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Pond Snail *Lymnaea stagnalis* L.: The Implication for Basic and Applied Research

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Abstract: The results show that it is possible to maintain marsh snail *Lymnaea stagnalis* L through the generations, under laboratory conditions, but with the first generation of animals bred in the laboratory from egg masses from the nature, is not infected with parasites. In conditions when egg masses are kept in Petri dishes in 50 ml of tap water at a temperature of about 20°C, embryonal development lasts 16 days, the survival rate of embryos is about 90%. During embryonal development, from the 8th day, it is possible to register a summary spon taneous bioelectrical activity of the embryonal nervous system (pedal ganglia). Embryonal growth (longitudinal) and postembryonal growth (longitudinal and weight) mathematically describes the sigmoid curve of growth and length-weight relationship describes the exponential growth function, which turns into a linear logarithmic dependence. The value of the parameter *b* (line slope of the linear relationship) in our experimental conditions is 2.68 and indicates that under these conditions, weight growth increases proportionally slower than the longitudinal growth (negative allometry of growth). Based on morphological, electrophysiological and pharmacological characteristics of the dorsal surface of the visceral and right parietal ganglia of adult snail identified 9 neurons. Marsh snail *Lymnaea stagnalis* L. is relatively good indicator (in- dicator weight G 3) of β- mezosaprobic zones (relatively pure water) of aquatic ecosystems, where it is most commonly found, but it also habitates in oligosaprobic (pure water) and α-mezosaprobic zone (pollution-regulated water).

Key words: Pond snail *Lymnaea stagnalis* L. · Laboratory culture · Embryonal and postembryonal development · Mathematical modeling of growth · Saprobiological investigation

INTRODUCTION

Due to its biological characteristics, pond snail *Lymnaea stagnalis* L. (systematic classification: Mollusca, Gastropoda, Pulmonata, Basommatophora, Lymnaeidae), as well as other representatives of mollusca species, is a suitable experimental animal for fundamental neuro-biological, embryological, biochemical, endocrinological and parasitological researches and also for applied ecological research (estimation of the pollution level of the water environment based on the distribution of indicator organisms - saprobiological analysis). Marsh

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snail is a hermaphrodite, with separate male and female genital opening, but self-insemination does not occur as the egg and sperm of the same individual mature at different times.

This paper presents some biological characteristics of the marsh snail and summarize our and literature results obtained while working with this species, as with other types of the fresh water molluscals, showing that fresh water mollusca, especially marsh snails are suitable experimental models in fundamental and applied-term tests.

Maintenance under Laboratory Conditions: Suitability for experimental work stems from the relatively low maintenance requirements under laboratory conditions. The snail can be kept in glass or plastic aquarium, which can be instantaneous or aired, but not necessary. As a stenotherm species, the snail does not tolerate large variations of temperature, but can be kept at a temperature of 5-25°C. In order to control the diet, it can be fed with lettuce, which this animal consumes well. Portion size depends on the temperature at which it is held. At lower temperatures the snail eats almost nothing and pulmonary respiration replaces breathing through the skin. At higher temperatures, it consumes all the food given to it and if the dead snails are not removed from the aquarium, cannibalism is observed.

The literature lists different data on the volume of water required by an individual snail: a 10-15 ml [1], 400 ml [2], 100 ml [3] (cited. [4]); 150-170 ml [5], 500 ml [6], 160 ml [7] and 5000 ml [8].

Our results showed that if the animals were kept in non-flow-up aquariums, a large number of egg masses were deposited withim an hour of changing the water, suggesting that an (increased oxygen concentration would stimulates laying of egg masses). The possibility of ob- taining a large number of egg masses, with a large number of fertilized eggs, enables conduction of various embryonal development tests (e.g. length growth, the emergence of the bioelectrical activity of the embryonal nervous system, the influence of different environments on embryonal development, development *in vitro*, etc).

Embryonal Development, Embryonal Development under *in vitro* Conditions, Embryonal Movements (Passive and Active) and Bioelectrical Activity of the Embryonal Nervous System: Developmental threshold temperature is 12°C. Long-term effect of 37°C temperature leads to the death of embryos, whereas shorter treatment, up to 30 min, leads to the formation of abnormal embryos [9]. Head region is specially sensitive. Sensitivity of embryos to higher temperature is highest after the 3rd trench [10].

