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**Year:** 2024

**Version:** Published version

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**Please cite the original version:**

Abdelsaleheen, O., Taskinen, J., & Kortet, R. (2024). Reproductive cycle, fecundity and growth of the freshwater mussel *Unio tumidus* (Bivalvia: Unionidae) from Lake Viinijärvi, Finland. *Journal of Molluscan Studies*, 90(3), Article eyae024. <https://doi.org/10.1093/mollus/eyae024>



## Reproductive cycle, fecundity and growth of the freshwater mussel *Unio tumidus* (Bivalvia: Unionidae) from Lake Viinijärvi, Finland

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(Received 23 March 2023; editorial decision 20 May 2024)

### ABSTRACT

Freshwater mussels have a substantial role in aquatic ecosystem function and provide valuable ecosystem services, including water filtration, nutrient cycling, habitat creation and sediment stabilization. While they face population declines globally that can negatively affect an ecosystem's health, such as reducing the water quality and increasing the turbidity, lack of information about the basic reproductive biology for most species makes their conservation much more difficult. Here, we monitored a population of *Unio tumidus* for a year to study its reproductive biology, gonad-dwelling parasites and growth rate in Lake Viinijärvi, Finland. Our data revealed that this species was dioecious with no evidence of hermaphroditism and was non-significantly female-biased (a male to female ratio of 1:1.3). Both sexes of *U. tumidus* had continuous gametogenesis during the study period with two peaks in the gonadosomatic index (GSI%) during November and May, followed by two clear declines in December and June. Gravidity period (brooding) of embryos or glochidia in the female's outer gills lasted from June to July. Therefore, this population of *U. tumidus* can be classified as a short-term breeder (tachytictic). The clear decline of GSI% in December might be a strategy that individuals of *U. tumidus* use to resorb resources back from the gonad for somatic maintenance during winter. Mean length of glochidia was  $391 \pm 0.049 \mu\text{m}$ , being among the largest known glochidia for Unionidae. No gonad-dwelling trematode parasites were detected and the population exhibited high fecundity (numbers of oocytes per female), with an average oocyte production of  $242,000 \pm 18,000$ . The studied population had a moderate growth rate with a maximum predicted age of 13 years and asymptotic length (the length at which growth slows down while the mussel continues to age,  $L_{\infty}$ ) of 114 mm. Our study provides the first quantitative data and histological analysis of the reproductive biology of *U. tumidus*, serving as a basis for future research and conservation.

### INTRODUCTION

Freshwater mussels constitute one of the largest groups of Mollusca and they are commonly found at high densities in both lotic and lentic waters (Negus, 1966; Strayer, 2008). They are sessile, benthic, filter-feeding organisms that have a considerable impact on aquatic ecosystems by connecting several trophic levels and affecting the macroinvertebrate diversity (Vaughn & Spooner, 2006; Strayer, 2017; Vaughn, 2018). Generally, mussels are key organisms that support benthic productivity, as they can transfer nutrients and energy from the water to the sediments through their filter-feeding activities. These activities reduce the density of suspended particles in the water and thus contribute to enhanced water quality. Mussels are commonly used as bioindicators in rivers and lakes due to their tendency to bioaccumulate contaminants from polluted wa-

ters (Negus, 1966; Naimo, 1995; Vaughn & Spooner, 2006; Vaughn, 2018).

Members of the Unionidae have unusual life histories, often characterized by generally long lifespans and high fecundities, and by the typical utilization of fish hosts for the early part of their life cycles, as their larvae are obligatory parasites on gills and/or fins of the fish (McMahon & Bogan, 2001; Haag & Staton, 2003). The females release their oocytes into the gills (brooding chambers or marsupia), where they are fertilized by the males' sperm filtered from the water. In the marsupium, embryos are incubated until they become mature glochidia that are then discharged through the exhalant siphons of the females (McMahon & Bogan, 2001; Haag & Staton, 2003). The duration that glochidia are kept in the marsupium has been used as a criterion to divide freshwater mussels

into two behavioural groups, short-term brooders and long-term brooders, which may differ among populations depending on geographic and environmental conditions (Haggerty & Garner, 2000). In addition, based on the timing of spawning and the release or retention of glochidia in relation to seasonal temperature changes, there are four distinct life history categories observed in unionoid populations (Dillon, 2000). (1) Summer tachytictic: these populations spawn during summer when temperatures rise and release glochidia before winter. (2) Winter tachytictic: these populations spawn during winter when temperatures fall and release glochidia in autumn or winter. (3) Summer bradytictic: these populations spawn during summer when temperatures rise and retain glochidia throughout winter. (4) Winter bradytictic: these populations spawn during winter when temperatures fall and retain glochidia throughout winter.

A released glochidium can live for only a few days before finding a suitable host fish, and after a period of encapsulation in the host's epithelium, metamorphoses into a juvenile, exits the host, falls to the substrate and begins its growth into an adult (McMahon & Bogan, 2001; Strayer, 2008). Unionid mussels have developed a large variety of strategies to attract and infect host fishes with glochidia and these different attraction strategies may indicate different dispersal abilities (Jokela & Palokangas, 1993; Schwalb, Morris & Cottenie, 2015; Soliman *et al.*, 2019). For instance, the thick-shelled river mussel, *Unio crassus*, which is an endangered species, uses a specialized behaviour called "larval spurling" to increase its chances of finding a suitable host fish for its parasitic larvae (Aldridge *et al.*, 2023). This behaviour has important implications for the conservation of *U. crassus* and highlights the need for conservation efforts that consider the impact of environmental stressors on animal behaviour.

Freshwater mussels are among the most imperiled organisms on the earth, as about 40% of the species were classified threatened in the 2015 IUCN Red List of Threatened Species (Lopes-Lima *et al.*, 2017). Unionidae form a major group of freshwater bivalves representing about two-thirds of all freshwater mussels (Lopes-Lima *et al.*, 2017). In Europe, much research attention has been given to members of the family Margaritiferidae, while members of the family Unionidae have received less attention (Graf & Cummings, 2007; Strayer, 2008). Many freshwater mussel populations have rapidly declined because of different factors such as pollution, water quality degradation, habitat destruction or alteration and invasive species, while the general lack of information on life history and the reproductive biology of most native species has hampered their conservation and management plans (Strayer *et al.*, 2004; Downing, Van Meter & Woolnough, 2010; Sousa *et al.*, 2022). Providing detailed information about reproductive traits and patterns such as reproductive strategy, timing of the reproductive cycle, fecundity, lifespan and generation length is key to the successful conservation and management of freshwater mussels (Ferreira-Rodríguez *et al.*, 2019). In the current study, we focused on the reproductive biology of the swollen river mussel *Unio tumidus* (Philipsson, 1788). This species shows a wide distribution range covering North Europe, Central Europe and Siberia (Lopes-Lima *et al.*, 2017). Previous studies have revealed that the glochidia of *U. tumidus* are flexible in their choice of host fishes in different locations (Berrie & Boize, 1985; Blazek & Gelnar, 2006; von Proschwitz & Wengström, 2021). This suggests that the size and shape of the glochidia of *U. tumidus* are not dependent on the size of the female mussel (Ćmiel *et al.*, 2021a). Additionally, the size and morphology of glochidia likely reflect the foraging habits and abundance of the hosts. The flexibility of glochidial size and shape may enable optimization towards site-specific host fishes, which could be an adaptive strategy for the mussel species that occupy a wide range of habitats and use a wide range of host fishes (Ćmiel *et al.*, 2021a). For example, the European perch, *Perca fluviatilis*, has been reported as the main host, as has the roach, *Rutilus rutilus*, in Swedish rivers, but the three-spined stickleback, *Gasteros-*

**Table 1.** The number of individuals of *Unio tumidus* collected monthly during the study period.

Month	Female	Male
Nov. 2020	13	7
Dec. 2020	15	8
May 2021	5	2
Jun. 2021	20	20
Jul. 2021	14	12
Aug. 2021	9	8
Sep. 2021	6	10
Oct. 2021	13	7

*teus aculeatus*, has been reported as the main host in the Thames River in the UK (Berrie & Boize, 1985; Blazek & Gelnar, 2006; von Proschwitz & Wengström, 2021).

