THE GONADS OF *MARGARITIFERA AURICULARIA* (SPENGLER, 1793) AND *M. MARGARITIFERA* (LINNAEUS, 1758) (BIVALVIA: UNIONOIDEA).

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ABSTRACT

A detailed study of the gonads of two endangered species of the genus *Margaritifera* living in the Iberian Peninsula, *Margaritifera auricularia* (Spengler, 1793) and *Margaritifera margaritifera* (Linnaeus 1758), based on macroscopic and histological observations is presented. In both species, gonadal tissue occurs within the visceral mass without a specific location. In hermaphrodite specimens, male and female acini are mixed in the visceral mass but can be clearly distinguished by detailed macroscopic study. The gonadal topography and cycle of *Margaritifera auricularia* is described for the first time, indicating that the species is a female hermaphrodite and that gametogenesis occurs from December to March. Fifty percent of *Margaritifera margaritifera* specimens studied were hermaphrodites and 50% females.

INTRODUCTION

Bivalve molluscs are considered to be typically dioecious (Coe, 1943; Fretter & Graham, 1964; Morton, 1991) although among freshwater bivalves there are some hermaphroditic families (i.e. Sphaeriidae) (Heard, 1965, 1977; Morton, 1991; Araujo, 1995). Historically, studies on Unionoidea indicate that hermaphroditism is a rare event (Bloomer, 1934; Tepe, 1943; Heard, 1970, 1975; van der Schalie, 1970; Smith, 1979; Kat, 1983) and that when hermaphrodite individuals exist, they occur in low frequencies (Heard, 1979). Among 220 North American species studied, hermaphroditism has only been documented as a predominant mode of reproduction in *Anodonta imbecilis* (Say, 1829), *Lasmigona subviridis* (Conrad), *L. compressa* (Lea, 1829), *Carunculina parva* (Barnes, 1823) and *C. pulla* (van der Schalie, 1969, 1970). Nevertheless, occasional functional hermaphroditism among Unionoidea is fairly common (van der Schalie, 1966, Heard, 1975; Dudgeon & Morton, 1983).

As regards the genus *Margaritifera* (Schumacher, 1816), data on sexuality exist for four species. The North American *M. falcata* (Gould 1850) is recorded as an hermaphrodite (Heard, 1970), and *M. hembeli* (Conrad, 1838) and *Margaritifera (Cumberlandia) monodonta* (Say, 1829) as dioecious (van der Schalie, 1970; Smith, 1988). The most ancient living species in the group is probably *M. margaritifera* (L, 1758), a Holarctic bivalve (Ziuganov *et al.*, 1994; Bauer, 1997) which, in Europe, lives from Spain to north-west Russia. Although considered as a dioecious species (Hendleberg, 1961; van der Schalie, 1966; Smith, 1979; Young & Williams, 1984; Ross, 1992; Hanstén, Pekkarinen & Valovirta, 1997), a few cases of hermaphroditism have been recorded (Bauer, 1987; Hanstén, Pekkarinen & Valovirta, 1997).

For the other European species, *M. auricularia* (Spengler, 1793), no data exist on gonadal topography nor sex ratio. *M. auricularia* was a relatively abundant species in large rivers of Western Europe (Iberian Peninsula, France, Italy, England, Germany) and Morocco (Preece *et al.*, 1983) although nowadays it is known only from Spain (Araujo and Ramos, 1996, 1998; Altaba, 1997; Ramos, 1998). Live specimens collected in 1991 are recorded from Morocco (Araujo & Ramos, 2000).

Both *Margaritifera* species, especially *M. auricularia*, are undergoing a great reduction in range, and population densities are decreasing alarmingly. In the IUCN Red List (IUCN, 1996) *M. auricularia* is included as Critically Endangered and *M. margaritifera* as Endangered, and both are listed under several European conservation laws and red lists (Bern Convention Council of Europe, 1979; Directive 92/43/ECC, Habitats Directive, 1992).

The Iberian Peninsula is the only area where both *M. auricularia* and *M. margaritifera* still live, allowing us to study their reproductive cycles. Histological examination of the gonads was performed to determine the sexual characteristics of these southernmost European populations of both species. These data will be essential to improve understanding of unionid biology (i. e. reproductive season and population sex ratio) and hence to design action plans aimed at conserving the populations.

