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Effect of transplantation on the gonad development of the freshwater pearl mussel, *Margaritifera margaritifera* (L.)

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In *Margaritifera margaritifera* (L.) individuals collected from the Ähtävänjoki river in July 1988, the histological structure of the pre-spawn ovaries and testes was neat, and a normal reproductive capacity could be predicted. The sex ratio was even, and no true hermaphrodites were present. The commonly occurring "microhermaphrodite" gonads with small nests of the germ cells of the opposite sex may show that the mussels are capable of hermaphroditism or sex reversal. The experimental transplantation of freshwater pearl mussels for conservation purposes 10 km upstream in the same river in 1987 did not disturb the mussels' gonad development. This was assessed from examination of the histological structure of the gonads in July, a year after the transfer. The sizes of testis follicles, the numbers of sperm morulae and oocytes per follicle, the occurrence of microhermaphrodite or out-of-phase follicles were similar both in endemic and transplanted mussels. From these results it could be concluded that transplantation of freshwater pearl mussels can be undertaken in a home river without reducing the gamete producing capacity of the mussels during the breeding season of the next year.

Introduction

During the last few decades, anthropogenic activities other than pearl fishing, have been found to destroy freshwater pearl mussel populations. In Finland, such activities have been e.g. cleaning and deepening processes of the pearl-mussel rivers, water level regulations, discharge of domestic and industrial waste waters, and run-off of fertilizers from field drainage (Brander 1957, Valovirta 1987, 1990a). The freshwater pearl mussel has disappeared or decreased in number in many rivers and brooks in Finland (Valovirta 1977, 1987, 1995ab).

The Ähtävänjoki river in western Finland still harbours a population of 50 000 specimens of freshwater pearl mussels (Valovirta 1987). Dur-