Trench in *Lymnaea* is holoblastic and spiral, with successive cleaving of deiotrop or leiotrop. First deiotrop grooving operations cover 8th degree of blastomere, but the next trenching is leiotropic, etc... [11]. Division is synchronized to the level of 256 cells (8th trench), after which the synchronization is lost.

In conditions when egg masses are kept in Petri dishes in 50 ml of tap water at a temperature of about 20°C, the embryonal development is 16 days and survival rate of the embryos is about 90% (Fig. 1A, curve 3). The presence of jelly mass (tunica interna) is necessary, because in the absence of jelly masses, the survival rate of the embryos in isolated egg membranes is about 40% (Fig. 1B, curve 3), while the absence of jelly masses has no influence on growth of the embryos in the isolated egg membranes (Fig. 2A and 2B, curve 3) [12].

Partial development of embryons under *in vitro* conditions, in artificial medium, also showed that the presence of egg membrane and perivitelline liquid in it, are necessary, whereas the results are statistically dependent on the age of embryos. Embryos were taken from the egg membrane on 6-th, 7-th and 8-th day of embryonal development. Only the embryos removed from the egg membrane on the 8-th day of embryonal development and further developed *in vitro*, reached the end of a normal embryonal development (16th day), in a high percentage (71%). Transferred to the tap water, all embryos died in the next 20 days. When extracted at 7th day of embryonic development, only 17% of embryos reached the end of the normal embryonal development (Fig. 3). Embryos of all age groups that are developing under *in vitro* conditions, had significant growth slowing compared to the group of the same aged embryos developed within isolated egg membranes and developed in artificial medium (Fig. 4) [13]. Similar outcomes, indicating the importance of the perivitelline liquid and the egg membranes were obtained during long-term culturing of decapsulated marsh snail embryos (*Helisoma trivolvis Say*) [14].

Perivitelline liquid has a nutritional role, as it contains carbohydrates and proteins [15-17]. At a very early embryonal stage, after gastrulation and loss of vitelline membrane [18], embryos start to use these substances, as partly as an energy source and partly as a building

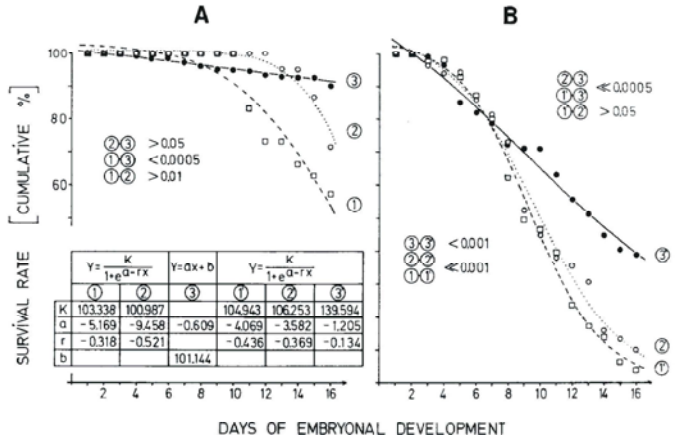

Fig. 1: Survival rates of embryos developed in intact egg masses (A) and isolated egg capsules (B). Curves 1 synthetic fresh-water (non-buffered), curves 2 synthetic fresh- water (buffered), curves 3 tap water. The initial number of embryos was 120 in each subgroup.