Although previous studies on *U. tumidus* have focused on behaviour, distribution, age structure, physiology and ecotoxicology (Annie, Ann, & Mats, 2013; Lurman, Walter & Hoppeler, 2014; Gnatsyhyna *et al.*, 2019; Abdelsaleheen, Kortet & Vornanen, 2022), few studies have focused on the details of the reproductive biology (Pekkarinen, 1993; Aldridge, 1999). To fill this information gap, we sampled a wild population of *U. tumidus* in Lake Viinijärvi, from November 2020 to October 2021. Based on these seasonal samples, we studied the reproductive biology and estimated the maximum predicted age for individuals of the population.

## MATERIAL AND METHODS

### Sampling

Random samples of wild populations of *Unio tumidus* were collected monthly by wading and snorkeling from Lake Viinijärvi, Eastern Finland (62°68.1601'N and 29°20.6887'E). This mesotrophic lake has moderate levels of alkalinity, nutrients and productivity (Lavento, 2021; Abdelsaleheen, 2023). A total of 169 individuals were collected from November 2020 to October 2021, except from January 2021 to April 2021 when the lake was covered with ice (Table 1). Temperature and pH data were recorded during the sampling period using a multiparameter instrument (Professional Plus, YLS, USA). Mussels were kept in the lake water and transported to the laboratory at the Department of Environmental and Biological Sciences, University of Eastern Finland (Joensuu), where they were kept in a thermally controlled stainless steel aquarium with continuous mechanical aeration until they were dissected within a day after collection. The aquarium's temperature was adjusted monthly to correspond to the lake water temperatures. Shell length, width and height (mm) were measured in the laboratory to the nearest 1 mm using a digital Vernier caliper. The specimens for each month were dissected and their total body flesh wet weights, gonad wet weights and shell weights were measured to the nearest 0.01 g using a precision digital balance.

### Histological examination of gonads

Histological sections of gonads were prepared as previously described in detail (Abdelsaleheen *et al.*, 2021, 2023). To reveal the male and female's gonadal structure, gonad samples of each sex were excised after dissection, fixed in 6% neutralized formalin (Sigma-Aldrich), dehydrated in an ascending series of ethanol (70%, 80%, 90% and 100%), cleared with xylene and embedded in paraffin wax. The samples were sectioned at 7 µm, mounted on glass slides, deparaffinized, rehydrated with descending ethanol concentrations and stained with haematoxylin and eosin (HE).

Photographs of the histological sections were captured using a Leica Mc 120 HD digital camera (Leica Microsystems CMS GmbH, Wetzlar, Germany). Different stages of oogenesis and spermatogenesis were measured (about 100 per stage) and identified based on the established morphological characteristics and histological features specific to the species under investigation. The identification process involved careful examination of cellular and tissue-level changes, such as the presence of specific cell types, nuclear morphology, chromatin condensation patterns and cellular arrangements within the gonads. These morphological criteria were consistent with the descriptions given by Park & Chung (2004), Çek & Şerefişan (2006), Hliwa *et al.* (2015) and Abdelsaleheen *et al.* (2023).

#### Determination of sex ratio and gonadosomatic index

The sex of each specimen was determined by microscopic examination of the gonad tissue for the presence of sperm or oocytes, and the gills for the presence of embryos or glochidia. Sex ratio was calculated as the proportion of males to females (M:F).

The gonadosomatic index (GSI%) is the most widely used quantitative approach to assess the reproductive activity in mussels. It was calculated monthly according to Çek & Şerefişan (2006) as follows:

$$\text{GSI}\% = \frac{G_W}{T_{WW}} \times 100,$$

where  $G_W$  = gonad wet weight and  $T_{WW}$  = total flesh wet weight.

#### Fecundity, oocyte diameters, glochidia and parasitism

Fecundity (i.e. number of oocytes in a female's gonads) provides a reliable measure of a female's reproductive capacity. Mature females with shell lengths ranging from 46 to 96 mm were studied. Three subsamples (2–10 mg) were taken from different areas of each female's gonad, weighed to the nearest 0.1 mg and submerged in a 20% aqueous glycerol solution. The number of oocytes was counted on a glass slide using a dissecting microscope. The equation below was used to extrapolate fecundity ( $F$ ) from the subsamples (De Croos, Pålsson & Thilakarathna, 2011; Abdelsaleheen, 2019).

$$F = \frac{\sum_{i=1}^n \frac{o_i}{w_i}}{n} \times W,$$

where  $o_i$  = number of oocytes in a subsample,  $w_i$  = weight of the subsample,  $n$  = number of subsamples and  $W$  = total weight of the gonad.

After estimating fecundity, photographs of each slide were captured at a magnification of 400× (Leica Microsystems CMS GmbH, Wetzlar, Germany) and processed with the image analysis programme Image J v. 1.38x (NIH, Bethesda, MD). For each specimen (three subsamples of 2–10 mg each were taken from different areas of each female's gonad per month), all gametogenic stages (at least 100 oogonia/oocyte) in the gonads were measured. This technique provided a summary of the monthly changes in the average diameter of developmental stages (oogonia or oocytes) over the study period. At the same time, a piece of gonadal tissue (about 4–8 mm in diameter) was squashed between two glass slides and viewed with a dissecting microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) to investigate the presence of any digenetic trematodes (Taskinen, Valtonen & Gibson, 1991). We focused only on gonads because the mussel's gonad is the most preferred site of infection (Taskinen, Mäkelä & Valtonen, 1997). Gills of females were examined for presence of glochidia, which were collected if found, and a random subsample of at least 100 glochidia were collected and microscopically photographed to measure the morphometric parameters (shell length, shell height and the length of hinge ligament for glochidia) by using Image J software.

#### Age and growth estimation

The age of each individual of *U. tumidus* was determined by measuring shell length (mm) at each annulus (external growth lines; Aldridge, 1999) with digital Vernier calipers. The von Bertalanffy equation was used to quantify growth (Bauer, 1992; Ziuganov *et al.*, 1994) as follows:

$$L_t = L_\infty [1 - \exp(-k(t - t_0))],$$

where  $L_t$  is the length of the mussel's annual ring at time  $t$  (age),  $L_\infty$  is the asymptotic length of the shell or the theoretical maximum length of the mussel would reach at infinite age,  $k$  is the growth constant and  $t_0$  is the age of the species for the annual ring  $t = 0$ . The coefficients ( $L_\infty$  and  $k$ ) of the von Bertalanffy equation were calculated by creating a linear regression line of a Walford plot using the yearly growth increments of  $L_{t+m}$  against  $L_t$  (Walford, 1946) as follows:

$$L_{t+m} = cL_t + d,$$

where  $L_{t+m}$  is the length of the shell at time  $(t + m)$ ,  $m$  indicates the measuring interval (selected to be each year; i.e.  $m = 1$ ),  $c$  is slope of the line and  $d$  is the  $y$ -intercept of the line. The asymptotic length ( $L_\infty$ ) and the growth constant ( $k$ ) can be calculated from coefficients  $c$  and  $d$  as follows:

$$L_\infty = \frac{d}{(1 - c)}; \quad \text{and } k = -\frac{\ln c}{m}.$$

The largest collected mussel recorded for this species was used to determine the maximum predicted age that might be expected at the studied site using the following equation (Ziuganov *et al.*, 1994):

$$T_m = -\frac{1}{k} \times \ln \left[ 1 - \left( \frac{L_m}{L_\infty} \right) \right],$$

where  $T_m$  is the maximum predicted age and  $L_m$  is the maximum length.