MATERIAL AND METHODS

Since we are dealing with two endangered species, the availability of specimens for research is highly restricted.

A total of 27 specimens were used for histological examination, including 8 specimens of *M. auricularia* collected at the 'Canal Imperial' (Ebro River, Spain) in February (4) and December (4) 1996. Five other specimens that died in the aquarium (2 after one year in captivity and 3 after one month) were also studied (Table 1). *Margaritifera margaritifera* samples were collected in October 1996 (11) and November 1997 (3) from different rivers in Galicia (NW Spain) (Table 2).

All specimens were fixed for 50 hours in Bouin's fluid, 10% formalin or 70% ethanol and then preserved in 70% ethanol. Voucher specimens are deposited in the 'Museo Nacional de Ciencias Naturales' collection (Madrid, Spain) (collection numbers: 15.07/5187–15.07/5213. Tables 1 & 2).

Length measurements of the specimens were performed with a dial caliper. Before histological examination, a detailed macroscopic study of the gonads was made with a stereomicroscope. The visceral mass of each specimen was inspected to locate the gonadal tissue and to distinguish male and female tissues. Different sections of the foot were cut for binocular observation, two of them always being kept for histological examination (Fig. 1). These wedges were dehydrated in a graded ethanol series, embedded in paraplast and serial sectioned (5–10 μm) with a microtome. All slides were stained with hematoxilin-eosin (Carazzi method).

RESULTS

Margaritifera auricularia

All specimens were adults larger than 14 cm (Table 1). Gonadal tissue was dispersed through the visceral mass without a specific location. Female and male tissues

Table 1. Sex and gonadal state of *M. auricularia* study specimens. * Mussels dead in aquarium after one year in captivity. + Mussels dead in aquarium after one month in captivity.

Ref. Num.	Collection Date	Fixation Date	Locality	Size (cm)	Sex	Gonadal state
15.07/5187	February 1996	February 1996	'Canal Imperial'	14.1	Hermaphrodite	Mature
15.07/5188	February 1996	February 1996	'Canal Imperial'	14.7	Hermaphrodite	Mature
15.07/5189	February 1996	February 1996	'Canal Imperial'	15.1	Hermaphrodite	Mature
15.07/5190	February 1996	February 1996	'Canal Imperial'	14.6	Female	Mature
15.07/5191	December 1996	December 1996	'Canal Imperial'	15.3	Hermaphrodite	Mature
15.07/5192	December 1996	December 1996	'Canal Imperial'	15.2	Hermaphrodite	Mature
15.07/5193	December 1996	December 1996	'Canal Imperial'	14.5	Hermaphrodite	Mature
15.07/5194	December 1996	December 1996	'Canal Imperial'	15.4	Hermaphrodite	Mature
15.07/5195 *	February 1996	May 1997	'Canal Imperial'	14.4	Female	Decayed
15.07/5196 *	February 1996	May 1997	'Canal Imperial'	14.7	Female	Decayed
15.07/5197 +	February 1998	March 1998	'Canal Imperial'	15.3	Hermaphrodite	Mature
15.07/5198 +	February 1998	March 1998	'Canal Imperial'	15.1	Hermaphrodite	Mature
15.07/5199 +	February 1998	March 1998	'Canal Imperial'	14.7	Hermaphrodite	Mature

Table 2. Sex and gonadal state of *M. margaritifera* study specimens.

