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Reproduction cycle, trematode parasitism and growth of freshwater mussel *Anodonta anatina* (Bivalvia: Unionidae)

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Abstract *Anodonta anatina*, a widely distributed Eurasian freshwater mussel, has dramatically declined and listed as endangered species in IUCN Red List in some parts of the Europe. To better understand its basic biology and possible sex-dependent effects of parasitism on reproduction, we investigated the reproductive traits, trematode parasitism and growth between November 2020 and October 2021. Our data from a Finnish population indicates male-biased sex ratio and the gonadal development lasting throughout the year with a bradytictic brooding pattern. Gonadosomatic index of both sexes peaked in May and

declined in June. Mature oocytes were smaller sized than those of previously studied unionids, fecundity was fairly low, and the maximum predicted lifespan was 14 years. Gonads were infected with digenean trematode parasites, *Rhipidocotyle campanula* being the dominant species. Interestingly, trematode infection rate was higher in females than in males. In July, when the water temperature reached its annual maximum, the trematode prevalence also peaked, which was accompanied with 16% of mussels being sterile. The trematode parasitism was thus negatively associated with reproduction, that might become even more affected if parasitism increases along with the predicted climate warming, which should be considered in possible future conservation and aquaculture efforts of this species.

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Introduction

Freshwater mussels are ecologically important organisms in aquatic ecosystems. For example, they comprise a significant proportion of the total biomass in freshwater benthic communities, play an important role in nutrient cycling, remove suspended detritus, clean the water, mix surficial sediments through bioturbation and serve as a food source for other aquatic organisms (Negus, 1966; Naimo, 1995; Strayer, 2017;

Vaughn, 2018). Freshwater mussels are aquacultured worldwide for a number of purposes, including conservation, food production, pearl production, and aquaculture fish feed production (Sicuro, 2015). Biogeochemical properties of mussels' shells and mussel growth rates can contribute to our understanding of environmental changes on both local and regional scales (Vaughn, 2018). Unfortunately, wild freshwater mussel populations have declined due to human actions, and, for example, species such as *Anodonta anatina* (Linnaeus, 1758) have been listed as endangered species in IUCN Red List in some parts of Europe (Lopes-Lima et al., 2017). Several conservation aquaculture efforts have been initiated to support and rehabilitate some of the threatened freshwater mussel species and populations (e.g., Haag & Williams, 2014; Ferreira-Rodríguez et al., 2019).

Unionid mussels (such as *A. anatina*) have distinctive reproductive characteristics with long lifespans and high fecundity, as well as internal fertilization that occurs in the gill chambers of females which are modified to form marsupia (McMahon & Bogan, 2001; Haag & Stanton, 2003). Unionid females release the oocytes from their gonads into the gill chambers, while males release their sperm into the water column (Bauer & Wächtler, 2001; Soliman et al., 2016). The fertilized eggs are brooded in the female's marsupia, where they develop into larvae that are known as glochidia. The duration of glochidia brooding in the marsupia divide mussels into two behavioral groups: tachytictic (short-term) and bradytictic (long-term) breeders (Kat, 1984; Bauer & Wächtler, 2001). Tachytictic breeders spawn in spring or summer and then release their larvae later in the same year, while bradytictic breeders spawn in summer or early fall and brood their glochidia overwinter in the marsupia until the next spring or summer (Kat, 1984; Hochwald, 2001). The duration of brooding may differ between populations or between years based on geographic and environmental factors (Garner et al., 1999; Haggerty & Garner, 2000). Glochidia are eventually released into the water column, where they must parasitize a certain host fish to complete their metamorphosis. After metamorphosis they develop into juveniles which leave the host to the substratum to grow into adult mussels (McMahon & Bogan, 2001; Strayer, 2008; Soliman et al., 2019).

Unionids are one of the most vulnerable groups of aquatic species under changing climatic and

environmental conditions (Strayer et al., 2004; Lopes-Lima et al., 2017; Sousa et al., 2022). While many unionid bivalves have become endangered, we still face a significant lack of knowledge about their basic biology, in particular the seasonality of their reproductive traits and associated parasitism, which makes their conservation much more difficult. It has been shown that environmental warming increases the larvae production of parasites of freshwater mussels (Choo & Taskinen, 2015) and also lengthens the seasonal period of larvae shedding (Taskinen et al., 2022). Climate warming has been predicted to be most prominent in the high latitudes, as in the present study area (Ruosteenoja & Jylhä, 2021). Mapping the seasonal aspects of reproduction and parasitism in the northern areas of mussel distribution range is of high importance, as the northern regions are thought to be the stronghold of many freshwater unionids (Lopes-Lima et al., 2017). The present study was carried out on a wild northern population of *A. anatina* (Unionidae), which is widely distributed in Euro-Siberian region, and a relatively common species in Finland (Lopes-Lima et al., 2017; Abdelsaleheen et al., 2021). The abundance and distribution of *A. anatina* varies across different water bodies as it is influenced by number of factors including water temperature, pH, distribution of host fishes, food availability and sediment composition (Schwalb et al., 2011; Kappes & Haase, 2012; Zieritz et al., 2014). For instance, in the Southern Finland lake areas, the density of *A. anatina* can be as high as 40 individuals/m² (Englund & Heino, 1994).

The known hosts for *A. anatina* glochidia include several fish species from different families (Jokela et al., 1991; Huber & Geist, 2019). Importantly, *A. anatina* serves as a first intermediate host for the digenetic trematodes *Rhipidocotyle fennica* Gibson et al., 1992 and *Rhipidocotyle campanula* (Dujardin, 1845). *Rhipidocotyle* spp. reproduce asexually in their first intermediate host (*A. anatina*) to produce branching sporocysts inside which develop cercariae that are shed into the water so as to infect the second intermediate host, roach [*Rutilus rutilus* (Linnaeus, 1758)], followed by the definitive host including pike (*Esox lucius* Linnaeus, 1758) and perch (*Perca fluviatilis* Linnaeus, 1758) (Taskinen et al., 1991; Gibson et al., 1992). These parasites can have negative impacts on the number of produced glochidia, growth rate, shell shape, energy storage and survival of the host (Jokela

et al., 1993, 2005; Taskinen & Valtonen, 1995; Taskinen, 1998; Zieritz & Aldridge, 2011). Recently, *A. anatina* has become vulnerable or near threatened in some parts of Europe, including Austria, Ireland and Germany (Lopes-Lima et al., 2017). Noteworthy, mussels such as *A. anatina* tend to reduce their reproductive and growth activities and reallocate energy for maintenance, defense, and repair mechanisms under stressful conditions (Jokela, 1997; Abdelsaleheen et al., 2022).

There is an urgent need to better understand the details of reproductive traits and seasonal patterns in many freshwater mussels, including *A. anatina*. Reproduction related traits have already been defined as one of the main research priorities in freshwater mussel conservation, being critical for successful conservation aquaculture actions (Ferreira-Rodríguez et al., 2019). The primary aim of the present study was to investigate the gonadal structure, gametogenesis, sex ratio, reproduction cycle, brooding patterns, fecundity, growth, lifespan, and possible presence of hermaphroditism for *A. anatina* (Lake Viinijärvi, Finland). Moreover, we tested the hypothesis that there would be sex-dependent effects of parasitism on host reproduction by examining the gonadal parasites (Jokela et al., 1993).

