

A Note on Growth and Fecundity of Long-term Selfing Population of *Lymnaea stagnalis* (Linnaeus, 1758)

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ABSTRACT

Lymnaea stagnalis is a freshwater pulmonate gastropod that has been maintained as an inbred selfing population for more than 10 years. The growth curves of both normal and trematode-infected animals are described. Selfing animals reach sexual maturity after 16 weeks, and live for about 40 weeks. Trematode infection results in increased growth and earlier sexual maturity after 12 weeks. Two subpopulations were observed: 'kastanea', which grows following the pattern of the uninfected animals, with a lifespan of about 60 weeks, and 'feiwo' animals that grow much faster and live for more than 72 weeks, reaching a length of more than 56 mm. Selfing animals lay egg clusters regularly, hatching after 13 - 17 days. A number of abnormalities were observed during the development: partially fertilized egg clusters, dead embryos, infected eggs, embryos without shells, empty eggs, or twins that fully mature and hatch.

Keywords: *Lymnaea stagnalis* Linnaeus 1758; Pulmonate gastropod; Selfing, Artificial habitat.

INTRODUCTION

Lymnaea stagnalis Linnaeus, 1758 is found in Europe and the temperate zones of Asia, Australia and North America. It is popular with its conchological variations, reasoning the reports of separate varieties, morphs, subspecies and even distinct species (Vinarski et al., 2020). Differs from other lymnaeids in its larger size: the adult shell length varies between 30 mm and 70 mm - gigantism, a result of parasitic trematode larvae infection (Ward et al., 1997; Zbikowska et al., 2006). *L.*

stagnalis displays both coiling directions in the wild: 98% dextral and 2% sinistral coiling. *L. stagnalis* inhabits standing, slowly moving, and temporary waters with rich vegetation. Prefers shallow places and is rarely found below 20 m water depth. Juveniles may often be found outside the water on plants. It is found in Switzerland (up to 1700 m) and Norway (up to 800 m), but not in southern Europe. It is used to monitor the quality of freshwater biotopes in Germany.

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Tolerates high pH values (pH 9.5), salinity - up to 7 ppt, and temperatures between 19 and 24°C. The animals do not surface for air supply below 5°C, egg clusters are not laid below 13°C, and mortality increases above 26°C. It is tolerant to desiccation and freezing. It lays egg clusters on plants, wood and stones. Two sister clades separated by a genetic distance of 0.085 are described: the first includes animals from Western and Central Europe and North America, and the second clade comprises snails living in Ukraine, Western Siberia and Asia Minor (Vinarski et al., 2015). In North America, *L. stagnalis* occurs south of the tree line from the St. Lawrence watershed to the Mackenzie and Yukon watersheds, and south to Colorado and the Mississippi system in North Dakota, Ohio, and Illinois (McAlpine et al., 2005). It has been described in Canada (Boag & Pearlstone, 2011).

L. stagnalis is diurnal, with a 3-4 hr feeding cycle in the activity pattern, and resting within 1 hr after darkness (Harris et al., 2012; Benjamin, 2012). There is no circadian modulation of the activity cycles, contrasting to *Aplysia* and *Octopus* (Stephenson & Lewis, 2011). *L. stagnalis* requires ~20 mg/L calcium to thrive, and low environmental calcium acts as a stressor blocking long-term memory formation (Lukowiak et al., 2014).

The heart and the locomotory contractions maintain the open vascular circulation of internally (buccal mass and stomach) and externally (ganglia and glands) vascularised organs (Bekius, 1971). Haemolymph, containing copper-containing haemocyanin and haemocytes, is expelled in the surrounding water during contraction of the foot within the shell, and later replaced with water from the surrounding environment (Rigby & Jokela, 2000). Haemocyanin synthesized mainly in the mantle tissue rhogocytes (Wood et al., 1981), is excreted through the slit apparatus into the haemolymph (~3 mg/ml) (Kokkinopoulou et al., 2015). Rhogocytes vary in size (2-30 µm) and shape, and are involved also in the transport and storage of nutrients, calcium mobilization for shell formation, immune defence, and heavy

metal detoxification (Boisseaux et al., 2016). However, trematode infection activates the defence system and increases the circulating haemocytes (Skala et al., 2019; Menendez, 2018), later (after 5 weeks) a suppression of the cellular immune response is observed (Horak & Kolarova, 2005). The trematode larvae can influence snails' behaviour, cause castration, compensatory fecundity, gigantism and increased longevity.