Ref. Num.	Collection Date	Fixation Date	Locality	Size (cm)	Sex	Gonadal state
15.07/5200	October 1996	October 1996	Arnego River	8.0	Hermaphrodite	Spent
15.07/5201	October 1996	October 1996	Arnego River	7.9	Hermaphrodite	Spent
15.07/5202	October 1996	October 1996	Arnego River	8.6	Hermaphrodite	Spent
15.07/5203	October 1996	October 1996	Mandeo River	9.6	Female	Spent
15.07/5204	October 1996	October 1996	Mandeo River	8.1	Female	Spent
15.07/5205	October 1996	October 1996	Mandeo River	9.3	Hermaphrodite	Spent
15.07/5206	October 1996	October 1996	Landro River	10.6	Female	Spent
15.07/5207	October 1996	October 1996	Landro River	9.1	Female	Spent
15.07/5208	October 1996	October 1996	Landro River	10.6	Hermaphrodite	Spent
15.07/5209	October 1996	October 1996	Oro River	10.3	Hermaphrodite	Spent
15.07/5210	October 1996	October 1996	Oro River	10.6	Hermaphrodite	Spent
15.07/5211	November 1996	November 1996	Oro River	9.3	Female	Spent
15.07/5212	November 1996	November 1996	Oro River	8.8	Female	Spent
15.07/5213	November 1996	November 1996	Oro River	10.4	Female	Spent

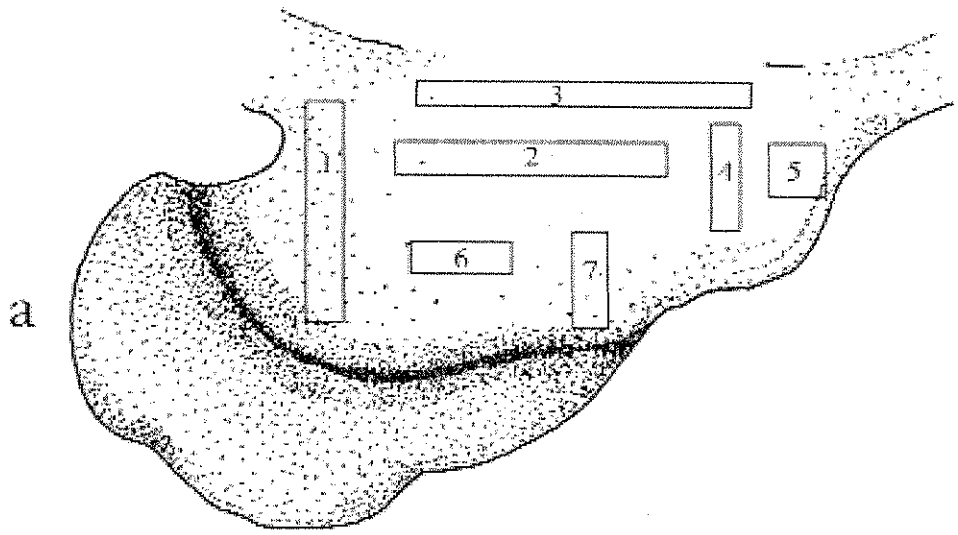

Figure 1. *M. margaritifera* and *M. auricularia*. Areas of the visceral mass of both species inspected in the macroscopic study. Area 5 was always used for histological examination in all specimens. The second area used was selected depending on macroscopic results. Abbreviations: a, anterior.

intermingled with the glandular digestive tissue and gut; in other words, no correspondence was found between a specific area of the visceral mass (Fig. 1) and a specific sex. Nevertheless, sex could be determined by macroscopic study of the visceral mass, male and female tissues being clearly distinguishable. Female tissue predominated over male and was arranged in brown clusters, while iridescent white male tissue was located among the female brown clusters.

Male and female gonads were mature (Fig. 2A) in the specimens from December, February and March, the latter after one month in captivity. Ten of the 13 specimens studied were hermaphrodites (Fig. 2A) and three had only female tissue (Fig. 2B). Specimens collected in February 1996 were hermaphrodites except for one female. Specimens from December 1996 and those that died after one month in the aquarium (March 1998) were all hermaphrodites (Table 1). The two specimens that died after one year in captivity (May 1997) presented only female cells although the gonadal tissue was seriously decayed after the long starvation, having many connective tissues around the acini (Fig. 2 C). Some oocytes, with the nuclear components lost, were found among the connective cells. All hermaphrodite specimens were predominantly female with simultaneous hermaphroditism.

Female and male tissues are organised in follicles (Fig. 2A), the walls of which consist of granular follicle cells (Fig. 2B). Hermaphrodite follicles were not observed. However, in some parts of the gonad of two hermaphrodite specimens the oocytes were surrounded by male cells and were not organised in mixed follicles (Fig. 2D). Female and male cells were also found inside the gut. Also, isolated male cells were found inside female follicles (Fig. 2E).

