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GROWTH AND REPRODUCTION OF *Planorbarius corneus* (Linnaeus, 1758) IN LABORATORY CONDITIONS

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ABSTRACT

This study presents the results of observation on growth and reproduction of *Planorbarius corneus* (Linnaeus, 1758) (Gastropoda, Pulmonata) over the course of several years of continuous rearing in the laboratory in order to use them as test organisms for toxicity testing of chemicals. Some growth and reproduction features (shell diameter increase, fecundity, hatching time and rate, age at first reproduction, juvenile survival), which could provide more information for culturing *P. corneus* in the laboratory, are presented. The quantitative results of growth and reproduction in laboratory conditions were obtained: heterogenous growth varied between 0.1 mm and 5.3 mm in individual snails, production of 0.6 egg masses per snail/day and 11 eggs per snail/day. A statistically significant negative correlation between initial snail size and growth was noticed. In the second part of the experiment, the reproductive output of 4 isolated snails was compared to that of permanently grouped snails. As a result, 2-fold decreased growth and 4-fold decreased reproductive output in the progeny of isolated animals was noticed.

How to Cite

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INTRODUCTION

Planorbarius corneus (Linnaeus, 1758) (Gastropoda, Pulmonata) or great ramshorn is a herbivorous freshwater pulmonate snail from the family Planorbidae, spreading from central Europe to western Siberia and central Asia (Kantor et al., 2009). In the IUCN Red List of Threatened Species, *P. corneus* is classified as Least Concern because it is widespread, abundant and highly opportunistic (Seddon and Van Damme, 2011). *P. corneus* inhabits near-shore zones of water bodies (Poznanska et al., 2015), woodland ponds (Spyra and Strzelec, 2014), fish ponds (Spyra et al., 2007) and can also be frequently found in other small or shallow bodies of water like pools, swamps, canals and ditches. Its typical habitat is still or slowly flowing water rich in dense vegetation.

The shell of this species is planispiral and large, the diameter of the shell is 35 mm, the thickness of the coil is 12 mm (Kriska, 2014), but the maximum shell diameter observed in the field and in laboratory culture is 28 mm (Costil, 1994; Costil and Daguzan, 1995a), which could be a consequence of different habitats or genetic variation.

P. corneus is a simultaneous hermaphrodite. Although outcrossing is the preferred mode of reproduction, *P. corneus* can reproduce by self-fertilization. In the field, the reproduction period begins when temperatures reach 15-16°C with two reproduction periods observed, vernal and estival-autumnal, with the remark that adaptive life-cycle variations can depend on annual climate variations (Costil and Daguzan, 1995a). Extensive interpopulation differences in reproduction such as higher or lower fecundity and shell diameter at maturation can occur within freshwater snail species in general. In the laboratory, the reproduction of *P. corneus* occurs continuously throughout the year (Costil and Daguzan, 1995b). The shell growth of freshwater snails is indeterminate, i.e. it continues until death. Growth is influenced by environmental factors like food availability and temperature, and the size is positively correlated with temperature. The optimum temperature for *P. corneus* is 20°C both for growth and fecundity, and the life span observed in the field is 18 to 20 months or up to more than two years (Costil, 1994; Costil and Daguzan, 1995b).

Freshwater pulmonate snails like *P. corneus* are easily reared and handled in the laboratory. Their shell size can be measured and they produce numerous eggs on a daily basis all year round. Egg masses and the number of eggs are easy to count, the development of eggs is easy to observe and maintenance is inexpensive. In partial life-cycle reproduction, toxicity tests conducted with *P. corneus* measuring fecundity proved to be a highly sensitive parameter to evaluate the toxicity of various substances (Mazuran et al., 1999).

