

# The Strange Case of the Tetrigenous *Anodonta anatina*

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## ABSTRACT

Unionoid freshwater mussels have a unique life cycle with a form of parental care where the larvae are developed and kept inside the gills until release, followed by an obligate parasitic stage on fish. The size and location of the marsupium have been used as important phylogenetic characters in unionoids and in Anodontini its location was described exclusively on the outer demibranchs. Two recent surveys in a lake in the North of Portugal revealed large anodontine mussels morphological identical to *Anodonta anatina* but with glochidia in both demibranchs and with an unusual large size. In order to establish the identity of these mussels, a barcoding approach was used and an anatomical description of the gills and glochidia was performed. These mussels were identified as *A. anatina* and presented an inner demibranch pair with tripartite tubes. The glochidial sizes were much higher than previously reported for the species reaching maximum (length × height) values of 566 × 552 μm. This species reveals a high ecological plasticity being able to change its size and anatomy to increase its fertility as well as infestation performance. *J. Exp. Zool.* 325A:52–56, 2016. © 2015 Wiley Periodicals, Inc.

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Freshwater mussels (Bivalvia, Unionoida) present complex life history traits adapted to life in fluvial environments that include parental care and specialized larvae, called glochidia (Barnhart et al., 2008; Douda et al., 2013). Once released from the females, glochidia have an obligate parasitic phase on aquatic vertebrates (primarily fish), which they have to attach and use for nutrition and dispersal (Wächtler et al., 2001). Before discharge, glochidia are kept inside modified demibranchs within water tubes (the interlammellar spaces of the ctenidia) for a variable time period, depending on the species (Haag and Staton, 2003). This form of parental care or brooding is common among other freshwater bivalve families such as the Cyrenidae and Sphaeriidae (Graf, 2013). Although the main freshwater mussel reproductive strategies may be more complex, two main patterns of brooding behavior are generally recognized among holarctic unionoida: short (tachytictic) or long (bradytictic) term (Heard, '98; Watters and O'dee, 2000). Tachytictic mussels, generally spawn their gametes in the spring with embryos being brooded in the female marsupial demibranchs and the discharge occurring immediately after their development into mature glochidia. On the other hand, bradytictic mussels usually spawn in the late summer and brood their glochidia overwinter, releasing the glochidia in the early spring long after they reached their maturity and infective capacity.

The sections of the female demibranchs that serve as brood spaces are called marsupia, their size being highly variable among taxa and have been used as a taxonomical character (Graf and Foighil, 2000). Depending on the taxa, marsupia may occupy from only a small portion to the entire outer demibranchs (ectobranchy), the entire inner demibranchs (endobranchy) or even all four demibranchs (tetrageny) (Heard, '98). Additionally, some groups present distinct structural features in the development of interlammellar connections among and within the different marsupial arrangements.

*Anodonta anatina* (Linnaeus 1758) is a widespread European freshwater mussel that belongs to the tribe Anodontini within the subfamily Unioninae (Froufe et al., 2014; Lopes-Lima et al., 2015). Regarding the reproductive traits, the main synapomorphies of this clade are: i) a long brooding period or bradytictic; ii) marsupium occupying only the outer demibranchs or ectobranchia; and iii) brooding ctenidial modifications, where the water tubes in the outer demibranchs are tripartite. In the modified demibranchs, the glochidia use only the internal middle tube leaving the outer tubes for gas exchange. Several studies carried out in Europe confirmed *A. anatina* has having all of these features (Bauer, 2001). Accordingly, the authors of the present study have also found these features in several *A. anatina* populations in Portugal (Hinzmänn et al., 2013). However, several large specimens putatively identified as *A. anatina* were found in Fermentelos Lake (Portugal) with distinct brooding behavior and morphological characteristics. Therefore, the aims of the present study were to confirm the identity of these specimens, and to

report and discuss the possible reasons behind these distinctive and unexpected features.

