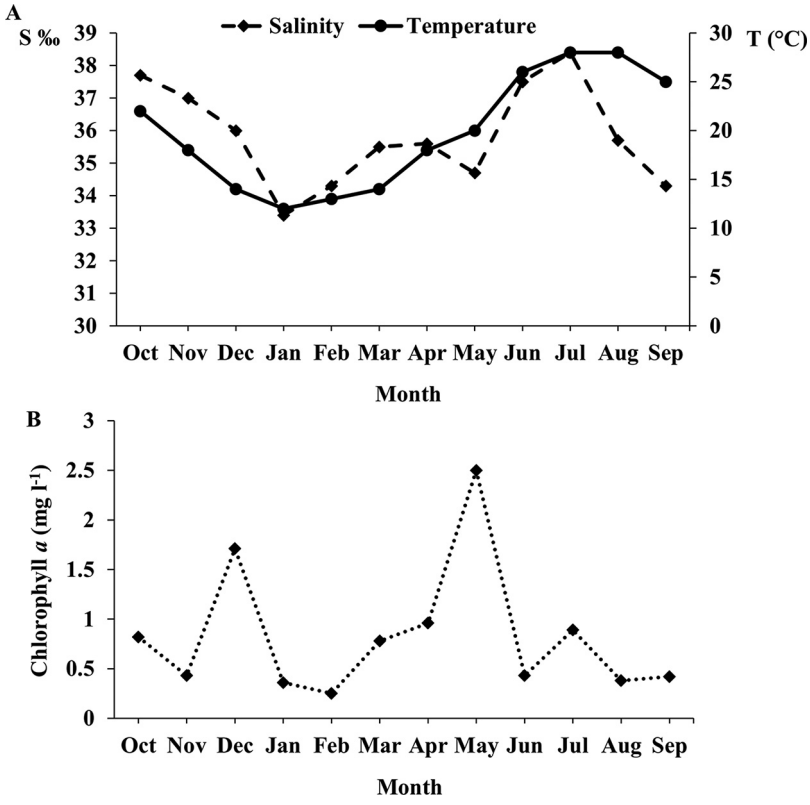

Figure 11. Monthly variation of (a) physico-chemical parameters, (b) chlorophyll *a* concentration (Chl *a*, μg l^{-1}) in the surface water of the Bizerte Lagoon.

$R^2 = 0.615$; $P < 0.05$). Together, the two regressors explained 62% of the BW_{adj} variance.

Discussion

The histological analyses of gonads coupled with the monthly succession of mature oocyte density suggested that, in the examined year, *Arca noae* from the Bizerte Lagoon underwent two reproductive phases, differing from each other in the pattern of gonad maturation as well as in the ensuing spawning. The October maturation was followed by a long spawning period, extending from November to March. A second ovarian maturation phase encompassed a three-month period, from April to June, and was followed by a comparatively rapid and intense spawning period from July to September.

The two spawning phases differed substantially from each other. The first one, extending throughout the autumn, winter and early spring, was characterised by protracted partial releases of gametes (i.e. multiple spawning). The second maturity/spawning phase, which started in April–May, involved all examined females. During this phase, the density of mature oocytes increased steadily, peaked in June and decreased gradually from July through September (Figure 8), thus showing that the animals were actively spawning from July to mid-September.

Temperature appeared to modulate the two gonad maturation/spawning phases. Comparatively low temperatures (12–14°C) were most probably responsible for a less intense release of gametes, in the winter spawning period, with respect to the summer, when temperatures were as high as 25–28°C (Figures 8 and 11(a)).

The fact that individuals of *A. noae* from the Bizerte Lagoon reproduced by multiple spawning, i.e. partial progressive release of gametes, is clearly shown by the progressive decrease of mature oocyte density during both spawning periods (Figure 8) as well as by the histological analysis of the gonads. Similarly, Peharda et al. (2006) reported the occurrence of partially spawned specimens in the two months following the period of gonad ripening in *A. noae* from the Adriatic Sea.