In 2010, the OECD (Organisation for Economic Co-operation and Development) published the Detailed Review Paper (DRP) on Molluscs Life-Cycle Toxicity Testing with the purpose of recommending toxicity testing methods with molluscs, which might be developed into OECD Guidelines for long-term toxicity tests for a regulatory assessment of the effect of chemicals. There is an increasing concern about endocrine-disrupting chemicals such as tributyltin, sex hormones, androgen antagonists and many other natural and man-made chemicals like polychlorinated biphenyls and polycyclic aromatic hydrocarbons which have severely affected molluscs worldwide and to which molluscs are very sensitive (Geiß et al., 2017). It is concluded that molluscs are probably most suitable for developing new long-term toxicity test methods that are sensitive to both endocrine disrupting and other chemicals. Another reason for attempting to develop testing guidelines for gastropods is that many are easy to culture and handle in the laboratory. Reproduction features such as fecundity, spawning time, hatching time, hatching success, juvenile mortality, growth and development should be established as primary endpoints in the environmental risk assessment because any impairment in reproductive output may have direct population-level consequences like a decline or extinction (OECD, 2010). There are three candidate test species for partial life-cycle and full life-cycle test methods recommended by OECD (gastropods *Potamopyrgus antipodarum* (Gray, 1843) and *Lymnaea stagnalis* (Linnaeus, 1758), and a bivalve *Crassostrea gigas* (Thunberg, 1793)). *Lymnaea stagnalis* reproduction test (Ducrot et al., 2014) and *Potamopyrgus antipodarum* reproduction test (Geiß et al., 2017) were validated, and *Potamopyrgus antipodarum* reproduction test was adopted as OECD Guidelines for the Testing of Chemicals in 2016 (OECD, 2016). An additional candidate for the use in standardised test protocols as a possible test species depends on whether or not a species can be easily induced to breed in the laboratory. One of the possible candidates is *P. corneus* which is easily bred in the laboratory, but there is no significant information on culture and laboratory testing available (OECD, 2010). Therefore, studies on laboratory cultures of candidate species like *P. corneus* could provide data for breeding and maintenance conditions. *P. corneus* and other freshwater snails have been used for decades in ecotoxicological research but mainly on specimens collected shortly prior to the experiments without laboratory culturing (Klobučar et al., 1997, 2001; Lajtner et al., 1996, 2003; Otludil et al., 2004; Pavlica et al., 2000; Špoljar et al., 2005).

The aim of this paper is to present the results of analyses on the reproduction and growth of *P. corneus* over the course of several years of continuous rearing in the laboratory, which was conducted in order to use them as a test organism for partial life-cycle and full life-cycle toxicity testing (Mazuran et al., 1999). The quantitative results of reproduction and growth in laboratory conditions are presented. The results were compared with literature results and could be useful in providing more information for laboratory rearing and research of *P. corneus* and in involving *P. corneus* in water quality assessments.

MATERIALS AND METHODS

The laboratory culture of *P. corneus* originated from ponds near the River Sava and was maintained continuously under laboratory conditions for five years. Eighty adult snails were collected from several ponds and our laboratory culture was started from the eggs they laid. The snails were reared in 35 L culturing tanks under natural photoperiod in dechlorinated tap water, containing 80 mg/L Ca^{2+} and 35mg/L Cl^{-}. Measured environmental conditions were as follows: conductivity 740 µS/cm, pH 6.8-7.3, temperature 23°C. In the breeding tank, one-half of the water was renewed weekly with already prepared dechlorinated tap water. The snails were fed daily on washed organic soft lettuce leaves ad libitum. Snails produced a constant number of egg masses in laboratory conditions throughout the year. 24 h old egg masses were occasionally placed into several 10 L tanks for the observation of hatching time and success, growth dynamics of the hatched snails and age at first reproduction. Culturing conditions were the same as in the breeding 35 L tank. From these tanks, with snails of equal age, equally sized individuals were selected for the experiments. The initial size of the groups of snails taken for the experiments ranged from 7 to 17 mm because mortality in that range never exceeded 10%, while increasing mortality was noticed with age and size for snails of shell diameter larger than 20 mm.

The snails assessed for growth and reproduction were placed into 36 differently sized groups. For each of 36 groups, a set of 20 equally sized and aged snails were used (720 specimens in total), each set placed in two 1000 mL glass beakers (10 snails/beaker). The observations lasted for six weeks. The water, feeding and other conditions were identical to those in the culturing tanks and water in the beakers was completely renewed weekly. Measurements of the shells were taken at the beginning of the experiments and at the end, after six weeks. The maximum diameter of the shell (width from the edge of the peristome across the umbilicus to the opposite periphery of the shell) was measured to 0.1 mm with callipers. Each week the egg masses attached to the surface of the beakers and to the lettuce leaves were removed and counted. Egg masses without eggs or with just a few eggs were rare and were not counted.