Materials and methods

Sampling and maintenance

A total of 184 wild *A. anatina* were randomly collected from Lake Viinijärvi, Eastern Finland (62° 68.1601' N and 29° 20.6887' E) by wading and snorkeling. The sampling commenced in November 2020 and continued until October 2021, except from January to April 2021 when the entire lake was fully frozen. Environmental parameters of lake water (temperature and pH; Fig. 1) were recorded using a multiparameter instrument (Professional Plus, YSL, USA). Live mussel samples were transported to the animal facilities of the University of Eastern Finland (Joensuu) in insulated plastic containers filled with lake water. In the animal facilities, mussels were maintained in thermally controlled stainless-steel tanks provided with continuous aeration until they were dissected. The water temperature in the tanks was monthly regulated following the lake

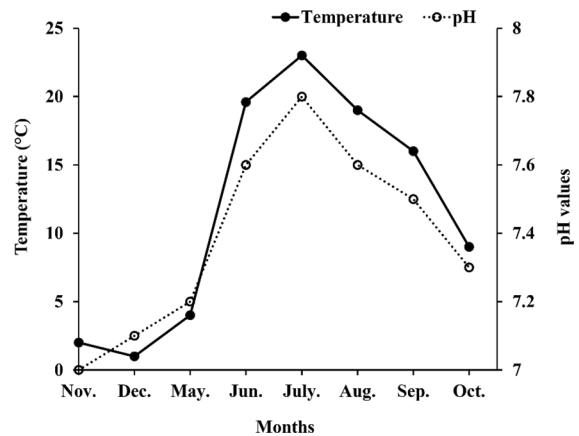


Fig. 1 Water temperature and pH in lake Viinijärvi during the sampling period between November 2020 and October 2021, except from January to April 2021

water temperature. Morphometric characteristics for the collected individuals [i.e., shell length, shell width and shell height (mm)] were measured to the nearest 1 mm using a digital Vernier caliper. Subsequently, the mussels were dissected monthly and the total body wet weight, gonad wet weight and shell weight were measured to the nearest 0.01 g using a precision digital balance.

Sex ratio and gonadosomatic index determination

Gonads were carefully dissected and separated from the body of the mussels. The sex of the mussels was determined through the microscopic inspection of gonad tissue for the presence of oocytes and gills for the presence of embryos or glochidia. Female to male sex ratio was calculated as the proportion of females to males. For the monthly numbers of sampled mussels, see Table 1; Fig. 2 and Supplementary Table 2.

The gonadosomatic index (GSI) is the most used quantitative approach for evaluating mussel reproductive activity. GSI% was calculated monthly according to Çek & Şerefişan (2006) using the following equation:

$$\text{GSI \%} = \left(G_w / T_{ww} \right) * 100,$$

where G_w is gonad wet weight, and T_{ww} is total flesh wet weight.

Table 1 The number of monthly collected mussels (females and males) of *A. anatina* during the study period

Months	Female	Male
November 2020	14	13
December 2020	19	15
May 2021	7	4
June 2021	16	18
July 2021	5	16
August 2021	4	13
September 2021	4	14
October 2021	9	8

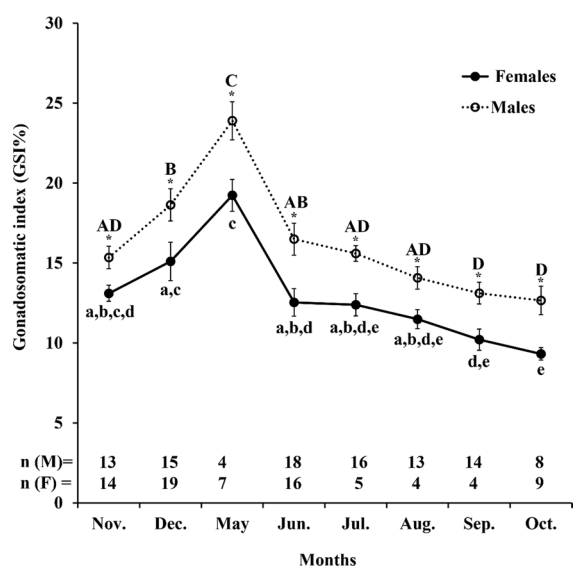


Fig. 2 The percentage values of GSI in males and females for each sampling period between November 2020 to October 2021, except from January to April 2021. An asterisk (*) indicates a statistically significant differences in GSI% between males and females each month (*t*-test; $P < 0.05$ for each month; Supplementary Table 2), while the dissimilar letters indicate a statistically significant differences in GSI% values between months for males (capital letters) and females (small letters) (two-way ANOVA; $P < 0.001$; Supplementary Table 1). The number of monthly collected samples for both sexes is shown near the X axis [male: n (M); female: n (F)]. The results of GSI are means \pm SEM

Fecundity and oocytes diameter monitoring

Three subsamples (about 10 mg) were collected from separate regions of each female's gonads (shell length 43.2–96.6 mm), weighed to the nearest 0.1 mg, and immersed in a 20% glycerol solution. The number

of oocytes were counted (to the nearest 100) under a dissecting microscope on a glass slide. Fecundity (F) was extrapolated from the subsamples using the following equation (De Croos et al., 2011; Abdelsaleheen, 2019).

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

where o_i is number of oocytes in a subsample; w_i is weight of the subsample; n is number of subsamples; and W is total weight of the gonad.

After the fecundity estimation, images of each glass slide were taken at a 400 \times magnification and analyzed using ImageJ software (NIH, Bethesda, MD, USA). At least 100 oocyte diameters of all gametogenic stages in the gonads were measured for each specimen. This method gave an overview on monthly variations of the average oocyte diameters.

Histological examination of gonads

To reveal the male and female gonadal structures, gonad samples of each sex in May 2021 were fixed in 6% neutralized formalin (Sigma-Aldrich), dehydrated in ascending series of ethanol (50%, 70%, 90% and 100%), cleared with xylene, and embedded in paraffin wax. The samples were sectioned at 7 μ m thickness, mounted on glass slides, dewaxed, rehydrated with descending ethanol concentrations, and stained with haematoxylin and eosin (HE) as described in detail by Abdelsaleheen et al. (2021). 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 as well as gonadal tissues were described according to Çek & Şereflışan (2006) and Şereflışan et al. (2013).

Parasite prevalence

For each dissected mussel, a piece (~6 mm in diameter) of gonadal tissue was squeezed between two glass slides (Taskinen et al., 1991), because gonad is the most preferred site of trematode parasite infection within the mussel body (Taskinen et al., 1997). The squeezed tissue was examined under a dissecting microscope to identify all the digenetic trematode infections, to determine the percentages of infected

males and females with one or more of these trematode species.