The spawned oocytes appeared to derive from one single cohort, contrary to what happens in other bivalves, e.g. *Venus nux*, in which several cohorts of oocytes were detected throughout the year (Tirado et al. 2011a). In this respect, Holopainen and Hanski (1986) suggested that populations of the same species might be either semeleparous or iteroparous

depending on the environmental circumstances that affect growth. These authors demonstrated in their work on *Pisidium* species that individuals living under favourable conditions are likely to be semelparous, while populations living under unfavourable conditions are likely to be iteroparous. A similar shift in reproductive modes was reported in *Musculium* species by Mackie et al. (1976a,b)) and Way et al. (1980). Therefore, the long, low-intensity late autumn to early spring multiple spawning period in *A. noae* from the Bizerte Lagoon may be related to the comparatively unfavourable conditions of that season (mainly low temperatures).

In the Bizerte Lagoon, the *A. noae* reproductive pattern showed peculiar features with respect to the other known Mediterranean areas. Indeed, the few existing studies on this topic were carried out on the Adriatic (sub)population (Valli & Parovel 1981; Peharda et al. 2006); several other papers have focused on accessory topics (Bello & Paparella 2001; Peharda et al. 2002, 2003, 2009; Bello et al. 2013; Župan et al. 2014; Puljas et al. 2015). Different reproductive patterns were described for the *A. noae* (sub)population of the Adriatic Sea, a comparatively small and closed sea. Peharda et al. (2006) showed that in Mali Ston bay (mid-eastern Adriatic Sea) this species reproduces by a single annual gametogenic cycle extending from October–November to April–May, and one spawning peak in the summer (July and August), with slight timing fluctuations from one year to another. A quite similar cycle was observed along the coast of Bari (south-western Adriatic Sea; P. Paparella and G. Bello, unpublished personal observations; see also Bello & Paparella (2001)). In the Gulf of Trieste (northern Adriatic Sea), according to Valli and Parovel (1981), *A. noae* reproduces throughout an extended season spanning from the spring to the autumn, with two spawning peaks in March and September, respectively. Two spawning peaks per year were also reported for the Gulf of Manfredonia (south-western Adriatic Sea) stock (Bello & Paparella 2001).

Contrary to the Adriatic reproductive patterns of *A. noae* with a single, more or less prolonged gametogenic cycle, either single- or two-peaked spawning, this study reports for the first time a rather different pattern of two discrete maturing/spawning phases differing in terms of both length and intensity. Fretter (1984), quoted by Baqueiro and Aranda (2000) in a detailed review of reproductive patterns of Mexican bivalves, classified them into two categories according to their reproductive strategies: tachitictic, with short and limited reproductive periods, and braditichtic, with an extended reproductive activity. Furthermore, the same population of a bivalve species can display differences in its reproductive activity from one year to another, i.e. either a short synchronic spawning or a long, partially asynchronous spawning or several partial spawnings (Bricelj & Malouf 1980; Baqueiro 1981; Jaramillo et al. 1993). Conversely, different spawning timings can be observed in the same bivalve from two geographically separate sites, depending on water temperature and pabulum concentration (Tirado et al. 2011a,b). The possibility of shifting bewteen braditichtic and tachitictic reproductive modes appears to pertain to *A. noae*, strategies which are sequentially exploited by this bivalve according to the season. Similar findings were reported about the oyster *Crassostrea* (= *Magallana*) *gigas* by Enríquez-Díaz et al. (2009), who observed different reproductive patterns in oysters cultured at different latitudes in the Atlantic Ocean. Those from the Baie des Veys (English Channel) showed a single partial spawning period in August, whereas those from Marennes-Oléron (Gulf of Biscay) displayed a partial spawning in July and a massive release of gametes during August. Enríquez-Díaz et al. (2009) assumed that the oyster spawning intensity was related to food availability. Interestingly enough, in the case of the Bizerte Lagoon, three Chl *a* peaks were observed, one between November and January; another one, the highest, between April and June; and the third one, lower, between June and August. The highest, spring peak corresponded with gonad ripening rather than spawning, whereas the other two peaks coincided with the two spawning periods, the slow winter one coinciding with the second highest Chl *a* peak.

The environmental peculiarities of the Bizerte Lagoon, chiefly the overall comparatively high chlorophyll *a* concentrations, were also related to the longer reproductive period in the oyster *Magallana gigas* cultured there with respect to an oyster stock cultured in an adjacent open sea site (Dridi et al. 2014).

