With all my thanks
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JOURNAL OF CONCHOLOGY (2003), VOL.38, NO.1 53 2003

LABORATORY PROPAGATION AND CULTURE OF JUVENILES OF THE ENDANGERED FRESHWATER MUSSEL *MARGARITIFERA AURICULARIA* (SPENGLER, 1793)

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Abstract *We infested three fish species, blenny* (Salaria fluviatilis)*, Adriatic sturgeon* (Acipenser naccarii) *and gambusia* (Gambusia holbrooki)*, with glochidia of the endangered freshwater mussel Margaritifera auricularia at different holding temperatures, and developed a system to grow and maintain cultured juvenile mussels. The sturgeon and blenny were successfully infested, although these fish sloughed the glochidia in the 24°C experiment. Release of juveniles began 30 days post-infestation for sturgeon and 31 days post-infestation for blennies, and excystment occurred over 8 and 10 days, respectively. The total number of juveniles cultured was 2,562. Although the mortality rate was high, juveniles were maintained alive for at least 4 weeks in the four test tanks. In the most successful tank, 13 juveniles were found after 6 weeks (39 days) of culture. Maximum dimensions for juveniles after culture were: length= 325 µm, width= 250 µm, height = 500 µm, indicating a mean increase of 51% in length, 60% in height and 23% in width.*

INTRODUCTION

Among the European freshwater mussels, *Margaritifera auricularia* is by far the most endangered species, currently living only in Spain (Canal Imperial, Canal de Tauste and Ebro River) and possibly in Morocco (Araujo & Ramos, 2000a). Although there has been a dramatic increase in published information on this species during the last decade (Araujo & Ramos, 2001), captive propagation for restocking, as recommended in the European Action Plan (Araujo & Ramos, 2001), has not been implemented.

Several papers have been published in the United States on captive breeding of freshwater mussels (Hudson & Isom, 1984; Yeager, Cherry & Neves, 1994; Gatenby, Neves & Parker, 1996; Gatenby, Parker & Neves, 1997; O'Beirn, Neves & Steg, 1998), but in Europe, only preliminary experiments on rearing juveniles of the endangered species *M. margaritifera* can be found (Buddensiek, 1995; Harsányi, 1999; Hruska, 1999).

The reproductive period of *M. auricularia* was previously studied (Grande, Araujo & Ramos, 2001). The successful production of juveniles of *M. auricularia* in *Acipenser baeri* and *Salaria fluviatilis* has been achieved (Araujo & Ramos, 2000b; Araujo, Bragado & Ramos, 2001) and gambusia has been recommended for captive breeding by López & Altaba (2000). In this study, we sought to improve upon these first experiments by: 1) using other fish species, 2) increasing the number of specimens, 3) testing different holding temperatures, and 4) developing a system to grow and maintain the juvenile mussels. Results could be applied in aquaculture facilities connected to the Ebro River (the natural habitat of *M. auricularia*), in order to rear thousands of juveniles for release into protected reserves.

MATERIAL AND METHODS

FISH INFESTATION

The experiment was conducted in the aquaculture facilities at Escuela Técnica Superior de Ingenieros Agrónomos (Universidad Politécnica de Madrid. Spain). Three

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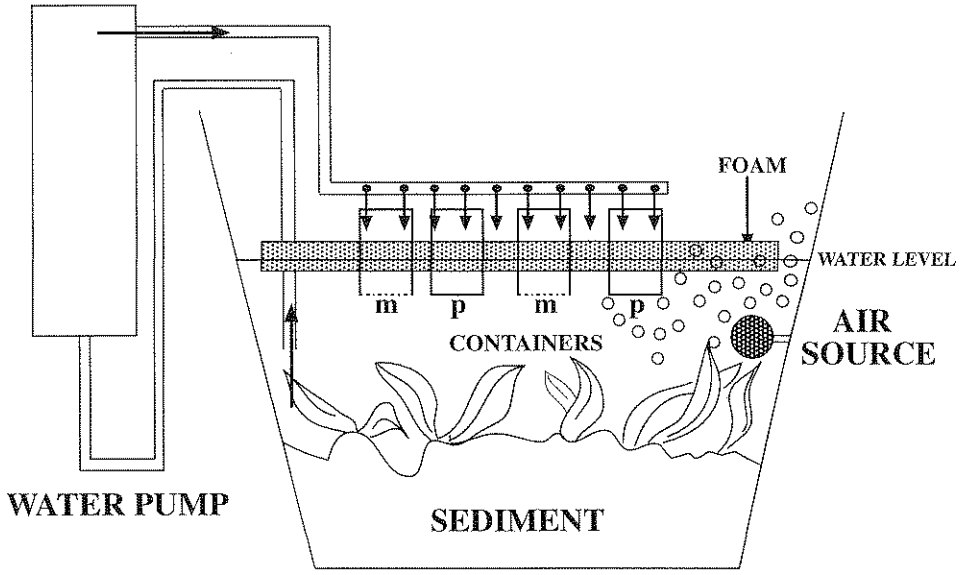