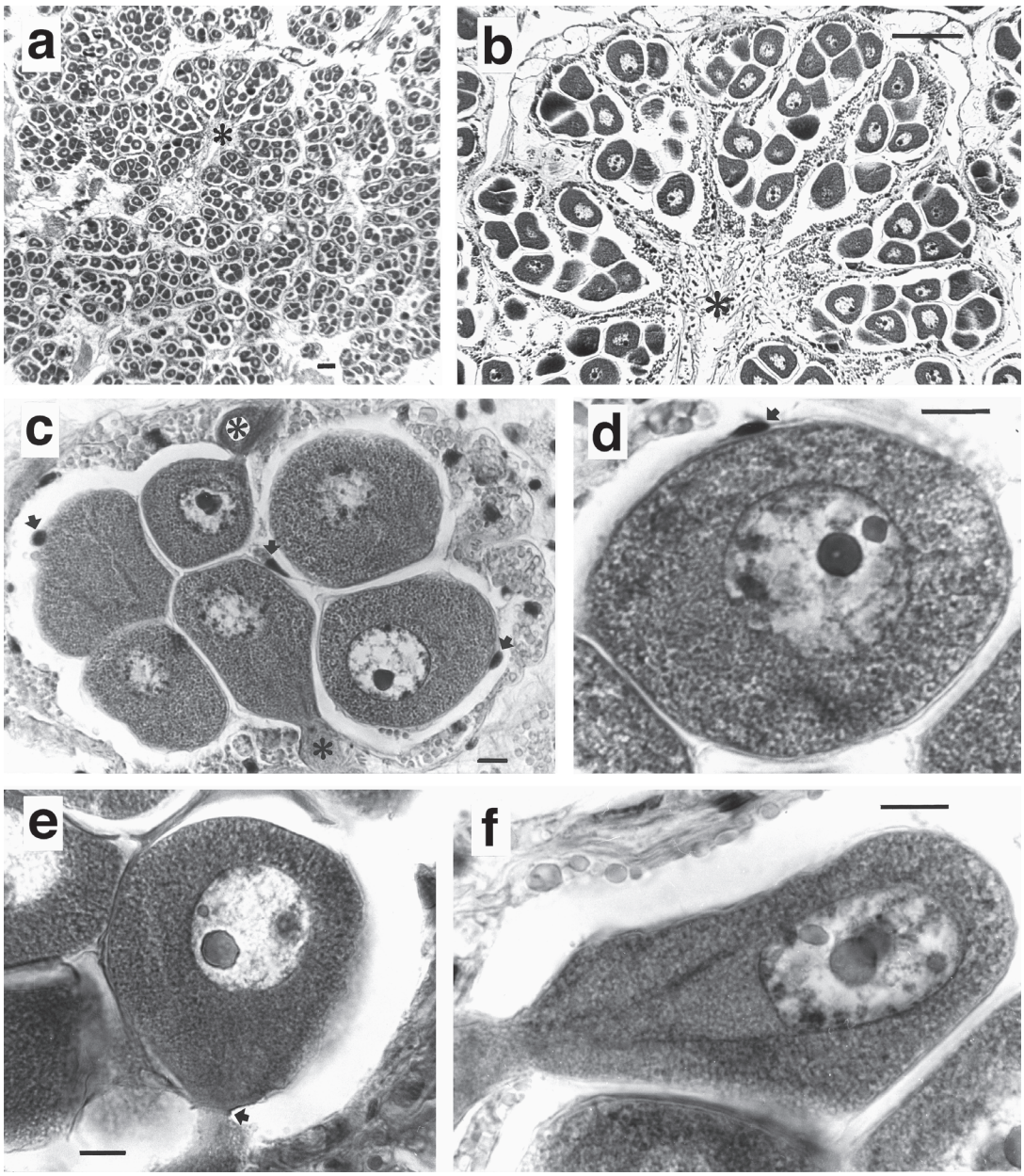


Fig. 1. Histology and stage of development of ovaries of transplanted (a and b) and control (c–f) *M. margaritifera* in July. — a: Ovarium in a general view. — b: A follicle system at a distal genital duct from Fig. 1a (asterisk). — c: A follicle with granular follicle cells and oocytes. The stalks and feet of two oocytes are visible (asterisks). Three follicle cell nuclei around the oocytes are marked with arrowheads. — d: An oocyte with a very thin follicle cell with a flattened nucleus (arrowhead). — e: A collar around the stalk at the future micropyle is visible (arrowhead). — f: Basophilic filaments lead from the nuclear area to the stalk of the oocyte. Stainings: Mowry's alcian blue + PAS + Mayer's haematoxylin (ABPH) in e, and Mayer's haematoxylin (H) and eosin (E) in a–d, f. Scale bars 100 μm (a and b), 10 μm (c–f).

ing many SCUBA dives it has been found that the mussel population has not, however, recruited juveniles during the past two decades (Valovirta 1990b). Moreover, there were plans to dig a wa-

ter channel for an electric power station in the river. In 1987, in order to protect the threatened mussels, 4 500 freshwater pearl mussels were transferred 10 km upstream in the river (Valovirta 1987, Valovirta 1990b).

One aim of this research was to study whether the freshwater pearl mussels in the Ähtävänjoki river are capable for producing gametes. The study includes histological descriptions of pre-spawn and spent gonads of endemic freshwater pearl mussels in the river. Another aim was to study whether the gametes of the transplanted mussels developed normally.

Material and methods

Origin of the mussels and treatment before histological sampling

The Ähtävänjoki river drains to the Gulf of Bothnia at the town of Pietarsaari (about 63°40´ N) in Finland. In the summer of 1987, 4 500 pearl mussels were collected by SCUBA from the rapid of Pölsfors and transferred about 10 km upstream in the river and set in the river bottom at Lappfors (site 12 in Valovirta 1987; Valovirta 1990b). In July 1988, 13 transplants were collected from the rapid of Lappfors and 12 control mussels from around the original site. The mussels were transferred in cold water to the laboratory. Before being examined the mussels were kept in charcoal-filtered, running and aerated tap water, at 12°C, for a few days. In order to see their reproductive development in autumn, one gravid female collected from the river in September, 1993, was transferred to the laboratory wrapped in cold moist paper and prepared following the same procedure as that used for the other mussels on the 1st of October (Pekkarinen and Valovirta 1996).

Processing and study of the histological samples

Samples for histological smears and squash preparations were taken with forceps from the gonads. The smears were stained with Giemsa diluted with neutral phosphate buffer (1+9). For histological sections, transverse slices were cut from the anterior part of the body (behind the anterior adductor muscle) and from the posterior part of the body (in front of the posterior adductor muscle). The slices were first prefixed for about two hours in Bouin's solution, and then they were trimmed with a sharp razor blade to a thickness of no more than 5 mm, put in cassettes and reimmersed in the fixation solution for about 24 h. After washing and dehydration the pieces were embedded in paraffin wax and processed according to standard procedures. The sections were stained with Mayer's haematoxylin and eosin (H and E), and some additional sections with Mowry's alcian blue - PAS - Mayer's haematoxylin (ABPH) (Pearse 1968).

