RESEARCH ARTICLE

Reproductive Cycle and Strategy of *Anodonta anatina* (L., 1758): Notes on Hermaphroditism

JOURNAL OF EXPERIMENTAL ZOOLOGY
PART A
ECOLOGICAL GENETICS AND PHYSIOLOGY
A Journal of Integrative Biology

MARIANA HINZMANN[1,2], MANUEL LOPES-LIMA[1,2]*, AMILCAR TEIXEIRA[3], SIMONE VARANDAS[4], RONALDO SOUSA[2,5], ANABELA LOPES[1,2], ELSA FROUFE[2], AND JORGE MACHADO[1,2]

[1]ICBAS-UP—Abel Salazar Biomedical Sciences Institute, University of Porto, Porto, Portugal
[2]CIMAR-LA/CIIMAR—Centre of Marine and Environmental Research, University of Porto, Porto, Portugal
[3]CIMO-ESA-IPB—Mountain Research Centre, School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, Bragança, Portugal
[4]CITAB-UTAD—Centre for Research and Technology of Agro-Environment and Biological Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[5]CBMA—Centre of Molecular and Environmental Biology, University of Minho, Campus de Gualtar, Braga, Portugal

ABSTRACT

Freshwater mussels have decreased dramatically in Iberia over the last decades. These animals are responsible for important ecosystem services such as recycling nutrients and improving water clarity. Under this view a better knowledge on the biological features of these animals is extremely important for future conservation and management actions. In this study the reproductive and gametogenic cycle of *Anodonta anatina* were studied during 2 years in one population as well as the sex ratio and hermaphroditism in six distinct populations, using standard histology. Gametogenesis was continuous in both sexes and germinal epithelium in early stages of development. Gametes were present throughout the reproductive cycle. Oogenesis and spermatogenesis occurred mainly between January and May. Larvae brooding occurred between September and March and main glochidia discharge occurred over a short period (2–3 weeks) in March. For the sex-ratio and hermaphroditism assessments a variable number of individuals were collected from several populations from lakes and rivers. Previous studies described *A. anatina* as mainly dioecious with only a few populations presenting occasional hermaphroditism. However, the present study indicates that *A. anatina* sexual behavior is influenced by environmental conditions, being mainly dioecious in rivers with increased hermaphroditism in standing waters. Although self-fertilization was not confirmed, additional studies with molecular characterization of larvae using fast evolving markers should be used in future studies to enlighten this process. Overall, this study indicates that for more efficient conservation actions and management plans, freshwater mussel reproductive biology should be studied at the population level mainly in the subfamily Anodontinae. *J. Exp. Zool. 9999A: 1–13, 2013.* © 2013 Wiley Periodicals, Inc.

How to cite this article: Hinzmann M, Lopes-Lima M, Teixeira A, Varandas S, Sousa R, Lopes A, Froufe E, Machado J. 2013. Reproductive cycle and strategy of *Anodonta anatina* (L., 1758): Notes on hermaphroditism. J. Exp. Zool. 9999:1–13.

Freshwater mussels (Bivalvia, Unionoida) are among the most critically threatened faunal groups globally (Neves et al., '97; Lydeard et al., 2004; Simberloff, 2012). The consequences of this catastrophic decline go far beyond the loss of species per se since freshwater mussels are responsible for critical trophic and non-trophic functions (Vaughn, 2010; Allen and Vaughn, 2011). In most European species very few studies have been done, either on the distribution and population structure but also on the study of their basic life cycle traits. This situation may be related with the complex and distinct life-history patterns of each species. Recently, biologists have made the first attempts to organize the life-history strategies of freshwater mussels into conceptual frameworks (Bauer and Wächtler, 2000; Dillon, 2000). However, the development of these frameworks is hampered by a lack of life-history information for most species, particularly for traits such as age at maturity, growth rate, longevity and fecundity (Strayer et al., 2004). This information is vital for the generation of testable hypotheses about the evolutionary, ecological, and management consequences of life-history variation. So, it is of primordial importance to increase fundamental knowledge on basic biology and habitat requirements of each species so that managers can more effectively conserve and manage the mussel fauna.

The reproductive biology of unionoids is distinctive: fertilization is internal and the fertilized eggs form larvae (glochidia) that are brooded in the gills of females until they are released in to the water column. To complete their development into juvenile mussels, they need to parasitize certain host fishes until complete metamorphosis into young adult mussels (Kat, '84). Recent studies within the subfamily Anodontinae have been carried on the reproduction development on related species (Sereflisan et al., 2009; Lima et al., 2012) to fill that gap of knowledge.

The present study is focused on *Anodonta anatina*, a freshwater mussel that is still widespread in Europe. Current distribution encompasses the Iberian Peninsula in the Southwest, the Scandinavia in the North and Russia in the East (Graf, 2007). Although widespread, this species already disappeared from several parts of the continent and is listed as threatened and protected in Germany (Zettler et al., 2006). In Iberia, all species of native unionoid mussels are decreasing both in number of populations and individuals (Araujo et al., 2009). These animals are extremely important ecologically serving important ecosystem functions (e.g., biomass transfer from the water column to the benthos, reduction of water turbidity, control of the concentration and composition of suspended particles, nutrient cycling and provision of habitat for other organisms; Vaughn, 2010; Sousa et al., 2011, 2012). All of these facts turn the research on the biological traits (mainly the reproductive characters) of these mussel species crucial for future conservation actions and management. Additionally, recent findings suggest that even host generalists such as Anodontinae mussels may be increasingly limited in their reproductive cycles by the host availability (Douda et al., 2013). Hence, precise data on the timing of glochidia production and the capability of hermaphroditism may be highly important for assessing the risk of host limitation in the field. Under this view, to increase the knowledge on the reproductive biological traits of *A. anatina*, the aim of this study was to describe in detail the histology of the sexual (spermatogenesis plus oogenesis) development of *A. anatina*. In addition, we verified the sex ratio and hermaphroditism from distinct habitats such as rivers, lagoons or impoundments to allow a more detailed evaluation on the sexual strategy of this species.

METHODS

Sampling

For the seasonal sexual development, from March 2009 to March 2011 six *A. anatina* were collected monthly in Tâmega River near Ribeira de Pena, Portugal (N 41°32′30″; W 7°47′15″; Fig. 1); all of these animals were also checked to determine the sex ratio. The mussels were transported to the laboratory on ice in a cooler box and processed within 24 hr. In the laboratory, bivalves were anesthetized in 2-phenoxyethanol solution 0.4% (v/v) for about 30 min and dissected for histology. To minimize eventual negative impacts, the number of animals sacrificed was reduced and the seasonal study was made in the larger population of *A. anatina* in Portugal.

Histological Procedure

Samples of gonad and gill tissue were excised from each animal and preserved in Bouin's solution (Panreac, Barcelona, Spain). After being preserved in Bouin's solution for a week, transverse sections of the gonads were dehydrated in graded ethanol, embedded in paraffin, sectioned at 5–8 μm and stained with hematoxylin and eosin for histological examination.

The sex was determined visually by the presence of male and female gonad tissue. The maturation level of the gonads was established using the classification stage table of the gamete development index (GDI) adapted from Barber ('96). Gills were

Grant sponsor: Portuguese Foundation for Science and Technology (FCT). grant numbers: PTDC/AAC-AMB/117688/2010 CONBI, PTDC/MAR/098066/2010 SYNERG.