L. stagnalis is a simultaneous hermaphrodite, where only one sexual role is performed within one copulation. It is able of self-fertilization, or selfing - the process of fusion of male and female gametes produced by the same individual. It reproduces from May to October, and egg-laying slows down and terminates in aged animals. Defined behavioural elements are described during copulation (el Filali et al., 2015; Benjamin & Kemenes, 2020). Allosperm is actively transported, and fertilizes the eggs at least 2 hr after deposition. Oviposition takes place after at least 2 hr from the time of ovulation, and depends on environmental factors (e.g., oxygen-rich water, clean surface, light) rather than copulation (Nakadera et al., 2014). Role-alternation and chain-copulations can be observed, where mating rates increase with population density. *L. stagnalis* can store received allosperm for about 2 months (62 days) (Marien et al., 2017; Nakadera et al., 2017; Hoffer et al., 2017). They can selectively use autosperm (produced in the ovotestis) in the process of selfing, despite the presence of allosperm. The female sperm storage along with the internal fertilization, and the multiple and polyandrous matings result in prolonged paternity gain (paternal longevity). *L. stagnalis* seminal fluid contains ovipostatin, a protein that suppresses the egg-laying activity of the recipients, and its influence depends on the body size of the recipients. Larger recipients have a shorter paternity longevity (Nakadera et al., 2014; Felmy-Burkli & Jokela, 2017).

The developing *L. stagnalis* embryos have been used to study the control of external symmetry and the left-right asymmetry of the internal organs. Chirality is maternally

inherited and controlled by *lsdia1*, an actin-related gene. The clockwise and anti-clockwise micromere rotation at the 3rd cleavage of the 8-cell-stage embryos is the earliest sign of chirality. Sinistral species, such as *P. acuta* and *I. exustus*, carry only the *lsdia2* gene. Mechanical manipulation of embryos (*L. stagnalis* and *P. acuta*) at the 3rd cleavage reverses the micromere rotational direction and results in the expression of *nodal/Pitx* genes (regulating the location of organs) at the opposite side. Handedness of the blastomere cleavage and that of the shell coiling at the organismal level are correlated. Molluscan shell matrix proteins are asymmetrically expressed. The *dpp* expression in the mantle edge switches sides between dextral and sinistral lineages of *L. stagnalis* (Kuroda & Abe, 2020; Abe & Kuroda, 2019; Kuroda et al., 2016).

Monitoring of lymnaeid populations is of medical and veterinary importance since they act as vectors of trematodes (Seppala et al., 2021; Zbikowska et al., 2006; Vorontsova et al., 2015), and accumulate heavy metals and pollutants (Kokkinopoulou et al., 2015; Otludil & Ayaz, 2020) that severely affect human populations and livestock. *L. stagnalis* has been a subject of ecoimmunological and global warming risk assessment studies (Fodor et al., 2020; Kuroda & Abe, 2020; Ricottone & Fernandes, 2021).

MATERIALS AND METHODS

The animals (*Lymnaea stagnalis* Linnaeus 1758) were obtained from a European trader, and an inbred selfing population was maintained in local tap-water (representative measurement: pH 7.42; GH 0.89 meq/L; conductivity 115.4 uS/cm, and Cl 0.11 mg/L; Sofiyska voda Ltd., Sofia, Bulgaria) at ambient temperature (18-22°C), 12/12 hr light/dark cycle, and fed on fresh lettuce (*Lactuca sativa*) leaves. The water and food were changed twice weekly. Shell length and egg cluster numbers were determined once every week. Light microscope images were acquired with a Nikon research microscope, equipped with a DXM1200c digital camera in the Department of Anatomy, Histology and

Embryology, Medical University - Sofia, Sofia, Bulgaria.

Data analysis: Results are given as weakly length (mm), average and +/-SD. Statistical analysis: Student's t-test ($p < 0.05$), and Z-test (Numbers for Mac 2020, Apple Inc. USA), where each value, including the mean, was tested against the data set for the respective day.