The gonad cycle of this species in Ireland described by Ross (1992) was used for comparison. To study the reproductive capacity of females, oocytes were counted in 15 randomly selected follicles per mussel from the posterior section, and then the means were used to test possible significant differences between the control and transferred females (*t*-test). To describe the reproductive fitness of males, sperm morulae were similarly counted in 15 follicles, and the means were compared between the groups (*t*-test). Additionally, the diameters of 15 testis follicles (the largest follicles in 15 randomly selected follicle groups) were measured and the means of the greatest lengths and breadths perpendicular to the lengths were obtained by using a calibrated ocular micrometer. Sperm head sizes ($N = 10$) were measured according to Pekkarinen (1991a) from a smear of a mussel gonad.

Results

Ovarian development

In July, the ovarian follicles were well ordered, situated radially around distal genital ducts (Fig. 1a and b). The epithelium of the ducts bore microvilli and cilia at the free surface, and often yellow-brown granules within the cells. There were alcianophilic mucous gland cells in the epithelium. The follicle walls were covered from the inside by coarsely granular follicle cells (Fig. 1c). The granules of the cells were eosinophilic and PAS-positive. In five females, very thin follicle cells occasionally still surrounded the oocytes (Fig. 1c and d).

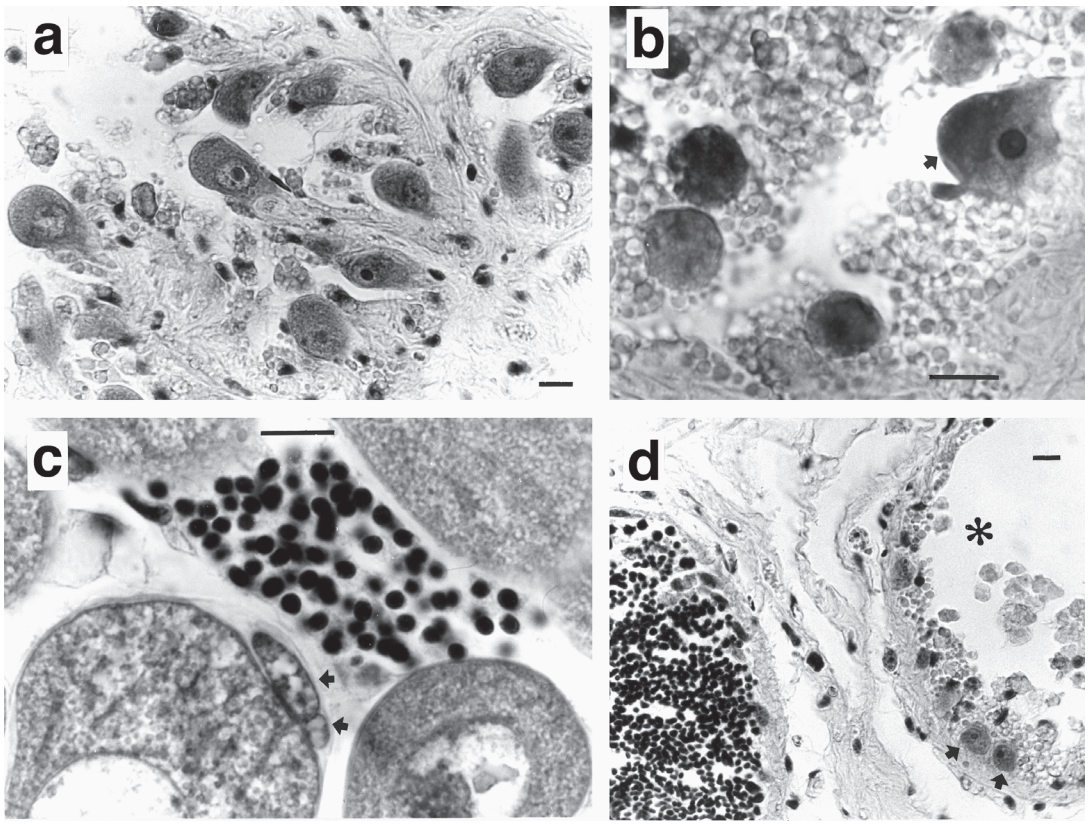


Fig. 2. Developing and "disturbed" gonad follicles of *M. margaritifera*.. — a: Normal developing ovarian follicles in a female in October. — b: An ovarian follicle with granular follicle cells, pyknotic clumps and a small oocyte (arrowhead) from a transplanted female with all its follicles disturbed in July. — c: A nest of spermatogenetic stages in an ovarian follicle of a control mussel in July. Note large polar-body-like structures (arrowhead). — d: An ovarian-like follicle (asterisk) in the testis of a transplanted mussel in July. Stainings: H and E (see Fig. 1). Scale bar: 10 μm.

In July the oocytes mounted in water under a coverslip measured 70–90 μm in diameter. In the ovaries the oocytes were still attached to the follicle walls with thin stalks and "feet" (Fig. 1c–f). The collars of the future micropyles were visible around the stalks (Fig. 1e). Although the vitelline coats or chorions were alcianophilic, the collars were PAS-positive. Thin basophilic filaments ran from the neighbourhood of the nuclear envelope to the stalk of the oocytes (Fig. 1f). There were no medium-size oocytes and small (10–20 μm) oocytes were few or lacking.

On 1 October 1993, in a gravid female all the oocytes were small, 10–20 μm in diameter (Fig. 2a).