*Correspondence to: Manuel Lopes-Lima, Abel Salazar Biomedical Sciences Institute, Applied Physiology Laboratory, Aquatic Production Department, University of Porto, Rua de Jorge Viterbo Ferreira No. 228, 4050-313 Porto, Portugal.
E-mail: lopeslima.ciimar@gmail.com

Received 15 June 2012; Revised 10 April 2013; Accepted 16 April 2013
DOI: 10.1002/jez.1801
Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

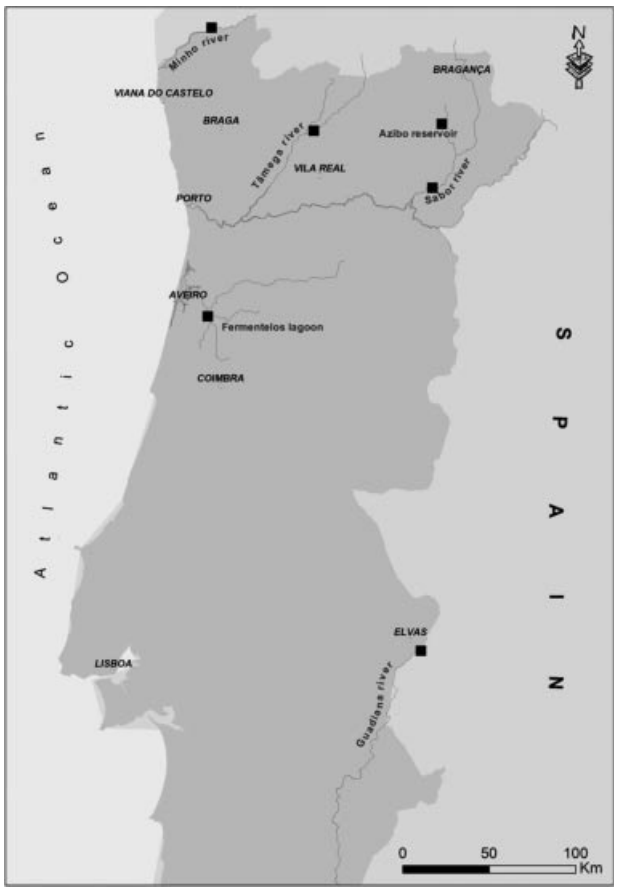

Figure 1. Map with the location of the sampled populations of *Anodonta anatina*.

observed for identification of the embryonic development periods and to complement sex determination.

Sex Determination

For sex ratio determination, we used a variable number of specimens ($15 < n < 32$) from the Minho (N 42°03′04″; W 8° 33′40″), Mondego (N 40°12′16″; W 8°21′39″), and Guadiana (N 38°39′56″; W 7°05′05″) rivers and from Azibo reservoir (N 41°33′55″; W 6°52′51″) and Fermentelos lagoon (N 40° 34′34″; W 8 31 37) (Fig. 1). Morphometric data (weight, length, height, width, and number of visible growth lines) were registered. Sex was determined using three criteria: presence of eggs or glochidia within the marsupial gill; observation of tripartite water tubes on the outer gill (Mcivor and Aldridge, 2007) and confirmed by observation of gonad tissue with a microscope. To test whether the sex ratios observed were significantly different from the expected sex ratios of the mussels a chi-square (χ^2) test was used. The comparison of dioecy and hermaphroditism among river and lagoon/reservoir populations were analyzed by one-way ANOVA followed by a Duncan multiple range test. Both tests were carried using JMP statistical package (SAS Institute, Cary, NC, USA).

Genetic Analysis

To avoid taxonomic uncertainties due to the usual misidentification between *A. cygnea* and *A. anatina*, identities were confirmed by genetic analysis. For this, foot tissue samples were collected and placed directly into 96% ethanol. The whole genomic DNA was extracted from small tissue samples pieces (2 mm^3) using a standard high-salt protocol (Sambrook et al., '89). A fragment of approximately 700 bp of the mtDNA cox1 gene was amplified by polymerase chain reaction (PCR) using the primers LCO22me2 (5′-GGTCAACAAAYCATAARGATATTGG-3′) and HCO700dy2 (5′-TCAGGGTGACCAAAAAAYCA-3′) (Walker et al., 2006, 2007), for all the samples (both species).

The PCR conditions (25 μL reactions) were as follows: each reaction contained 2.5 μL 10× Invitrogen PCR Buffer, 0.5 μL 10 mM of each primer, 1.5 μL 50 mM $MgCl_2$, 0.5 μL 10 mM dNTP's, 0.1 μL Invitrogen Taq DNA Polymerase and approximately 100 ng per μL DNA template. The cycle parameters were: initial denaturation at 94°C for 3 min, denaturation at 94°C (30 sec), annealing at 48°C (45 sec), and extension at 72°C (45 sec) repeated for 35 cycles and a final extension at 72°C for 5 min. Amplified DNA templates were sequenced, for three individuals of each population by a commercial company—Macrogen Europe, Amsterdam, The Netherlands. Chromatograms were checked by eye using ChromasPro 1.41 (technelysium.com.au). The sequences were aligned with ClustalW using Bioedit v. 5.0.9. (Hall, '99) and adjusted manually, resulting in a final alignment of 590 bp. Two additional sequences from both species *A. anatina* and *A. cygnea*, plus the genetically close species, *Pseudanodonta complanata* available in GenBank were incorporated into our analyses (Table 1). All the new sequences obtained in this study were submitted to GenBank (Table 1).

To describe the diversity of DNA sequences, basic descriptive statistics and genetic diversity parameters, namely haplotype diversity (h), nucleotide diversity (p), and characterization of polymorphic sites were calculated using the software DnaSP v5.00.04 (Librado and Rozas, 2009). The parameters of genetic similarity and distances of the compared sequences were estimated according to the observed variation (p-distance) using MEGA 5 software (Tamura et al., 2011). The same program was used to estimate their evolutionary relationships, that is, we used neighbor-joining (NJ) analysis with random sequence addition (10 replicate heuristic searches) with the support for nodes being estimated using the bootstrap technique with 1,000 replicates. Additionally, and in order to reduce the costs, the remaining individuals collected ($n = 225$) were subject to a PCR-based molecular identification key (PCR/RFLP analysis) following Gerke and Tiedemann (2001): a fragment of the ITS-1 region was

Table 1. List of specimen samples sequenced (COI) and GenBank accession numbers.

Reference	Population	Genbank no.	Country
AA1	Tâmega river	KC583446	Portugal
AA3	Tâmega river	KC583454	Portugal
AA11	Sabor river	KC583447	Portugal
AA12	Sabor river	KC583448	Portugal
AA13	Sabor river	KC583449	Portugal
AA14	Guadiana river	KC583450	Portugal
AA16	Guadiana river	KC583452	Portugal
AA17	Guadiana river	KC583453	Portugal
AA42	Minho river	KC583458	Portugal
AA51	Minho river	KC583459	Portugal
AA61	Minho river	KC583460	Portugal
AA31	Azibo reservoir	KC583455	Portugal
AA32	Azibo reservoir	KC583456	Portugal
AA33	Azibo reservoir	KC583457	Portugal
AC1	Fermentelos lagoon	KC583461	Portugal
AC3	Fermentelos lagoon	KC583462	Portugal
AC4	Fermentelos lagoon	KC583463	Portugal
Anodonta anatina		GU230745	Poland
Anodonta cygnea		GU230749	Poland
Pseudanodonta complanata		DQ060172	Sweden

amplified and the PCR products were then incubated with the *Hin*fI restriction enzyme for a period of at least 6 hr.

