LIFE CYCLE AND GROWTH OF *BULGARICA CANA* (HELD, 1836) UNDER LABORATORY AND NATURAL CONDITIONS

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Abstract *Members of the family Clausiliidae have relatively uniform shell structure, but are highly diverse in terms of life strategies. However, the life cycles of the majority of clausiliids are still unknown. One of the poorly known species is* Bulgarica cana *(Held, 1836), a species listed on the national Red Lists in 10 European countries.*

The life cycle of B. cana *was studied on laboratory cultures between the years 2004 and 2010. The growth of this species was observed under laboratory and natural conditions. Under laboratory conditions, the hatching success was 86.3%. The eggs of* B. cana *are resistant to short periods of adverse environmental conditions, i.e. drying out or flooding.*

The species is oviparous. Its eggs are oval in shape, less often spherical, their size depends on the parent's size; the mean number of eggs per batch is 8.4 (SD=3.97). Mean incubation time at room temperature is 16.2 days (SD=3.09).

Individuals that completed shell building were considered adults, while the others as juveniles. Hatchling shells have a mean of 2.63 whorls (SD=0.32), and adult shells 12.42 whorls (SD=0.49). In the laboratory, growth to adult size took on average 6.8 months (SD=2.1). Mean growth rate was 1.6 whorls/month (from 0.75 to 2.67 depending on the individual). Various shell deformations were observed in 12.8% of the individuals. Juvenile mortality in the laboratory was 32.1%, adult mortality in consecutive years of life ranged from 7 to 32%. Under natural conditions, juveniles grew (built their shells) only during the four warmest months of the year, namely from June to September. Mean growth rate in summer was 0.71 whorl/month. Yearly growth rate was 0.21 whorl/month. At this rate, shell growth completion took at least 40 months (more than 3 years).

Sexual maturation was delayed in relation to shell growth completion by at least 3.5 months. The longest observed reproductive period in the laboratory was more than 4 years. Snail pairs produced on average 60.74 eggs per year. Bulgarica cana *is a long-lived species; its maximum lifespan both under laboratory and natural conditions was 9 years.*

Key words *Life history, Gastropoda, doorsnails, Clausiliidae, terrestrial molluscs*

INTRODUCTION

The clausiliids (family Clausiliidae) form a monophyletic, species-rich group, with 1,278 living and 156 extinct species; about 50 species have yet to be described (Nordsieck, 2007). They usually have a sinistral, fusiform shell, with the closing apparatus consisting of a movable clausilium and folds restricting its movement (Likharev, 1962; Nordsieck, 2007). Despite the relatively uniform structure of the shells and the reproductive system (Likharev, 1962; Nordsieck, 2007), clausiliids are very diverse in terms of life strategies and as such are particularly good objects to study the evolution of these strategies. Although in the last 10 years, there has been a significant progress in research on their life cycles (e.g. Maltz & Sulikowska-Drozd, 2008; 2010; 2011; 2014; Maltz & Pokryszko, 2009; Sulikowska-Drozd & Maltz, 2012a,b; Sulikowska-Drozd, Maltz, & Stachyra, 2012), the vast majority of clausiliid species remain completely unknown in this respect.

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The species *Bulgarica cana* was originally described by Held (1836) in the genus *Clausilia*. According to the current taxonomy (Bouchet, Rocroi, Hausdorf, Kaim, Kano, Nützel, Parkhaev, Schrödl & Strong, 2017) it belongs to the subfamily Clausiliinae, tribe Baleini, genus *Bulgarica* and subgenus *Strigilecula*. *Bulgarica cana* is a European species occurring in the central and eastern parts of the continent (Likharev, 1962; Kerney, Cameron & Jungbluth, 1983; Welter-Schultes, 2012; Marzec, 2017). It inhabits moist deciduous and mixed forests in the mountains and foothills, less frequently lowland forests (Likharev, 1962; Kerney *et al.*, 1983; Horsák, Juřičková & Picka, 2013), with a strong preference for well-preserved and undisturbed tree stands. It is a strictly dendrophilous species (Horsák *et al.*, 2013), living on trunks and in bark crevices of standing or fallen trees, less frequently found in the litter (Likharev, 1962; Kerney *et al.*, 1983; Sulikowska-Drozd, 2005; Horsák *et al.*, 2013).