In the second part of the experiment, the reproductive output of 4 isolated snails was compared to that of permanently grouped snails from the experiment described above under the same laboratory conditions. Four 6 mm long snails (named B, J, N T by choice) were isolated from laboratory culture in four separate 1000 mL beakers. We wanted to prevent them from group mating and expected that most of the produced eggs would be the result of selfing. When they started spawning, egg masses were collected for three weeks. The collected egg masses were transferred to separate tanks, the eggs were counted and hatched snails were observed until the adult age. The four groups of adult offspring (B, J, N, T) were divided by size into subgroups of 10 equally sized siblings and each subgroup was placed in a 1000 mL beaker and kept under the same conditions as described above. The reproductive output and growth were observed for six weeks and compared with the growth and reproduction of 36 groups of 20 snails described above.

Statistical analyses were performed by Statistica 12.0 and set to $p<0.05$ (StatSoft, Inc., USA). Prior to the tests, all the data were normalized by log-transformation. The normality of the data was tested by Shapiro–Wilk's W test. Homogeneity of variance for each variable was tested by Levene's Test. The possible difference in the mean values of the growth rate parameters and the number of egg masses among the groups and subcategories was assessed by one-way ANOVA, followed by Newman-Keuls post-hoc comparison test. Regression analysis was used for the assessment of the influence of the initial diameter on the percentage of diameter increase. To assess the dependence of growth rate on initial shell diameter, the shell growth results were divided into four subcategories: A (7 - 7.9 mm), B (8 - 9.9 mm), C (10 - 13 mm) and D (14 - 17 mm).

RESULTS

Growth rate

The initial shell diameter of 36 groups of 20 snails ranged from 7 to 17 mm, and the final diameter after 6 weeks of growing ranged between 8 and 19 mm. The mean shell diameter increase of all the groups of snails ranged from 0.4 to 4.6 mm in six weeks and the shell increase of individual snails ranged from 0.1 to 5.3 mm. The percentage of diameter increase during six weeks varied among groups between 2% and 37% (19% ± 10%), depending on the initial size. The simple bivariate correlation between the initial and final diameter as well as between the initial diameter and the percentage of diameter increase (Fig. 1A, B) showed a high, statistically significant negative correlation between the initial diameter and the percentage of the diameter increase ($R= - 0.85$; $p<0.0000$). Those results confirmed the statistically significant influence of the initial shell diameter on their growth rate.

The mean values and standard deviations for the three variables and four subcategories depending on the initial diameter are presented in Fig. 2. The snails from the category of the lowest initial diameter showed the highest percentage of diameter increase, which ranged between 21% and 37% (31% ± 4%), while just the opposite was found in the case of the subcategory of the highest initial diameter where the percentage of diameter increase varied between 2% and 9% (5% ± 3%). The results suggested that the shorter the initial diameter, the higher the growth rate of snails.

The analysis of variance showed statistically significant variance and the results of the Newman-Keuls test showed a statistically significant difference between all pairs of subcategories except between subcategories B and C (Fig. 2).

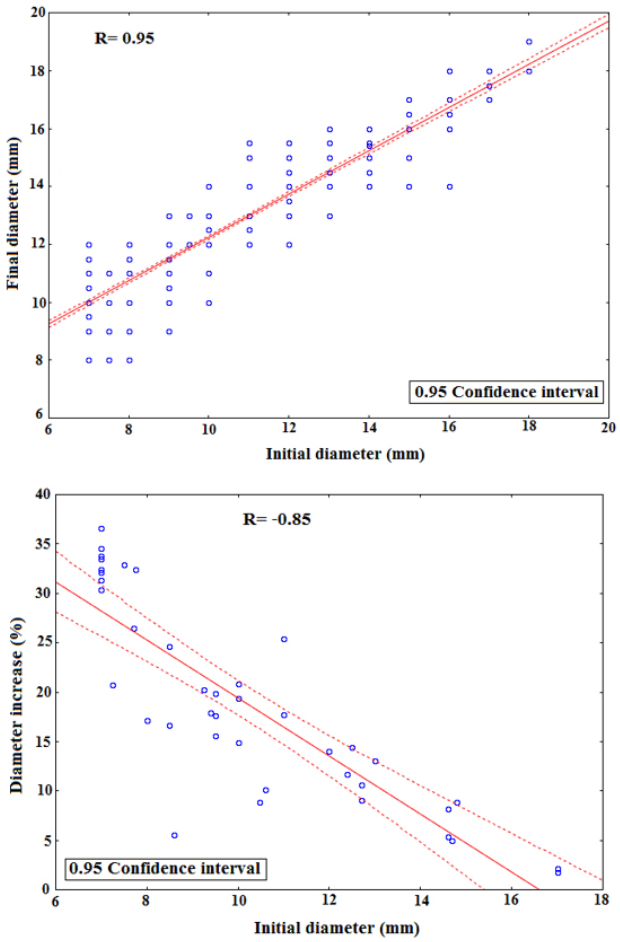

Fig 1. Results of bivariate correlation between the variables. A) the initial and the final shell diameter; B) the initial diameter and the percentage of diameter increase during six weeks of growing under the same conditions