RESULTS

General Structure of the Gonads

The gonads of *A. anatina* are enclosed in the foot and involving the glandular digestive tissue and the gut. It is a diffuse organ, consisting of highly branched acini surrounded by connective and muscular tissue, which may vary in density according to the gamete development stage. Four types of gonad structure were identified in *A. anatina*, three for rivers and one for standing waters, according to their size and histology. In the first type of gonad (Type I), the ovarian acini dominated significantly, the mussel were functionally female and the male tissue was not observable. In the second type of gonad (Type II), the male acini dominated significantly, and the mussel were functionally male. Intensive spermatogenesis was observed, whereas female tissue was not detectable. In the third type of gonad (Type III, ♀ ≫ ♂), the demarcation of the two sexual acini was easily discernible microscopically (Fig. 2). Female acini clearly predominated over male and was arranged in independent acini, whereas iridescent white male tissue was located among the female acini but as a separated acinus. In this type of gonad, hermaphroditism was not simultaneous but specimens were predominantly female. The fourth type (Type IV, ♀ ≈ ♂), occurred only in Azibo impoundment and Fermentelos lagoon where gonads contained both eggs and spermatozoa. Detailed observations showed that hermaphroditic condition was in balance, with equal proportions of separate male and female acini (Fig. 2).

Demibranchs

Ninety-six percent of individuals with type I gonad females and type III gonad hermaphrodites from Tâmega, Mondego, and Guadiana rivers either were gravid or presented tripartite marsupial outer demibranchs. In the remaining 3%, additional water tubes either were not present or were not detected. All type IV gonad hermaphrodites also presented tripartite marsupial outer demibranchs.

Gametogenesis and Reproductive Cycle

A careful study of sections revealed that different gonad acini from the same organism may exhibit different degrees of development and when comparing different organisms this variation was even more evident, being difficult the establishment of the static gametogenic stages. The description of the (GDI) classification stages (Table 2) were based on the majority of observed cases in the respective time period, which may be broader than a month, since these transformations were gradual.

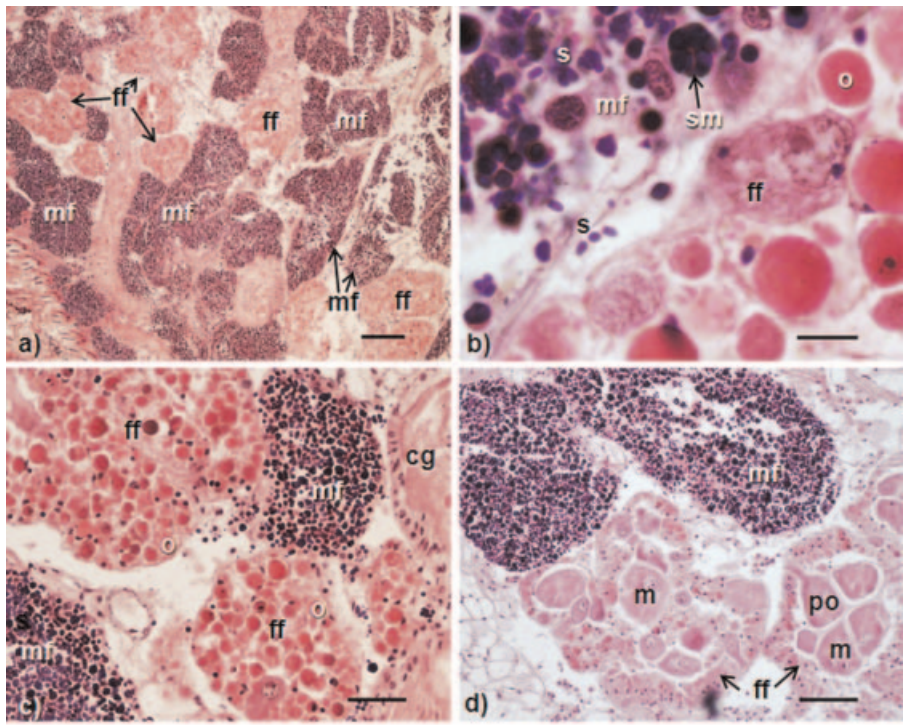

Figure 2. Histological sections from the hermaphrodite gonads of *Anodonta anatina* stained with H&E (eosin and hematoxylin). (**a**) General aspect of accidental hermaphroditism male and female gonads organized in acini (ma and fa respectively). (**b**) Portion of male and female acini in April, the acini show gonads at different stages. The male acinus is in a more advanced stage of maturation with: spermatogonia (sg), spermatocyte (sc), spermatid (st), sperm morulae and free spermatozoa (s) (scale bar 10 μm). (**c**) Hermaphrodite condition in April with male acini in a more advance stage of maturation (free spermatozoa (s)), female acini only with early stages of maturation (oogonia (o)) the ciliated gonoduct (cg) is also visible. (**d**) Hermaphrodite condition in June with female acini in a more advanced stage of maturation, with pedunculated oocytes (po) and mature oocytes (m), male acini with different stages of maturation but without free spermatozoa. a: scale bar 200 μm; b: scale bar 10 μm; c: scale bar 50 μm; d: scale bar 100 μm.

Gametogenesis was continuous in both sexes, since germinal epithelium in early stages of development of gametes was always present; no inactive stages were found during the study. Intense maturation and posterior elimination of both female and male gametes occur progressively from February to June; in hermaphrodite organisms the maturation of both acini is lagged with the male gonad tissue developing first. Gravid females were collected from September to March. Glochidia from this species need a long time of maturation in the gill, being the main release discharge from February to March.

Oogenesis

The oogenesis of *A. anatina* (Fig. 3) was mainly divided in five continuous stages that occur consecutively: (i) oogonia; (ii) previtellogenic oocytes; (iii) early oocytes; (iv) oocytes; and (v) mature oocytes. Maturation period goes from winter to spring, without any resting period being less active in the other months (Table 2). In the females, all developmental stages were present in the winter and beginning of spring and oogenesis was intense. In fact, in January, the ovarian acini were well-ordered, being located radially around the distal genital ducts. (i) Oogonia cells (Fig. 3a) were present in the female acini of *A. anatina* throughout the reproductive cycle. Spherical oogonia which are located among the stroma, had a diameter between 10 and 20 μm, presenting dispersed chromatin, basophilic nucleus and acidophilic cytoplasm. As oogonia grow they develop into previtellogenic oocytes (Fig. 3b). (ii) At the previtellogenic oocytes stage, the nucleus increased in size. The cells in this stage were generally located at the periphery of the germinal vesicle. (iii) In the early vitellogenic oocytes stage, the cytoplasm was markedly stained with eosin, with diameters varying from 25 to 35 μm. At the same period undifferentiated mesenchyma and eosinophilic granular cells were also abundant on the follicular wall. Connective tissue was always widely distributed between acini. The vitellogenic oocytes (Fig. 3d) were located in the center of the lumens acinus. The nucleus of vitellogenic oocytes consists of many nucleoli and

Table 2. Classification of the gamete development index (GDI) stages for evaluation of gametogenic activity of *Anodonta anatina*.