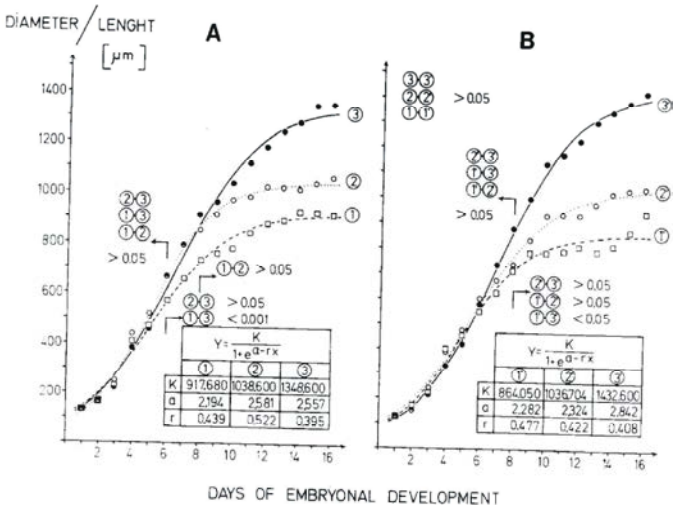

Fig. 2: Mean values of the longutudinal growth of embryos developed in intact egg masses (A) and isolated egg capsules (B). Curves 1 synthetic fresh-water (non-buffered), curves 2 synthetic fresh-water (buffered), curves 3 tap water. The initial number of embryos was 120 in each subgroup.

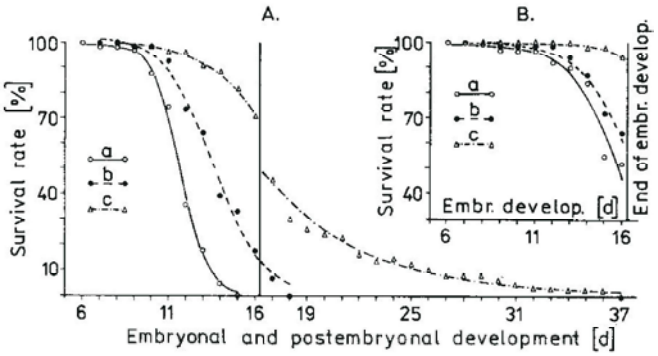

Fig. 3: Survival rates of embryos developed in the absence of the egg capsule (A) and of embryos developed in isolated egg capsule (B) from the 6th (a) (initial number of embryos was 62), 7th (b) (initial number of embryos was 64) and 8th (c) (initial number of embryos was 73) days of embryonal development in the artifitial medium.

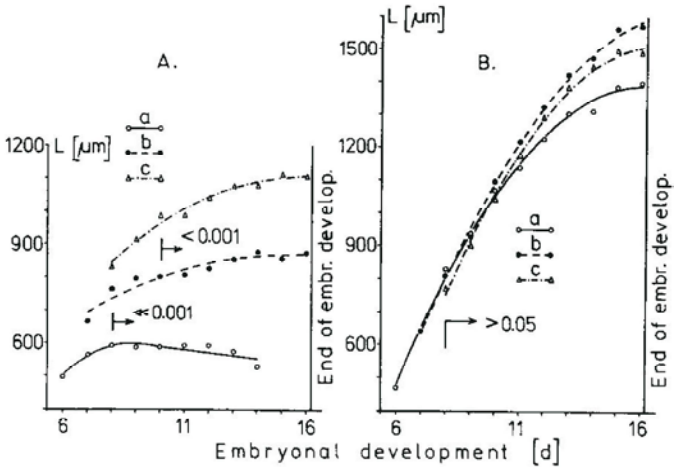

Fig. 4: Mean values of the longutudinal growth of embryos developed in the absence of the egg capsule (A) and of embryos developed in isolated egg capsule (B) from the 6th (a) (initial number of embryos was 62), 7th (b) (initial number of embryos was 64) and 8th (c) (initial number of embryos was 73) days of embryonal development in the artifitial medium.