Spermatogonia, which occur at the follicle walls, are oval cells (7.7–9.2 μm long; 4.6–5.4 μm wide) with a nucleus full of chromatin granules. The nucleolus (one or, more frequently, several) are located on the nucleus boundaries. Spermatocytes are spherical cells with a large homogeneous nucleus (3.8–4.6 μm in diameter). Spermatids are polyhedral (3.0 μm long) with an homogeneous nucleus. The heads of the spermatozoa are about 2.3–3.0 μm long (Fig. 2F, 2G). Sperm morulae were found in all specimens.

Oogonia (5.3–6.1 μm in diameter) are spherical and with dispersed chromatin and they are very similar to spermatogonia. Mature oocytes are large cells (66.5–83.6 μm in diameter) with several nucleoli (Fig. 2B, 2E). At this stage, there is no evidence of nutritional cells.

Among female follicles it was common for many spheres (diameter 1.0–2.0 μm) to be spread in the visceral mass (Fig. 2H). Larger structures (4.6–7.7 μm) of the same kind were also found below the body epithelium in all specimens (Fig. 3A). Both kinds of spheres were stained purple with hematoxylin-eosin.

Margaritifera margaritifera

All specimens were larger than 8 cm (Table 2). As in *M. auricularia*, gonadal tissue spreads without having a specific location, female and male tissues being mixed