Month	Female gonad (♀)	Male gonad (♂)	Female Gills (♀)
January to February	Stage 1—Early active—acini contain oogonia and primary oocytes Stage 2—Late active—free oocytes	Stage 1—Early active—acini contain spematogonia and primary spematocytes Stage 2—Late active—spermatids, spermatid morulae and some spermatozoa	Full of glochidia
March	Stage 2	Stage 2 Stage 3—Mature—mature spermatozoa fill the acini	Gradual release of glochidia
April	Stage 2 Stage 3—Mature—mature oocytes fill the acini	Stage 3	Gradual accumulation of calcium concretion
May to June	Stage 3	Stage 4—Spawned—acini contain spaces, mostly devoid of gametes	Empty—full of calcium concretions
July to September	Stage 4—Spawned—acini contain spaces, mostly devoid of gametes	Stage 4	Gradual accumulation of eggs and reabsorption of calcium concretions
October	Stage 4	Stage 4 Stage 5—Resorption—acini have shrunken and contain products of resorption	Full of immature glochidia
November to December	Stage 5—Resorption—acini have shrunken and contain products of resorption and Stage 1	Stage 5 and Stage 1—early active	Full of glochidia

Complemented by outer gill content of female organisms during reproductive cycle. Adapted from Barber ('96).

continue to enlarge, becoming very irregular in shape; length can vary between 55 and 80 μm. This stage was mainly observable at the end of spring and beginning of summer. The fully grown oocytes were intensely eosinophilic (Fig. 3c and d) and the nucleus was located in the center of the oocytes. (iv) At the oocytes stage the nucleus showed a significant number of projections into the cytoplasm (ooplasm), and its membrane was highly coiled in the sections. The nuclear membrane disintegrated, the nucleus was

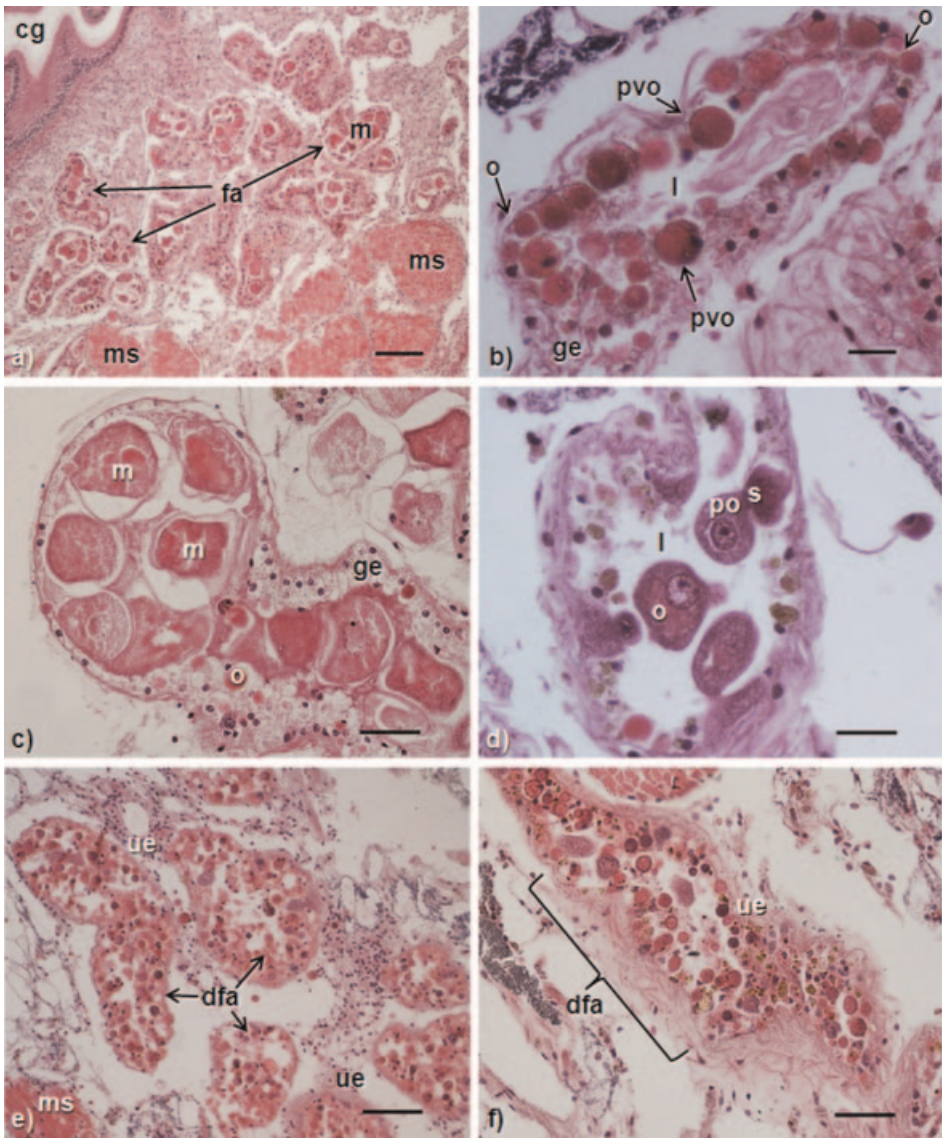

Figure 3. Histological sections from the female gonads of *Anodonta anatina* stained with H&E (eosin and hematoxylin). (a) General aspect of female gonads (fa) organized in acini; in June the acini show gonads at different stages of oogenesis, with several mature oocytes (m) in the lumen, the ciliated gonoduct (cg) and muscle tissue (ms) are also visible. (b) Female acini in February with earlier stages of oogenesis: oogonia (o), previtellogenic oocytes (pvo) and the lumen (l) visible as well as the germinal epithelium (ge) surrounding the germinative cells. (c) Female acinus in April with mature oocytes (m) in the lumen and oogonia (o) in the germinative epithelium. (d) Mature female acinus in a postspawned period in June; oocyte (o) in the lumen and oocyte still connected to the germinal wall by a stalk (s) designated by pedunculated oocytes (po). (e) Female gonads in the postspawned period in September, the retraction of acini, entering in a degenerative stage—degenerative female acini (dfa) which are surrounded by an undifferentiated epithelium (ue). (f) Degenerative female acini (dfa) in November with some oogonia and resorption products surrounded by undifferentiated epithelium (ue). a: scale bar 200 μm; b: scale bar 20 μm; c: scale bar 50 μm; d: scale bar 50 μm; e: scale bar 100 μm; f: scale bar 50 μm.

smaller in size, and the nucleoli were smaller than the previous stage and hardly distinguishable in the nucleus. Each oocyte, at the beginning of cytoplasmic growth, was attached by an egg stalk to the walls of the oogenic acinus, the respective eggs retained their attachment to the germinal epithelium by the same basal stalk (Fig. 3d) until an advanced stage of development when they move to the lumen before spawning. (v) The ovaries reached their maximum gravid stage in May and June. At this time they were filled with fully grown mature oocytes (eggs) and the interacinar space was minimal. After the onset of spawning, July to September, the eggs were loosely arrayed and the acini were less crowded, moving into the ciliated gonoduct. This event last through the spawning season, during the end of summer and autumn months. At the end of the breeding period, in November, all the mussels had spent gonads containing few or no oocytes and the gonad tissue was fairly reduced and almost totally replaced by connective tissue; the acini were in degenerative state presenting numerous undifferentiated cells. In the winter gametogenesis restarts.