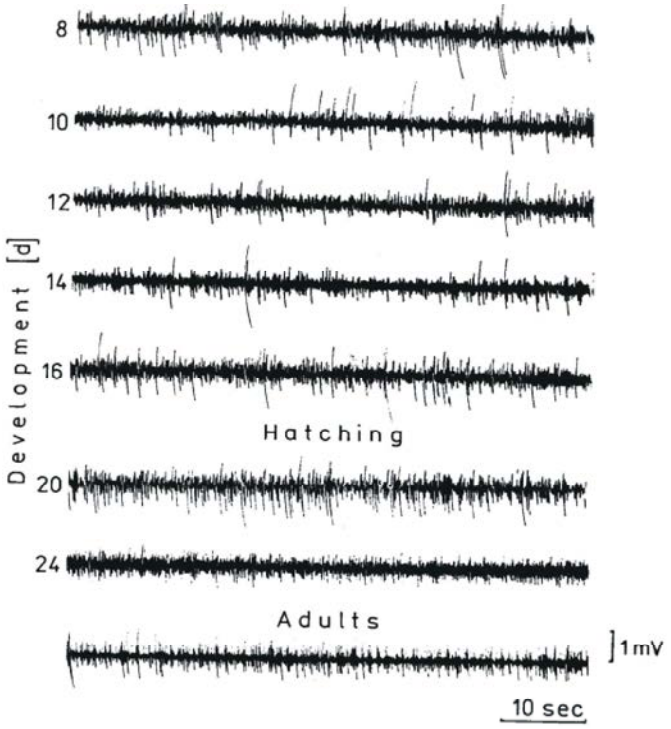

Fig. 5: Recording of the spontaneous bioelectrical activity of the pedal ganglia during embryonal development and after hatching and in adult animals of the pond snail *Lymnaea stagnalis* L.

material [19]. Early startup of using the deposits of perivitelline liquid reserves is the result of the fact that the egg *Lymnaea telolecit*, contains a small amount of vitellus in the vegetative pole, that is used up to the gastrulation phase [6].

During embryonal development, which takes place entirely inside the egg membrane, within the egg mass, it is possible to follow the longitudinal growth of the embryo and organs development. While the embryo is spherical, its diameter is measured, when bilateral

elongation of an embryo starts, longer diameter is measured, when the shell appears, the length of the shell is measured. Growth of the embryo can be traced from the fertilized egg through its first distribution, from the appearance of micromeres and macromeres and so on [6].

The elements of the nervous system are formed from a quarter of micromeres. *Lymnaea* nervous system is conceived in the form of two cephalic plates. From the cephalic plates the cerebral ganglia is formed first and then the body parts that they innervate (eyes and tentacles) [20].

During embryonal development, starting from the 8th day, it is possible to register summary spontaneous bioelectrical activity of the embryonal nervous system (pedal ganglia) (Fig. 5) [21]. Pedal ganglia can be observed on the 6-th day of embryonal development, when active movements of the embryo in the form of spontaneous light contractions and relaxations of foot basis first appear. Embryonal nervous tissue and other tissues are colourless, but the pedal ganglia can be identified by perceiving the statocysts, with white statolite. Statocyst is located near the pedal ganglia.

In further development, spontaneous contractions of the foot base gradually grow into reflex movements in the form of contractions and relaxations of the foot parts, which is increasingly emerging as a response to touching of the inner side of the egg membrane. This is the second phase in the development of movement function, so-called "active movements", when the embryo actively changes and restores the shape of the body.

"Active movements" during the development phase are preceded by the so-called "passive movements" consisting in two forms of rhythmic movement: 1) rotation of the embryo around its axis, without changing the position within the egg membrane, 2) rotation of the embryo dur- ing which the position changes. Passive movements are caused by the movements of prototro-cha, when the whole embryo moves and there is no change and the restoration of body shape. Our results show that the first form of rhythmic movement occurs around the 3rd day of embryonal development and the second form on 5th day, when it starts to lengthen bilaterally. Rotation of the embryos is influenced by complex interactions of abiotic environmental factors (the most important ones being temperature and oxygen concentration, but also pH and conductivity) [22] and is also influenced by dopamine and serotonin [23, 24].

Postembryonal Development, Longitudinal and Weight Growth, Rates of Longitudinal and Weigth Growth, Mathematical Modeling of Growth and Length-weight Relationship of Growth: Postembryonal growth was followed up to the 155-th day of development. In our experimental conditions (first 2 weeks of postembryonal development of 10 animals in Petri dishes in 50 ml of tap water and then 10 animals in 1600ml of tap water, room temperature), the survival rate is about 20% and 28% of animals reach sexual maturity at about 83-day of our postembryonal development. Literature data show that in summer season adult snails become sexually mature in less than 3 months and during the winter period in 4 months [4]. During postembryonal development, inflection point of the longitudinal growth is the 80-day (Fig. 6) and 103 day for weight growth (Fig. 7). Inflection point represents the highest gains in longitudinal and weight growth [7]. After reaching inflection points, longutudinal and weight growth will continue, but the growth rate starts decreasing to reach a final maximum possible value for the given conditions of growth (upper asymptote of mathematical models of growth). In our experimental conditions, the maximum length of shell growth was 47 mm. Under natural conditions, we found some individuals with shell-length over 60 mm (7.1% of the total number of animals collected), although the most common length of the shell is 50 to 60 mm (70.8%) [7].