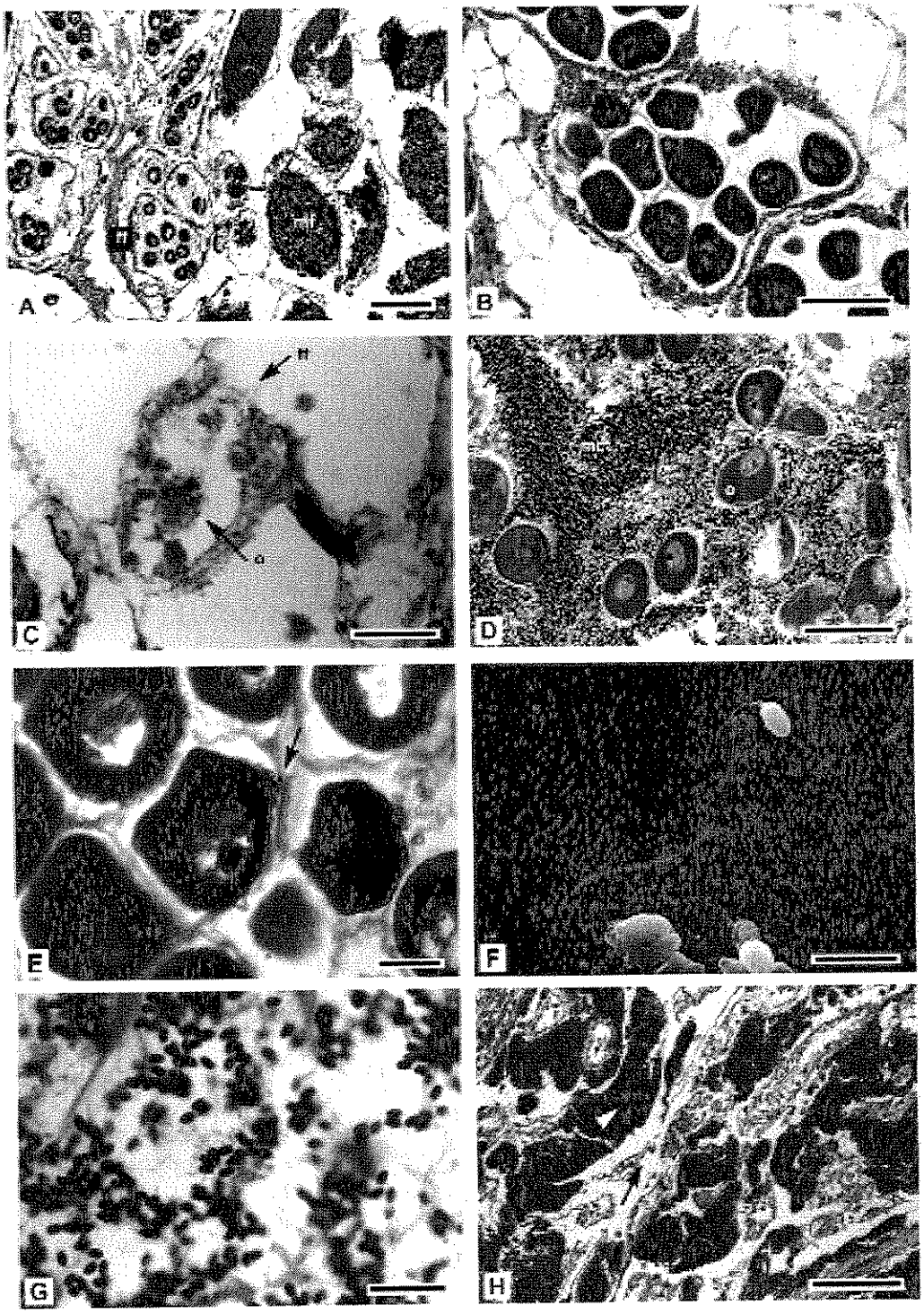

Figure 2. *Margaritifera auricularia*. A-H (except F): histological sections. **A**. General aspect of the gonad showing female and male follicles. Scale bar = 200 μm. **B**. Female follicle with mature oocytes. Scale bar = 100 μm. **C**. Spent female follicle. Specimen dead after one year in captivity. Scale bar = 25 μm. **D**. Male and female cells without follicle organisation. Scale bar = 100 μm. **E**. Oocytes inside a female follicle. The arrow indicates spermatozoa close to the oocyte. Scale bar = 25 μm. **F**. Spermatozoon of *M. auricularia*. Scale bar = 10 μm. **G**. Male cells inside male follicle. Scale bar = 20 μm. **H**. Concretions in connective tissue. Scale bar = 100 μm. (*White* arrow indicates concretions; *black* arrow indicates connective tissue). Abbreviations: ff, female follicles; mc, male cells; mf, male follicles; o, oocyte.