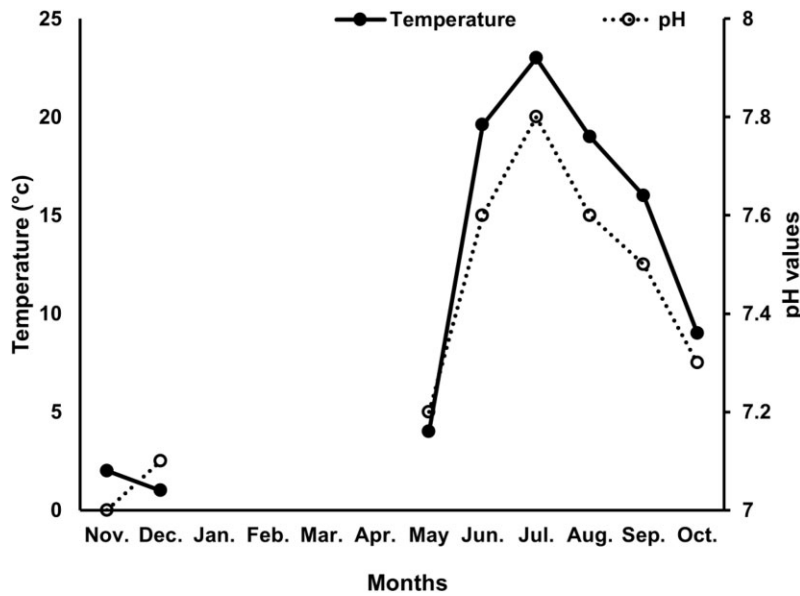
#### Statistical analysis

After checking the normality and homogeneity of data, a two-way ANOVA with least significant difference (equal variances assumed) and Dunnett's T3 (equal variances not assumed) *post hoc* tests were used to check the significance differences in GSI% in both sexes during the study period, while a Student's *t*-test was applied to compare GSI% values between females and males during the study period. A chi-square test ( $\chi^2$ ) was used to assess whether the sex ratio (M:F) obtained was significantly different from the expected ratio (1:1). Pearson's correlation was used to check the correlation between fecundity and age or shell length. The von Bertalanffy equation was calculated using the R language for statistical computing v. 3.6.3 (R Core Team, 2019). The statistical significance differences were tested by the statistical software SPSS (IBM software version 27.0). Data were presented as mean values  $\pm$  SE and the differences between the mean values were considered statistically significant if  $P < 0.05$ .

## RESULTS

#### Environmental variables

Water temperature and pH followed almost the same seasonal trend (Fig. 1). The maximum water temperature and pH were recorded in July (23.0 °C and 7.8, respectively), while the minimum value for water temperature was recorded in December (1 °C) and for pH in November (7.0).



**Figure 1.** Water temperature and pH in Lake Viinjärvi during the sampling period between November 2020 and October 2021, except from January to April 2021.

#### Gametogenesis

The gonads in *Unio tumidus* appeared as diffuse organs surrounding the digestive system. Gametes were grouped in acini surrounded by connective and muscle tissue in the gonads of both sexes. In both male and female gonads, the presence of different stages within the acini was found to differ depending on the phase of gamete maturation (Figs 2A, 3A).

In males, spermatogenesis was divided into five main stages: spermatogonia, spermatocytes, spermatids, spermatozoa and spermatid morulae (Fig. 2A–D). Spermatogonia appeared spherical (3.6–4.8  $\mu\text{m}$  in diameter; Fig. 2B). Each spermatocyte (2.5–3.8  $\mu\text{m}$  in diameter; Fig. 2C) was spherical with a large heterogeneous nucleus that stained intensively with haematoxylin. Spermatids were spherical with highly stained nuclei (2.1–2.7  $\mu\text{m}$  in diameter; Fig. 2D). Early spermatids were clustered to form sperm morulae (sperm morulae: 2.8–8.3  $\mu\text{m}$  in diameter; Fig. 2C). These structures appeared in spring. Spermatozoa were bullet-shaped with a head diameter of about 2.1  $\mu\text{m}$  (Fig. 2D).

We recognized five developmental stages of oogenesis: oogonia, previtellogenic oocytes, vitellogenic oocytes, late vitellogenic oocytes and mature oocytes (Fig. 3A–F). Oogonia were spherical in shape, with each having a spherical nucleus and a noticeable nucleolus. They were found in clusters connected to the inner wall of the acinus (4.0–9.0  $\mu\text{m}$  in diameter; Fig. 3B). Previtellogenic oocytes were characterized by the increased size of the germinal vesicle and prominent eosin staining of the cytoplasm (30.3–34.1  $\mu\text{m}$  in diameter; Fig. 3B). Vitellogenic oocytes were found in the lumina of the acini. Each was distinguished by the presence of a nucleus in its center containing a nucleolus (34.9–39.3  $\mu\text{m}$  in diameter; Fig. 3C). Each late vitellogenic oocyte was characterized by the presence of the nucleolus at the oocyte's margin (42.2–46.7  $\mu\text{m}$  in diameter; Fig. 3D). Each oocyte was attached by an egg stalk by that adhered it to the follicular wall until it transferred to the follicle's lumen as a mature oocyte before spawning (Fig. 3F). Mature oocytes were spherical and were distinguished by a collapsing germinal vesicle (48.8–63.0  $\mu\text{m}$  in diameter; Fig. 3E, F).

#### Sex and sex ratio

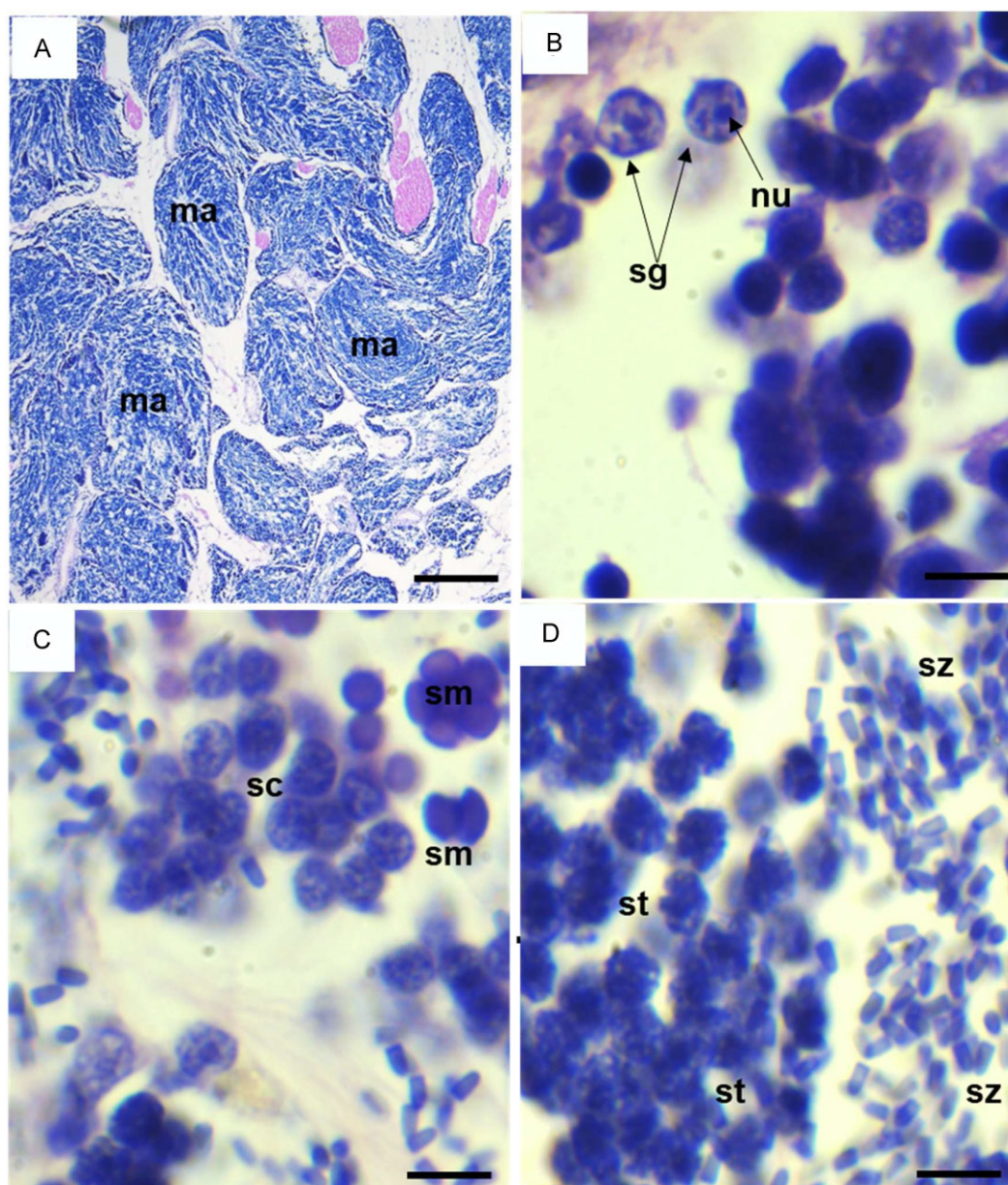
The mean shell length, shell height and shell width for the collected mussels were  $75.3 \pm 0.91$ ,  $38.5 \pm 0.40$  and  $26.9 \pm 0.50$  mm, respectively. Microscopic investigation of gonads revealed that the study population was dioecious with the development of gonadal tissue throughout the studied period. Out of 169 mussels examined, 74 (43.8%) were males and 95 (56.2%) were females, with no observations of hermaphroditism during the study period. The sex ratio was non-significantly biased to females from the expected ratio of 1M:1F, where the ratio of male (M) to female (F) was 1M:1.3F ( $n = 169$ ;  $df = 1$ ;  $\chi^2 = 2.609$ ;  $P > 0.05$ ).