Table 1. Comparison of reproductive capacity and stage of ovaria and testes between transplanted and control freshwater pearl mussels in July 1988. *N* = number of mussels (no. of follicles), *P* = probability arising for *t*-test.

	Transplanted (Mean ± *S.E.*)	*N*	Control (Mean ± *S.E.*)	*N*	*P*
No. oocytes/follicle	6.8 ± 0.6	8 (15)	7.7 ± 0.9	6 (15)	0.412
Diam. of testis follicle (μm)	443 ± 33	5 (15)	436 ± 21	5 (15)	0.839
No. of morulae/follicle	11.2 ± 1.9	5 (15)	13.3 ± 2.9	5 (15)	0.550

Histology of the ovaries in control and transplanted mussels in July

The histological structures of the ovaries of five control mussels and seven transplanted mussels were similar. The reproductive capacity (mean number of oocytes/follicle section) of the transplanted mussels did not differ significantly from that of the control mussels (Table 1, $P = 0.412$ *n.s.*).

In the ovaries of both groups some "disturbed" follicles could be seen. In such follicles, only granular follicle cells, very small oocytes and pyknotic clumps were found. In one, transplanted, individual, all the follicles were "disturbed" (Fig. 2b). This female had an exceptional orange colour. Moreover, three of the females in the control group and one in the transplanted group had clusters of spermatozoa developing in a few ovarian follicles (Fig. 2c). In five individuals in both mussel groups large polar-body-like structures were in rare cases observed on oocyte apices (Fig. 2c). They were not always obviously in the neighbourhood of the spermatogenetic stages as they are in Fig. 2c.

Testicular development in July

The testicular follicles were neat and regularly arranged (Fig. 3a and b). Yellow-brown granules were a common feature of the follicles. Minute yellow-brown granules were also often seen in the epithelia of the genital ducts (Fig. 3c). The epithelia of the genital ducts also bore alcianophilic mucous gland cells and, occasionally, also PAS-positive cells.

The follicles contained a quantity of sperm, which in many males was flooding into the genital ducts. In squashes, the spermatozoa stuck together, and their tails moved only slightly. The length of the sperm head (Fig. 3d) was 3.58 ± 0.05 µm and the breadth 1.88 ± 0.04 µm. Small numbers of sperm morulae and spermatogenetic cysts were still present in the follicles (Fig. 3e).

Histology of the testes in control and transplanted mussels in July

The histological structures of the testes of the seven control mussels and five transplanted mussels were nearly similar. The only difference was the occurrence of spermatozoa groups, similar in size to the largest sperm morulae, in the control mussels. Such groups, containing numerous spermatozoa, were from none to a few or many in number per follicle section in six control mussels and very rare in one control mussel.