Age and growth estimations

Age of *A. anatina* was determined by measuring shell length (mm) at each annulus (growth line) with digital Vernier caliper. The continuity of the annulus around the entire valve distinguished the real rings from the false ones (Aldridge, 1999). The growth estimation of the *A. anatina* population was based on the von Bertalanffy growth equation (Bauer, 1992; Zyuganov et al., 1993):

$$L_t = L_\infty (1 - e^{-k(t-t_0)}),$$

where L_t is the length of the shell at time t ; L_∞ (asymptotic length) is the theoretical maximal length obtained by an individual at infinite age; and k is the growth constant which determines the rate at which L_∞ is reached.

The coefficients of the von Bertalanffy equation (k and L_∞) were measured by creating a Walford plot (Walford, 1946) of L_{t+m} against L_t , and the following equation was applied 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 ($=e^{-km}$) is the slope of the line; d ($=L_\infty(1-c)$) is the y-intercept of the line. Therefore, the growth constant and asymptotic length can be calculated from coefficients c and d as follow: $k = -\ln c/m$ and $L_\infty = d/(1-c)$.

The maximum age (T_m) of *A. anatina* that might be expected at Lake Viinijärvi was calculated using the following equation (Zyuganov et al., 1993):

$$T_m = -1/k * \ln [1 - (L_m/L_\infty)],$$

where T_m is the maximum age; and L_m is the maximum length.

Statistical analysis

All data were expressed as mean values \pm SEM. After checking the normality and homogeneity of data, two-way ANOVA with LSD (equal variances assumed) and Dunnett's T3 (equal variances not

assumed) post hoc tests were used to test for significant differences in GSI changes and in both sexes over the study period. A Student's t test for independent samples was applied to compare the monthly GSI changes between females and males. Chi-square (χ^2) Goodness of Fit test was used to assess the significance differences of sex ratio between the sexes. Chi-square (χ^2) contingency test were used to assess the monthly changes of parasite prevalence in the mussels, the differences in the prevalence of *Rhipidocotyle* spp. infection between male and female mussels, and the differences between the prevalence of *R. campanula* and *R. fennica*. To compare the parasites' prevalence between seasons, the monthly collected data of the infected mussels were combined into four different seasons (i.e., winter: November and December; spring–early summer: May and June; late summer: July and August; autumn: September and October) and compared pairwise by Chi-square (χ^2) contingency test. Pearson's correlation was used to check the correlation between fecundity and age or shell length. The statistical software program SPSS (IBM, version 27.0) was used for aforementioned statistical comparisons. The von Bertalanffy equation was calculated using the R version 3.6.3 (R Core Team, 2020). 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 was recorded in July (23.0 and 7.8 °C, respectively), while the minimum value for water temperature was recorded in December (1 °C) and for pH in November (7.0) (Fig. 1).

Sex ratio

Microscopic examination of females' gonads revealed that the studied population of *A. anatina* was dioecious, and no hermaphroditism was observed. Out of 184 specimens examined, 78 (42.4%) were females, 101 (54.9%) were males and 5 (2.7%) remained undefined (i.e., the gonad's tissues were fully destroyed by severe parasite infections). The sex ratio was biased

to males, where the ratio of females (F) to males (M) was 1:1.3, which differs significantly from the expected ratio of 1:1 ($\chi^2=81.9$; $df=2$; $P<0.0001$).

Gonadosomatic index (GSI%)

The variation in gonadosomatic index (GSI%) had similar trend in both sexes throughout the year (Fig. 2). GSI% revealed remarkable variation between months in both sexes (two-way ANOVA; $n=179$; $df=7$; $F=14.7$; $P<0.001$ between months) and gradually increased from November 2020 and peaked in May 2021 before dramatically declining from June to October 2021 (Fig. 2; Supplementary Table 1). The highest values of GSI% were observed in May ($19.2\pm 1.2\%$ for females and $23.9\pm 1.5\%$ for males), while the lowest values were detected in October ($9.3\pm 0.6\%$ for females and $12.7\pm 1\%$ for males). GSI% was significantly higher in males than in females during the study period (t -test; $P<0.031$ in all months; Supplementary Table 2).

Brooding pattern and spawning

In the current species, the released mature oocytes from female's gonads were occupied only on the outer-demibranchs of gills (marsupia). The development of embryos to mature glochidia was gradual and recognizable by the color of female gills. The coloration of marsupia changed from creamy beige in early embryos to orange-brown for mature glochidia before spawning (Fig. 3A). The gravidity of marsupia in females of *A. anatina* was described according to the presence of embryos (e) or glochidia (g), and as non-gravid in absence of both from marsupia. *Anodonta anatina* had a long brooding period, lasting from July to December (Fig. 3B). Embryos [Gravid (e)] gradually filled the marsupia from July (40%) until full in September (100%), and then developed into mature glochidia [Gravid (g)] in October (100%) (Fig. 3B). The release of glochidia started already in November, so that the proportion of females carrying mature glochidia decreased from 100% to 64.3% and 47.4% in November and December, respectively (Fig. 3B). No presence of embryos or glochidia in marsupia (non-gravid, Fig. 3B) were observed during May, indicating continuity of gradually discharged glochidia during winter and early spring months to be ready for loading the new mature oocytes in July.

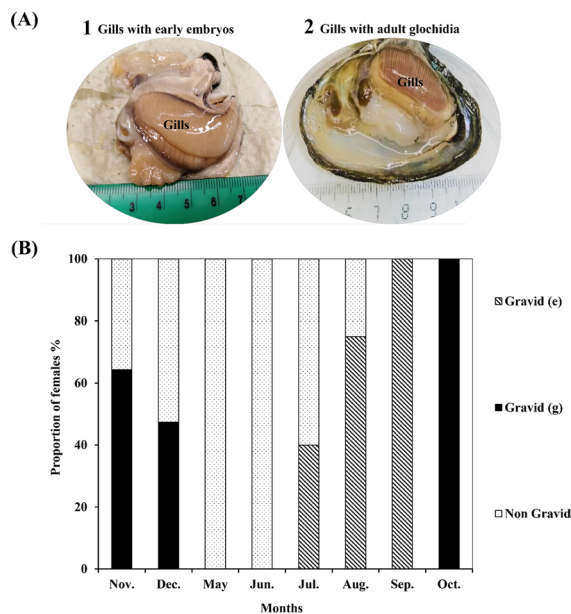


Fig. 3 The gravidity of gills in females of *A. anatina* during the sampling period between November 2020 and October 2021, except from January to April 2021. **A** Dissected *A. anatina* showing the alteration in the color of gills from creamy beige in early embryos (1) to orange-brown for mature glochidia (2). **B** The gravidity percentage of gills in females depending on the presence of embryos (e) or glochidia (g), and non-gravid in absence of both from gills

Fecundity and oocyte size-frequency

Fecundity of *A. anatina* ranged from 10,400 oocytes (43.2 mm shell length) to 53,000 oocytes (96.6 mm shell length) with an overall average (\pm SEM) of $31,200\pm 1,800$. Fecundity was strongly positively correlated with the shell length (Pearson correlation; $n=78$; $R^2=0.98$; $P<0.001$) and age (Pearson correlation; $n=78$; $R^2=0.93$; $P<0.001$) and no decline in the fecundity was observed in the mussels of largest size (Fig. 4).