Spermatogenesis

The spermatogenesis had the same continuous pattern as seen in females occurring all year although it was more evident during winter and spring (Table 2). The different stages were more difficult to follow since distinct stages were generally found in the same organism or acinus simultaneously, but four main stages of development can still be determined (Fig. 4): (i) spermatogonia; (ii) spermatocytes; (iii) spermatids that may be organized in clusters forming spermatid morulae; and (iv) spermatozoa which are the mature gametes, developing only under suitable environmental conditions. The spermatozoa have a rod shape and flagellum, being this visible mostly in fresh samples; this last stage is very short in time. The early stages and spermatid morulae were always present through the year. Spermatogenesis in male *A. anatina* had the same continuous pattern seen for the females. Additionally, the testes contained sperm at various stages of development with clusters of spermatocytes and spermatids along the male follicular wall. The testicular acini were neat and regularly arranged in winter months. (i) Spermatogonia cells were present in the male acini of the *A. anatina* throughout the reproductive cycle. Spermatogonia were oval and the largest cells (Fig. 4b–d), growing out of the acini wall and were approximately 6–7 μm in diameter and had relatively little cytoplasm. These cells divided mitotically and formed spermatocytes. (ii) Spermatocytes were spherical cells with a large homogeneous nucleus (Fig. 4b and d). However, they had no visible nuclear membrane and their nucleolus was not clearly detected. Spermatocytes were smaller (4–5 μm) than the spermatogonia and developed into (iii) spermatids, which were darkly stained with hematoxylin and distributed in the middle of the lumen of the acini. They were polyhedral and the nucleus was completely homogeneous. Their recorded diameter was 2–3 μm. The spermatids developed into spermatozoa. (iv) Spermatozoa were smaller than the spermatids and were strongly basophilic with a diameter of 1.5–2.0 μm. Yellow-brown granules were a common feature of the male acini in the end of summer months. Minute yellow-brown granules were also frequently seen in the epithelia of the genital ducts. In May–June these acini contained a quantity of sperm, which in many males was flooding into the genital ducts. It was clear that mature spermatozoa exit a male acinus through a ciliated gonoduct. Sperm morulae, clusters of early spermatids, with 7–10 μm of diameter, were detected through the reproductive cycle in most studied specimens. Males spawned mainly in May extending until August. In October, the acini still contained some mature spermatozoa, but were mainly in a degenerative stage. By November, the male acini were almost empty and spermatogonia were located at the male acinus periphery. The entire male gamete cell line from the spermatogonia to the spermatozoa was present in winter months.

Brooding Cycle of *Anodonta anatina*

From the end of September to March glochidia can be found in the marsupial gills (outer gill of female and hermaphrodite organisms) of *A. anatina*. The maturation is gradual from egg to mature glochidia, visible macroscopically by the coloration of the outer gill of females that change from a creamy beige color with early embryos to orange-brown before glochidial parturition. In each sampling period some females contained clusters of early embryos while others brooded glochidia, and even in the same organism both forms could be found, being the majority mainly of one type. The main glochidia discharge is concentrated in a short period of time (2–3 weeks in March). The number of glochidia accumulated is very high and the release is gradual.

Sex Ratio

The sex ratio and hermaphroditism varied among populations (Tables 3 and 4) being hermaphroditism significantly ($P < 0.05$) lower in rivers than in our studied standing waters—the Azibo reservoir and Fermentelos lagoon (in this site all the collected organisms were hermaphrodites). Additionally the difference in the proportions of females and males in the four rivers was significant ($P < 0.01$) concluding that the rivers (Minho, Sabor, Tâmega, and Guadiana) influence the sex of individuals.

Hermaphroditism

The ratio between female and male tissue in hermaphrodites also varied being highly disproportional (occasional hermaphroditism) in rivers and of similar amounts in standing waters populations.

Genetic Analysis

The COI alignment contained 18 new sequences corresponding to three samples of *Anodonta* sp. individuals from six localities (Table 1) with 28 variable positions and 14 parsimony-informative ones. Haplotype diversity was high ($h = 0.895 \pm 0.07$), while nucleotide diversity was moderately low ($p = 0.01 \pm 0.003$).