#### GSI% and oocyte diameter frequency

Temporal changes in GSI% from November 2020 to October 2021 were significant in both sexes during the study period ( $n = 169$ ;  $df = 168$ ;  $F = 41.89$ ;  $P < 0.05$ ; Fig. 4). Variation of GSI% had a similar trend in both sexes, but GSI% was significantly higher in male than in female during the studied period except in July ( $n = 169$ ;  $df = 168$ ;  $F = 0.063$ ;  $P < 0.05$ ). GSI% synchronously peaked in November ( $34.1 \pm 0.6\%$  and  $31.1 \pm 0.9\%$  for males and females, respectively), was high in May ( $37.7 \pm 0.6\%$  and  $30.1 \pm 0.7\%$  for males and females, respectively) and dropped drastically in December ( $30.8 \pm 0.85\%$  and  $23.7 \pm 0.75\%$  for males and females, respectively) and in June ( $28 \pm 0.73\%$  and  $25.4 \pm 0.67\%$  for males and females, respectively). The lowest recorded values for GSI% were in July ( $23 \pm 0.91\%$  for males and  $21.3 \pm 0.78\%$  for females), when gonads of both sexes were almost empty.

Size-frequency distributions of the diameters of female gametes changed monthly during the sampling period (Fig. 5). Consistent with the GSI% patterns, the greatest percentages of the largest oocytes ( $>40$ – $42$   $\mu\text{m}$ ) were observed in November and May, with strong declines in December and June. Throughout the study period, the acini exhibited different developmental stages of immature oocytes ( $<18$   $\mu\text{m}$ ), indicating the continuity of the development of gametes in the ovaries.





**Figure 2.** Histological sections of male gonads of *Unio tumidus* show different developmental stages. **A.** Overall view of the testis. **B.** Enlarged portion of the testis showing spermatogonia and the nucleolus. **C.** Spermatocytes and sperm morula. **D.** Spermatids and spermatozoa. Abbreviations: ma, male acinus; nu, nucleolus; sc, spermatocyte; sg, spermatogonia; sm, sperm morula; st, spermatids; sz, spermatozoa. Scale bars: **A** = 400  $\mu$ m; **B–F** = 5  $\mu$ m.

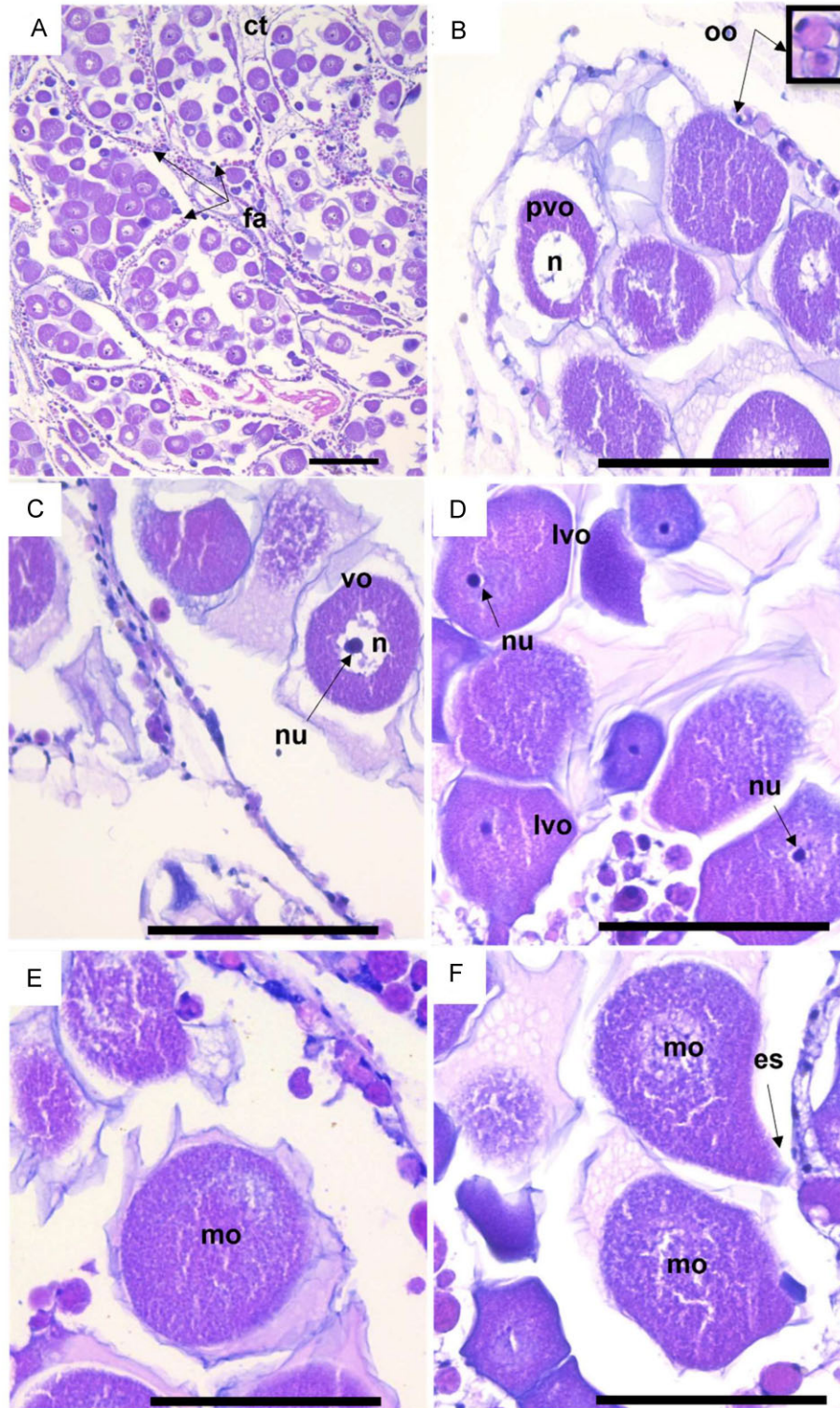
#### *Fecundity and parasites*

Fecundity (estimated numbers of oocytes per female) ranged from 69,200 (produced by the smallest collected specimen) to 424,400 oocytes (produced by the largest collected specimen), with an average oocyte production of  $242,000 \pm 18,000$ . Fecundity was strongly positively correlated to both shell length ( $n = 95$ ;  $R^2 = 0.97$ ;  $P < 0.05$ ; Fig. 6A) and age ( $n = 95$ ;  $R^2 = 0.94$ ;  $P < 0.05$ ; Fig. 6B). The relationship between fecundity and shell length or age was best represented by a power function, in which the rate of rise in fecundity increased over the entire observed range of shell length ( $R^2 = 0.97$ ; Fig. 6) and age ( $R^2 = 0.94$ ; Fig. 6). Microscopic investigations of gonads revealed no presence of trematode parasite infection during the study period.