Size-frequency of oocyte diameters was changed monthly from November 2020 to October 2021 (Fig. 5). Consistent with the spawning of mature oocytes, the large percentage of the biggest oocytes ($>42\ \mu\text{m}$) was only observed in May, which strongly declined in June. On the contrary, the large frequency of small oocytes ($10\text{--}16\ \mu\text{m}$) was observed in September and October indicating the production of new oocytes (beginning of oogenesis). Presence of different stages of the immature oocytes in all months

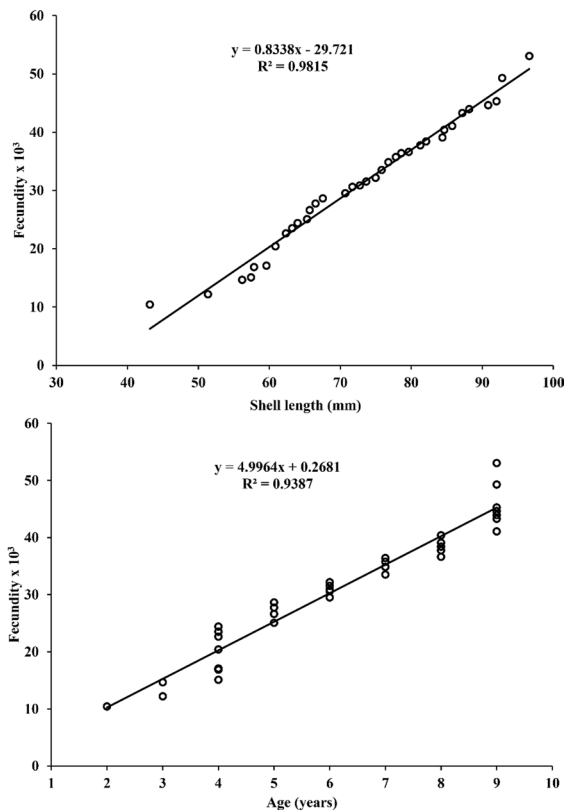


Fig. 4 Relationship between fecundity (numbers of produced oocytes per female) and shell length/age of *A. anatina* using a linear regression model ($n=78$ females) during the sampling period between November 2020 and October 2021, except from January to April 2021

revealed the continuity of the gametogenic cycle of *A. anatina* throughout the year.

Gametogenesis

Female and male gametes were organized in follicles surrounded by connective and muscular tissues, the density of which varied according to the developmental stages of gametes (Figs. 6A and 7A).

In females of *A. anatina*, oogenesis was divided into five stages: oogonia, previtellogenic oocytes, vitellogenic oocytes, late vitellogenic oocytes and mature oocytes (Fig. 6). Oogonia appeared in clusters attached to the inner follicle wall. They were rounded to oval shaped, with spherical nuclei and a prominent nucleolus (4–9 μm ; Fig. 6B). Thereafter, oogonia developed into previtellogenic oocytes, in which the germinal vesicle increased in size and the

cytoplasm was clearly stained with eosin (25–30 μm ; Fig. 6B, C). The vitellogenic oocytes were present in the lumina of the follicles and characterized by the presence of a nucleus at their center which contains two asymmetric nucleoli (35–40 μm ; Fig. 6C, D). Late vitellogenic oocytes were characterized by large yolk granules and a nucleus displaced to the periphery of the oocyte (41–49 μm ; Fig. 6D, F). Each oocyte contained an egg stalk to which the follicular wall adheres until it is transferred to the lumen for spawning as a mature oocyte. The latter is spherical in shape and distinguished by a collapsing germinal vesicle and the presence of a nucleus to the margin of the oocyte (50–60 μm ; Fig. 6E, F).

Spermatogenesis was recorded as four distinct developmental stages as follows: spermatogonia, spermatocyte, spermatids and spermatozoa (Fig. 7). Spermatogonia were oval-shaped and developed from the follicular wall (germinal epithelium) (3.4–4.7 μm ; Fig. 7B). Mitotic division of spermatogonia results in the formation of spermatocytes, which are spherical and have heterogeneous nucleus stained intensively with haematoxylin (2.4–3.8 μm ; Fig. 7C, D). Spermatocytes were smaller than spermatogonia and developed into spermatids which are polyhedral in shape and have homogeneous highly stained nuclei (1.8–2.7 μm ; Fig. 7D). Thereafter, spermatids metamorphosed into spermatozoa with bullet-shaped structures and smaller than spermatids; its head length was found to be about 2 μm (Fig. 7C, E, F). The male follicles of this species showed the existence of early spermatid clusters known as sperm morulae (4.1–9.1 μm ; Fig. 7D, F).

Trematodes (*Rhipidocotyle* spp.) prevalence

Microscopic investigations revealed that gonads of *A. anatina* were infected by the bucephalid trematodes *Rhipidocotyle campanula* and *R. fennica*. The overall prevalence of *R. campanula* infection (31%) was significantly higher than *R. fennica* (7.1%) in *A. anatina* ($\chi^2=27.7$; $n=70$; $\text{df}=1$; $P<0.001$; Fig. 8A). Moreover, female gonads were significantly more frequently infected by *Rhipidocotyle* spp. than males ($\chi^2=7.4$; $n=65$; $\text{df}=1$; $P=0.007$; Fig. 8B, C). The prevalence of *Rhipidocotyle* spp. infection went through significant monthly changes ($\chi^2=69.4$; $n=179$; $\text{df}=7$; $P<0.001$; Fig. 8A). From June to September, all (100%) collected females were infected by