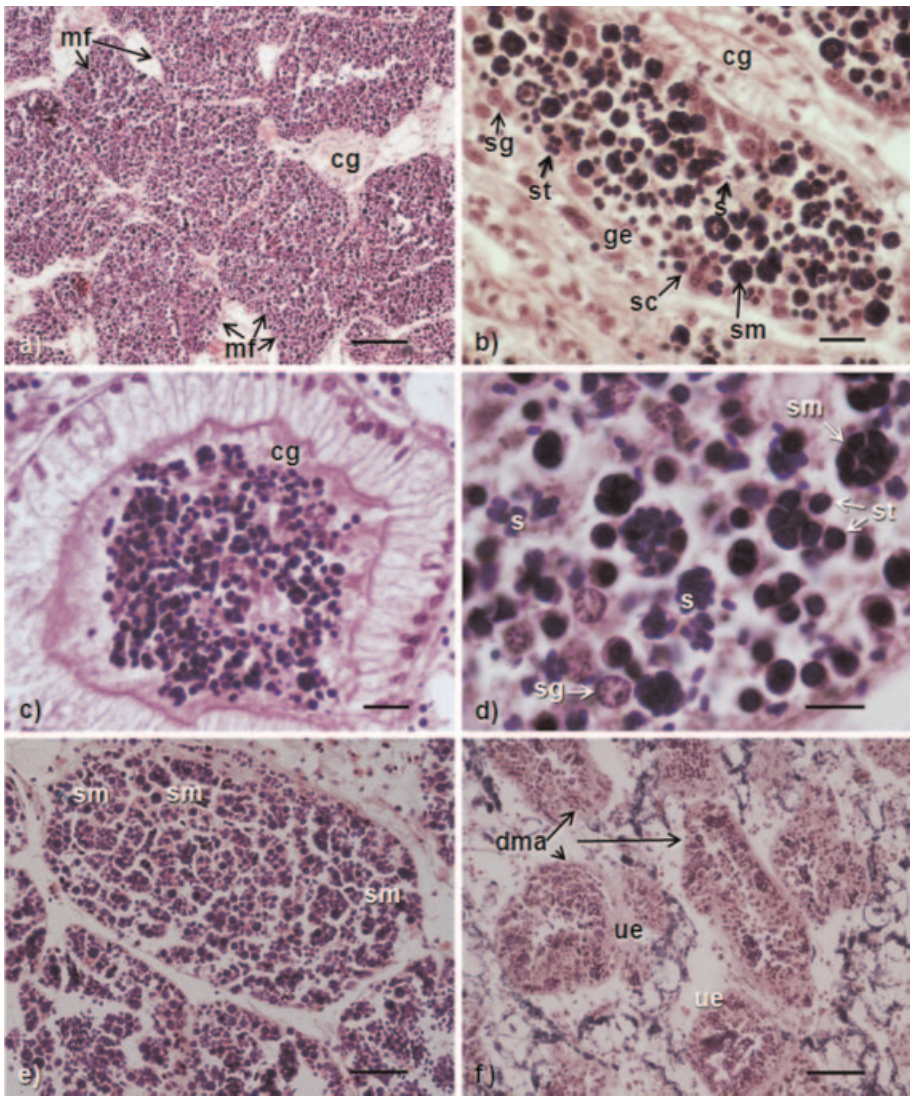

Figure 4. Histological sections from the male gonads of *Anodonta anatina* stained with H&E (eosin and hematoxylin). **(a)** General aspect of male gonads organized in acini (ma); in June the acini show gonads at different stages of spermatogenesis, the ciliated gonoduct (cg) is visible. **(b)** Male acinus surrounded by germinal epithelium (ge), the lumen is full of gonads in different stages of maturation in February: spermatogonia (sg), spermatocyte (sc), spermatid (st), spermatozoa (s), and sperm morulae (sm). **(c)** Mature spermatozoa (s) and other stages of spermatogenesis in male ciliated gonoduct (cg) in March. **(d)** Detail of a male acinus where different stages of maturation are visible in March: spermatogonia (sg), spermatocyte (sc), spermatid (st), spermatozoa (s) and sperm morulae (sm). **(e)** Male acinus in postspawning period (September), lumen with many free spaces and the dominant stage of gonads is the sperm morulae. **(f)** Degenerative male acinus (dma) in November, postspawned male acini surrounded by undifferentiated epithelium (ue) and few gonad cells. a: scale bar 100 μm; b: scale bar 20 μm; c: scale bar 20 μm; d: scale bar 10 μm; e: scale bar 50 μm; f: scale bar 100 μm.

When analyzing all the specimens together with the three outgroups sequences, all the new sequenced individuals grouped together in a single clade with maximum bootstrap support, including the *A. anatina* sequence from Genbank (Fig. 5). Both *A. cygnea* and *P. complanata* individual sequences cluster outside of this clade. Moreover, the mean overall genetic difference with the *A. anatina* specimens is only 1% and with a mean genetic distance of 9% to the *A. cygnea* sequence and 7% to *P. complanata*. The same result was achieved by the ITS sequences for all the remaining 144 individuals subjected to the PCR/RFLP analysis, presenting all the *A. anatina* RFLP profile (Fig. 6). Taking altogether, all the individuals analyzed here are a match to the

Table 3. Sex distribution in different populations of *A. anatina*.

Population	*N*	♀ (%)	♂ (%)	♀♂ (%)	[a]♀♂ (♀ vs. ♂)
Tâmega river	144	34.7	40.3	22.2	♀ ≫ ♂
Sabor river	15	60.0	20.0	20.0	♀ ≫ ♂
Guadiana river	16	50.0	50.0	0.0	–
Minho river	32	12.5	65.6	15.6	♀ ≫ ♂
Azibo reservoir	15	26.7	20.0	53.3	♀ ≈ ♂
Fermentelos lagoon	22	0.0	0.0	100.0	♀ ≈ ♂

[a]Male/female tissue balance in hermaphroditic gonads.

previous species identification based on morphological characters and are confirmed without any doubts as being *A. anatina*.

DISCUSSION

General Gonad Structure

Sereflisan et al. (2009) has divided the gonad structure of *Anodonta pseudodopsis* in three main types. (Type I) Typically female with a high dominance of female tissue, (Type II) typically male with a high dominance of male tissue, and (Type III) hermaphroditic but with a high prevalence of female over male tissue. In the present study a fourth type was described (Type IV) with approximate amounts of female and male acini interspersed in the whole gonad. This fourth type was found in animals only from the standing water populations and it is possibly found in species that use alternative sexual strategies such as in the present study and also in *A. cygnea*.

Reproductive Cycle

A. anatina completes its reproductive cycle in approximately 10 months. The gametogenesis is long as well as the spawning period. Sperm and egg production peak in early spring and spawning occurred synchronously between sexes and relatively soon after gamete production between April and September. In the end of summer a decrease on gonad content occurs followed immediately by a new regeneration of the gonads and the cycle is never completely interrupted. These results are in agreement with what occur in other anodontine species in Europe (Table 5) which generally have long periods of gametogenesis. The reason for the presence of large number of gametes throughout the year and outside the spawning season is not clear, the fate of unspawned gametes is not known, but it seems to be a characteristic among *Anodonta* species (Heard, '75). The larvae are accumulated in the outer gills of females from October to March a period similar to the other native European Anodontines. In fact, *P. complanata* broods glochidia from September to April (Mcivor and Aldridge, 2007) with probable main discharge on late April and *Anodonta cygnea* from November to March with the main discharge in February to March (Giusti et al., '75). This fact may implicate a long period of maturation in the marsupial outer gill, having a brooding season of approximately 6 months. In March the release of mature glochidia occurs gradually in clusters but very concentrated in time, 1- to 2 months maximum. However it contrasts with the

Table 4. Total mean proportion of sex and hermaphroditism in different aquatic environments.

	Mean ♀	Mean ♂	Mean ♀♂
Rivers	39.30	43.98	14.45
Lakes	13.35	10.00	76.65

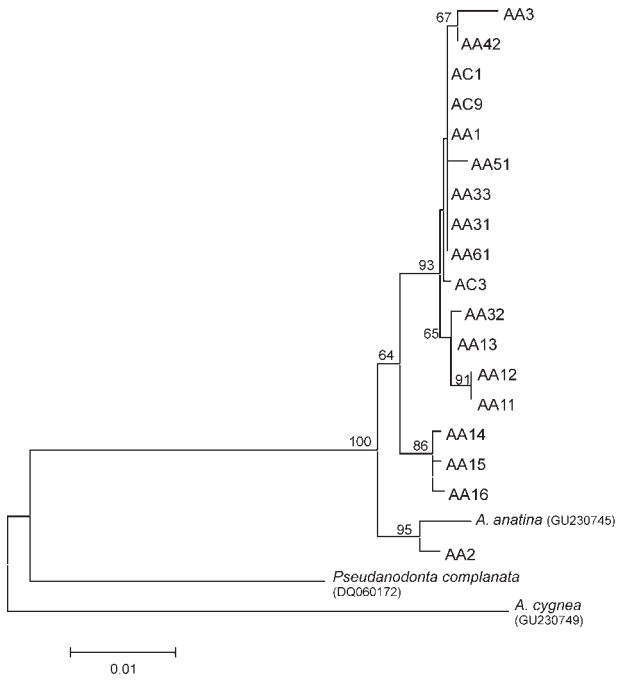

Figure 5. Phylogenetic relationships as shown by the neighbor-joining (NJ) analysis between haplotypes of COI gene. Bootstrap values are indicated above nodes. Information on each haplotype origin and polymorphisms is found in Table 1 and main text.

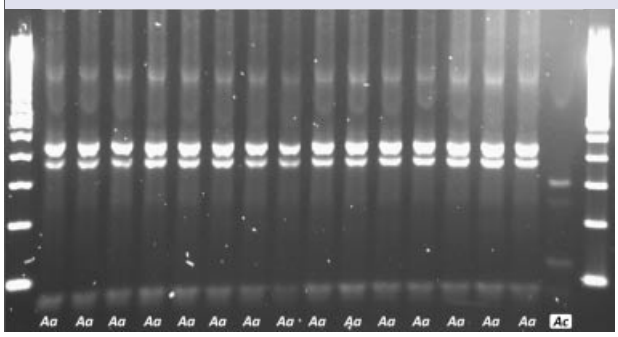

Figure 6. RFLP bands after enzymatic digestion of ITS-1 PCR products for adult specimens of *A. anatina* (Aa) from six populations across Portugal and one *A. cygnea* as control sample (Ac). For details please see Table 1 and main text.

invasive species *Sinanodonta woodiana* which main glochidia discharge period is from February to July in its native tropical home range (Dudgeon and Morton, '83) but shifts up to September in central Europe (Douda et al., 2012).