## METHODS

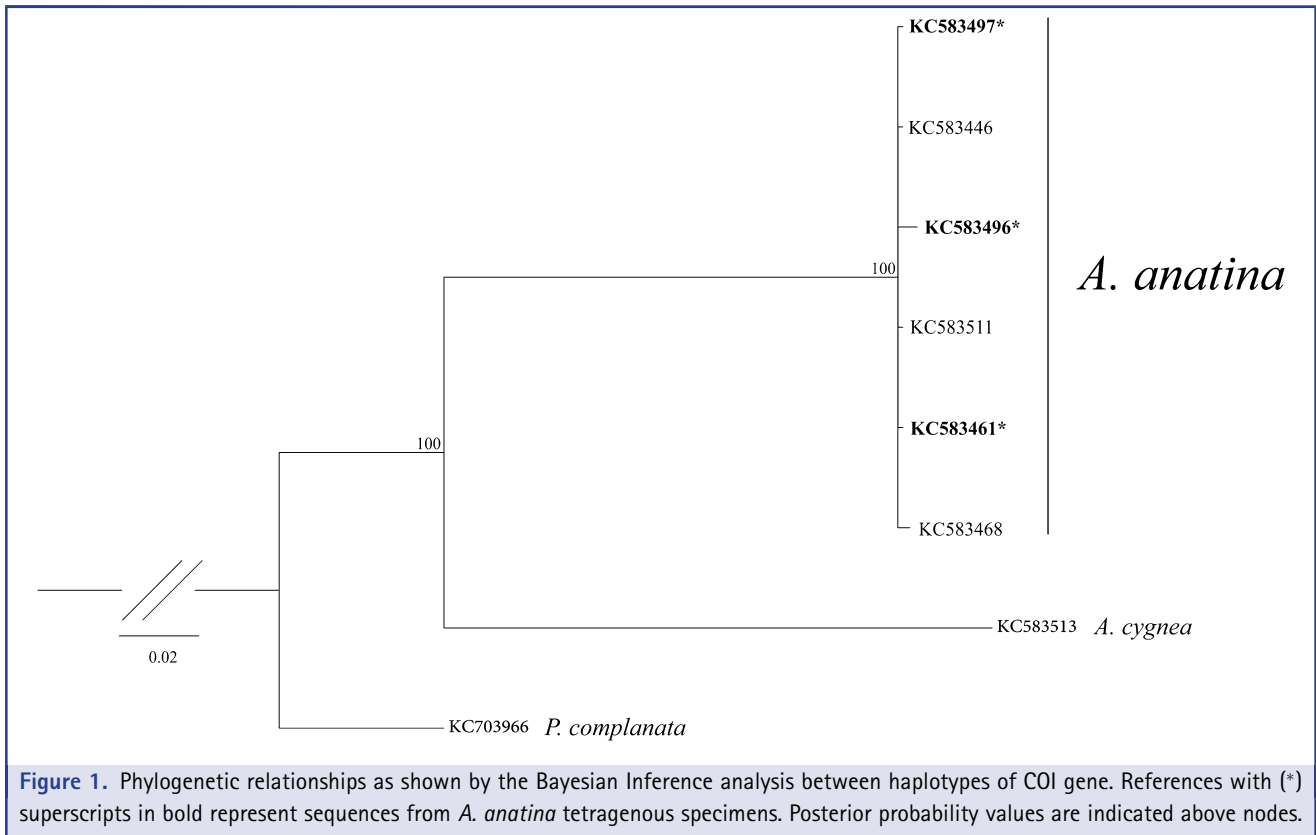
Two surveys were carried out in March 2011 and December 2012, by wading on the south bank of Fermentelos Lake. This is a shallow water body in the North of Portugal (N 40.573983, W -8.514636) with approximate 2.6 km<sup>2</sup> and recognized as an important fishing and recreation area. Ten and 27 specimens of *Anodonta* sp. were collected on the first and second surveys, respectively. Three gravid *A. anatina* specimens were also collected from the Tâmega River (N 41.541608, W -7.787269) for comparative studies. Mussels were immediately transported to the laboratory on ice in a cooler box and processed within 24 hr. All of the animals were measured and weighed, anesthetized in 2-phenoxyethanol solution 0.4% (v/v) for about 30 min and dissected for anatomical analysis and histology.

Samples of gonad and demibranch tissues were excised from each animal and preserved in Bouin's solution for a week (Panreac, Barcelona, Spain). Afterwards, transverse sections of the gonads and demibranchs were dehydrated in graded ethanol, embedded in paraffin, sectioned at 5–8 mm and stained with hematoxylin and eosin for histological examination. The sex was visually verified by the presence of male and female gonad tissue. Glochidia from the Tâmega River and Fermentelos Lake were collected in eppendorf tubes with 96% ethanol from three distinct places of both demibranchs (one on each demibranch tip and one in the middle) per bivalve. A pool of 50 glochidia was selected randomly from each replicate and measured under the microscope.