Figure 1 Detail of the culture tank. m= meshed bottom container. p= plastic bottom container.

fish species were used: 14 specimens of blenny (*Salaria fluviatilis*), 15 Adriatic sturgeon (*Acipenser naccarii*) and 9 gambusia (*Gambusia holbrooki*). Sturgeon were obtained from the fish hatchery at Riofrío (Granada, Spain), blennies from the Centro de Experimentación Piscícola El Palmar (Valencia, Spain), and gambusia from an irrigation pond in Madrid (Spain). Fish were maintained at two temperatures (see below) in aerated 200 litre tanks in a recirculating well-water system equipped with biological and mechanical filtration. They were fed frozen mosquito larvae daily. We used ambient light levels for the photoperiod regime.

Ten gravid specimens of *Margaritifera auricularia* (Spengler) were collected at the Canal Imperial (Zaragoza, Spain) in February 2001, and maintained in two aquaria: six specimens in water from the Canal Imperial, and four specimens in Canal Imperial water with 10 cm of sediment.

For induced infestations, mature glochidia were collected with a pipette through the exhalant aperture of the gravid mussels and rinsed with aerated water for 20 min in a glass jar containing the isolated fish. Once infested, the fish were maintained in the aerated tanks, but were isolated from the recirculating system. They were fed for the first 28 days. During this period, they were regularly anaesthetized with 3-Aminobenzoic Acid Ethyl Ester (MS222) to examine the occurrence of glochidia in the gills.

Two different experiments were conducted:

- Low temperature. Infested fish were maintained in four tanks: A1: 6 blennies infested on March 12. A2: 2 blennies infested on March 12. A3: 6 small (<12 cm) sturgeon infested on March 13. A4: 3 large (> 15 cm) sturgeon infested on March 13. The experiment was set at a water temperature of 16-17 °C, but due to a malfunction of equipment, the temperature increased to 21°C.
- High temperature. B1: 2 small sturgeon infested on March 14. B2: 4 small sturgeon infested on March 14. B3: 9 gambusia infested on March 14. B4: 6 blennies infested on March 13. Water temperature was maintained at 24 °C during the experiment.

On days 27-29 after glochidial inoculation, fish were isolated in aquaria without substratum and with a 5 mm mesh plastic net on the bottom to separate the fish from

TABLE 1

Fish from which the juveniles were obtained, container type and number of juveniles cultured in each tank. * Only three containers with juveniles obtained from blennies were suspended in this tank.

FISH	CONTAINER	TANKS			
		T1	T2	T3*	T4
Blenny	Plastic bottom	12	23	-	414
Blenny	Mesh bottom	144	33	112/54/393	267
Sturgeon	Plastic bottom	34	126	-	14
Sturgeon	Mesh bottom	308	394	-	152

excysted juveniles. Water was maintained at the same temperature as in the tanks, and no food was added. The bottom water layer was pumped through a 60 µm mesh every day until no more juveniles were found.

JUVENILE MUSSEL CULTURING

Recently released juveniles, recovered from each aquarium in the 60 µm mesh, were pipetted and put in plastic containers with 1 cm of clean fine substratum (particle size: 1–2 mm) from the Canal Imperial. All juveniles were counted but not tested for viability. Although all were used for experimentation, it is probable that not all were alive.

Two kinds of containers were used: one with a plastic bottom and the other with a 60 µm gauze mesh bottom. Juveniles collected from sturgeon were separated from those taken from blenny, and both were tested in the two containers. In this way, a total of 15 containers were suspended from foam platforms in four tanks (Table 1). The tanks were filled with well and green water, sediment from the Canal Imperial, soil, and vegetation from the Ebro River; and were continuously aerated (Fig. 1). In all tanks except T1, a pump was installed to flush tank water over the containers holding the juveniles. In T1, no pump was installed, but aeration of the water was greatly increased. Because we were testing the survival capability of juveniles in nutrient laden-water, we did not add any extra food to the tanks.