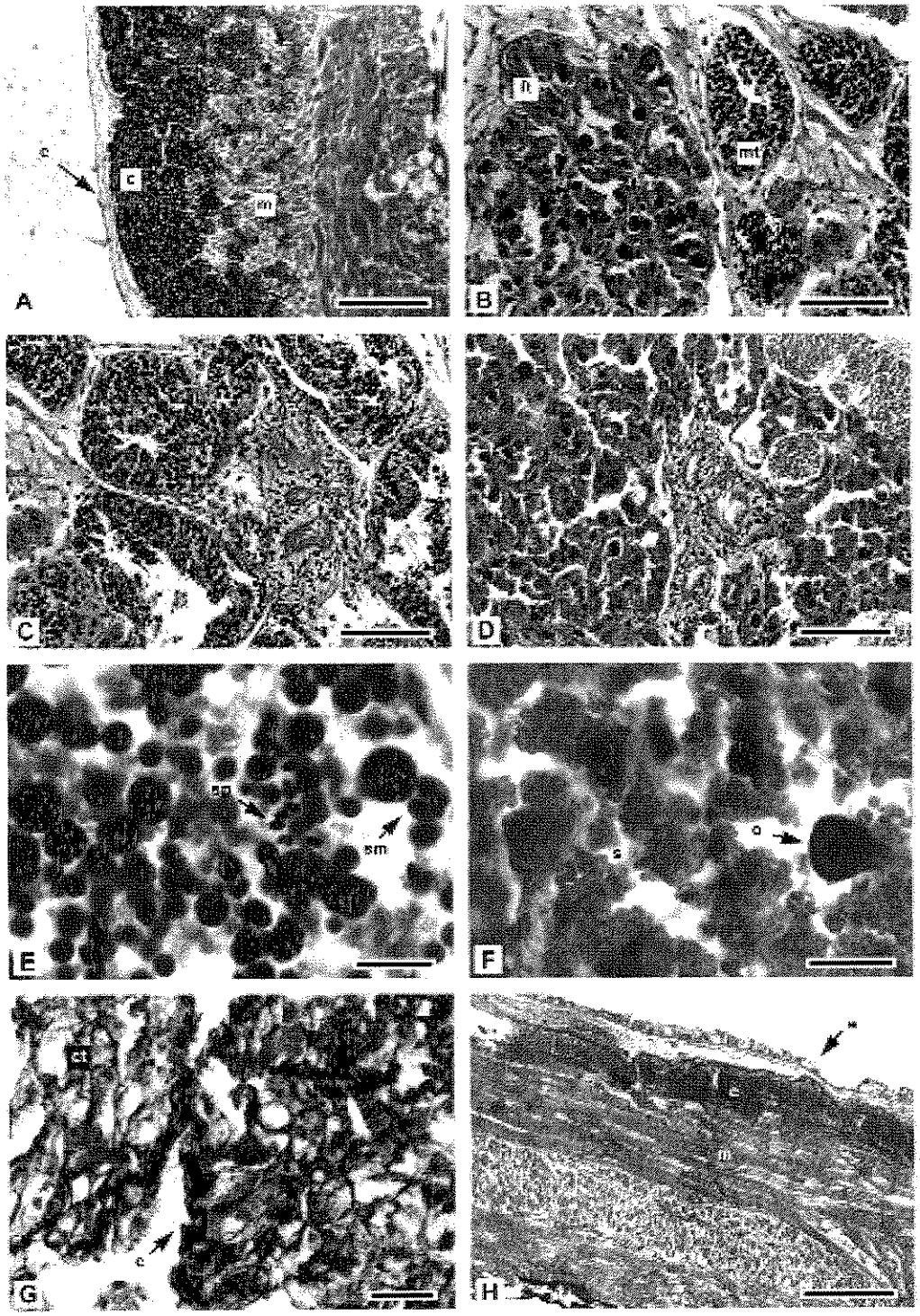

Figure 3. **A.** *M. auricularia.* Concretions below the epithelium of *M. auricularia* body. Scale bar = 100 μm. **B-H:** *M. margaritifera.* Histological sections. **B.** General aspect of gonad showing female and male tissues. Scale bar = 100 μm. **C.** Male follicles. Scale bar = 100 μm. **D.** Female tissue without follicle organisation. Scale bar = 100 μm. **E.** Male cells inside male follicle. Scale bar = 20 μm. **F.** Oocyte included in a disperse stroma. Scale bar = 25 μm. **G.** Concretions among connective tissue. Scale bar = 25 μm. **H.** Concretions below the epithelium of the body. Scale bar = 100 μm. Abbreviations: c, concretions; ct, connective tissue; e, foot epithelium; ft, female tissue; m, muscle; mt, male tissue; o, oocyte; s, stroma; sm, sperm morulae; sp, spermatozoa.

into the visceral mass. The glandular digestive tissue and the gut were also surrounded by the gonad. Determination of sex can also be made macroscopically.

Seven of the 14 study specimens were hermaphrodites (Fig. 3B), the other seven being females. In relation to collection dates, seven of the 11 specimens from October 1996 were hermaphrodites and the rest females. All mussels collected in November 1997 were females (Table 2). Hermaphroditism was always simultaneous and specimens were predominantly female.

In *M. margaritifera*, only male tissue was organised in follicles (Fig. 3C). Female gonads are always dispersed (Fig. 3D) and fill spaces between male tissue and muscular bundles. No hermaphrodite follicles were observed.

In specimens collected in October, the gonadal tissue was intermingled with connective tissue, the ovocytes were small and surrounded by cellular remains. In November, the gonadal tissue seems to disappear and is replaced by connective tissue.

In October, spermatogenesis takes place. Spermatogonia are oval cells (7.7–9.0 μm long; 4.6–5.0 μm wide) growing out of the acinar wall. The nucleus is full of chromatin granules and shows one or, more frequently, several nucleoli situated on the nucleus boundaries. Spermatocytes are spherical cells with a large homogeneous nucleus (3.8–4.6 μm in diameter). Spermatids are polyhedral (2.5–3.0 μm long) and the nucleus is completely homogeneous. Spermatozoa are 2.0–2.3 μm long. Sperm morulae were found in all study specimens (Fig. 3E).