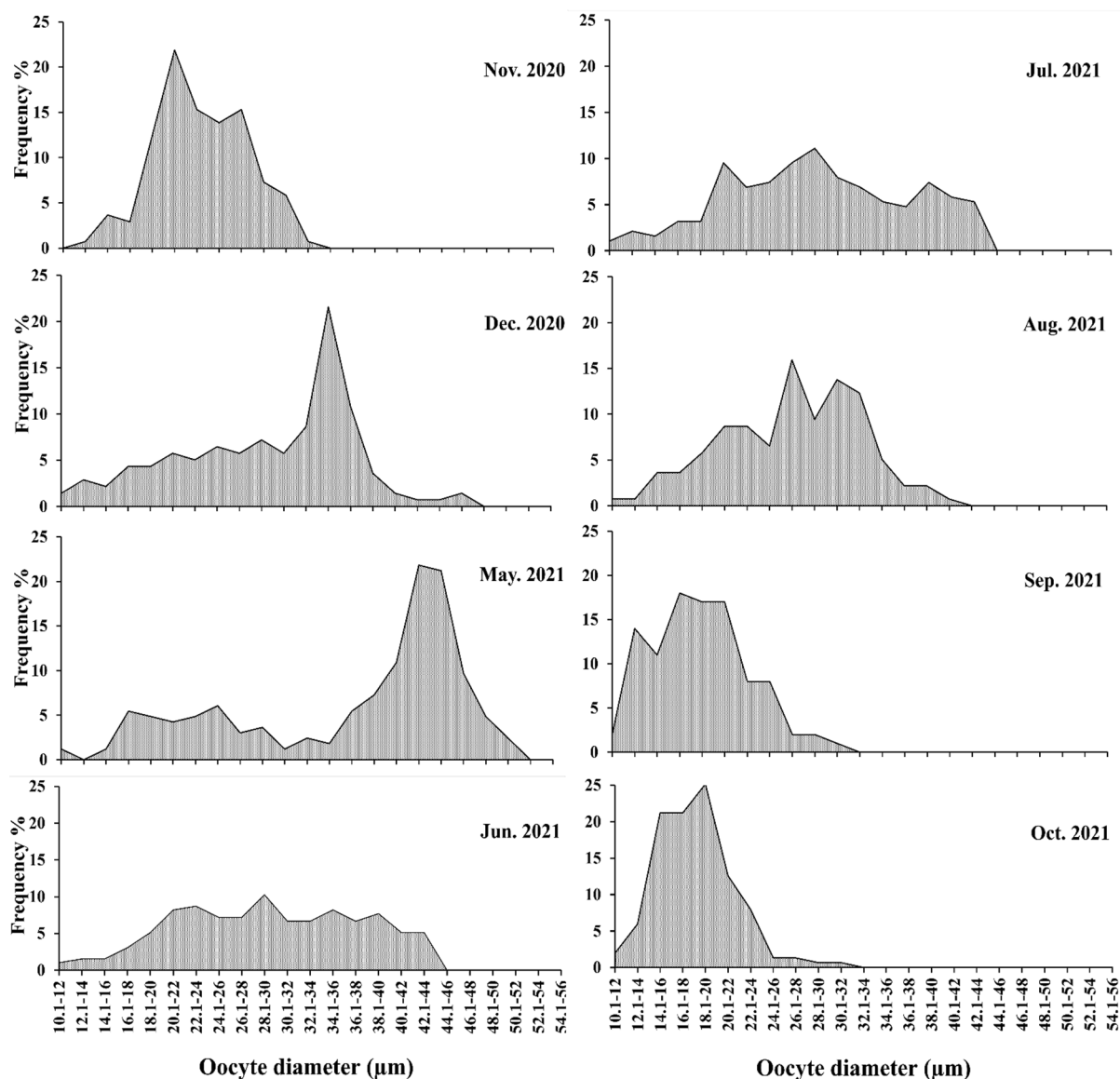


Fig. 5 Relative distribution of oocyte size-frequency of various size classes (grouped in 2 μm size classes; $n \geq 100$ oocytes) in female's gonads of *A. anatina* during the sampling

period between November 2020 and October 2021, except from January to April 2021

Rhipidocotyle spp. and in May and October 57.1% and 44.4% were infected, respectively (Fig. 8B). In July, 75% of collected males were infected, while 33.3%, 38.5% and 35.7% were infected in June, August and September, respectively (Fig. 8C). No infections were recorded in November and December for females (Fig. 8B), and in May and from October to December for males (Fig. 8C). *Rhipidocotyle* spp. infection peaked at the highest recorded water

temperature (late summer: July–August) (vs. winter: $\chi^2 = 56.6$; $n = 99$; $df = 1$; $P < 0.001$; vs. spring–early summer: $\chi^2 = 1$; $n = 83$; $df = 1$; $P = 0.312$; vs. autumn: $\chi^2 = 7.2$; $n = 73$; $df = 1$; $P = 0.007$), and its prevalence was the lowest at coldest temperatures (winter: November–December) (vs. spring–early summer: $\chi^2 = 46.7$; $n = 106$; $df = 1$; $P < 0.001$; vs. late summer: $\chi^2 = 56.6$; $n = 99$; $df = 1$; $P < 0.001$; vs. autumn: $\chi^2 = 26.2$; $n = 96$; $df = 1$; $P < 0.001$). In July, the

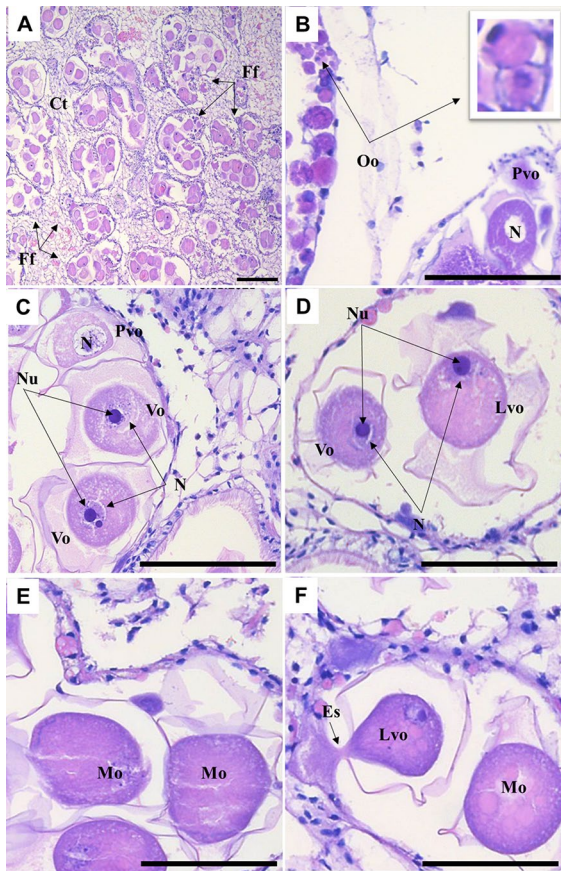


Fig. 6 Histological sections of gonads of *A. anatina* females show different developmental stages: overall view of ovarian female follicles and connective tissue (A), enlarged portion of gonad showing oogonia, previtellogenic oocytes and nucleus (B), previtellogenic oocytes, vitellogenic oocytes, nucleus and nucleolus (C), vitellogenic oocytes, late vitellogenic oocytes, nucleus and nucleolus (D), late vitellogenic oocytes, mature oocytes and egg stalk (E and F). *Ff* female follicle, *Ct* connective tissue, *Oo* oogonia, *Pvo* previtellogenic oocytes, *Vo* vitellogenic oocytes, *Lvo* late vitellogenic oocytes, *Mo* mature oocytes, *N* nucleus, *Nu* nucleolus, *Es* egg stalk. Scale bar: A 400 μm and B–F 70 μm

prevalence of *Rhipidocotyle* spp. infection peaked and the identification of mussel's sex was impossible in 4 out of 25 collected mussels (16%), where the gonadal follicles were completely degenerated due to parasitism.