To avoid taxonomic uncertainties due to the unusual anatomical features found, identities were confirmed by genetic analysis. For this, foot tissue samples from three tetragenous individuals were collected and placed directly into 96% ethanol. COI sequences were obtained from these individuals and have been included in previous genetic diversity studies (Hinzmänn et al., 2013, Froufe et al., 2014). Three additional sequences from *A. anatina* (KC583446, KC583511, KC583568), one from *Anodonta cygnea* (Linnaeus 1758) (KC583513), and one from *Pseudanodonta complanata* (Rossmässler 1835) (KC703966), were aligned with the tetragenous specimens sequences (KC583461, KC583496, KC583497) using ClustalW, in Bioedit 7.2.5 (Hall, '99), resulting in a final alignment of 577 bp. This data set was then analyzed using Bayesian Inference using the model GTR+I+G with  $2 \times 10^6$  generations. All sequence references are available on GenBank.

## RESULTS

The obtained phylogenetic tree (Fig. 1) revealed that the tetragenous animals are indeed *A. anatina*. The three gravid females collected on the Tâmega River presented the regular ectobranchous condition. From those collected on the

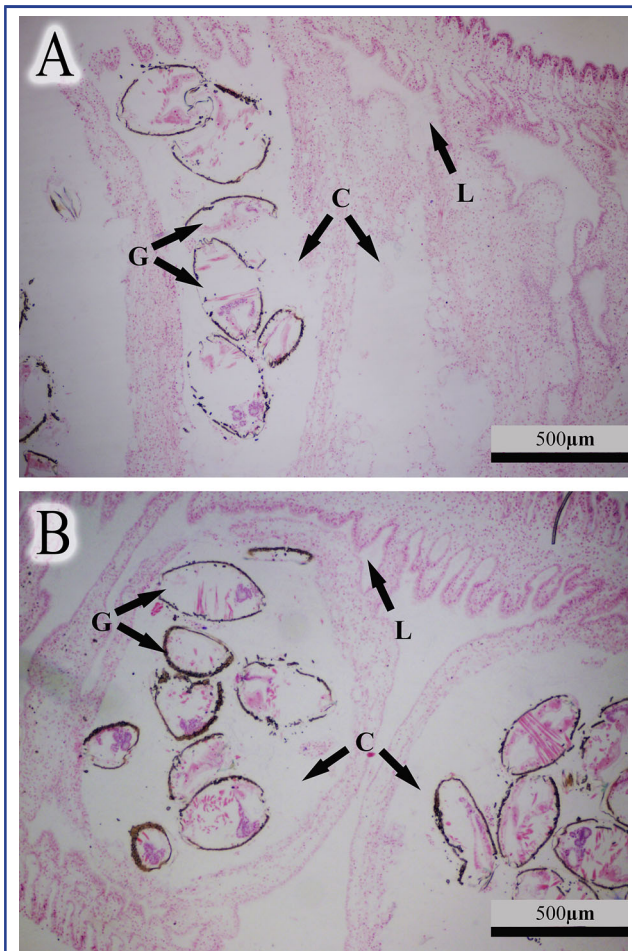


Fermentelos Lake, all specimens were hermaphroditic, three out of the ten specimens on the first survey were gravid with two presenting a regular ectobranchy condition (glochidia in the outer demibranch pair) and one tetragenous (glochidia in both demibranch pairs) (33%). From the second sampling period all 27 animals were hermaphroditic and 26 were gravid, 5 being tetragenous (19%). In tetragenous animals both the external and internal pair of demibranchs presented tripartite modified water tubes (Fig. 2), where the central tube was covered with glochidia. Size and weight of these *Anodonta* specimens were very high for the species, with several individuals passing 19 cm and 500 g wet weight, respectively. The glochidia presented triangular shaped valves with a ventral hook filled with spines. The mean size ( $\pm$ SD) of the glochidia were  $501 \pm 82 \mu\text{m}$  (length) and  $487 \pm 91$  (height) in the Fermentelos Lake and  $339 \pm 17 \mu\text{m}$  (length) and  $377 \pm 19 \mu\text{m}$  (height) in Tãmega River (Table 1).