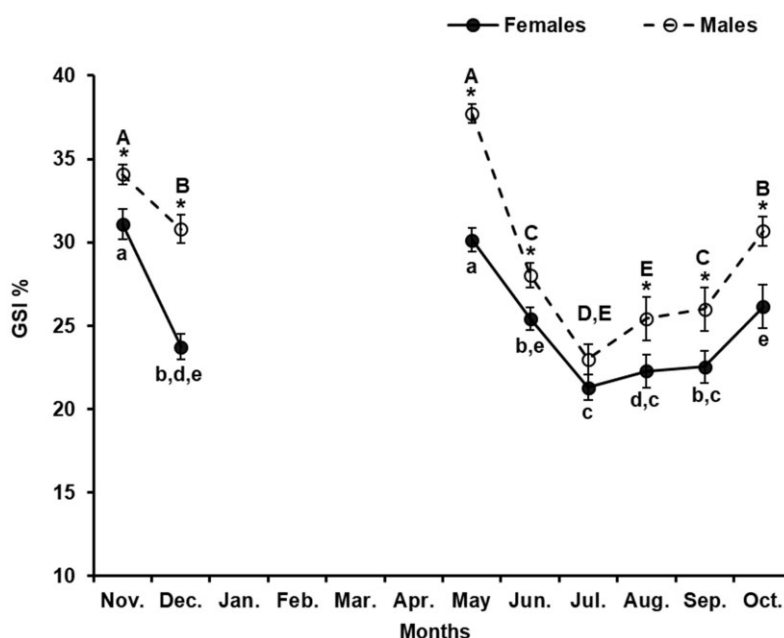
#### *Brooding and glochidia*

Examination of gills revealed that females incubated mature glochidia with D-shaped shells in the outer demibranches (marsupia) (Fig. 7A). In the current study, the mature glochidia were observed only in June and July. D-shaped larvae (glochidia) had two valves connected dorsally by a straight hinge ligament (Fig. 7B–D). The valves had spiny hooks and numerous surface pores. Larval threads “byssal threads” were observed coming out between the two valves (Fig. 7B, C). The mean shell length, height and the length of hinge ligament for the D-shaped larvae (glochidia) were  $0.391 \pm 0.049$ ,  $0.324 \pm 0.013$  and  $0.294 \pm 0.027$  mm, respectively. There were no significant differences ( $P > 0.05$ ) in glochidia measurements among different females, regardless of their shell length, during June and July.





**Figure 3.** Histological sections of female gonads of *Unio tumidus* showing different developmental stages. **A.** Ovarian acini and connective tissue. **B.** Portion of gonads showing oögonia, previtellogenic oocytes and nucleus. **C.** Vitellogenic oocytes, nucleus and nucleolus. **D.** Late vitellogenic oocytes and nucleolus. **E.** Mature oocytes. **F.** Mature oocytes and egg stalk. Abbreviations: ct, connective tissue; es, egg stalk; fa, female acinus; lvo, late vitellogenic oocytes; mo, mature oocytes; n, nucleus; nu, nucleolus; oo, oögonia; pvo, previtellogenic oocytes; vo, vitellogenic oocytes. Scale bars: **A** = 400  $\mu$ m; **B–F** = 70  $\mu$ m; inset in **B** = 40  $\mu$ m.



**Figure 4.** Gonadosomatic indices (GSI%; means  $\pm$  SE) of males and females of *Unio tumidus* during the sampling period between November 2020 and October 2021 except from January to April 2021. An asterisk indicates a statistically significant difference ( $t$ -test:  $n = 169$ ;  $F = 0.063$ ;  $P < 0.05$ ) between the sexes for a given month, while the dissimilar letters indicate a statistically significant difference between the monthly GSI% values for each sex (uppercase letters for males and lowercase letters for females).

#### Predicted growth and longevity

From November 2020 to October 2021, the shell lengths (mm) of 169 living mussels were measured at all the growth interruption lines. For the present species, the estimated parameters of the growth model from the Walford plot ( $Y = 15.96 + 0.86x$ ) and the von Bertalanffy equation are growth constant (the indicator of growth rate that represents the rate at which a species approaches its asymptotic length;  $k$ ) =  $0.15 \text{ year}^{-1}$  and theoretical lengths (the length at which growth slows down while the mussel continues to age;  $L_{\infty}$ ) = 114 mm. The fitted von Bertalanffy growth curve of this population of *U. tumidus* was illustrated as a relationship between shell length and age (Fig. 8). The growth curve revealed that the population of *U. tumidus* in Lake Viinijärvi had a moderate growth constant ( $k = 0.15 \text{ year}^{-1}$ ), in which the shells of the population were approaching their theoretical maximum lengths ( $L_{\infty} = 114$ ) at slightly moderate rates. The maximum predicted age ( $T_m$ ) and length ( $L_m$ ) for the entire population of the studied species were 13 years and 98.4 mm, respectively.

## DISCUSSION

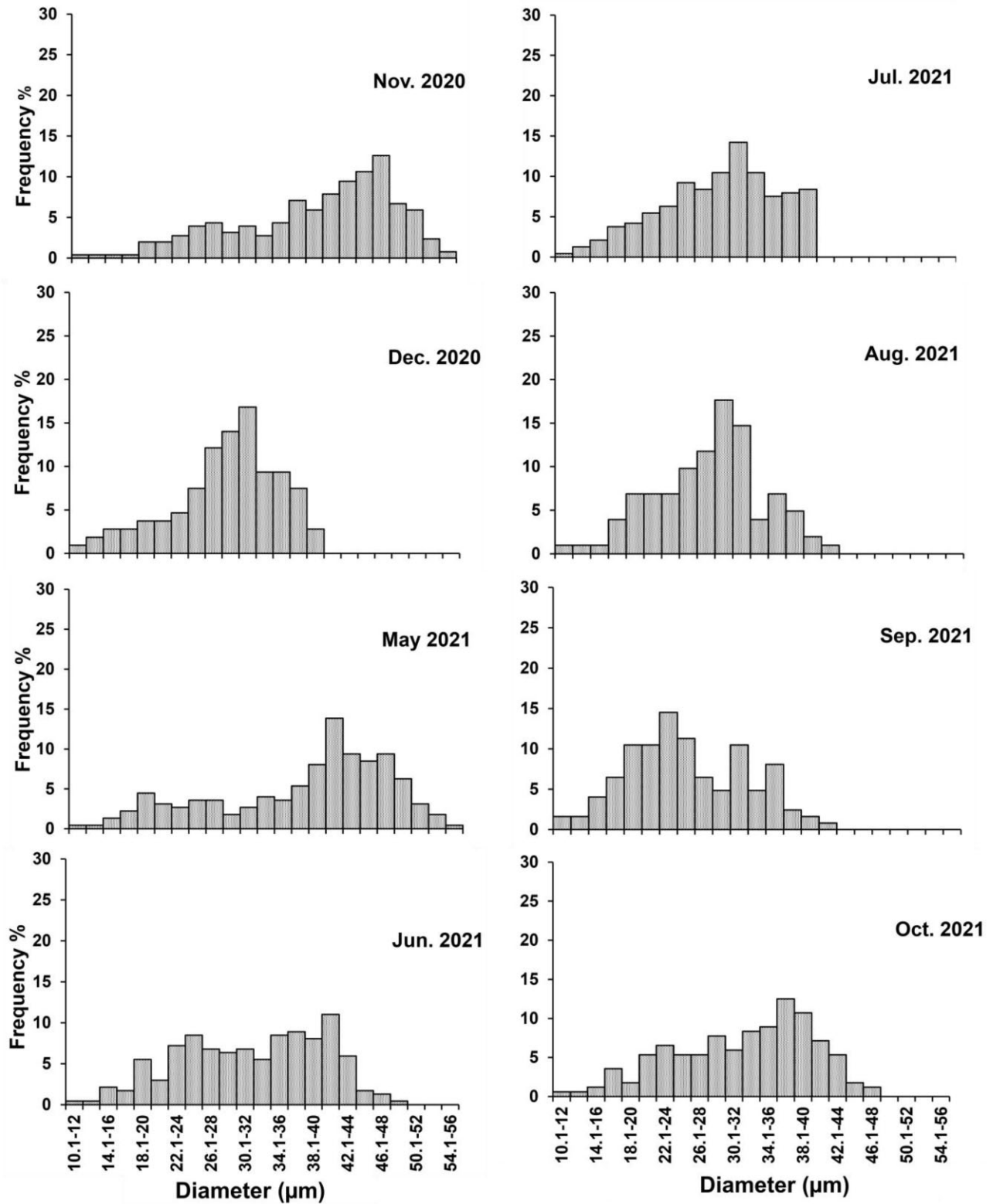
Understanding the details of reproductive biology for a species is needed to determine the life history and population dynamic, which are crucial for successful management and conservation (Ferreira-Rodríguez *et al.*, 2019). To the best of our knowledge, the current study represents the first quantitative analysis and histological study of the reproductive biology of *Unio tumidus*. This information could be useful in establishing conservation actions for those populations of *U. tumidus* that will become threatened due to the changing environment and warming climate.