Mussel age and growth estimations

From November 2020 to October 2021, the shell lengths (mm) of 184 living mussels were

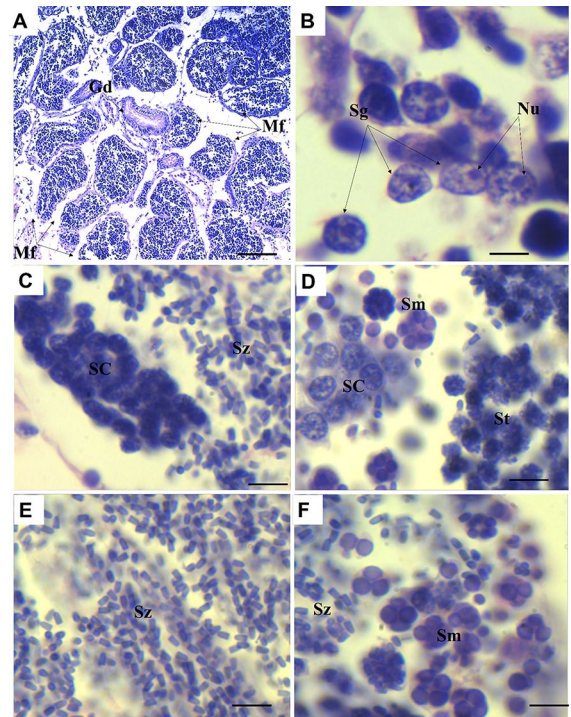


Fig. 7 Histological sections of gonads of *A. anatina* males show different developmental stages: overall view showing testis of male follicles and the gonadal duct (A), enlarged portion of testis showing spermatogonia and the nucleolus (B), spermatocyte and spermatozoa (C), spermatocyte, spermatids and sperm morula (D), spermatozoa (E) and spermatozoa and sperm morula (F). *Mf* male follicle, *Gd* gonadal duct, *Sg* spermatogonia, *Nu* nucleolus, *SC* spermatocyte, *St* spermatids, *Sz* spermatozoa, *Sm* sperm morula. Scale bar: A 400 μm and B–F 5 μm

measured at all the growth interruption lines. For the present species, the values of growth constant and asymptotic length were $k=0.16 \text{ year}^{-1}$ and $L_{\infty}=106.38 \text{ mm}$; respectively (Table 2). The fitted von Bertalanffy growth curve of *A. anatina* species was illustrated as a relationship between shell length and age (Fig. 9). The growth curve revealed that the population of *A. anatina* in Lake Viinijärvi has moderate growth constant ($k=0.16 \text{ year}^{-1}$; Table 2), where the shells of *A. anatina* population were approaching their theoretical maximum lengths ($L_{\infty}=106.38$) at slightly moderate rates. The maximum predicted age (T_m) for the entire population of the studied species was about 14 years (Table 2).

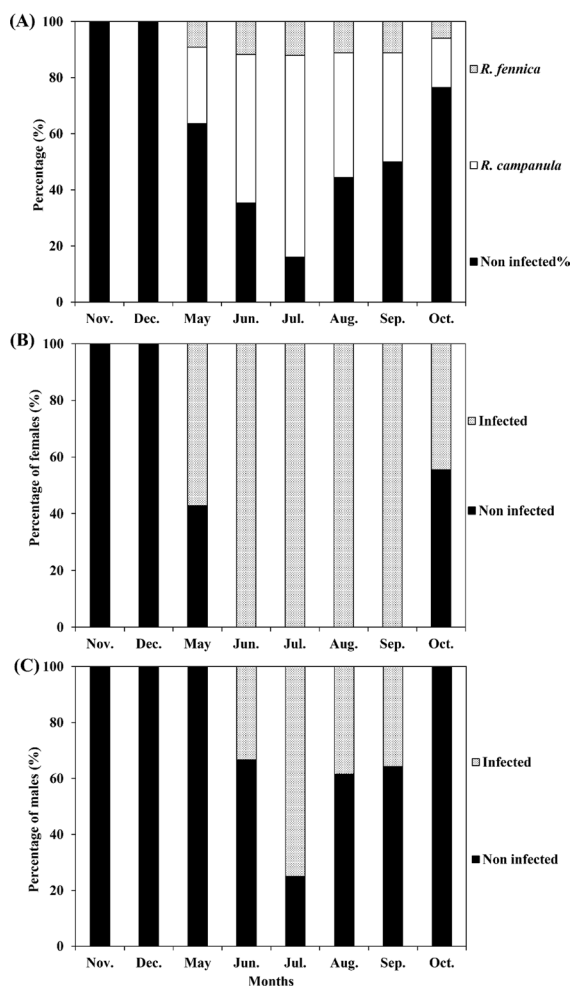


Fig. 8 Percentage (%) of non-infected and infected individuals by trematodes (*Rhipidocotyle campanula* and *Rhipidocotyle fennica*) of *A. anatina* during the sampling period between November 2020 and October 2021, except from January to April 2021. **A** The percentage (%) of non-infected and infected mussels (both sex) by *R. campanula* and *R. fennica*. **B** The percentage (%) of non-infected and infected females by *Rhipidocotyle* spp. **C** The percentage (%) of non-infected and infected males by *Rhipidocotyle* spp

Discussion

Previous studies on *A. anatina* have described the species as strictly dioecious with occasional hermaphroditism in some populations (Kat, 1983; Bauer, 1988). Other studies have suggested that *A. anatina* has a high prevalence of hermaphroditism only in standing waters while remaining mostly dioecious in running rivers and streams (Hinzmann et al., 2013;

Table 2 Growth parameters for *A. anatina* from Lake Viinijärvi, Finland

Walford plot	Growth constant (k)	Asymptotic length, L_{∞} (mm)	Maximum length, L_m (mm)	Maximum age, T_m (year)
$Y = 16.38 + 0.85x$	0.16	106.38	96.6	14

L_{∞} is calculated from the Walford equation, L_m is the observed maximum length in the field, and maximum age (T_m) is calculated from L_m

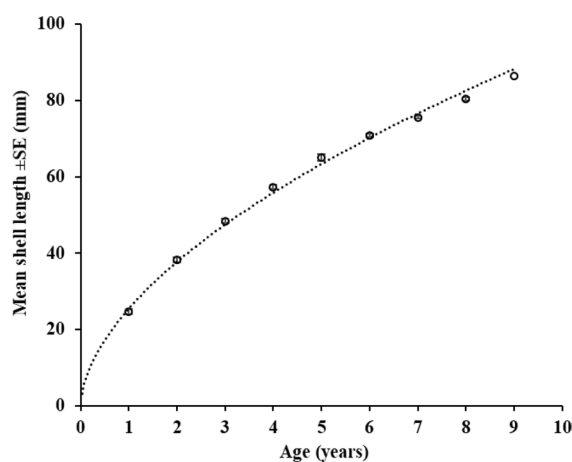


Fig. 9 Von Bertalanffy growth curve for *A. anatina* population ($n = 184$ individuals) in lake Viinijärvi from November 2020 to October 2021, except from January to April 2021

Yanovych, 2015). However, we found no evidence for hermaphroditism in our standing water population (Lake Viinijärvi), and our survey indicates that the studied population is completely dioecious with a male biased sex ratio (1F:1.3M). Our results are similar to those found for other populations of dioecious unionids having male biased sex ratios (Yokley, 1972; Aldridge, 1999; Şereflişan et al., 2013; Müller et al., 2021). The overabundance of males in relation to females in the present population could be explained by sex-specific differences in mortality. Due to the high cost of gonadal maturation, females are at greater risk of mortality during glochidia discharge (Barnhart et al., 2008; Strayer, 2008) and have higher rate of trematodes infection in their gonads (Taskinen & Valtonen, 1995; Yusa, 2007; Müller et al., 2015). Alternatively, a biased sex ratio may illustrate just a phase in the population's evolution for hermaphroditism

(Kat, 1983; Yusa, 2007). Absence of hermaphroditism and female-preference of trematode parasites in the *A. anatina* population in Lake Viinijärvi could support especially the first possibility.