Ethics statement: The snail *L. stagnalis* is abundant in Europe, and neither an endangered nor a protected species. All animal work has been conducted according to national guidelines. There was no need to involve an ethics committee in the case of these gastropods.

RESULTS AND DISCUSSION

The growth of *Lymnaea stagnalis* was determined following shell length measurements once every week (Figure 1). After initial exponential growth, their growth slows after the 26th week (~29 mm) without reaching a plateau, continuing to increase until they die after about 40 weeks.

Selfing is a unique mode of reproduction of simultaneous hermaphrodites, where eggs are fertilized by their own sperm (autosperm), not by allosperm from their partners (Nakadera et al., 2014). Several generations of selfing animals were raised for a period of more than 10 years without any effects on growth and reproduction. Selfing animals reach sexual maturity after 16 weeks, laying their first egg clusters (Figure 2).

Trematode parasites are common among freshwater snails, causing inhibition of immunity and reproduction, an increase in calcium content, and stimulation of growth and gigantism. Trematode prevalence increases with snail size in the wild, where larger individuals (more than 30 mm) are most infected (Zbikowska et al., 2006). Unlike *L. stagnalis*, *L. peregra* show no gigantism, and *L. ovata* display only a 10% increase in the growth of infected snails (Ward et al., 1997). The augmented growth of the animals was attributed to a trematode infection, where 2 subpopulations were observed, referred to as 'kastanea' and 'feiwó' (Figure 1). While both

reach sexual maturity (selfing) earlier after 12 weeks (Figure 2), 'kastanea' growth follows that of the uninfected 'stagnalis' animals, living longer for about 60 weeks. They differ from 'stagnalis' with their darker brownish shell colouration. The 'feiwo' animals grow much faster and live longer for more than 72 weeks, reaching a length of more than 56 mm. *L. stagnalis* produces 2-3 egg clusters every week, 2-6 cm long, containing 50-150 transparent oval eggs (Fodor et al., 2020). Egg clusters produced by selfing animals contain less eggs (Jarne et al., 1991). Selfing animals laid egg clusters regularly that hatch after about 13 - 17 days (Figure 3). The cells of the embryo divide rapidly, forming an actively rotating and moving in the egg fluid trochophore during day 2 of development. The

veliger secretes the initial shell after the 5th day. The eyes and the heart are easily recognizable after the 7th day.

A number of abnormalities were observed during the development (Figure 4). Partially fertilized egg clusters are frequent. Eggs with normally developing embryos were found in the same egg cluster with eggs with altered development - death embryos (arrested at different developmental stages), infected eggs (white fluffs within the egg fluid), embryos without shells, empty eggs, or twins that fully mature and hatch. Different agents, like actinomycin D (Morrill et al., 1976) and colchicine (Mahobiya, 2020), and many others have been reported to affect embryonic development of *Lymnaea stagnalis* and many other mollusks.

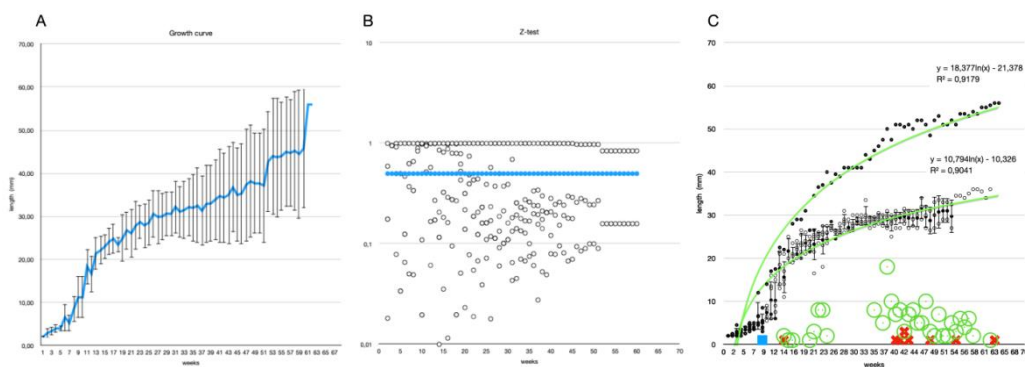


Figure 1: Growth and longevity of *Lymnaea stagnalis* (Linnaeus, 1758). A) A growth curve of the selfing population (blue line, mean length; and SD). B) Z-test of the values from A. C) Growth of 'stagnalis', 'kastanea' and 'feiwo' animals. The blue square marks the time when the juveniles start to explore the environment and leave the containers. The total number of egg clusters is shown with green circles. Deaths are marked in red.