Sexual Strategy

The freshwater mussels are generally considered as mainly dioecious (gonochoristic) with only a few species found to be usually hermaphroditic (monoecious or ambisexual) (Kat, '83). A third type of species may develop a sexual strategy that is dependent on their habitat characteristics, being predominantly hermaphroditic or dioecious accordingly (Bauer, 2001). Previous studies on *A. anatina* described this species as strictly dioecious with occasional hermaphroditism (with very low content of male gonad tissue) in some populations (perhaps due to infection of the gonads with trematodes or developmental defects; Kat, '83; Bauer, '88). However, the present study showed a completely different pattern since our results indicated that *A. anatina* had a high prevalence of hermaphroditism in standing waters but retaining its mainly dioecious state in flowing rivers and streams; these results are corroborated by Yanovych et al. (2010) which found a hermaphroditic ratio on this species of 37.5 ± 9.9% on Ukrainian water reservoirs. In the same vein, early in the 20th century Weisensee ('16) suggested a correlation between habitat type and reproductive mode for *A. cygnea* where dioecious individuals occurred predominantly in rivers, while hermaphrodites were encountered mainly in standing waters. In fact, most *A. cygnea* populations studied from standing or slow moving waters consist mainly of a large proportion of hermaphrodites, a lower proportion of females, and very few males (Franke, '93; Teutsch, '97; Lopes-Lima, personal observation). The discrepancy between our results and earlier studies with *A. anatina* may have two distinct explanations: (i) *A. anatina* from Iberia presents a distinctive sexual strategy than other European studied populations (Bloomer, '36, '39; Franke, '93; Teutsch, '97; Aldridge, '99), maybe due to its genetics differentiation. This seems unlikely due to the fact that the mean genetic difference between the Iberian *A.*

Table 5. Reproductive and glochidia discharge period of several anodontine species.

Species	Region	Gametogenesis	Glochidia brooding	Main glochidia discharge	Authority
Anodonta anatina	Portugal	Throughout the year Stronger (January–May)	October–March	April	This study
Anodonta cygnea	Italy	Throughout the year Stronger (July–March)	October–March	November–April	Giusti et al. ('75)
Anodonta pseudodopsis	Turkey	December–April	–	–	Sereflisan et al. (2009)
Pseudanodonta complanata	UK	May–August mainly	August–April	April	Mcivor and Aldridge (2007)
Sinanodonta woodiana	Hong Kong	♀, Throughout the year ♂, Early winter	January–July –	February–July –	Dudgeon and Morton ('83) –
	Czech Republic	–	–	September	Douda et al. (2012)

anatina populations and the Central European ones is only 1.5% (COI) implying that their isolation is still recent and therefore seems unlikely to be the cause of the patterns described here; or (ii) Since *A. cygnea* preferential habitat is slow flow or standing waters, sampling and population studies are more likely carried on these habitats whereas with *A. anatina* the opposite pattern prevails. This might bias sexual ratio studies and render the sexual behavior of *A. cygnea* as mainly hermaphroditic and *A. anatina* as mainly dioecious. The most long-standing explanation for the development of hermaphroditism in animal populations is that it would increase the reproductive success of individuals found at low population densities (Tomlinson, '66; Ghiselin, '69; Puurtinen and Kaitala, 2002). This would double the encounter probability with an individual of the "opposite sex." If sperm survival time is short (but further studies are needed on sperm survival and dispersion on these organisms), one might expect, that in a spatially heterogeneous population, dioecious organisms might increase their reproductive success by being more frequently found in dense aggregations (Strayer et al., 2004). However, the Fermentelos lagoon population has higher densities than those found on most rivers (Lopes-Lima, personal data) so the explanation might reside on the increased available energy to both male and female reproductive function (Locher and Baur, 2002) or on the best dispersion rates of sperm in flowing waters. In fact, Jokela ('96) indicated that *A. anatina* ranked their energy demands so that allocation to maintenance is the top priority, allocation to reproduction the second priority, and allocation to growth is least important. So, if the availability of nutrients is higher (e.g., in lowland lakes or standing waters) it is expected that both the energy allocation for reproductive function and growth would be maximized.

Another interesting fact not studied in the present work but related with sperm dispersion is the possible self-fertilization changes on animals under different flow conditions. Selfing have been suggested by a noticeable higher fertility of sparse populations in *Margaritifera margaritifera* (Bauer, '87). The higher proportion of female over male tissue may also indicate that hermaphrodites spend less on male than on female functions and that these spermatozoa may be used for self-fertilization (Kat, '83). In fact this feature was suggested recently for *A. pseudodopsis* (Sereflisan et al., 2009) and a few laboratory studies were carried by Bloomer ('40) that indicate that self-fertilization may occur in the related species *A. cygnea*. In the present work the different proportion of males and females in distinct rivers and lakes may indicate also that self-fertilization, if present, may also have distinct proportions. Recent molecular techniques on larvae of freshwater mussels using fast evolving markers (e.g., microsatellites) allow for the establishment of multiple parenting (Ferguson et al., 2013) and eventually self-fertilization rates. These techniques should be included in future investigations on eventual changes of self-fertilization depending on the aquatic habitat.

As a conclusion this study established successfully the seasonal reproductive cycle on *A. anatina* and indicates a possible shift of the previous established sexual strategy from essentially dioecious to a more plastic sexual strategy that may be highly dependent on habitat characteristics. In addition, the information gathered could be a valuable help in future *A. anatina* conservation since the sexual strategy and reproductive cycle of this species was clearly established for Southern European populations.

ACKNOWLEDGMENTS

The authors thank the laboratory of anatomy and cellular biology from ICBAS—Oporto University for facilitating the use of their histological equipment as well as António Rocha for his laboratorial expertise.

LITERATURE CITED

Aldridge DC. 1999. The morphology, growth and reproduction of Unionidae (Bivalvia) in a fenland waterway. J Mollus Stud 65: 47–60.

Allen DC, Vaughn CC. 2011. Density-dependent biodiversity effects on physical habitat modification by freshwater bivalves. Ecology 92: 1013–1019.

Araujo R, Reis J, Machordom A, et al. 2009. The naiades of the Iberian Peninsula. Iberus 27:7–72.

Barber BJ. 1996. Gametogenesis of Eastern oysters, *Crassostrea virginica* (Gmelin, 1791) and Pacific oysters, *Crassostrea gigas* (Thunberg, 1793) in disease-endemic lower Chesapeake Bay. J Shellfish Res 15:285–290.

Bauer G. 1987. Reproductive strategy of the freshwater pearl mussel *Margaritifera margaritifera*. J Anim Ecol 56:691–704.

Bauer G. 1988. Threats to the freshwater pearl mussel *Margaritifera margaritifera* in central Europe. Biol Conserv 45:239–253.

Bauer G. 2001. Life-history variation on different taxonomic levels of naiads. In: Bauer G, Wächtler K, editors. Ecology and evolution of the freshwater mussels Unionoida, Vol. 145. Berlin: Springer-Verlag Ecological Studies. p 83–91.