As in other freshwater mussels, the reproductive cycle of *A. anatina* passes through two phases to form new glochidia: gonadal development (gametogenesis) in the gonad and embryonic maturation to glochidia in gill marsupia (Çek & Şereflişan, 2006; Şereflişan et al., 2013; Soliman et al., 2016). The present northern *A. anatina* population exhibited a long reproductive cycle throughout the year that was characterized by gradual gonadal growth within a single spawning season. The synchronization in gonadal production and maturation of eggs and sperm in this population, which peaked in May and discharged in June, which likely increase the success rate of fertilization in *A. anatina* along with the slow water velocity of the lake. Monthly variations in size frequency of oocytes, reflecting the maturation progress of oocytes, and the presence of small oocytes in all months, indicate the continuity of oogenesis in *A. anatina* throughout the year. Moreover, the discharged mature oocytes in June were rapidly compensated by the onset of oogenesis in September. The cause for the existence of a substantial number of gametes throughout the year and outside of the spawning season, and the fate of non-spawned gametes, is yet mostly unknown, although it appears to be a feature within Anodontine species (Heard, 1975). Consistent with the previous findings, mature oocytes occupied the outer gills in females for fertilization and matured to glochidia within 2–3 months (July–September) (Aldridge, 1999; Mcivor, 1999; Hinzmann et al., 2013). On the contrary, glochidia are only restricted to the inner demibranches in *Hyridella depressa* (Lamarck, 1819) and *Anodontites trapesialis* (Lamarck, 1819) (Jupiter & Byrne, 1997; Silva -Souza et al., 2011), while the incubation of glochidia occurred in all the four demibranches (both inner and outer gills) in *Nitia teretiuscula* (R.A. Philippi, 1847) (Soliman et al., 2016). In *Mycetopoda legumen* (E. von Martens, 1888), the glochidia occupied only the anterior half of the inner or outer demibranches (Watters & O’Dee, 1998). In the current study, *A. anatina* had a long brooding period (bradytic breeding), where the females’ outer gills were gravid during the midsummer and early winter months (July–December). The brooding could have possibly extended until March–April,

and the absence of glochidia in May is consistent with the previous studies (Jokela et al., 1991; Pekkarinen, 1993; Aldridge, 1999; Hinzmann et al., 2013).

The timing of glochidia release can vary among unionid species and within the same species at different environments (Watters & O’Dee, 1998; Aldridge, 1999). For example, in the current study, glochidia were gradually released from November to probably April, which corresponds to the earlier findings from Finland (Jokela et al., 1991), while in the Tâmega River, Portugal, *A. anatina* gradually released glochidia from March to April (Hinzmann et al., 2013).

Fecundity is an essential life-history trait, indicative of the mussel’s fitness. It is affected by shell length (size) and age of unionids, where large individuals are expected to have the highest contribution to the reproductive output of the population (Bauer, 1988; Hochwald, 2001; Haag and Stanton, 2003). In the present study, the fecundity was strongly related to length and age of *A. anatina* (i.e., individuals with the maximum size and age have higher egg production, larger gills, and a higher capacity for glochidia brooding). Gonads of *A. anatina* produces a large number of eggs 10,434–53,058 per individuals of 43.2–96.6 mm shell length, respectively. Despite the large number of eggs produced by the current species, it is much lower compared to other freshwater unionids species as *Pleurobema decisum* (I. Lea, 1831) (27,860–69,553), *Utterbackia imbecillis* (Say, 1829) (25,500–146,500) and *N. teretiuscula* (26,000–208,000) (Haag and Stanton, 2003; Haag, 2013; Abdelsaleheen, 2019). In Zhytomyr, Ukraine, the number of produced glochidia in *A. anatina* per female was 288,000–694,000 in 1939, and dramatically declined to 36,600–319,950 in 1990s, and to 5,500–40,400 by 2015 (Troitskiy, 1939; Yanovych et al., 2010; Yanovych, 2015). The reduction in reproductive output might be the case in the current population in Lake Viinijärvi, unfortunately this is the first study, and no previous data is available for comparison with. Infection of the gonads with trematode parasites and/or prevailing food, toxic substances and temperature conditions in their habitat might be reasons for the dramatic decline in reproductive output (Jokela et al., 1993).

Histological investigations of gonads from *A. anatina* confirmed that female oogenesis can be classified into five stages, which is consistent with the previous findings in other unionids with minor differences

in the oocyte's diameter (Jones et al., 1986; Çek & Şereflişan, 2006; Hinzmann et al., 2013; Şereflişan et al., 2013; Hliwa et al., 2015; Soliman et al., 2016). The mature oocyte diameter of *A. anatina* was among the smallest recorded oocytes diameters across the studied unionid species. The mature oocyte diameter of *A. anatina* was found to be smaller than that of *Unio terminalis delicatus* (I. Lea, 1863) (80–100 µm; Çek & Şereflişan, 2006), *Anodonta woodiana* (I. Lea, 1834) (75 µm; Dudgeon & Morton, 1983) and *Nitidulites teretiuscula* (63–93 µm; Soliman et al., 2016).

Male spermatogenesis of *A. anatina* can be classified into four stages (spermatogonia, spermatocyte, spermatids and spermatozoa) which are consistent with those observations in other unionids (Çek & Şereflişan, 2006; Hliwa et al., 2015; Soliman et al., 2016). The morphology of spermatozoa for *A. anatina* males are characterized by a simple structure which resembled that of most Unionidae species (Matos et al., 1998; Hliwa et al., 2015). Spermatozoa have a bullet-shaped head and a uniflagellate tail with a very small acrosome, however, the acrosome can only be seen with a transmission electron microscope that was not applied in the current study (Rocha & Azevedo, 1990; Shepardson et al., 2012). Spermatozoa of *A. anatina* were within the average size of other Unionidae. The spermatozoa head length of *A. anatina* in the current study was ca. 2 µm, while it has been found to vary from 2.65 to 4.2 µm in other studied unionids (Matos et al., 1998; Hliwa et al., 2015; Soliman et al., 2016). Spermatogenesis of *A. anatina* revealed the presence of sperm morula, whose function remains unclear (Hliwa et al., 2015; Soliman et al., 2016). Some studies have suggested that sperm morulae are a collection of spermatids that, under certain environmental conditions, transform into mature sperm (Matos et al., 1998; Çek & Şereflişan, 2006; Şereflişan et al., 2013). Shepardson et al. (2012) reported that the presence of sperm morula under certain conditions was evidence for an abnormal (atypical) spermatogenetic pathway. It is noteworthy that we found *A. anatina* to follow an atypical pathway of spermatogenesis with absence of Sertoli cells as described by Shepardson et al. (2012). This fact was consistent with previous findings in *Sinanodonta woodiana* (I. Lea, 1834), *Venustaconcha ellipsiformis* (Conrad, 1836) and *N. teretiuscula* (Shepardson et al., 2012; Hliwa et al., 2015; Soliman et al., 2016). Therefore, more research is needed,

particularly using transmission electron microscopy, to get more information about the developmental phases of sperm morula and its role in *A. anatina*.