Increasing rates of growth up to reaching inflection points is the result of multiplicative growth, with the number of cells increasding exponentially, leading to an increase in body size. After reaching the inflection points, multiplicative rates of growth is reduced as a consequence of desynchronization of divisions [25]. Tracking postembryonal growth of *Lymnaea stagnalis* L. under laboratory conditions enables us to observe the whole postembryonal development, because in nature one can not find a snail smaller than 20 mm. Shell length of 20-30 mm is found in 2.6% of the total number of collected animals.

Embryonal growth (longitudinal) and postembryonal growth (longitudinal and weight) are mathematically described with a sigmoidal curve. Criterion for choosing the most appropriate mathematical model of growth is the size of square error and it depend on experimental data. A variety of mathematical models describe the sigmoid growth curve: 1) different forms of logistics functions: a) 3-parameter Verhulst's function [26, 27], b) 3-parameter

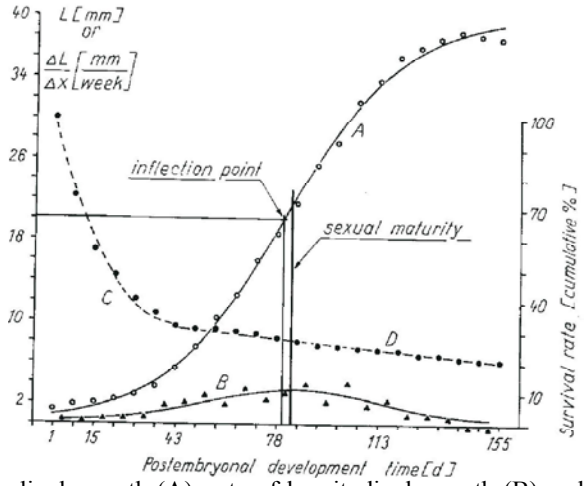

Fig. 6: Mean values of longitudinal growth (A), rate of longitudinal growth (B) and survival rate (C and D) during postembryonal development of *Lymnaea stagnalis* L. The initial number of snails was 300.

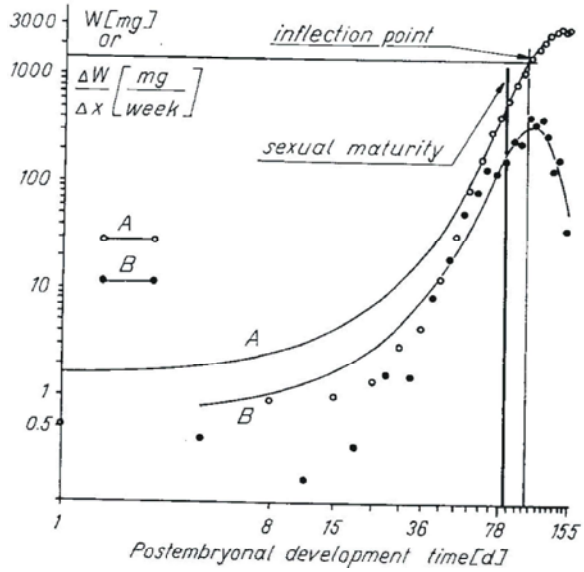

Fig. 7: Mean values of weigth growth (A) and rate of weigth growth (B) during postembryonal development of *Lymnaea stagnalis* L. The initial number of snails was 300.