The experiment ran for 6 weeks, and all containers were regularly inspected for growth and survival of juveniles. The sediment of each container was screened through two sieves of 500 µm and 60 µm mesh. Juveniles were pippeted, counted, and when possible, measured (length, height and width). Dead juveniles were removed at each sampling, and the sediment was cleaned or changed. The inspection of each container took an average of 3 hours.

RESULTS

All glochidia used in the experiment were obtained from the mussels held in the aquarium with sediment. Release of mature glochidia occurred for 3 days only, between March 12 and 14. Mussels in the aquarium without sediment released only embryos and not mature glochidia.

Although all fish were successfully infested with glochidia, fish sloughed the glochidia in the 24°C tanks. Up to 8 days after infestation, glochidia were encapsulated on the gills of at least one of the 9 gambusia, but no glochidia were present by the day 15. Glochidia were observed on the blennies for the first few days, but none were

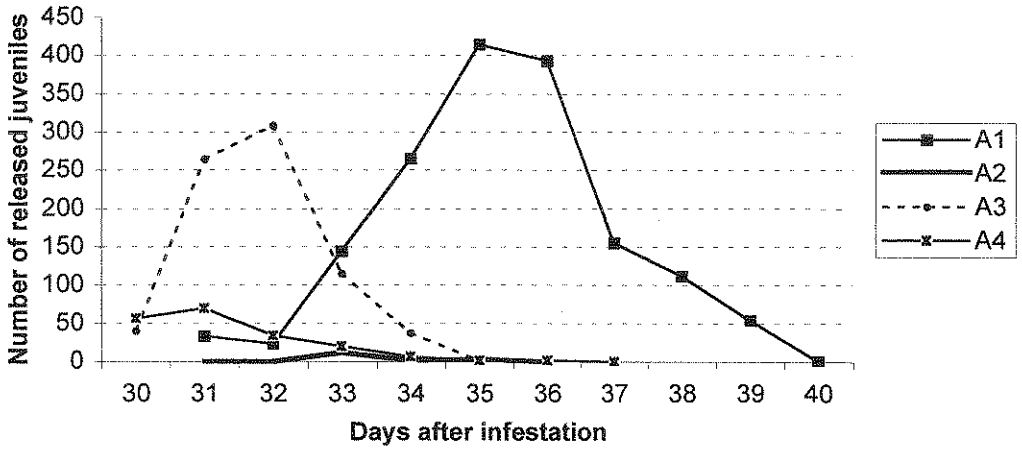

Figure 2 Release of 2,562 juveniles from the infestated fish. A1, A2= blenny. A3, A4= sturgeon. Temp = 21°C.

present 20 days after infestation. Sturgeon also maintained encysted glochidia for the first several days, but they were inspected less carefully for glochidia than the other fish species. For this reason, they were isolated 26 days after infestation in aquaria equipped with a net on the bottom, to collect any juveniles that transformed. No juveniles were found. Although unlikely, juveniles could have excysted in the tanks before the fish were isolated in aquaria.

Because the experiment at 24 °C was not successful, all the juveniles used for culture were taken from the infestation experiment at 21 °C. Only one fish in this experiment, a female blenny from tank A2, was not successfully infested with glochidia.

The release of juveniles began 30 days post-infestation for sturgeon and 31 days post-infestation for blennies (Fig. 2). Excystment occurred over 8 days for sturgeon and over 10 days for blennies.

The total number of juveniles used in the culture experiment was 2,562. Seven hundred seventy-six juveniles were excysted from the five small sturgeon, peaking on day 3 with 308 released juveniles. One hundred eighty-five juveniles were obtained from the three large sturgeon. Blennies produced more juveniles than did sturgeon. Five blennies in one aquarium released a total of 1,594 juveniles (peaking on day 5 with 414), but only 17 juveniles were released by the male blenny in the other aquarium (Fig. 2).