Histological and microscopical investigations of gonads revealed that individuals of *U. tumidus* in Lake Viinijärvi are strictly dioecious. There was no evidence of hermaphroditism. In contrast, Pekkarinen (1993) reported the presence of hermaphroditism in a

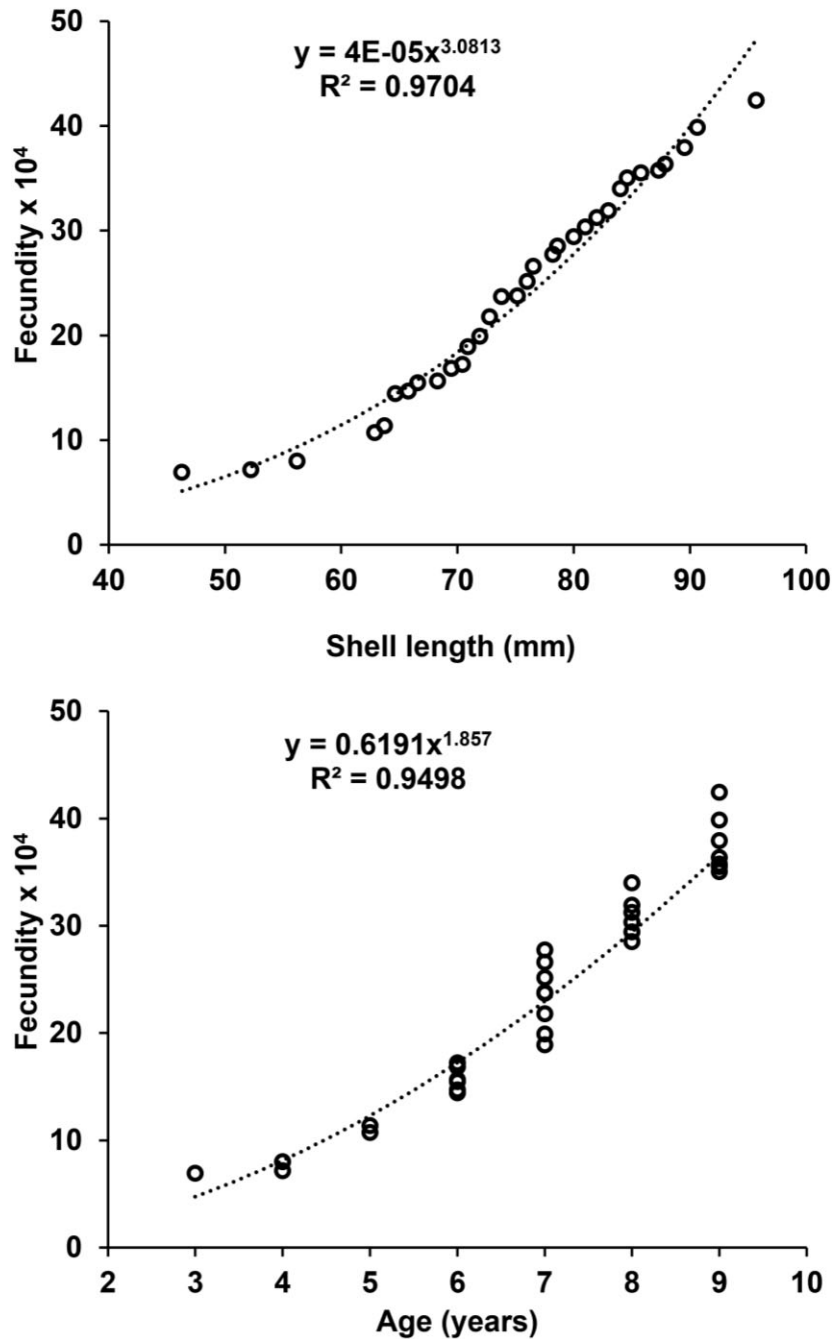
population of *U. tumidus* in the Vantaa River, South Finland. The prevalence of hermaphroditism in this lake was 8% in individuals that were smaller in size, while it was only 0.6% in those that were the largest.

We divided spermatogenesis in *U. tumidus* into four stages, spermatogonia, spermatocyte, spermatids and spermatozoa, which are consistent with those found in other unionids but with slight differences in the spermatozoan head sizes (Çek & Şerefişan, 2006; Hliwa *et al.*, 2015; Soliman *et al.*, 2016; Lopes-Lima *et al.*, 2020). Unionid spermatozoa are generally characterized by a very small size, where the shortest head length was recorded in *Unio terminalis* (2  $\mu\text{m}$ ; Çek & Şerefişan, 2006) and the longest head length in *Sinanodonta woodiana* (4.3  $\mu\text{m}$ ; Hliwa *et al.*, 2015). In *Unio delphinus*, the diameter varies between 2.0 and 2.5  $\mu\text{m}$  (Lopes-Lima *et al.*, 2020). Our data demonstrate that spermatozoan head length of *U. tumidus* is short (2.1  $\mu\text{m}$ ) and considered within the smallest head lengths recorded in unionids, being about the same size as those of *U. terminalis* and *Nitidulana teretiuscula* (Çek & Şerefişan, 2006; Soliman *et al.*, 2016; Abdelsaleheen, 2019). As in other unionids, each spermatozoan of *U. tumidus* has a bullet-shaped head and a uniflagellate tail with a very small acrosome (Rocha & Azevedo, 1990; Hliwa *et al.*, 2015). The acrosome was not detected in the current study as it can only be seen with the transmission electron microscope (Rocha & Azevedo, 1990; Shepardson *et al.*, 2012). Our observation of spermatogenesis in *U. tumidus* revealed the presence of sperm morulae. The role of these structures remains unclear (Hliwa *et al.*, 2015; Soliman *et al.*, 2016). Some authors have suggested that sperm morulae are a collection of spermatids that under certain environmental conditions transform into mature sperm (Çek & Şerefişan, 2006; Şerefişan, Menderes & Soylu, 2009), while others have proposed that sperm morulae may be evidence of an abnormal (atypical) spermatogenic pathway that occurs under certain conditions (Shepardson *et al.*, 2012). The males of *U. tumidus* we observed followed an atypical pathway of spermatogenesis, which is consistent with previous findings in some species such as *N. teretiuscula* (Abdelsaleheen, 2019). On the other hand, previous studies have shown that *S. woodiana* and *Venustaconcha ellipsiformis*





**Figure 5.** Relative distribution of the size-frequency of female gametes (grouped in 2  $\mu\text{m}$  size classes;  $n \geq 100$  female gametes) of *Unio tumidus* during the sampling period between November 2020 and October 2021 except from January to April 2021.