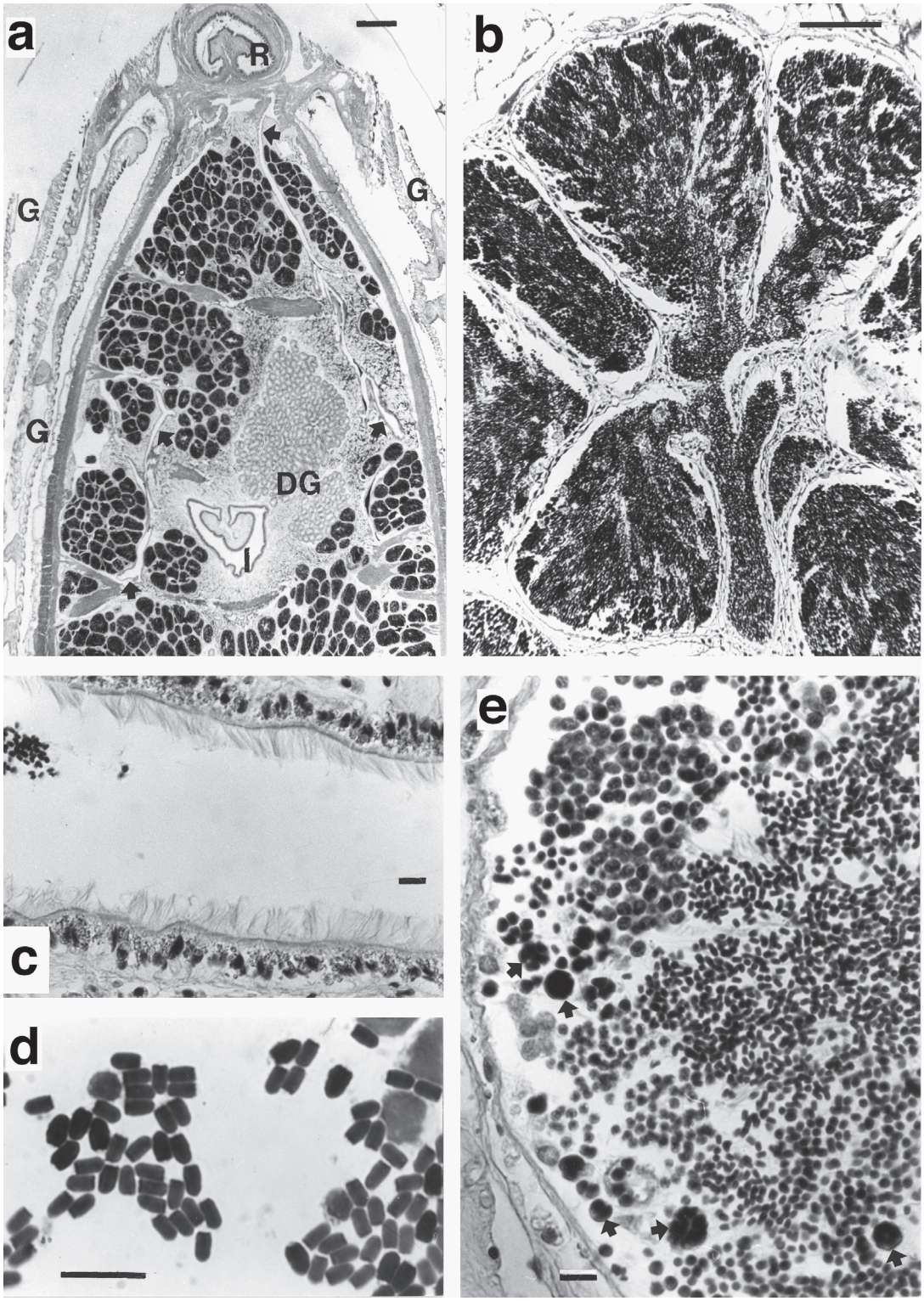
Among the transplanted mussels, in one out of five there were very rare sperm groups in the testis. Although sperm morulae, a sign of atypical spermatogenesis, were more frequent in the control males, the difference between the two mussel groups was not significant (Table 1). The follicles were of similar size in both mussel groups (Table 1). Two males in the control group and one in the transferred group had apparently smaller follicles than the others. Occasional disturbed follicles were found in five out of seven control males and in three out of five transferred males. Such follicles suggested immature or degenerating ovarian follicles with granular follicle cells (Fig. 2d).