Although with varying degrees of success, juveniles were kept alive for at least 4 weeks in the four test tanks (Fig. 3). Juveniles survived up to 6 weeks in tanks T1 (4%) and T3 (11%) (Fig. 3). Survival was highest in the containers with gauze-meshed bottoms, although some juveniles remained alive until the fourth week in plastic-bottomed containers (Fig. 3D). In the most successful tank (T3), 13 living juveniles were found after 6 weeks (39 days) of culture. In the four tanks, mortality was greatest during the first 2 weeks. This may be due to the fact that not all juveniles were viable initially. We observed a substantial size increase in juveniles from both the sturgeon and blenny hosts (Fig. 4) (Table 2). Maximum dimensions for cultured juveniles were: length= 325 µm, width= 250 µm, height = 500 µm. The largest juvenile was measured in the sixth week of culture and was 350 µm height and 325 µm long (Figure 5). Although shell measurements were not taken of all juveniles, it appears that they grow more successfully in containers with gauze-mesh bottoms than in those with plastic bottoms.

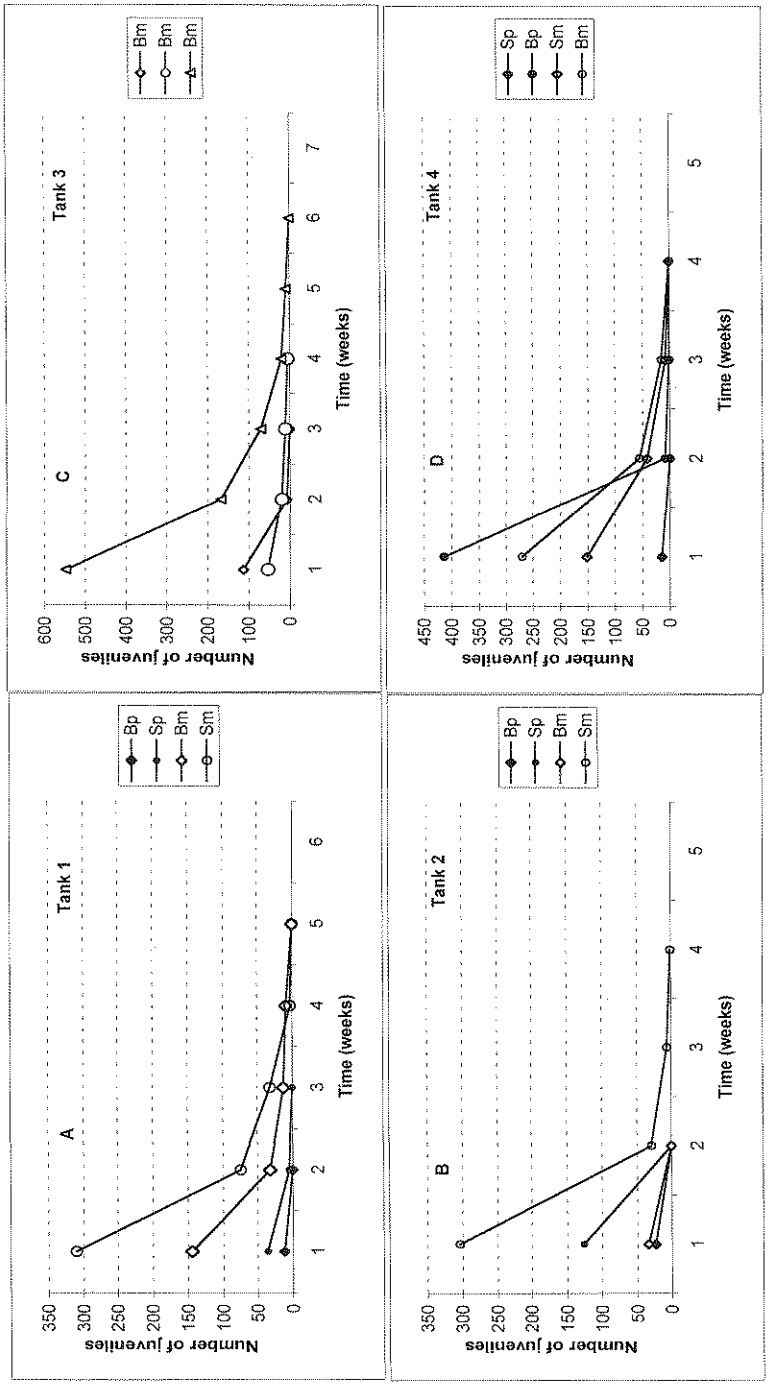

Figure 3 Survival of juveniles in containers in the four test tanks. B= blenny. S= sturgeon. m= meshed bottom container. p= plastic bottom container.