Table 1: Mathematical models used for the estimation of experimental data

Model No.	Function (Y)	Name of functions
1.	$\frac{K}{1+e^{a-rx}}$	3-parameter Verhulst logistic function
2.	$\frac{K}{1+ae^{-rx}}$	3-parameter function
3.	$\frac{K}{1+10^{a+rx}}+C$	4-parameter function
4.	$\frac{K}{1+e^{a+rx+cx^2+dx^3}}$	5-parameter function
5.	$a + rx + cx^2 + dx^2$	Polynomial function
6.	$Ke^{-ae^{-rx}}$	Gompertz's function
7.	$K(1-e^{r(a-x)})$	Bertalanffy's function

function [28], c) 4-parameter function [29], d) 5- parameter function [30, 31], 2) 3-parameter polynomial functions, 3) Gompertz's function [26, 32, 33], 4) Bertalanfi's function [32] (Table 1). Their graphical presentation, together with presenting their maximal and minimal fitting values, we obtain a "corridor" of growth and its rate of growth (Figs. 8 and 9) [34]. Mathematicaly expressed, rate of growth is the first derivative of the function of growth.

In both 3- and 4-parameter functions as well as in Gompertz and Bertalanffy's functions, the parameter *K, C, a* and *r* have t he following mathematical or biological

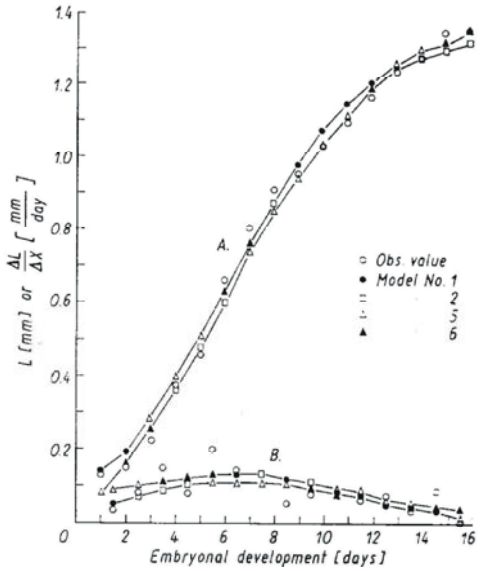

Fig. 8: The corridors of longitudinal growth (A) and rate of longitudinal growth (B) during embryonal development.

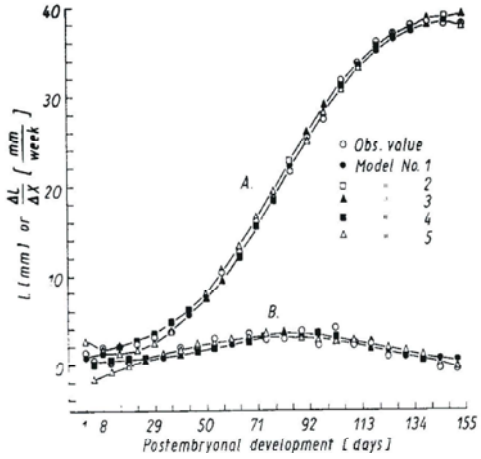

Fig. 9: The corridors of longitudinal growth (A) and rate of longitudinal growth (B) during postembryonal development.

meanings: *K* is the upper asymptote or the highest growth value, *C* is the lower asymptote, *a* is the position of the curve for x=0 and *r* is cpecific growth factor. The interpretation of the nature of polynomial function is not possible while in 5-parameter logistic function only parameter *K* has both mathematical and biological meaning (the upper asymptote or the highest growth value) [34].

The length-weight relationship is described by the exponential growth function:

$$W = aL^b \quad (1)$$

where *W* is the weight, *L* the length. Logarithm of the exponential function confers a linear dependance:

$$\log W = \log a + b \log L \quad (2)$$

which is the common form of linear equation:

$$Y = a + b X \quad (3)$$

Values of the parameters *a* (Y axis intercept) and *b* (line slope) are determined from experimental data. The parameters *a* and *b* have the following biological meaning: *a* is the density and shape of the body, *b* is the change in body shape during growth [35]. Value of the parameter *b* in our experimental conditions is 2.68 and indicates that under these conditions, the weight of growth increases proportionally slower than the longitudinal growth (negative allometry of growth, the value of the parameter *b* is less than 3.00) (Fig. 10) [7]. If the value of the parameter *b* is greater than 3.00 (positive allometry of growth) the increase in weight of growth increases proportionally faster than the longitudinal growth and the value of parameters *b* 3.00 indicates that the longitudinal growth is proportional to the longitudinal growth (isometry of growth) [33, 36]. The value of parameter *b* in some freshwater mollusca may depends on environmental factors and can have values from 2.6 to 3.2 [37] and on the degree of environmental pollution (saprobic index values of aquatic ecosystems) [38].