In many countries, *Bulgarica cana* is listed on the national Red Lists, where it has obtained the following categories of threat (according to the

IUCN classification): CR – Austria (Reischütz & Reischütz, 2007) and Switzerland (Rüetschi, Stucki, Müller, Vicentini & Claude, 2012); EN – Czech Republic (Beran, Juřičková & Horsák, 2005), Germany (Jungbluth & Von Knorre, 2009) and Finland (Rassi, Hyvärinen, Juslén & Mannerkoski, 2010); VU – Sweden (Gärdenfors, 2000) and Slovakia (Šteffek & Vavrová, 2006); LC – Ukraine (Balashov, 2016); DD – Estonia (Red Data Book of Estonia, 2008) and Norway (Kålås, Viken & og Bakken, 2006). In Latvia, *Bulgarica cana* is protected by law (Pilāte, 2003); additionally, it is listed in the category of threat 3 – rare (Spuris, 1998). In other countries, this species is not considered as threatened.

The biology of *Bulgarica cana* has only been studied fragmentarily: its breeding biology (Mamatkulov, 2005; 2007) and habitat requirements (Sulikowska-Drozd, 2005) are partly known. In this context, the aim of my research was to obtain detailed knowledge about the life cycle of this species.

Material and Methods

Laboratory culture

The initial material for the laboratory culture consisted of 52 adult specimens collected from two natural sites in the Romincka Forest (NE Poland: N54°19.554 'E22°41.313' and N54°20.72' E22°32.597'). Snails were kept at room temperature (ranging from 16°C in winter to 25°C in summer) and at a natural light regime (disturbed during winter by artificial lighting while working in laboratory rooms). All specimens were kept in plastic semi-transparent containers with a capacity of 0.5 or 1.2 litres. The containers were lined with lignin soaked in water, which provided a constant, high humidity. The slots in the cover allowed air exchange. Bark pieces of deciduous trees were placed in each container as a shelter and source of food (algae, fungi and microorganisms inhabiting the wet bark). Bark from dead trees was collected in the Romincka Forest, dried in the sun to eliminate most of the hiding organisms (also eggs) and stored in airy sacks. Before being placed in the containers, the bark was soaked in cold water for about 30 minutes. Every 10 days, all the lignin and bark was replaced and the containers were cleaned with dish washing detergent. Snails from each container were checked and observed at different intervals depending on the specific experimental requirements, at least every 10 days.

Freshly laid eggs were transferred to separate containers with the bark pieces on which they were laid. Eggs were observed every 1–2 days. The *Bulgarica cana* juvenile period was observed on 358 individuals that hatched from eggs under laboratory conditions. In a single container (0.5 l), there were 4 to 51 juveniles of the same age. The size of the individuals, expressed in the number of whorls, was determined every 10 days. In the case of 18 individuals, the height of the shells was also measured.

Bulgarica cana is a land snail with determinate growth. The fully developed shell has a fusiform shape, tapering at both ends and diamond-shaped aperture with a broad white lip and clearly visible two (inferior and superior) lamellae. Individuals that completed shell building and displayed these characteristics were considered adults and transferred to new containers. Adults were kept in pairs. To check the ability of self-fertilisation, selected specimens (n=5) were kept individually from the early stages of development (unfinished shell) until death. Data on 182 specimens that obtained a typical shell structure were used for most analyses of the growth period and growth rate.

Observations requiring magnification were made using a PZO Warszawa stereoscopic microscope with variable magnification: 4, 6.3, 10, 15, and 25 times. Measurements were made using callipers or a measuring eyepiece with an accuracy of 0.05mm.

In the years 2005–2009, in the winter period, i.e. from November to March or April, some of the individuals were moved into a cellar located in the forest to imitate winter conditions in nature. Snails were placed in a 10-litre bucket with a thick layer of soil and sand and a large number of soft and wet pieces of deciduous tree bark. The bucket was covered with cloth, which allowed air circulation, but prevented the snails from escaping. During the harsh winter of 2005/2006, the cellar was frozen, and the frost also persisted in the bucket with snails. This was confirmed by frozen bark pieces and pieces of ice that were found there on 4.03.2006 (the end of the winter season). The actual temperature in the bucket with snails was measured only in the winter of 2008/2009: mean temperature was 4.676°C (range: 1.26–10.76°C) and mean humidity was 99.23% (range:

95.2–100%). Measurements were made every hour using the HOBO Pro v2 U23–001 automatic temperature and humidity recorder.