Primary oocytes are included in a stroma consisting of different components, including oogonia, portions of oocytes, yellow cellular remains and sperm morulae (Fig. 3F). Oogonia are like spermatogonia, they are spherical (3.5–5.0 μm in diameter), with dispersed chromatine and located among the stroma. The primary oocytes are small and spherical (19.0–30.0 μm); no evidence of nutritive cells was found.

Spherical structures (1.0–2.0 μm diameter) like those in *M. auricularia* were also found between female follicles (Fig. 3G) and below the body epithelium, the latter having larger diameters (3.9–5.4 μm) (Fig. 3H).

DISCUSSION

Sexing unionoids has always been a difficult task. Until now, the only accurate method of checking the sex of a mussel was by histological studies, an inadvisable method for endangered species. Thus, the method commonly used, at least to study *M. margaritifera*, consisted of puncturing the foot to extract tissue fluid, which was then microscopically inspected for spermatozoa and oocytes (Vlastov, 1956; Bauer, 1987). Knowing the chaotic distribution of the gonadal tissues inside the mussel foot, it is easy to imagine that previous results obtained by this method were unreliable, since only a few parts of the gonad can be studied. Another difficulty stems from the possibility of sex change in Unionoidea as has been recorded for the freshwater pearl mussel *M. margaritifera,* almost exclusively among females (Bauer, 1987). These arguments may suffice to explain the reported high sex variability, both at population and individual levels, in unionoids (Weisensee, 1916; Bloomer, 1930, 1934, 1935, 1939; van der Schalie, 1970; Heard, 1975; Gordon & Smith, 1990).

This study shows that macroscopic dissections of the visceral mass are sufficient to determine the sex of freshwater mussels, but the sacrifice of a few specimens is necessary to obtain an accurate idea of gonadal topography. In fact, our initial observations working with a random sampling of gonadal tissues yielded erroneous results, indicating as females specimens that were in fact hermaphrodites.

In both *Margaritifera* species, gonadal tissue intermingles with the digestive cells as in other unionoids in which gonadal tissue occurs among gut loops and even enveloping the digestive gland (Mackie, 1984).

With only one exception, all *M. auricularia* specimens fixed directly in the field or after one month in captivity were hermaphrodites, but the two specimens fixed after a year in captivity showed very damaged gonadal tissue with only female cells. Even though these results refer to a relict population of about 2,000 individuals (Araujo & Ramos, unpublished), they may suggest the possibility of sex change (i.e. male reabsorption) under unnatural conditions. If *M. auricularia* was not a strictly hermaphroditic species, the observed high ratio of hermaphroditism in this population might be explained by the effect of low population size or by energetic factors, as has been reported for *M. margaritifera* by Bauer (1987) and for brooding organisms by Heath (1979), respectively. Changes in sex ratio with age in bivalve species are discussed in Morton (1991).

In the Spanish *M. margaritifera* populations, 50% of the specimens were hermaphrodites and the other 50% females. In central New England, Smith (1979) did not find any case of hermaphroditism among 52 specimens of *M. margaritifera*, all of which were dioecious. Moreover, this species is recorded as dioecious in Finland (Hanstén, Pekkarinen & Valovirta, 1997) although some cases of 'microhermaphroditism' ('occasional nets of gametocytes or follicles of the opposite sex occurring in the gonad') have been described (Hanstén, Pekkarinen & Valovirta, 1997). Occasional hermaphroditism

has also been recorded in German populations (Bauer, 1987). This variability among populations has been attributed to the interaction between multiple hereditary sex differentiating mechanisms and environmental factors (van der Schalie, 1970), although further studies are needed to test this hypothesis.

Although data on Spanish *M. margaritifera* populations are not yet conclusive, results for both species suggest that hermaphroditism was female and simultaneous, i.e. female tissue prevails and both male and female gametes develop in the gonad at the same time.

Gametogenesis of *M. auricularia* in the 'Canal Imperial' probably occurs from December to March, the glochidia being released from February to March (Araujo & Ramos, 1998; Araujo, Bragado & Ramos, 2000). The fact that we found oocytes surrounded by some spermatozoa inside female follicles suggests that intrafollicular fertilisation could occur as described for other freshwater bivalves (Araujo & Ramos, 1997).