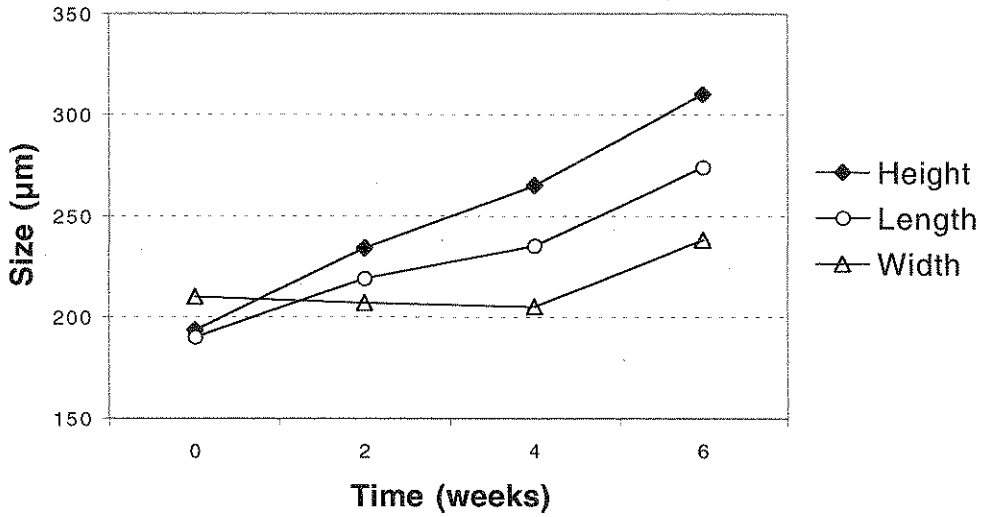

Figure 4 Mean juvenile shell size increase during culture. See Table 2 for details.

DISCUSSION

Results of these experiments show that captive propagation and juvenile culture of the critically endangered *Margaritifera auricularia* may be feasible in the very near future.

It is recommended to use gravid mussels and maintain them in the most natural conditions possible (i. e., water and sediment from the natural habitat). It is also advisable to harvest them quickly in order to obtain the required number of glochidia. Knowledge of the reproductive season of the species is critical, making the need for more basic research on the reproductive biology of the species imperative.

Results of the present study show that blenny, and the Adriatic sturgeon, can host the transformation of the glochidia of *M. auricularia*. As *A. naccarii* is the second species of sturgeon shown to support glochidial metamorphosis of this species (Araujo & Ramos, 2000b), it is likely that other species of *Acipenser* are suitable for captive breeding. Small sturgeon (approx. <12 cm) are highly recommended because they host more juveniles than large specimens and are more easily managed. Because our data on *gambusia* are not conclusive, experiments must be repeated with this species. Its distribution range does not overlap with that of *M. auricularia*, which is suspected of being stenotopic in terms of suitable glochidial hosts. Nevertheless, the period of time that elapsed between the infection and sloughing of the glochidia was longer in *gambusia* (8 days) than it was

TABLE 2
Standard deviation and mean juvenile shell size (µm) increase during culture.

WEEKS	0	2	4	6
LENGTH	190 n=1	219±19.93 n=15	235.45±27 n= 11	274.17±39.23 n= 18
HEIGHT	193.50±48.06 n= 4	234.06±32.18 n= 65	265.09±31.09 n= 54	309.62±69.45 n= 39
WIDTH	210±48.94 n= 4	207±18.01 n= 15	205±11.18 n= 5	238±17.68 n= 2