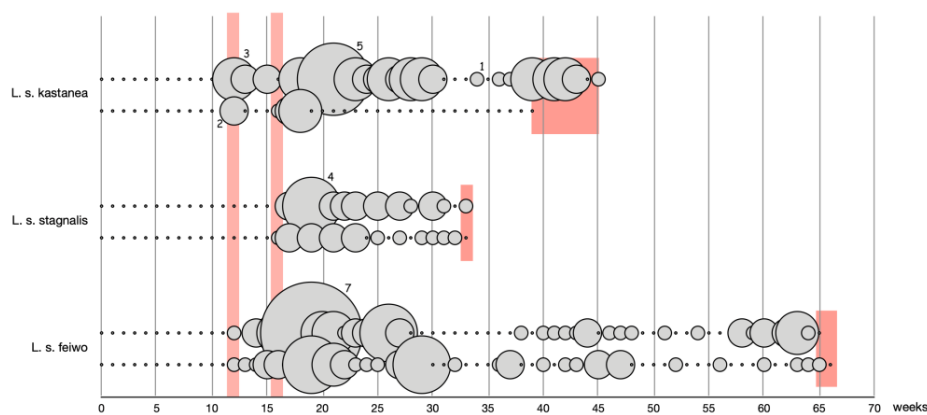


Figure 2: Fecundity of *Lymnaea stagnalis* (Linnaeus, 1758). The productiveness of 6 representative selfing animals shows the earlier sexual maturity of 'kastanea' and 'feiwo' animals, and their longer lifespan. The circles represent the number of egg clusters per week for each animal.