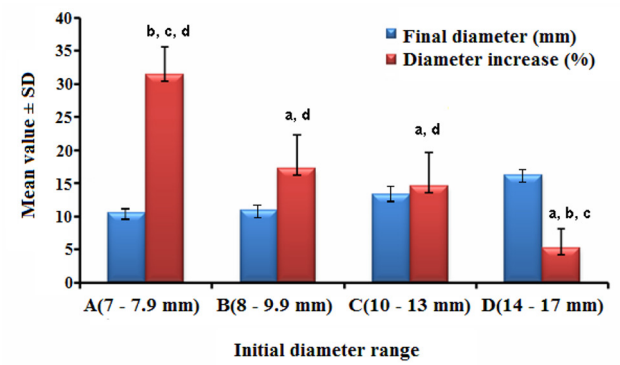

Fig 2. The mean values and standard deviations for the final shell diameter in mm and the percentage of the diameter increase during six weeks of growing under the same conditions. A, B, C, D = initial shell diameter: A (7-7.9 mm), B (8-9.9 mm), C (10-13 mm), D (14-17 mm)

Reproduction

Snails from our laboratory culture sexually matured (produced their first egg masses) at the age of 14 weeks when their shell diameter was between 7 and 8 mm (Table 1). The mean reproductive output of 36 groups of 20 snails after six weeks of rearing under the same conditions ranged from 186 – 311 egg masses (244.86±40.54). The mean weekly number of egg masses per snail after six weeks of observation ranged from 3.89±5.02 to 4.33±5.1, with an average of 4.12±5.1, 0.6±0.7 per snail/day (Table 1). The hatching rate of laboratory-reared snails was very high (97%±4) and the most of young snails always hatched after 14 days. Only a small number left egg masses within the next seven days (Table 1).

Table 1. *P. corneus* main reproduction features in laboratory conditions at 23°C

Age at first reproduction (weeks)	14 - 15
Mean shell diameter at the onset of sexual maturity (mm)	7.5 ± 0.5
Mean number of egg masses per snail / per week	4.1 ± 0.5
Mean number of egg masses per snail / per day	0.6 ± 0.7
Mean number of eggs per egg mass	22.4 ± 3.9
Mean number of eggs per snail / per day	11 ± 2
Mean hatching rate (%)	97 ± 4
Mean hatching time (days)	14 - 21
Juvenile survivorship (%)	80 - 90

The connection of the number of egg masses with the initial diameter and the percentage of diameter increase was analysed, but the results of the analysis of variance and Newman-Keuls showed no significant difference among those variables.

Survey of isolated snails' offspring

In the second part of the experiment, four isolated snails B, J, N and T produced a similar number of eggs in three weeks: snail B produced 229 eggs, snail J produced 234 eggs, snail N produced 220 eggs and snail T produced 232 eggs (228.7±6.2; 10.89 ±0.29 eggs per snail/day). The hatching rate was 90% and juvenile survival was 98%. The basic statistical parameters for the growth rate, expressed as the final diameter after six weeks of growing as well as the percentage of the diameter increase, and for the egg mass production during the same growing period for four different groups of the snails, divided into subgroups by size, are presented in Table 2. In groups B and J, the similar values of the percentage of diameter increase were found, while groups N and T showed a significantly lower growth rate for all subcategories compared to the first two groups (Table 2).

Table 2. Study of isolated snails' offspring. The final shell diameter after six weeks and the percentage of diameter increase depending on the group of snails (B, J, N, T) and on the initial shell diameter; the number of egg masses in each group of snails (B, J, N, T) depending on initial shell diameter; X-mean value; SD-standard deviation; N-number of sets of 10 snails; a, b, c, d - significant difference (one-way ANOVA followed by Newman-Keuls post-hoc comparison test, $p<0.05$) among the size categories of the same group; *-significant difference in diameter increase between B and J compared to N and T (one-way ANOVA followed by Newman-Keuls post-hoc comparison test, $p<0.05$)