Arca noae from the Bizerte Lagoon showed the presence of mature gonads during most of the year, which makes the reproductive pattern of this (sub) population more similar to that of tropical bivalves (e.g. the tropical oyster, *Crassostrea corteziensis*; Rodríguez-Jaramillo et al. 2008) than to the other known Mediterranean *A. noae* (sub)populations. This large flexibility confirms that bivalve reproductive strategies are not strictly species-specific since they are driven by complex interactions of environmental and genetic factors (Jovanovich & Marion 1989; Baqueiro & Aranda 2000).

In addition to Chl *a*, the second most important environmental factor to which mature oocyte density is significantly correlated, namely temperature,

appears to modulate the gametogenic cycles. Comparatively low temperatures (12–14°C) are most probably the cause for a slower release of gametes in the winter spawning period with respect to the summer, when temperatures are as high as 25–28°C (Figures 8 and 11(a)). Mature oocyte density is also multiple correlated to temperature and salinity except in the month of September, when oocyte density reached its lowest value following total gamete release in most specimens.

"Body condition" – in any of its many expressions found in the literature – is generally deemed a good descriptor of bivalve reproductive cycles (e.g. Peharda et al. 2006; Tirado et al. 2011a,b). In the present study the customary "soft tissue mass/shell mass" ratio (or similar expressions of "body mass/body size"), without any further transformation, was deemed inappropriate to describe body condition because it does not compensate for the effects of body size (Trippel & Hubert 1990; Labocha et al. 2014). In fact, in our case, (a) specimens of a comparatively large size range were used; (b) more importantly, body mass (*BW*) was not isometric to shell mass (*SW*) in most months (the slope of monthly ln *BW*/ln *SW* regression equations significantly differed from 1); and (c) the monthly ln *BW*/ln *SW* regression lines significantly differed from each other either in slope and/or position. That is why "body condition" was expressed as mean adjusted soft tissue dry mass BW_{adj}.

The monthly progression of "body condition" for *A. noae* from the Bizerte Lagoon is in agreement with the seasonal trend of mature oocyte density. However, the June "body condition" relative negative peak conflicts with the peak of mature oocyte density (Figures 8 and 12). This may be explained by the concomitant massive reduction of phytoplankton in the lagoon, as shown by the sudden drop of chlorophyll *a* concentration (Figure 11(b)), which in turn caused a dramatic decrease in weight of the soma, and hence of the June BW_{adj}. This supposition is markedly supported by the statistically significant multiple correlation of BW_{adj} with both mature oocyte density and chlorophyll *a* concentration. It is also corroborated by the fact that the June BW_{adj} variance was the lowest in the whole year; that is, all June specimens employed in the "body condition" analysis had rather similarly reduced soft tissue mass, hence they similarly suffered a shortage of food resources. Whatever the cause, the June decrease of BW_{adj} seemingly did not affect the reproductive cycle. In addition, the release of oocytes from November to March is not paralleled by the decrease of BW_{adj} likely because the release of gametes was compensated by the somatic mass growth; this is particularly evident in March, when BW_{adj} showed a significant increase concomitantly with phytoplankton early spring blooms.

To conclude, caution should be exerted when using "body condition" seasonal progression as a proxy for the reproductive cycle (see also Matozzo et al. 2005; Boussoufa et al. 2015) since this index changes according to both gonad and somatic mass variations.

With regard to the occurrence of hermaphroditic *A. noae* individuals, the protandric sex change was generally deemed occasional (Valli & Parovel 1981; Peharda et al. 2006) or obligate in this bivalve (Bello et al. 2013). In the present case as well, the male fraction in the examined samples was found to be smaller in size (*SL*) than the female fraction, which corroborates the obligate hermaphroditism