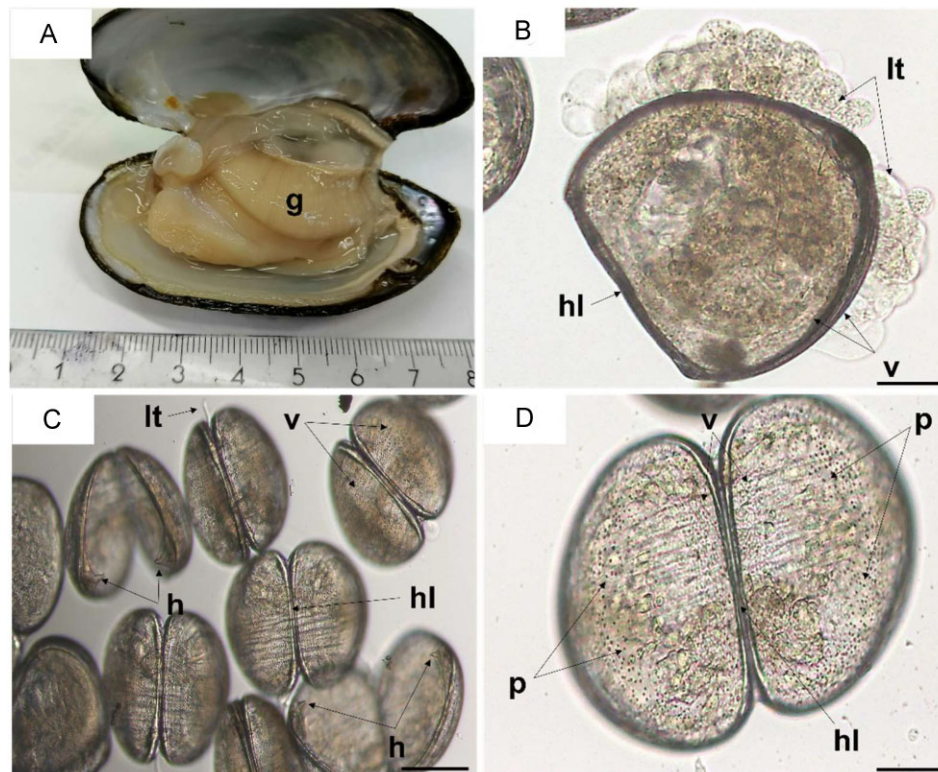
**Figure 6.** Relationships between fecundity (numbers of oocytes produced per female) and shell length (A) and fecundity and age (B) of *Unio tumidus* using a power regression model ( $n = 95$  females) during the sampling period between November 2020 and October 2021 except from January to April 2021.

have two distinct pathways for spermatogenesis (Shepardson *et al.*, 2012; Labecka & Domagala, 2019). In one pathway in *S. woodiana*, testicular follicles develop into primary spermatocytes, which then undergo meiosis to produce secondary spermatocytes and ultimately spermatozoa. This pathway is similar to the typical process of spermatogenesis seen in most animals.

The process of oogenesis of *U. tumidus* was consistent with that of other unionids (Jones, Simpson & Humphrey, 1986; Çek & Şereflışan, 2006; Soliman *et al.*, 2016). The diameters of mature oocytes in *U. tumidus* were 49–63  $\mu\text{m}$ , which is within the average range of unionid species, which varies from 40  $\mu\text{m}$  in *Cucumerunio novaehollandiae* (Jones *et al.*, 1986) to 75–100  $\mu\text{m}$  in *S. woodiana*, *U. ter-*

*etensis*, *Leguminaia whaetleyi* and *N. teretiuscula* (Dudgeon & Morton, 1983; Çek & Şereflışan, 2006, 2011; Soliman *et al.*, 2016).

Our study demonstrated that the population of *U. tumidus* in Lake Viinijärvi is slightly female-biased (1M:1.3F), but this ratio was not statistically significantly different from the expected ratio of 1:1. The population of *U. tumidus* in Wicken, Cambridgeshire, UK, was shown to be slightly female-biased (1M:1.13F) (Aldridge, 1999). Other unionids, including *U. pictorum*, *U. terminalis*, *S. woodiana* and *N. teretiuscula*, have been shown to have female-biased populations (Aldridge, 1999; Çek & Şereflışan, 2006; Hliwa *et al.*, 2015; Soliman *et al.*, 2016; Labecka & Domagala, 2018). The reasons behind these variations in sex ratios are not yet fully understood, but factors such



**Figure 7.** Light microscopic photos of dissected samples and the D-shaped larvae of *Unio tumidus*. **A.** The outer demibranches of gills (brooding chambers) filled with glochidia. **B.** The D-shaped larvae with two valves connected by a hinge ligament and the presence of larval threads. **C.** The D-shaped larva with two valves connected by a hinge ligament and presence of larval threads and hooks. **D.** Outer surface of a D-shaped larva having numerous pores. Abbreviations: g, gills; h, hooks; hl, hinge ligament; lt, larval threads; p, pores; v, valves. Scale bars: **A.** see ruler in image; **B, D** = 100  $\mu$ m; **C** = 200  $\mu$ m.

as genetics, ecological factors and interactions with other species could potentially play a role (Hamilton, 1967; West, Reece & Sheldon, 2002). For instance, Hamilton (1967) reported that genetic factors can influence sex ratios by affecting the linkage between sex-determining genes and genes that impact the species' fitness. Additionally, food availability has been found to trigger sex reversal in some bivalve species, such as *Mytella charruana* in the southeastern USA (Stenyakina *et al.*, 2010). Further research is needed to explore the genetic and environmental factors that underlie the observed variations in sex ratio in the present species and to determine the potential ecological implications of female-biased populations.

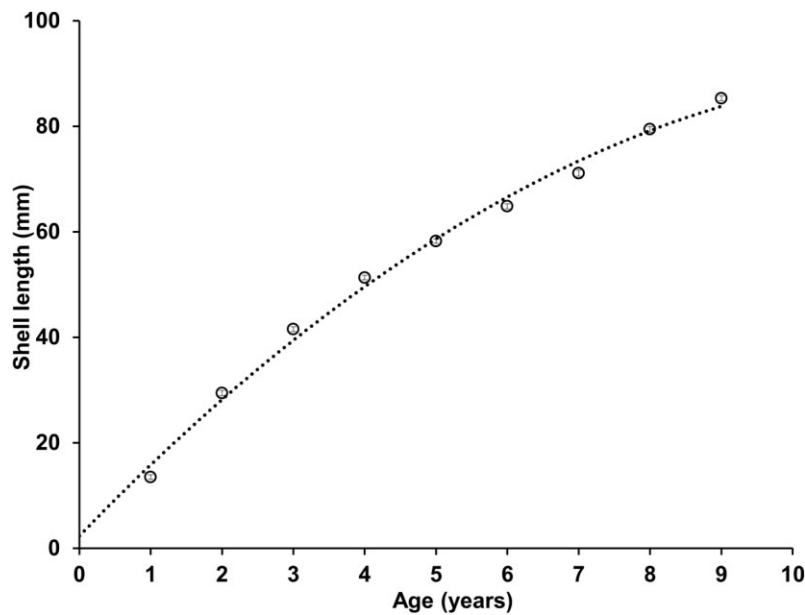
Earlier studies have reported fecundities for various unionids (Haag & Staton, 2003; Moles & Layzer, 2008; Abdelsaleheen, 2019), but to the best of our knowledge, the current study provides the fecundity of *U. tumidus* for the first time. Fecundity values provide basic information for the management of mussel populations and are needed to assess the reproductive and commercial potentials of mussel stocks. The estimated fecundity of *U. tumidus* ranged between 69,200 and 424,400 oocytes per individual of 46.3–95.7 mm shell length. Variations in fecundity with respect to shell length and age are commonly reported in mussels, and fecundity is considered to be a good indicator of a population's reproductive potential (Hochwald, 2001; Haag & Staton, 2003; Haag, 2013). In *U. tumidus* there is generally a strong relationship between fecundity and shell length, and between fecundity and age in a power function form, where large individuals are expected to contribute highly to the reproductive output of the population (i.e. individuals with the maximum size and older age have higher egg production, larger gills and higher capacity for brooding glochidia) (Hochwald, 2001; Haag & Staton, 2003; Haag, 2013).