As the parameter *b* of the length-weight relationship of growth, after logarithmic transformation of the exponential function to a linear function, represents the slope of the linear function (i.e. regression coefficients), it is possible to compare the length-weight relationship growth by comparing the slopes using analysis of variance [39].

Neurobiological Researches: Adults of marsh snail, as well as other types of mollusca, are particularly suitable models for a variety of neurobiological researches, because of their relatively simple nervous system with the following features: the number of nerve cells is relatively limited, nerve cell bodies are lo- cated on the surface of the ganglion and have relatively large diameter and a relatively constant position in the same species, while

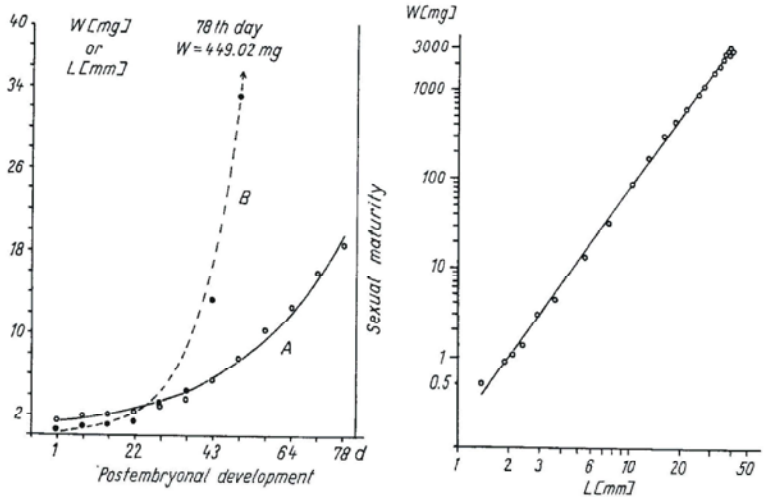

Fig. 10: Mean values of longitudinal growth (A) and weigth growth (B) during postembryonal development (from hatching to sexual maturity) and the length-weigth relationship during postembryonal development.

their axons belong to the peripheral nerves and form synapses in the interior of the ganglia. The size of the nerve cells enables electrophysiological testing, where microelectrodes are introduced into the nerve cells. The large size of the nerve cells also allows introduction of multiple microelectrodes in the same nerve cell. This further enables neuron mapping and tests can be performed continuously on the same neurons, identified neurons, with well known morphological, electrophysiological and pharmacological characteristics. Unlike neuronal bodies of other gastropods, in adult specimens of marsh snail, perikarya are pigmented. They contain two pigments: red and yellow hematoprotein, protein-carotene, found in granules in the neuronal body. The granules contain one or both pigment [40]. The importance of pigments is not known, but pigmentation of neural bodies makes them visible and suitable for identification.

Based on the electrophysiological characteristics, identified neurons can be divided into: a) neurons that do not generate action potentials spontaneously (so-called silent neurons), b) neurons with the rhythmic endogenous bioelectric activity without synaptic potentials, c) endogenously active neurons whose bioelectrical activity modifies synaptic potentials and d) spontaneously active neurons, whose action potentials cause activation of synaptic inputs [41]. There is also a categorization of neurons based on responsiveness to transmitters: acetylcholine, dopamine, norepinephrine and 5-hydroxytryptamine [42].

Table 2: Mean ± S.D. values for electrophysiological properties (Resting Membrane Potential (RMP)(mV), Amplitude of Action Potential (AP amplitude)(mV) and Frequency of Àction Pîtential (AP Frequency) (imp./sec.)