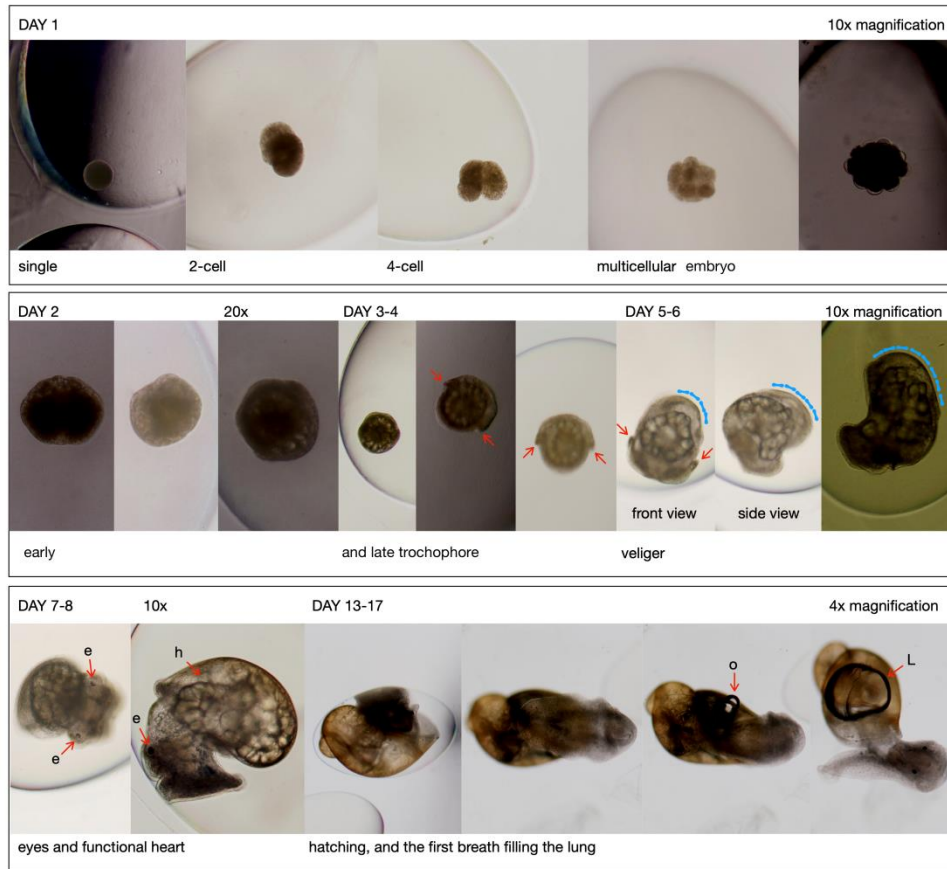


Figure 3: Intra-capsular development of *Lymnaea stagnalis* (Linnaeus, 1758). The rapidly dividing cells of the fertilized egg are easily observed during the 1st day. The movements of the trochophore are detectable after day 2 of development. The secretion and growth of the veliger shell are visible after day 5. The eyes (e) and beating heart (h) are obvious after day 7. Hatching, and the first breath of air through the pneumostome (o), filling the lung (L).

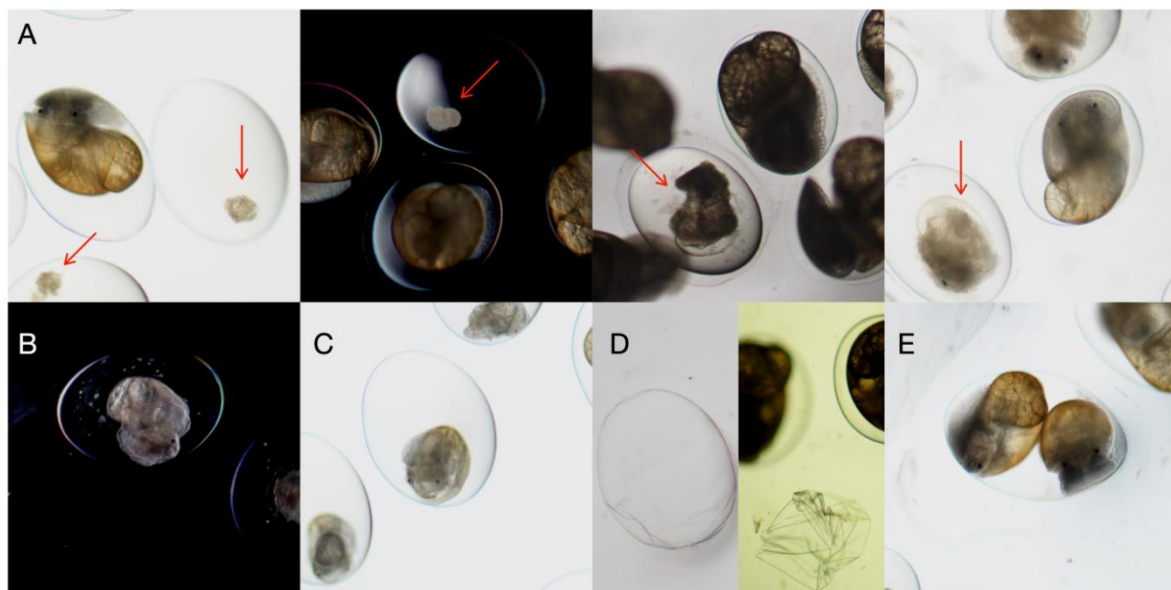


Figure 4: Altered embryo development of *Lymnaea stagnalis* (Linnaeus, 1758): A) Blocked at different stages of development; B) Matt flakes in the egg fluid, suggesting a possible infection; C) Embryos without a shell; D) Empty and hatched eggs; and E) Twins.

CONCLUSIONS

Lymnaea stagnalis Linnaeus 1758 is an easily maintained animal. Long-term selfing does not affect the health and longevity of the population, although some developmental alterations have been observed over the years. Sexual maturity depends largely on individual size rather than individual age. Maintaining a selfing population makes these animals an attractive supplement for the artificial habitats without the difficulties from their copious proliferation.

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Conflict of Interest:

There is no such evidence of conflict of interest.

Author Contribution:

All authors have participated in critically revising of the entire manuscript and approval of the final manuscript.

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