The gonadal examination for the current species revealed no parasite infections during the study period. Our data are consistent with Taskinen *et al.* (1991) and Pekkarinen (1993), who also reported that *U. tumidus* populations in Lake Saravesi and in River Vantaanjoki, Finland, did not host any infections by gonad-dwelling bucephalid trematodes (*Rhipidocotyle fennica* and/or *R. campanula*), in contrast to co-occurring populations of *Anodonta anatina*.

The spawning season in unionids varies among species and can be influenced by factors such as location, environmental conditions and specific reproductive strategies (Price & Eads, 2011; Ćmiel *et al.*, 2021b). For example, Price & Eads (2011) investigated the brooding patterns and timing of reproduction in three species of *Elliptio* in the Broad River, SC, USA. Their study revealed distinct brooding strategies among the species, with *Elliptio complanata* exhibiting long-term brooding, *Elliptio dilatata* displaying short-term brooding and *Elliptio icterina* engaging in tachytictic brooding. Another study focusing on nine freshwater mussel species from Poyang Lake, China, found variations in reproductive traits among the species, including differences in timing of reproduction and brooding patterns (Ćmiel *et al.*, 2021b). In the present study, the gravidity of *U. tumidus* seems to be short and lasts for 2–3 months with a single spawning season as in some other unionids (Aldridge, 1999). The synchronization of gonadal discharge (spawning) in both sexes along with aggregation of species and the slow current of lake water might increase the success rate of fertilization in *U. tumidus*.

Our data demonstrate that mature oocytes occupy the outer demibranches of gills in females for fertilization and maturation to glochidia similar to *Pseudanodonta complanata* and *Anodonta cygnea* (Aldridge, 1999; McIvor, 1999).





**Figure 8.** The von Bertalanffy growth curve for a population of *Unio tumidus* ( $n = 169$  individuals) in Lake Viinijärvi from November 2020 to October 2021 except from January to April 2021.

Previous studies from Finland and England have reported that *U. tumidus* has a single reproductive season with a short gravid period in which embryos appear in gills between April and May and glochidia appear from late May to July (Pekkarinen, 1993; Aldridge, 1999). The presence of mature glochidia from June to July in the present study is in line with these earlier studies and indicates no major geographic differences in phenology of production of mature glochidia between England and Finland. The current findings on GSI% suggest that the spawning (gonad released mature oocytes) of *U. tumidus* in spring occurs in the period from June to July. It seems that individuals of *U. tumidus* have a strategy to allocate energy between reproduction and body function maintenance over different seasons. This strategy is consistent with the capital and income breeding theory, where animals use different strategies to finance the costs of reproduction (Jönsson, 1997). The theory is relevant to freshwater mussels, where the timing of reproduction and variation in reproductive effort can vary depending on the species and environmental factors. In summer, *U. tumidus* likely benefits from warm water conditions and abundant nutrients to mature gametes in the gonads and glochidia in the gills. Therefore, the females release the matured glochidia, which frees the gills to increase respiration rate. In late autumn and winter, individuals of *U. tumidus* tend to slow down the gamete maturation in gonads during the coldest period. Size-frequency diameters of oocytes supported these findings, where the largest group size of oocytes ( $>42 \mu\text{m}$ ) was observed in gonads in May but was not present in July. Presence of the smaller group size of oocytes ( $<18 \mu\text{m}$ ) throughout the study indicated that oocyte production continued throughout the year. The GSI% decline in December suggests a strategy in which *U. tumidus* resorbs resources back from the gonads to somatic maintenance during winter. Additional studies are needed to confirm whether the decline in GSI% and the largest group size of oocytes during December is because of the degeneration of oocytes during the study period or resource allocation during winter for somatic maintenance or whether it is explained, for example, by release of mature oocytes from gonads to form glochidia in gills. The later explanation is unlikely, as we did not detect embryos or mature glochidia in gills during December.

The shells of the glochidia larvae of *U. tumidus* are D-shaped and are characterized by the presence of hooks, attachment threads and

numerous pores on the outer surfaces of the shells. These findings are consistent with previous studies of unionids such as *N. teretiuscula*, *Anodonta imbecilis*, *Anodonta cataracta*, *Margaritifera margaritifera*, *Utterbackia imbecilis* and *Hyriopsis bialatus* (Rand & Wiles, 1982; Schwartz & Dimock, 2001; Chumnanpuen *et al.*, 2011; Soliman *et al.*, 2016). Some unionoids, including *A. imbecilis*, *Unio gibbus*, *Hyriopsis myersiana*, lack hooks, have semi-oval valves and lack threads (filamentous structures that serve multiple functions during the early stages of mussel development and facilitate attachment of the glochidia to substrates in the aquatic environment) (Rand & Wiles, 1982; Uthaiwan *et al.*, 2001; Araujo, Toledo & Machordom, 2009). Bauer (1994) reported that the length of the glochidia in Unionidae can range between 0.05 and 0.45 mm. The glochidia of *U. tumidus* are among the largest known for Unionidae, and the presence of hooks suggests that the glochidia are well adapted to attach to the tissues of the host fish. This flexibility allows them to optimize their chances of successfully attaching to specific host fish in specific environments (Ćmiel *et al.*, 2021a).

The growth constant  $k$  of the von Bertalanffy equation is often used to describe the growth of bivalves (Negus, 1966; Bauer, 1992; Ziuganov *et al.*, 1994; Aldridge, 1999). Lifespan is negatively related to the growth constant, which explains the large variations in longevity between different species and populations. The growth constant of *U. tumidus* ranged between 0.075 and 0.38 per year, while the maximum predicted age ( $T_m$ ) ranged between 7 and 21 years (Negus, 1966; Aldridge, 1999; Zotin, 2009; Czerniejewski *et al.*, 2021). The individuals of *U. tumidus* in Lake Viinijärvi showed a slightly moderate growth rate ( $k = 0.15$ ) in comparison to the highest recorded growth constant and maximum predicted age for *U. tumidus* (Aldridge, 1999; Zotin, 2009). The contradictory findings for different populations of *U. tumidus* can be explained by several factors including habitat type, ecological factors, food availability, water temperature and geographical latitude (Negus, 1966; Aldridge, 1999; Zotin, 2009). Furthermore, Müller *et al.* (2021) reported that the growth patterns of *A. anatina* vary with sex and mortality in populations, and the growth curves of unionid mussels can differ among lakes and between sexes. The variability of growth patterns within and between populations suggests that other factors such as environmental conditions, availability of suitable hosts and population density can also impact their growth and survival. The

reproductive capacity of unionids is highly influenced by their maximal size and age. Individuals with longer lifespans will reproduce more times, resulting in a higher lifetime fecundity than those with a shorter lifespan (Aldridge, 1999).

## ACKNOWLEDGEMENTS

The authors thank Pyry Pihlasvaara and Lena Herz for their help in sampling mussels during the study.

## CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this study.

## FUNDING

This research has been supported by the Finnish Cultural Foundation, North Karelia Regional Fund and OIVI foundation (to O.A.) and by Nordic Centre of Excellence for Sustainable and Resilient Aquatic Production, SUREAQUA (to R.K.).

## AUTHORS' CONTRIBUTIONS

O.A.: conceptualization, methodology, formal analysis, investigation, resources, visualization, writing of original draft and review and editing. J.T.: conceptualization, review and editing. R.K.: conceptualization, resources, supervision, review and editing.

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