Neurons	RMP (mV)	AP Amplitude (mV)	AP Frequency (imp./sec.)
A	-34 ± 3.8 (N=79)	55 ± 5.7 (N=81)	0.5 ± 0.2 (N=59)
A1	-32 ± 4.4 (N=15)	51 ± 7.6 (N=16)	0.5 ± 0.2 (N=10)
A2	-32 ± 4.8 (N=24)	54 ± 7.1 (N=26)	0.4 ± 0.1 (N=17)
B	-32 ± 2.3 (N=22)	55 ± 4.8 (N=21)	0.4 ± 0.2 (N=18)
C	-33 ± 3.2 (N=18)	58 ± 6.3 (N=18)	0.1 ± 0.1 (N=13)
D	-33 ± 7.3 (N=23)	54 ± 2.5 (N=23)	0.6 ± 0.2 (N=17)
E	-37 ± 3.6 (N=13)	59 ± 3.1 (N=14)	0.5 ± 0.2 (N=13)
N1	-51 ± 6.5 (N=26)		
N2	-49 ± 7.5 (N=17)		

By Arambašić and Pašić [43] based on their morphological, electrophysiological (Table 2) and pharmacological properties, neurons were identified on the dorsal side of the visceral and right parietal ganglion of the pond snail *Lymnaea stagnalis* L. The total of 9 neurons were identified out of which 7 (A, A1, A2,B, C, D and E) were found to be responsible for spontaneous genesis of action potential and 2 (N1 and N2) were "silent". Among the spontaneously active neurons 4 (A, A1, B and D) were involved rhythmical genesis of action potential and the other 3 (A2, C and E) were characterized by intermittent bioelectrical activity.

Parasitological Researches: *Lymnaea stagnalis*, as well as other fresh water snails, is the first intermediate host in the developmental cycle of various species of parasitic trematodes. Animals that are taken from na- ture are

abundantly infected with redias of parasitic trematodes [44]. This does not pose a danger to persons working with *Lymnaea*, because for the complete cycle of trematodes development, it is necessary for the cerkaria to leave the redia and find a second interme diate host (eg, fish) or end-host (eg, fish, birds like waterflow or mammal), where trematodes development ends and poses as threat to humans. Literature data show that it is possible to infect a marsh snail with a parasite (*Elaphostrogylus rangiferi Mitskevich*) that does not naturally live in it. *Lymnaea stagnalis* is not a natural intermediate host; natural hosts are land snails [45]. Our results show that the first generation of animals grown in the laboratory, from egg masses of population of animals collected in nature, was not infected with parasites.

Saprobiological Analysis: Marsh snail *Lymnaea stagnalis* L. is a relatively good indicator of β-mezosaprobic zone (relatively pure water) of aquatic ecosystems, where it is most frequently found, however, but it can be also found in oligosaprobic (pure water) and α-meso-saprobic zone (contaminated water) [46]. Saprobic marsh snail has the following characteristics: the weight indicator *G* is 3 (a fairly good indicator, because it is found in 3 saprobic zones. Saprobic valences are as follows: oligosaprobic: β-mezosaprobic : α-mezosaprobic - 2:3:1), saprobic index of the species **s** 1.85, *G* 3 [47], saprobic valences oligosaprob.: β-mezosaprob. : α-mezosaprob. - 2:6:2, saprobic index *s* 2.00 [48], with 1.85, *G* 3 [49], *s* 1.9 [50], *s* 2.0 [51]. There was no information in the literature about finding *Lymnaea stagnalis* L. in xenosaprobic (very pure water) and polysaprobic (highly polluted water) areas.

Based on the presence of *Lymnaea stagnalis* L. and other mollusca indicator species [52] by using the formula:

$$S = \frac{\sum_i s_i h_i}{\sum_i h_i} \quad (4)$$

by Pantle and Buck [53], where: *s* is the saprobic index of indicator species, *h* is relative presence of the indicator species, *S* is saprobic index of aquaoes ecosystem), or expanded formula:

$$S = \frac{\sum_i s_i h_i G_i}{\sum_i h_i G_i} \quad (5)$$

[54], where: *s* is the saprobic index of indicator species, *h* is relative presence of the indicator species, *G* is indicator weight of indicator species, *S* is saprobic index of water ecosystem) one can calculate saprobic index of a water ecosystem i.e. estimate the pollution degree of a water system and rank the quality of water into one of the 7 zones (4 classes and 3 subclasses:, I-II, II, II-III, III, III-IV and IV) [55, 56]. Saprobic index of a water ecosystem can be calculated on the basis of the presence of all indicator species (plants and animals) found in the tested ecosystem, including the fresh water mollusca [57].

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