Laboratory cultivation of *B. cana* was carried out from October 5, 2004, to March 29, 2010. At the end of the breeding experiment, most of the snails were moved into their natural habitat to the sites where the founders of the culture came from. The oldest individuals hatched in the laboratory were kept until their natural death. The last snail died in 2014.

For statistical analyses, i.e. univariate analyses, average values comparison and covariates comparison, the PAST program was used (Hammer, Harper & Ryan, 2001).

Field observations

Field observations were carried out in the Romincka Forest. It is a compact forest covering an area of about 360km^2, situated east of the town of Gołdap (54°18'22''N 22°18'13''E) on both sides of the Polish–Russian (Kaliningrad District) border. Romincka Forest is a part of the East European Lowland and belongs to the Lithuanian Lakeland macroregion (Kondracki, 2000). In the geobotanical division of Poland, Romincka Forest belongs to the northern Section, Augustowski-Suwalski land (Matuszkiewicz, 2007). This area is characterised by the most severe climate in the Polish lowlands, with the lowest average annual temperature (6.2°C), the lowest average temperature in February (–5.5°C), the lowest number of days per year with an average temperature over 0°C (247 days), the shortest growing season: number of days per year with an average temperature over 5°C (194 days), the highest number of days per year with a maximum temperature under 0°C (66 days), the highest number of days per year with a minimum temperature under –10°C (36 days), and the highest number of days per year with snow cover (100 days) (Matuszkiewicz, 2007). Among the forest types, the typical dry-ground forest (Tilio-Carpinetum) with a large amount of spruce dominates. There are also many patches of mixed forests and wet or swampy forests. As a result of forest management, forest types are not always equivalent to habitat types. In the Romincka Primeval Forest, spruce (41%), oak (22%) and pine (20%) dominate; birch accounts for 8%, alder for 7%, other species (including ash, hornbeam, elm, linden, poplar, willow, larch) for 2%. The average age of forest stands is about 50 years. The Romincka Forest is protected as a Natura 2000 site (PLH280005) and in the scope of the Romincka Forest Landscape Park. The forest contains a rich land snail fauna (60 species) with 12 clausiliid species (Marzec, 2010).

The research area (1,600m^2) was located in the valley of the Duży Budier stream (54°19,554'N 22°41,313'E) in an alder-ash forest (Fraxino-Alnetum). Alder at the age of about 60 years dominates there, interspersed with birch, spruce and oak, with hazel as an undergrowth. The ground flora is partly dense and dominated by nettle (*Urtica dioica*); growth in the shade of trees is considerably poorer. Specimens of *B. cana* were marked individually. Three coloured stripes (encoded numbers) were painted with nail varnish on the shells of the snails. From 2005–2008, 809 adults were marked, of which 485 were recaptured (results concerning the activity of individually marked snails will be published elsewhere). In 2007–2008, 100 juveniles with 6.8 to 11.3 whorls were marked, of which 46 were recaptured. At each capturing, the number of whorls was determined and marked.

RESULTS

Eggs, incubation period & hatching success

Bulgarica cana is oviparous. Its freshly laid eggs are snow-white and opaque, becoming darker during the incubation period. Eggs are partly calcified, with numerous calcium carbonate crystals densely packed in a gelatinous suspension; this ensures durability and elasticity. Egg shape is usually oval but sometimes spherical (7.6% of 79 eggs). The size of eggs depends on the size of the parent. Eggs laid by larger specimens raised in the laboratory were longer than eggs laid by smaller, wild specimens (i.e. individuals collected in natural conditions as adults), while the width of the eggs was the same. As a result, the egg shape was different – more elongated eggs were laid by bred specimens (Table 1).