Group	N	Initial diameter (mm)	Final diameter (mm) X±SD	Diameter increase (%) X±SD	Number of egg masses X±SD
* B	6	< 8 (a)	8.2±0.5	14.3±5.5	60.7±15.9 b
		8 (b)	9.3±0.9	13.3±8.2	90.5±20.5 a
		9.5 (c)	11.2±1.3	14.3±9.7	80.0±0.0
* J	6	< 8 (a)	8.4±0.6	16.0±6.0	77.0±3.6
		8 (b)	9.3±0.8	13.0±7.6 c	95.0±0.0
		8.5 (c)	10.2±0.7	16.3±5.9	84.0±0.0
		9.5 (d)	11.5±2.2	18.3±14.1 b	78.0±0.0
N	7	< 8 (a)	7.8±0.5	10.1±5.3 b, c	36.8±6.9 c
		8 (b)	8.4±0.5	4.4±5.0 a	50.5±0.7
		9.5 (c)	9.8±0.3	1.3±2.5 a	65.0±0.0 a
T	16	< 8 (a)	7.7±0.6	8.2±6.6	40.0±20.7 c, d
		8 (b)	8.6±0.5	7.1±5.5	59.3±5.1 c, d
		9-(c)	9.9±0.7	8.9±5.6	110.3±18.9 a, b
		10 (d)	10.4±6.2	6.9±5.2	112.8±7.1 a, b

The reproduction results showed a lower number of egg masses in groups N and T compared to the first two groups, with the exception in subcategories 9 and 10 mm in group T. Significant differences in growth and reproduction were also noticed among some groups of siblings of different initial sizes (Table 2). When comparing the results of the study on isolated snails' offspring with the study conducted on 36 groups of snails (Table 3), a significant difference was found for both the growth rate and the number of egg masses. When the snails of a similar initial diameter were compared, the results of the study on isolated snails' offspring showed a two-fold lower percentage of the diameter increase, while the number of egg masses was approximately four-fold lower compared to 36 groups of snails study.

Table 3. Comparison of the growth rate and number of egg masses of the snails during six weeks of growing under the same environmental and feeding conditions from both parts of the study; X-mean value; SD-standard deviation; *(t-test, $p<0.05$)

Category	Isolated snails' offspring		36 groups of snails	
	Diameter increase (%) X±SD	No. of egg masses X±SD	Diameter increase (%) X±SD	No. of egg masses X±SD
<8 mm	11.6±6.6*	51.4±20.7*	31.5±4.2	226.6±46.0
8-10 mm	9.2±8.1*	73.9±16.2*	17.2±5.1	252.7±40.5

DISCUSSION

An ideal test species for a reproduction toxicity test should exhibit a steady reproductive output in laboratory conditions throughout the year and reproduction endpoints should be easy to measure. Our *P. corneus* culture continuously produced an average of 4 egg masses weekly per snail at 23°C, which is 0.6 per snail/day or 11 eggs per snail/day with an average of 22.4 eggs per egg mass. Other studies on *P. corneus* reported similar results: mean egg mass number of 0.9 weekly per snail and maximum egg mass number of 8.5 in two weeks per snail at 20°C (Costil and Daguzan, 1995b); one egg mass per snail/day and 13.12 eggs per snail/day at 25°C (Parveen et al., 2019). The number of eggs and egg masses among laboratory cultures can differ as a result of different origin of populations of snails and also different culturing conditions. Differences in numbers of eggs and egg masses, which can vary considerably even between individuals of the same species, are a consequence of inherent variability in molluscs (OECD, 2010).

Egg production features found in our population of *P. corneus* showed to be also very similar to those observed in other species of the Planorbidae family. For instance, Norton and Newman (2016) reported approximately one egg mass per day per snail and on average 22.9 eggs in egg mass in *Helisoma trivolvis* (Say, 1817), but the number of eggs per mass was much greater than found in the previous study (Norton and Bronson, 2006), which was explained by differences in the origin of the snail population. At temperature between 20 – 25°C, the mean age at onset of sexual maturity was 17 and 15 weeks at minimum shell diameters of 7.6 mm and 7.8 mm, respectively, with a 94% hatching rate (Costil and Daguzan, 1995b), which agrees with our findings at 23°C (14 weeks at 7.5 mm, hatching rate 97%).