Discussion

Histology of the gonads and reproductive stages of the mussels in July and October

The footed stalks and microtubules of the oocytes of the Finnish *M. margaritifera* were similar to those described in an American *Anodonta* species (Beams and Sekhon 1966). A micropyle remains on the oocyte's chorion at the site of detachment as has been found in *Anodonta* and *Unio* species (Schierholz 1889, Lillie 1895, Beams and Sekhon 1966). In the oocytes of *Unio elongatus*, a crater, which may be for sperm entrance, forms on the pole opposite to that which is attached to the follicle wall (Focarelli *et al.* 1990).

The follicle cells of *M. margaritifera* ovaries contained eosinophilic and PAS-positive granules. The eosinophilia may indicate the presence of basic proteins and the PAS-positivity may mean that there are neutral glycoproteins. The follicle cell granules of an *Anodonta* species were also eosinophilic (Weisensee 1916), and the granules have been thought to provide nutrition for the oocytes (Weisensee 1916, Beams and Sekhon 1966). The genital canals of *M. margaritifera* had ciliated cells and mucous cells. The mucous cells were mostly basophilic (alcianophilic) as they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were in *Anodonta* (stained with haematoxylin; Weisensee 1916).

In July, the intraovarian oocytes of the freshwater pearl mussels, measured in a fresh condition in a water mount, were about 70–90 µm. According to Smith (1976), during release the oocytes of *M. margaritifera* are about 100 µm in diameter. In some females from the Ähtävänjoki river, oocytes surrounded by thin follicle cells were still visible, indicating immaturity (Pipe 1987). Because the oocyte apices were free even in the ovary studied in October, the flat cells may be abnormal vestiges of follicle cells. Because all the oocytes were nearly of similar size, the ovaries thus are assumed to be nearly mature. Ross (1992) described a five-stage gonad cycle of *M. margaritifera* in Ireland. At stage 5 the gonads were fully mature, but Ross (1992) did not present a very detailed description of the appearance of the gonad follicles.

The testis follicles in Finnish *M. margaritifera* in July were smaller than those measured by Ross (1992) even in stage 1 testes in Irish freshwater pearl mussels. However, the number of spermatozoa and the degree of flooding of sperm in the Finnish mussels suggested near-mature testes. Occasional sperm morulae and spermatozoa groups were visible but no sperm balls or spermatozeugmata were recorded (cf. Pekkarinen 1991b, Lynn 1994). The occurrence of sperm morulae in certain bivalves is evidence of atypical spermatogenesis (Mackie 1984, Kotrla 1989). Their appearance is seasonal and their function is unknown; they are least abundant during the period of normal spermatogenesis (Heard 1975). The sperm heads of *M. margaritifera* measured in this study were of similar length but slightly thicker than those of *Unio tumidus* (Pekkarinen 1991a).

In October 1993, in a gravid female from the Ähtävänjoki river, a new batch of oocytes was developing. Large oocytes were lacking. The stage may be approximately equivalent to stage 1 as determined by Ross (1992). In 1978, a specimen of *M. margaritifera* from the Ruonanjoki river, in southern Finland was already gravid in July, brooding early embryonic stages (Valovirta and Pekkarinen, unpublished observation). In the present study, a female mussel bore mature glochidia at the beginning of October. The same mussel had early embryos in its marsupia three weeks earlier (Pekkarinen and Valovirta 1996). Thus, the glochidia have needed at least three weeks for their development. The lengths of individual gravid periods of *M. margaritifera* have been reported to range from 16 to 49 days (Harms 1907, 1909, Smith 1976, Bauer 1987, Ross 1992).

The stages of gonadal and embryonal development in Finnish *M. margaritifera* are well in line with the timing of the gamete release and gravidity of this species described in the literature to occur in late summer or early autumn (Wellman 1938, Hendelberg 1959, Björk 1962, Smith 1976, Young and Williams 1984, Bauer 1987, Nagel 1991, Hruška 1992, Ross 1992, Nezlin *et al.* 1994). Interannual differences in the timing of fertilisation and glochidia release occur (Björk 1962, Ross 1992), as they occur in the reproduction of unionid mussels in Finland (Pekkarinen 1993). Young and Williams (1984) reported both interannual and geographical differences in the glochidial release of *M. margaritifera* in Scotland.