Eggs are laid in batches; the mean number of eggs per batch is 8.4 (SD=3.97) (Fig. 1). Within a batch, the eggs are gently stuck together via a mucus, but they can be easily separated without any damage. Single eggs are laid rarely (0.7% of 2,190 eggs). In the laboratory culture, the eggs were always laid in damp, drought-protected places. Among the 334 batches, most were

Table 1 Relationship between egg and adult size. Measured adults were not parents of these eggs, but belonged to the same population of wild or bred snails as real parents.

	Adults collected at natural sites		Adults raised in laboratory culture		Comparison of average values (mean or median)*	
	mean	range	mean	range	t or U	P
EGGS, n:	33		46			
Egg length (major diameter) [mm]	1.61	1.35–2.0	1.79	1.5–2.1	t=5.52	P<0.001
eggs width (minor diameter) [mm]	1.43	1.1–1.75	1.46	1.25–1.7	t=1.52	P=0.13
Egg slenderness [length/width]	1.13 (Me 1.11)	1–1.45	1.23 (Me 1.21)	1–1.68	U=386	P<0.0001
ADULTS, n:	38		45			
Shell height [mm]	16.08	15.02–18.26	17.51	15.3–19.5	t=-7.48	P<0.00001
Shell width [mm]	3.66	3.4–3.87	3.60	3.3–3.9	t=2.08	P=0.04
Mouth height [mm]	3.49	3.2–3.9	3.54	3.2–4.1	t=-1.41	P=0.16
Mouth width [mm]	2.46	2.2–2.71	2.48	2.3–2.7	t=-0.51	P=0.61
n of whorls	11.77	11–12.4	12.87	12–14.2	t=-10.17	P<0.00001
RELATIVE EGG SIZE (mean egg length/mean shell height)	0.100		0.102			

*The Student's t-test, preceded by the F-test for equality of variance, was used for mean comparisons. If the normality of the distributions was not confirmed (at least by Shapiro-Wilk or Jarque-Ber tests), the Mann-Whitney U test, comparing differences in medians, was used.

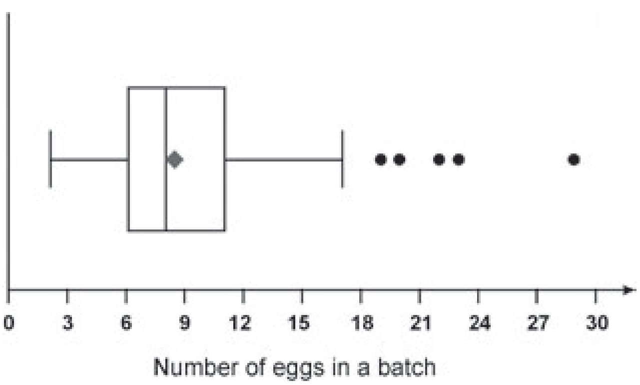


Figure 1 Size of batches laid by snails kept in pairs; quarter spread; diamond – mean value, circle – outliers; n=260 batches (2,174 eggs)

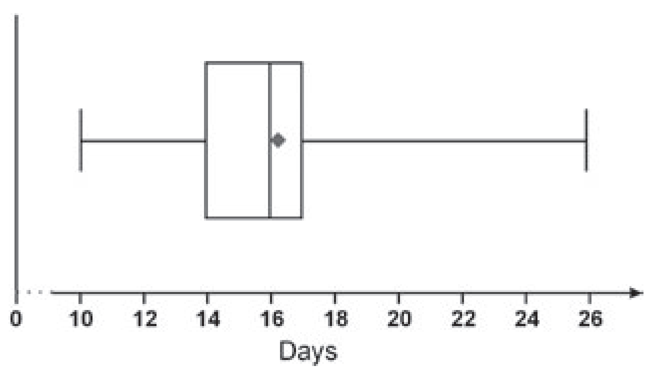


Figure 2 Period of egg incubation under laboratory conditions; quarter spread; diamond – mean value; n=187 eggs

placed between bark pieces (96.7%), while others were laid on the lignin below the bark (2.1%) or wrapped in decayed leaves (1.2%). There was not a single case of egg-laying in places exposed to drying out. In natural conditions, I found *B. cana* eggs (the species was confirmed by growing adult snails from eggs) twice in June, in a fine rot of dead hazel. In natural conditions, eggs are probably also laid in places where I could find clusters of juveniles at an early stage of development, i.e. moist holes of living and dead trees (e.g. bark crevices or miniature hollows created after the breaking of branches).