M. margaritifera specimens collected in October showed characteristic post-spawning features. The ovocytes were small, surrounded by a great mass of picnotic clumps and unspawned gametes in various stages of development or cytolysis. In November, reproductive tissue had been completely reabsorbed. During these months the *M. margaritifera* female gonad was not arranged in follicles. This may be explained by the reabsorption of the follicle components once the gonad is spent or as a characteristic of the Spanish populations due to the isolation resulting from their peripheral distribution at the southernmost limits of its range.

Although we have no conclusive data on the reproductive period of *M. margaritifera* in Spain, gametogenesis probably takes place in spring-summer as glochidia are released in July-August (San Miguel, personal communication). This concurs with data for the species in New England (Smith, 1979), Finland (Hanstén et al., 1997) and Scotland (Young & Williams, 1984), where gametogenesis has been recorded in May, June and July. In north-west Ireland, *M. margaritifera* males and females undergo gametogenesis throughout the year (Ross, 1992). Biannual gametogenesis has been described in North America for *M. margaritifera* (Smith, 1978) and *M. falcata* (van der Schalie, 1970). Sexual features of the genus *Margaritifera* are shown in Table 3.

Sperm morulae were found in all the hermaphrodite specimens. Although their function is unknown, Coe & Turner (1938) explained cytolysis of sperm morulae in *Mya arenaria* as a possible way of supplying nutrients, and according to Mackie (1984) and Kotrla (1989) these structures are evidence of abnormal spermatogenesis in certain bivalves. Heard (1975) suggested that some sperm morulae become mature sperm although their viability is unknown. Van der Schalie & Locke (1941) and Heard (1969, 1975) found sperm morulae in June in *Anodonta grandis*, but by July all sperm

Table 3. Sexual characteristics of the genus *Margaritifera* in the world.

Species	Locality	Sex	Gonad maturation	References
M. auricularia	Spain	Hermaphrodite and dioecious	December–March	This study
M. margaritifera	USA (Wyoming)	Hermaphrodite and dioecious	–	van der Schalie, 1970
	USA (Massachusetts)	Dioecious	Mid-winter	Smith, 1978
	USA (Oregon)	–	Before April	Karna & Millemann, 1978
	USA (New Enland)	Dioecious	May–July	Smith, 1979
	Scotland	Dioecious	May–July	Young & Williams, 1983
	Germany	Hermaphrodite and dioecious	–	Bauer, 1987
	Ireland	Dioecious	Thoughout the year	Ross, 1992
	Russia (Kola Peninsula)	–	May–July	Ziuganov, 1994
	Finland	Hermaphrodite and dioecious	May–July	Hanstén *et al.* 1997
	Spain	Hermaphrodite (50%) and dioecious (50%)	–	This study
M. falcata	USA	Hermaphrodite	May–July	Heard, 1970
M. (Cumberlandia) monodonta	USA (Wyoming) USA (Missouri)	Dioecious	–	van der Schalie, 1970 Gordon & Smith, 1990
M. laevis	Japan	Dioecious	April–May	Awakura, 1968 Naito, 1988
M. hembeli	USA (Louisiana)	Dioecious	October–February	Smith, 1988

morulae had disappeared and the acini were full of mature sperm.

Spherical structures found among female follicles and below the body epithelium are similar to calcified concretions recently reported by Pekkarinen & Valovirta (1997). They found calcified concretions in mussel gonadal tissues of *M. margaritifera*, particularly in the testes, as a source of lipofuscin and Fe^{3+} (Pekkarinen & Valovirta, 1997). According to our own observations, these spherules are larger when situated below the body epithelium and smaller when they appear among gonadal tissues. The ability to gather calcium ions in hard and soft waters would help adult *M. margaritifera* individuals to survive periods of unfavourable water quality (Heming *et al* 1988, Pynnönen, 1990).

Although information concerning mussel reproduction has been improving over recent years, more work is still needed to establish useful bases in order to formulate effective plans to protect species of *Margaritifera* in Europe.

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