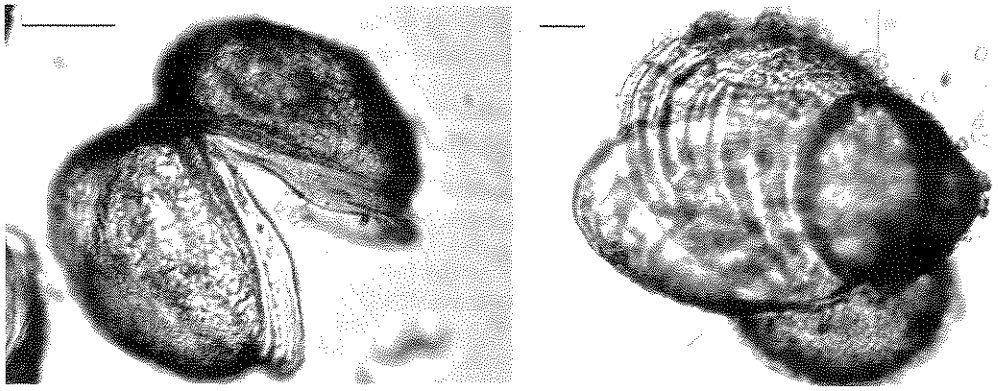

Figure 5 Juvenile shells of *M. auricularia* at release and at 6 weeks old. Scale bar: 40µm.

in other non-host fish that were tested (Araujo, Bragado & Ramos, 2001).

Water temperature is one of the main factors controlling the rate of metamorphosis of freshwater mussel glochidia in host fish. Araujo, Cámara & Ramos (2002) have demonstrated that the rate of metamorphosis of *M. auricularia* glochidia accelerates when water temperature is raised to 20°C, but does not accelerate at several degrees higher. Present data suggest that above 21°C this process is interrupted, although Araujo and Ramos (2000b) did observe the complete metamorphosis of *M. auricularia* glochidium in *Acipenser baeri* in 30 days at 23-24°C (ca. 700 degree-days). Thus, the possibility of an unobserved rapid metamorphosis in our experiment exists, but it is not very probable.

Although temperatures in the Ebro River never rose above 20°C in the months following the release of *M. auricularia* glochidia (March-April), conducting experiments at temperatures greater than 20°C could be of interest. Higher temperatures may accelerate juvenile growth and minimise production costs in future culture experiments of naiads.

Without redundant food and by maintaining "natural" conditions for culture, survival of some juveniles exceeded 1 month. Mean measurements of recently released *M. auricularia* juveniles are: length= 190 µm, width= 210 µm and height= 193 µm (Araujo, Cámara & Ramos, 2002). During six weeks of culturing, the (mean) dimensions of juveniles in our experiment increased by 51% in length, 60% in height and 23% in width, suggesting that at least some juveniles grow successfully during culturing.

Although we did not attempt similar experiments in containers without sediment, it seems that sediment is a very important factor for survival (Gatenby, Neves & Parker, 1996; O'Beirn, Neves & Steg, 1998). Survival is probably related to pedal-feeding behavior and juvenile stability. Our study suggests that some nourishment (probably detritus) was present in the culture containers, which increased suitability of the sediment. In our experiments, juveniles survived for 6 weeks, and pedal movements of the juveniles were observed, indicating that surviving juveniles were feeding. Due to their small size and fragility, it is improbable that recently released *M. auricularia* juveniles could survive without exogenous food as has been suggested for species of *Lampsilis* (Gatenby, Neves & Parker, 1996).

Oxygen may also play an important role in successful culturing. Our results showed that the system of circulating water over the cultured containers can be effectively replaced by simply increasing the oxygen levels in the tanks, thereby improving the oxygen flow through the containers.

Similar experiments using a nutrient-rich suspension generated directly from the river have been successful with the European margaritiferid *M. margaritifera* (Hruska,

1999). However, juveniles in these experiments were maintained for 5 years in semi-natural conditions in the river where the mussels previously survived.

Results appear promising for unionoid restocking using juveniles cultured in facilities that are connected to the species' natural habitat, or in facilities that duplicate the water conditions and food sources of these natural habitats. Nevertheless, further research on the relative importance of detritus, algae and bacteria in the freshwater mussel diet (adults and juveniles) is needed.

ACKNOWLEDGEMENTS

We wish to thank the following projects for their support: Demografía, hábitat y ciclo vital de *Margaritifera auricularia* (MIMAM-CSIC) and the Fauna Ibérica Project (DGES PB95-0235). We are also indebted to Prof. J. M. Rodríguez Alvariño for allowing us to conduct our experiment at the E.T.S.I. Agrónomos aquaculture facilities, and to F. Berdejo for his assistance during the experiment. Thanks also to the fish hatchery at Riofrío (Granada, Spain) and the Centro de Experimentación Piscícola El Palmar (Valencia, Spain) for the loan of specimens of sturgeon and blenny, respectively. Dr. Dick Neves and one anonymous referee kindly reviewed and improved the manuscript, and James Watkins corrected the English version.

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