## DISCUSSION

The population of *A. anatina* from Fermentelos Lake is exclusively hermaphroditic. This fact has been attributed to the increased available energy for both male and female reproductive functions (Hinzmann et al., 2013). The main features of the glochidia do not differ from what was previously described for several European *A. anatina* populations with the

exception of size which is much larger (Table 1; Nagel, '85; Niemeyer, '92). In contrast, the tetragenous condition and anatomical features of the marsupium here described are unusual and have never been reported. In fact, the presence of tripartite water tubes on the inner demibranch pair has never been described for *A. anatina* nor for any other studied anodontine species (for a review see Heard, '75). These unusual characteristics might be explained by two major reasons i) the population of Fermentelos Lake is unique in these features due to its genetic characteristics, and/or ii) *A. anatina* brooding ability is plastic and these animals are capable to adjust the inner demibranch morphology to increase the number of marsupia, and therefore fertility, when inhabiting areas with high nutrient levels. The present study confirms the identity of those specimens as *A. anatina*. However, in order to evaluate in detail the genetic influence on this behavior, a quantitative trait loci analysis should be carried on these animals. Fermentelos Lake is a shallow water body that sustains an important wetland area. In the last decades this area was subjected to high nutrient inputs, mainly from agriculture runoffs, which lead to its current eutrophic to hypereutrophic status (De Figueiredo et al., 2007). This situation was responsible for the high nutrient levels and the massive development of phytoplankton and macrophytes, including the presence of the invasive water hyacinth (*Eichhornia crassipes*



**Figure 2.** (A and B) Histological sections from the inner demibranchs of two tetragenous *Anodonta anatina* specimens stained with H&E (eosin and hematoxylin), showing the tripartite condition being (G) glochidia; (C) central water tubes; and (L) lateral water tubes.

[Mart.] Solms) (Almeida, 2006). Among molluscs, and freshwater bivalves in particular, growth and metabolism may be adjusted to the availability of food resources (Bauer et al., '91). In fact, some freshwater mussels have a resource allocation strategy in favor of somatic functions being only the energetic surplus applied to reproduction (Bauer, '98). The high quantity of food resources and the mild and stable temperatures of Fermentelos Lake might explain the high size and weight of adults and glochidia. In addition, the above mentioned conditions can also explain the high fertility with the occupation of an additional pair of marsupia for brooding. The marsupia of unionoids are usually tightly filled and considerably swollen (i.e., the number of glochidia seems to be determined largely by the space available in the marsupia and thus by the size of the mussel; Niemeyer, '92). However, this fact does not explain the glochidia larger size. According to Bauer ('94) there is no relationship between body size and offspring size and bigger offspring size naturally decreases fertility. So, if on one hand, the presence of large glochidia from these *A. anatina* specimens reduces fertility, on the other hand the use of both demibranchs almost doubles the area available. It is possible that the host can also play an important role in this aspect since the size of the host range is positively related with glochidia size (Bauer, '94). Additionally, an important physiological constraint is the duration of the parasitic stage, which is inversely related to glochidia size. The largest glochidia are highly developed when released by the parent (Harms '08, '09). So, these *A. anatina* specimens present increased fertility probably with a higher survival rate due to increased host spectrum and a shorter parasitic period.

In conclusion, the *A. anatina* individuals found in the highly eutrophic Fermentelos Lake exhibit an extraordinary ecological plasticity. Their large body sizes and the ability to change the morphology of the inner demibranch pair to accommodate an extra pair of marsupia substantially increase fertility. Additionally, their exceptional glochidia size may allow for better survival due to an increased host range and a shorter parasitic stage.

**Table 1.** Glochidia size ranges of *Anodonta anatina* reported from this and other previous studies with the exception of those with (\*) superscripts which relate to mean size

Country	Length ( $\mu\text{m}$ )	Height ( $\mu\text{m}$ )	Reference
Germany	360*	340*	Niemeyer ('92)
Germany	350*	—	Harms ('09)
Germany	360*	350*	Hüby ('88)
Finland	335–382	329–368	Pekkarinen and Englund ('95)
Russia	350–365	340–365	Antonova and Starobogatov ('88)
Portugal (Tâmega)	340–422	320–380	This study
Portugal (Fermentelos)	450–566	422–552	This study

Further studies involving ecological (focusing on the importance of food resources) and genomics (centered on a quantitative trait loci analysis) on both tetragenous and ectobranchous animals should help to clarify these findings.

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