Our observations on the growth of 36 differently sized groups of 20 snails growing under identical conditions during six weeks showed that growth was very variable among groups, depending on initial size (0.4 – 4.6 mm) and also among individuals of the same sized groups (0.1 – 5.3 mm). The snails with the lowest initial shell size showed the highest growth rate (approximately 3 mm or 31% in six weeks, 0.5 mm weekly), while the snails with the highest initial size grew 0.8 mm or 5% in six weeks, 0.13 mm weekly. Both results are comparable to values recorded by Moriarty (1978) and Costil (1994). Moriarty (1978) found that the mean rate of shell increase in *P. corneus* at 20°C was 0.125 mm per day (which is approximately 0.9 mm per week) and that growth rates were significantly higher for snails kept in separate jars. Costil (1994) found that the mean growth increase in two weeks at 20°C in *P. corneus* was 0.29 mm (which is 0.14 per week) and 0.51 mm in two weeks at 25°C. Some authors found a negative relationship between the growth of freshwater snails and their reproductive output (Norton and Bronson, 2006; Norton and Newman, 2016) but it was not observed in our *P. corneus* laboratory culture.

The study of isolated snails' offspring showed differences among and inside groups, which revealed heterogeneity of growth and reproduction: N and T groups had significantly lower growth than B and J groups, and there were significant differences in growth and egg mass production among some groups of siblings of different initial sizes. Two-fold reduced growth and 4-fold reduced fecundity were noticed in comparison to the laboratory population from which the isolated parents and 36 observed groups originated. Slower growth led to slower maturation, i.e. longer time to achieve sexual maturity. Puurtinen et al. (2004) demonstrated the connection between low genetic variability and slow maturation and low fecundity by measuring genetic variability in natural populations of basommatophoran snail *Lymnaea stagnalis*. The decrease in fecundity between the least and the most fecund population was dramatic: 5.14 times.

The laboratory population of snails reared for several years without renewing the stock can be viewed as a genetically isolated population with possible frequent mating among relatives and thus with less genetic variability. Four snails isolated at an early age a few weeks before the beginning of spawning probably did not have the opportunity to mate with multiple partners and possibly self-fertilised, which resulted in less variable offspring with decreased growth, maturation and fecundity.

P. corneus was easily bred and handled in the laboratory at room temperature at low cost. The growth rate in 36 groups was very variable among individuals and significantly higher in snails with the lowest initial diameter than those with the highest initial diameter. It produced a large and steady number of countable egg masses and eggs continuously throughout the year, irrespective of the season (11 eggs per snail/day). It was easy to observe and measure the development of eggs and hatchlings. Isolated animals, which possibly self-fertilized, and with possibly less genetic variability produced progeny with decreased growth, maturation and fecundity. Based on the criterion of whether or not a candidate species for partial and full life-cycle toxicity assessment can be easily induced to breed in the laboratory (OECD, 2010), *P. corneus* proved to be a possible good test species for long-term reproduction toxicity testing.

RAST I RAZMNOŽAVANJE VRSTE *Planorbarius corneus* (Linnaeus, 1758) U LABORATORIJSKIM UVJETIMA

SAŽETAK

Ovo istraživanje obuhvaća rezultate istraživanja rasta i razmnožavanja vrste *Planorbarius corneus* (Linnaeus, 1758) (Gastropoda, Pulmonata) tijekom višegodišnjeg

kontinuiranog uzgoja u laboratoriju u svrhu njihovog korištenja kao test organizama za testiranje toksičnosti kemikalija. Prikazane su neke značajke rasta i razmnožavanja (rast kućice, plodnost, vrijeme izlijeganja i brojnost izleženih puževa, vrijeme početka razmnožavanja, preživljavanje mladih) koje bi mogle pružiti više informacija za laboratorijski uzgoj vrste *P. corneus*. U laboratorijskim uvjetima dobiveni su kvantitativni rezultati rasta i razmnožavanja: heterogeni rast varirao je između 0.1 mm i 5.3 mm u pojedinih puževa; proizvodnja od 0.6 mrijestova po pužu dnevno i 11 jaja po pužu dnevno. Uočena je statistički značajna negativna korelacija između početne veličine puževa i rasta. U drugom dijelu pokusa četiri puža su izolirana iz laboratorijske kulture kako bi se usporedila njihova reprodukcija s puževima u skupinama. Uočeno je dvostruko smanjenje rasta i četverostruko smanjenje reproduktivnog učinka u potomstvu izoliranih životinja.

Ključne riječi: laboratorijska kultura, proizvodnja jaja, dinamika rasta, ponašanje potomaka izoliranih puževa

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