Bauer G, Wächtler K. 2000. Ecology and evolution of the freshwater mussels Unionoida. Berlin: Springer-Verlag.

Bloomer HH. 1936. A note on the sex of *Anodonta Anatina*. J Mollus Stud 22:129–134.

Bloomer HH. 1939. A note on the sex of *Pseudanodonta* Bourguignat and *Anodonta* Lamarck. J Mollus Stud 23:285–297.

Bloomer HH. 1940. Experiments on self-fertilization in *Anodonta cygnea* (L.). J Mollus Stud 24:113–121.

Dillon RT. 2000. The ecology of freshwater Molluscs. Cambridge, United Kingdom: Cambridge University Press.

Douda K, Vrtílek M, Slavík O, Reichard M. 2012. The role of host specificity in explaining the invasion success of the freshwater mussel *Anodonta woodiana* in Europe. Biol Invasions 14:127–137.

Douda K, Lopes-Lima M, Hinzmann M, et al. 2013. Biotic homogenization as a threat to native affiliate species: fish introductions dilute freshwater mussel's host resources. Divers

Distrib Available online ahead of print 17 Jan 2013; DOI: 10.1111/ddi.12044

Dudgeon D, Morton B. 1983. The population dynamics and sexual strategy of *Anodonta woodiana* (Bivalvia: Unionacea) in Plover Cove Reservoir, Hong Kong. J Zool 201:161–183.

Ferguson CD, Blum MJ, Raymer ML, Eackles MS, Krane DE. 2013. Population structure, multiple paternity, and long-distance transport of spermatozoa in the freshwater mussel Lampsilis cardium (Bivalvia: Unionidae). Freshwater Sci 32:267–282.

Franke G. 1993. Zur Populationsokologie und Geschlechtsbiologie der Teichmuscheln *A. anatina* L. und *A. cygnea* L [MS Thesis]. Bayreuth, Germany: University of Bayreuth.

Gerke N, Tiedemann R. 2001. A PCR-based molecular identification key to the glochidia of European freshwater mussels (Unionidae). Conserv Genet 2:285–287.

Ghiselin MT. 1969. The evolution of hermaphroditism among animals. Q Rev Biol 44:189–208.

Giusti F, Castagnolo L, Farina LM, Renzoni A. 1975. The reproductive cycle and the glochidium of Anodonta cygnea L. from Lago Trasimeno (Central Italy). Monit Zool Ital 9:99–118.

Graf DL. 2007. Palearctic freshwater mussel (Mollusca: Bivalvia: Unionoida) diversity and the comparatory method as a species concept. Proc Acad Nat Sci Phila 156:71–88.

Hall TA. 1999. Bioedit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98.

Heard WH. 1975. Sexuality and other aspects of reproduction in *Anodonta* (Pelecypoda: Unionidae). Malacologia 15:81–103.

Jokela J. 1996. Within-season reproductive and somatic energy allocation in a freshwater clam, *Anodonta piscinalis*. Oecologia 105:167–174.

Kat PW. 1983. Sexual selection and simultaneous hermaphroditism among the Unionidae (Bivalvia: Mollusca). J Zool 201:395–416.

Kat PW. 1984. Parasitism and the Unionacea (Bivalvia). Biol Rev 59:189–207.

Librado P, Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Lima P, Monteiro SM, Sousa M, Machado J. 2012. A histological study of oogenesis in the freshwater mussel *Anodonta cygnea* (Linnaeus, 1758) in Mira Lagoon, Portugal. Malacologia 55:251–261.

Locher R, Baur B. 2002. Nutritional stress changes sex-specific reproductive allocation in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Funct Ecol 16:623–632.

Lydeard C, Cowie RH, Bogan AE, et al. 2004. The global decline of nonmarine mollusks. BioScience 54:321–330.

Mcivor AL, Aldridge DC. 2007. The reproductive biology of the depressed river mussel, *Pseudanodonta complanata* (Bivalvia: Unionidae), with implications for its conservation. J Mollus Stud 73:259–266.

Neves RJ, Bogan AE, Williams JD, Ahlstedt SA, Hartfield PW. 1997. Status of aquatic mollusks in the southeastern United States a downward spiral of diversity. In: Benz GW, Collins DE, editors. Aquatic fauna in peril: the southeastern perspective. Decatur, GA: Southeast Aquatic Research Institute. p 45–86.

Puurtinen M, Kaitala V. 2002. Mate-search efficiency can determine the evolution of separate sexes and the stability of hermaphroditism in animals. Am Nat 160:645–660.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Press.

Sereflisan H, Çek S, Sereflisan M. 2009. Histological studies on gametogenesis, hermaphroditism and the gametogenic cycle of *Anodonta Gabillotia Pseudodopsis* (Locard, 1883) in the Lake Gölbasi, Turkey (Bivalvia: Unionidae). J Shellfish Res 28:337–344.

Simberloff D. 2012. Sustainability of biodiversity under global changes, with particular reference to biological invasions. In: Weinstein MP, Turner RE, editors. Sustainability science: The emerging paradigm and the urban environment. New York: Springer. p 139–157.

Sousa R, Ilarri M, Souza AT, Antunes C, Guilhermino L. 2011. Rapid decline of the greater European peaclam at the periphery of its distribution. Ann Limnol Int J Lim 47:211–219.

Sousa R, Varandas S, Cortes R, et al. 2012. Massive die-offs of freshwater bivalves as resource pulses. Ann Limnol Int J Lim 48: 105–112.

Strayer D, Downing JA, Haag WR, et al. 2004. Changing perspectives on pearly mussels, North America's most imperiled animals. BioScience 54:429–439.

Tamura K, Peterson D, Peterson N, et al. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739.

Teutsch S. 1997. Populationsbiologische Untersuchung an *Anodonta*: Fortpflanzungsstrategien und Charakterisierung von Teilpopulationen innerhalb eines Stillgewassers [MS Thesis]. Freiburg, Germany: University Freiburg.

Tomlinson J. 1966. The advantages of hermaphroditism and parthenogenesis. J Theor Biol 11:54–58.

Vaughn CC. 2010. Biodiversity losses and ecosystem function in freshwaters: emerging conclusions and research directions. BioScience 60:25–35.

Walker JM, Curole JP, Wade DE, et al. 2006. Taxonomic distribution and phylogenetic utility of gender-associated mitochondrial genomes in the Unionoida (Bivalvia). Malacologia 48:265–284.

Walker JM, Bogan AE, Bonfiglio EA, et al. 2007. Primers for amplifying the hypervariable, male-transmitted COII-COI junction region in amblemine freshwater mussels (Bivalvia: Unionoidea: Ambleminae). Mol Ecol Notes 7:489–491.

Weisensee H. 1916. Die Geschlechtsverhaltnisse und der Geschlechtsapparat bei Anodonta. Z Wiss Zool 115:262–335.

Yanovych LM, Pampura MM, Vasilieva LA, Mezhherin SV. 2010. Mass hermaphroditism of Unionidae (Mollusca, Bivalvia, Unionidae) in the Central Polissya region. Rep Nat Acad Sci Ukraine 6:158–167.

Zettler ML, Jueg U, Menzel-Harloff H, et al. 2006. Die Land- und Süßwassermollusken Mecklenburg-Vorpommerns. Schwerin, Germany: Obotritendruck GMBH.