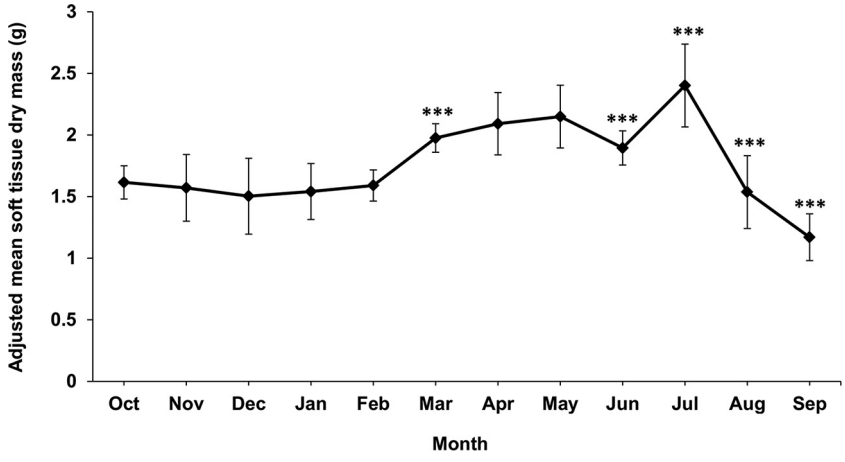

Figure 12. Monthly progression of adjusted mean soft tissue dry weight, BW_{adj}, for *Arca noae* from the Bizerte Lagoon. Significance levels: ***, $P < 0.001$.

hypothesis. However, data about *A. noae* hermaphroditism are too scanty to positively delineate a precise model of sex change, whether occasional or obligate and whether depending, to some extent at least, on the socio-demographic structure of the population, as has been clearly shown in other molluscs, e.g. *Patella ferruginea* (Rivera-Ingraham et al. 2011). Most likely, hermaphroditism is a potential feature in *A. noae*, which is expressed when and in the proportion needed to maintain population homeostasis, under the influence of either biotic or abiotic environmental factors (cf. review by Breton et al. 2017). Incidentally, in this case as well, the sex change occurred in a restricted period of time, shortly before the late spring massive gamete maturation. Moreover, the corrected percentage of hermaphroditic individuals in the Bizerte Lagoon sample (33.3%) is very close to that computed for the Croatian (sub)population (35%) and higher than the Apulian one (20%) (Bello et al. 2013).

The reproductive flexibility observed herein is not the only trait of *A. noae*'s extreme adaptability. The gonads and the gonad duct system are strictly associated and combined with other visceral organs as well as with mantle muscle fibres, which is a feature common to many bivalve species (Baccetti et al. 1991). During the reproductive cycle, gonads and the gonadal duct system undergo remarkable seasonal modifications in volume and structure, which are connected with changes in the volume and structure of visceral organs. In particular, the relative surfaces of the histological sections covered by the digestive and gonadal components vary on a seasonal basis: spent gonads are associated with a well-developed digestive gland system, and conversely gonad ripening is associated with a reduction of the digestive gland volume. Furthermore, the gonadal duct system develops during the spawning season at the expense of the digestive gland. The latter displays adenomere cell degeneration and proliferation of new lining epithelial cells that assemble the gonadal duct system. The immunohistochemical analysis showed anti-PCNA positive, i.e. proliferating, cells within digestive gland adenomeres when gonad maturity reached its annual peak. This proliferating activity is most likely associated with the development of gonadal ducts.

To the best of our knowledge, no data are available in the bivalve literature on transdifferentiation – that is, the process of transformation of differentiated somatic cells into different cell types. Indeed, several recent studies demonstrate that aquatic invertebrate taxa, including cnidarians, platyhelminthes, echinoderms and urochordate ascidians, exhibit widespread multiple cell types with stem cell attributes; hence, transdifferentiation is ubiquitous in both anatomically simple and highly evolved invertebrates (Rinkevich et al. 2009). In the present study anti-Pou5f1 immunopositive cells – that is, cells sharing stemness characteristics – were distributed around and within digestive gland adenomeres. These cells may retain the potentiality to differentiate and proliferate, giving rise to diverse cell lineages, so their occurrence can be related to the hypothesised, albeit highly probable, tissue transdifferentiation in *A. noae*. Therefore, the observed modifications in the *A. noae* reproductive and digestive gland systems should be interpreted as a mosaic piece of a wider and astonishing anatomical and physiological plasticity widespread in invertebrate organisms.

Conclusions

Arca noae has evolved a flexible reproductive strategy that makes it capable of exploiting diverse environmental situations in consonance with exogenous factors, mainly pabulum availability and water temperature. The present study, carried out on an unexploited (sub)population, widened the frame of the *A. noae* reproductive strategies and further corroborated it. Present results represent a landmark for a future management of the Bizerte Lagoon *A. noae* stock and, for the first time, offer interesting insights into mechanisms underlying seasonal changes in gonad and visceral organs of a bivalve species.

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