Duck mussels (*A. anatina*) are filter feeders that extract food particles from the water around them, which makes them susceptible to exposure to parasites such as the miracidium larvae of digenetic trematodes (Nichols et al., 2005). However, the invasive zebra mussel [*Dreissena polymorpha* (Pallas, 1771)] can negatively impact native species like the duck mussel by settling on their shells, interfering with their feeding and growth. Zebra mussels themselves are highly efficient filter feeders and can remove significant amounts of suspended particles from the water. A recent study by Müller et al. (2021) found that zebra mussels with heavier biofouling (i.e., the accumulation of organisms on their shells) tend to have a smaller abundance of parasites, including water-borne trematode larvae. This suggests that the zebra mussels' filtering activity may be reducing the prevalence of parasites in the water, indirectly affecting the populations of duck mussels and other species that rely on those parasites as part of their diet. Previous studies have reported that trematode parasites can have negative impacts on freshwater mussels, including altering their phenotype and clearance rates, reducing reproductive output, decreasing longevity, and changing their energy storage and shell shape (Jokela et al., 1993, 2005; Taskinen & Valtonen, 1995; Taskinen, 1998; Zieritz & Aldridge, 2011; Brian et al., 2022). However, Brian and Aldridge (2022) reported that 21% of the estimated parasite fauna is in imminent danger of extinction, which is likely to have a severe impact on freshwater ecosystems due to the important roles that parasites play in community structure and function, affecting also freshwater mussels. Furthermore, parasites have a high productivity and unique nutritional composition, making them an attractive food source for predators (Johnson et al., 2010). Additionally, the abundance and diversity of parasite cercariae can be used as indicators of ecosystem health, and changes in their populations can provide early warning signs of environmental stressors (Morley, 2012).

The trematode parasites were here commonly found in the gonads of *A. anatina*. *Rhipidocotyle* spp. (*R. campanula* and *R. fennica*) are generally the most dominant trematodes in *A. anatina* (intermediate host) gonads (Taskinen et al., 1991). Cercarial

production of *Rhipidocotyle* is positively influenced by increasing temperature (Choo & Taskinen, 2015; Taskinen et al., 2022). Previously, the cercariae shedding rate, intensity of infection, as well as proportion of different developmental stages of the cercaria and the sporocysts of *Rhipidocotyle* spp. were observed to undergo seasonal changes (Taskinen et al., 1994, 2022), but seasonality in the prevalence of infection has not been previously observed. A remarkable seasonal variation in the prevalence of infection was evident in the present study. The prevalence of *Rhipidocotyle* spp. increased rapidly during spring-early summer to reach the highest infection rate in July (late summer), decreasing correspondingly quickly thereafter so that in November–December (winter) no infections were observed. *R. campanula* was the dominant parasite species in the study population. This species shows more pronounced changes also in other seasonal aspects of infection than *R. fennica* (Taskinen et al., 1994) and imposes much higher parasite-induced host mortality than *R. fennica* (Jokela et al., 2005; Taskinen et al., 2022), which may indeed manifest in the observed drop of *R. campanula* prevalence in autumn. It is worth to note that the infection of mussels is seasonally timed to take place in autumn, but the development of new infections becomes visible in mussels in the spring (Taskinen et al., 1991). Results by Taskinen et al. (2022) suggest that the development of *R. campanula* with mussels in spring is much quicker than that of *R. fennica*. In our data, the infection with trematodes peaked at the highest recorded temperature in July (late summer), and the identification of host sex was difficult after completely degeneration of gonadal tissues in 16% of the collected samples that were classified as sterile. This result is well in line with the hypothesis that parasites can alter host reproduction (Jokela et al., 1993).

Our findings revealed that *Rhipidocotyle* spp. preferred gonads of females over gonads of males, since all collected females were infected during high temperature months (June–September) while only about 35% of collected males were infected with exception of July (75%). This fact might, at least partly, explain our results on male biased sex ratio and the dramatically reduced fecundity of females along with smallest mature oocytes in comparison to other unionids. Moreover, Taskinen and Valtonen (1995), and Müller et al. (2015), reported that females of an *A. anatina* were more heavily infected by trematodes than males

and infected females brooded fewer glochidia than uninfected ones.

The lifespan and growth rate varies considerably among unionids species and the von Bertalanffy equation's growth constant k is often used to describe bivalves' growth (Alimov, 1981; Bauer, 1992; Aldridge, 1999). In Anodontinae, the growth constant (k) ranged between 0.072 and 0.35 year⁻¹, while the maximum predicted age ranged between 6 and 37 years, and the maximum theoretical length ranged between 60.4 and 150.5 mm (Comfort, 1957; Økland, 1963; Negus, 1966; Alimov, 1981; Aldridge, 1999; Ollard & Aldridge, 2022). *A. anatina* showed a moderate growth rate ($k=0.16$ year⁻¹) in Lake Viinjärvi. The contradictory findings for growth rate of *A. anatina* in different sites might be caused by the sample size and by several environmental and ecological factors, including habitat type, food availability, water temperature and geographical latitude (Negus, 1966; Aldridge, 1999; Ollard & Aldridge, 2022). Significant differences in the maximum ages have previously been recorded in different populations of unionids. In *Unio tumidus* Philipsson, 1788 and *U. pictorum* (Linnaeus, 1758), the maximum recorded age was between 8 and 15 years (Negus, 1966), and in *Margaritifera margaritifera* (Linnaeus, 1758) it was between 30 and 132 years (Bauer, 1988, 1992). The reproductive capacity of unionids is strongly influenced by their maximal size and age, individuals with a longer lifespan generally reproduce more frequently, resulting in a higher lifetime fecundity than those with a shorter lifespan (Aldridge, 1999). Additional research should be conducted for other mussel species to explain the possible relationships between variation in growth patterns and the mussel's habitat.

In conclusion, our study provides novel and comprehensive data on reproductive traits of *A. anatina* population in North Europe that can be potentially applied for aquaculture and conservation actions (captive breeding, juvenile propagation) of freshwater mussels. The gonadal developmental stages of *A. anatina* last throughout the year with a bradyctictic brooding pattern and a single spawning season. In Lake Viinjärvi, the population of *A. anatina* was significantly male-biased and we found no evidence for hermaphroditism. Gonads of *A. anatina* were more frequently infected by *Rhipidocotyle* spp. than males. Moreover, the infection prevalence increased with increasing temperature and 16% of collected samples

were sterile at the highest recorded temperature in July due to parasitism. The negative direct impacts of parasites on reproductive traits might compromise the reproductive capacity of *A. anatina*, and the situation might become worse with the predicted climate warming. Overall, along with providing the first data on gamete production in *A. anatina*, our results support earlier observations on reproduction biology. Our data also suggest that parasitism can have notable negative effects, which should be considered in possible future conservation aquaculture efforts on this species.

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Author contributions OA: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Visualization, Writing—original draft, Writing—review and editing. JT: Conceptualization, Review and editing. JK: Review and editing. RK: Conceptualization, Resources, Supervision, Review and editing.

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Data availability The original data of the study is available upon request from the corresponding author.

Declarations

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.

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