Mean incubation time at room temperature was 16.2 days (SD=3.09) (Fig. 2). Hatching is generally synchronous: most of the juveniles leave the eggshell at the same time, but some individuals may need more time for development. Out of 23 egg batches observed every day, 12 were hatched synchronously, while among 11 batches, single individuals hatched from 1 day (six cases) to even 4 days (one case) later than siblings.

Under laboratory conditions, the hatching success, i.e. the percentage of eggs from which juveniles hatched, was 86.3% (n=513 eggs). The only recognized cause of hatching failure was egg cannibalism (4.9%), which means egg eating by individuals hatched earlier from the same batch. The juveniles hatch from the egg by eating the egg envelope. After that, they continue consuming the remains of their own eggs and sometimes also those from their siblings' eggs. Cannibalistic behaviour is more frequent than damage caused by this phenomenon. If the embryo inside the egg is already developed sufficiently to survive, losing the egg envelope does not lead to the death of this juvenile. Cannibalistic behaviour is not obligatory. Eggs were found intact by the previously hatched young, and normally developed snails hatched later from these eggs. Egg cannibalism was observed only among juvenile individuals. Adults did not eat eggs of their own or related species (Table 2).

Bulgarica cana eggs are resistant to short-term unfavourable environmental conditions. Three eggs were accidentally left for 6 hours at room temperature without any protection; they dried up and lost their original shape, becoming flat and concave. When they were placed back under conditions suitable for incubation (between wet bark pieces, in a container with constant high humidity), they resumed their natural shape and all of them hatched after 10 days. Eggs can also be resistant to complete flooding. Eight eggs were placed in a container of cold water, where they all fell to the bottom. After 19 days, two individuals hatched, but were unable to climb the wall of the container to reach the water surface. No juveniles hatched from the remaining eggs.

Juvenile period, growth under laboratory conditions
Hatchling shells had mean of 2.63 whorls (SD=0.32, range 1.5–3.2 whorls, n=96 individuals), while adult shells had on average 12.42 whorls (SD=0.49, range 11.3–14,0 whorls, n=182 individuals). The height of the shells, depending on the number of whorls, is shown in Fig. 3.

Under laboratory conditions, 59.2% (n=358) reached the final size and typical shape of the shell. Growth to adult size (completed shell growth) took on average 6.8 months (SD=2.1, range 4–14 months, n=182 individuals) (Figs 4, 5). Examples of individual growth are shown in Fig. 6. Differences in the growth period between individuals are caused by the different growth rates, growth interruptions and differences in the final sizes of the shells.

Mean growth rate was 1.6 whorls/month (from 0.75 to 2.67 depending on individual, n=182). Individuals with a greater growth rate finished

Table 2 Eggs cannibalism among juveniles and adults.

Tested individuals			Time of observation		Eggs			
species	age	n	days	date	species	available	eaten	notes
Bulgarica cana	juveniles: 7.8–8.9 whorls	5	10	25.09–05.10.2007	*B. cana*	10	10	1 or 2 eggs are eaten each day; after consuming the egg, the juvenile goes away
Bulgarica cana	juveniles: 10.0–10.7 whorls	5	10	25.09–05.10.2007	*B. cana*	10	0	no interest in eggs; new juveniles hatched (7), 1 unhatched egg, 2 eggs eaten by newly hatched individuals
Bulgarica cana	adults	8	4	3–7.06.2006	*B. cana*	10	0	no interest in eggs
Bulgarica cana	adults	8	4	3–7.06.2006	*M. ventricosa*	10	0	no interest in eggs
Macrogastra ventricosa	adults	8	4	3–7.06.2006	*B. cana*	10	0	no interest in eggs
Macrogastra ventricosa	adults	8	4	3–7.06.2006	*M. ventricosa*	10	0	no interest in eggs