Sex ratio and hermaphroditism

The ratio of males and females in the population in the Ähtävänjoki river was even. According to Itkonen (1963), among freshwater pearl mussels in some rivers in Finland there were only 5% males. The occasional nests of gametocytes or follicles of the opposite sex found in the gonads in the present study may be signs of the capacity

Fig. 3. Histology and stage of development of testes in transplanted (a and c) and control (b, d and e) *M. margaritifera* in July. — a: Transverse section from the body of a mussel showing testis follicles (dark) and proximal and distal genital ducts (arrowheads), digestive gland (DG), intestine (I), rectum (R) and gills (G). — b: A testis follicle system around a distal genital duct (sperm is flooding to the duct). — c: Longitudinal section through a genital duct. The epithelial cells contain small granules and bear cilia. — d: Giemsa-stained sperm heads in a gonad smear. — e: Spermatozoa and spermatogenetic cysts in a follicle. A few sperm morulae (arrowhead) are also visible. Stainings: H and E (except for d). Bars: 1 mm (a), 100 µm (b), 10 µm (c–e).

of this species for hermaphroditism or sex reversal. Such "microhermaphrodite" individuals were also found, in addition to true hermaphrodites and normal males and females, among *Unio tumidus* in the Vantaanjoki river in southern Finland (Pekkarinen 1993). According to Bauer (1987), females of *M. margaritifera* in some rivers in Germany became hermaphrodites. When he transferred freshwater pearl mussels upstream, many females, according to gonad punctures, became hermaphrodites and on the other hand some hermaphrodites became females. Only three males changed their sex. Generally, the prevalence of hermaphrodites among *M. margaritifera* has been very low (eg. according to Wellman 1938 1/80, Hendelberg 1961 0/20, Ross 1992 0.9%). Occasional functional hermaphroditism among bivalves is fairly common (van der Schalie 1966, Heard 1975). In contrast, *Margaritifera falcata* (Gould) according to Heard (1970) is normally monoecious.

Comparison of gonad development in control and transplanted mussels

The reproductive capacity (no. of oocytes/follicle section) of normal females in both groups was similar. In males, sperm morulae on average were more frequent in the control group, but the difference was not significant between the mussel groups. The follicles in both groups were of similar size. Oogenesis and spermatogenesis in the transplanted mussels thus do not seem to be disturbed.

Because on the whole, the gonads in both groups looked normal, smaller disturbances were carefully looked for in the gonads. Microhermaphroditic gonads with occasional follicles containing gametes of the opposite sex and out of phase (degenerating) ovarian follicles were found. The total number of individuals with such occasional "disturbed" follicles was 9 among 12 control mussels and 6 among 13 transplanted mussels (Fisher's Exact Test $P = 0.526$, *n.s.*). These disturbances were so common that they can be considered normal. In the transplanted mussel group there was, however, one individual with all its ovarian follicles "disturbed". One abnormal individual in such a small sample is not a reliable argument against the success of the transplantation. The female may simply be out of phase or "resting". It is natural that some females take a rest from reproduction some years (Young and Williams 1984, Bauer 1987, Ross 1992).

Perspectives

This study showed that the first reproductive phase, the development of the gametes of *M. margaritifera* in the Ähtävänjoki river seems to proceed normally. In the future, the next phases, the fertilisation of eggs, the embryonic development, free and parasitic phases of glochidia, and survival of juvenile mussels should be studied.

When a relatively large part of a river is spoiled, transplantation upstream in the same river system could then save at least some mussels (Young and Williams 1983). The transplantation of freshwater pearl mussels in the Ähtävänjoki river did not affect the reproductive capacity of the transplants, assessed by the development of the mussels' gametes a year after the transfer. However, nothing is known about the mussels' reproductive capacity in the late summer and autumn immediately after the transfer.

The requirements for the quality of the environment, contributing to successful development of the later reproductive stages of the mussel and flourishing of the host fish should be studied. These aspects have already been tentatively considered by Bauer (1988). A viable environment should be offered to original mussel populations and host fish, and when this is not possible, the mussels should be transplanted to other suitable sites in the same river system.

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