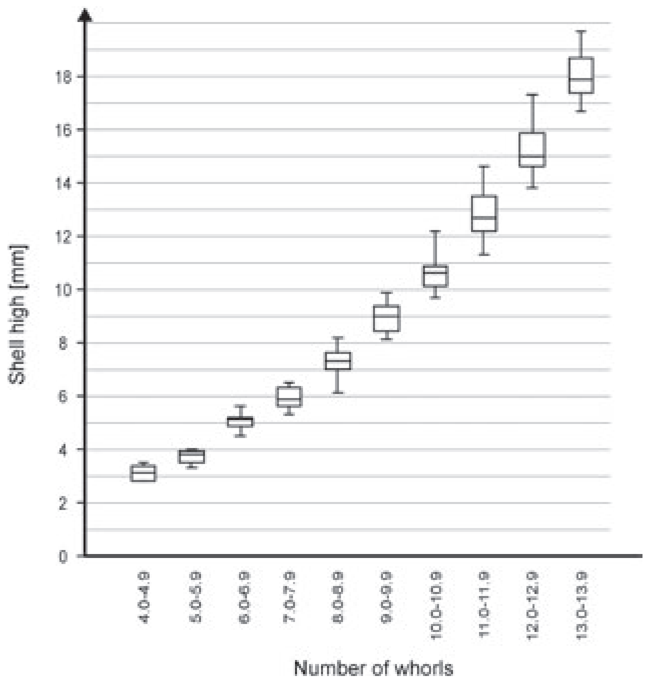


Figure 3 Diversity of the size of *B. cana* shells during growth. The height of the shell (quarter spread) depends on the number of whorls (n=243 measurements of 18 individuals; measurements were made every 2 weeks)

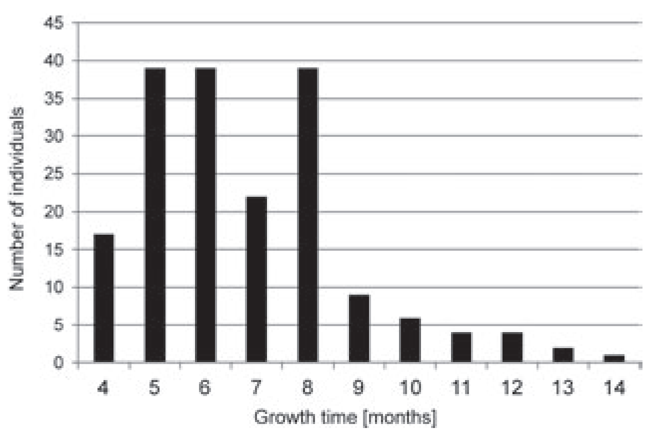


Figure 4 *B. cana* growth period from hatching to the final shell size; n=182 individuals

shell building faster (correlation coefficient r=-0.9 p<0.0001, n=182), and thus, their growth period was shorter (Fig. 7). The growth rate varied. Individuals showed minimal (between 0.1 to 2.2 whorls/month) and maximal (between 1.4 to 4.0 whorls/month) growth rates at various stages of growth (Fig. 8). However, growth rate tended to decrease with increased shell size (r_s=-0.53, p<0.0001 n=981 measurements of 182 individuals). A weak relationship was found between

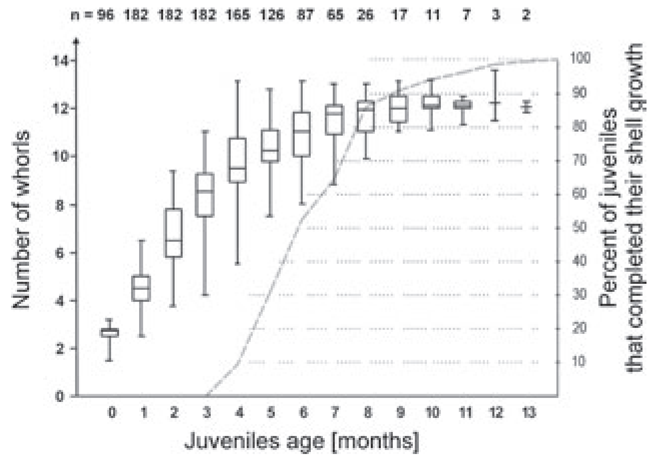
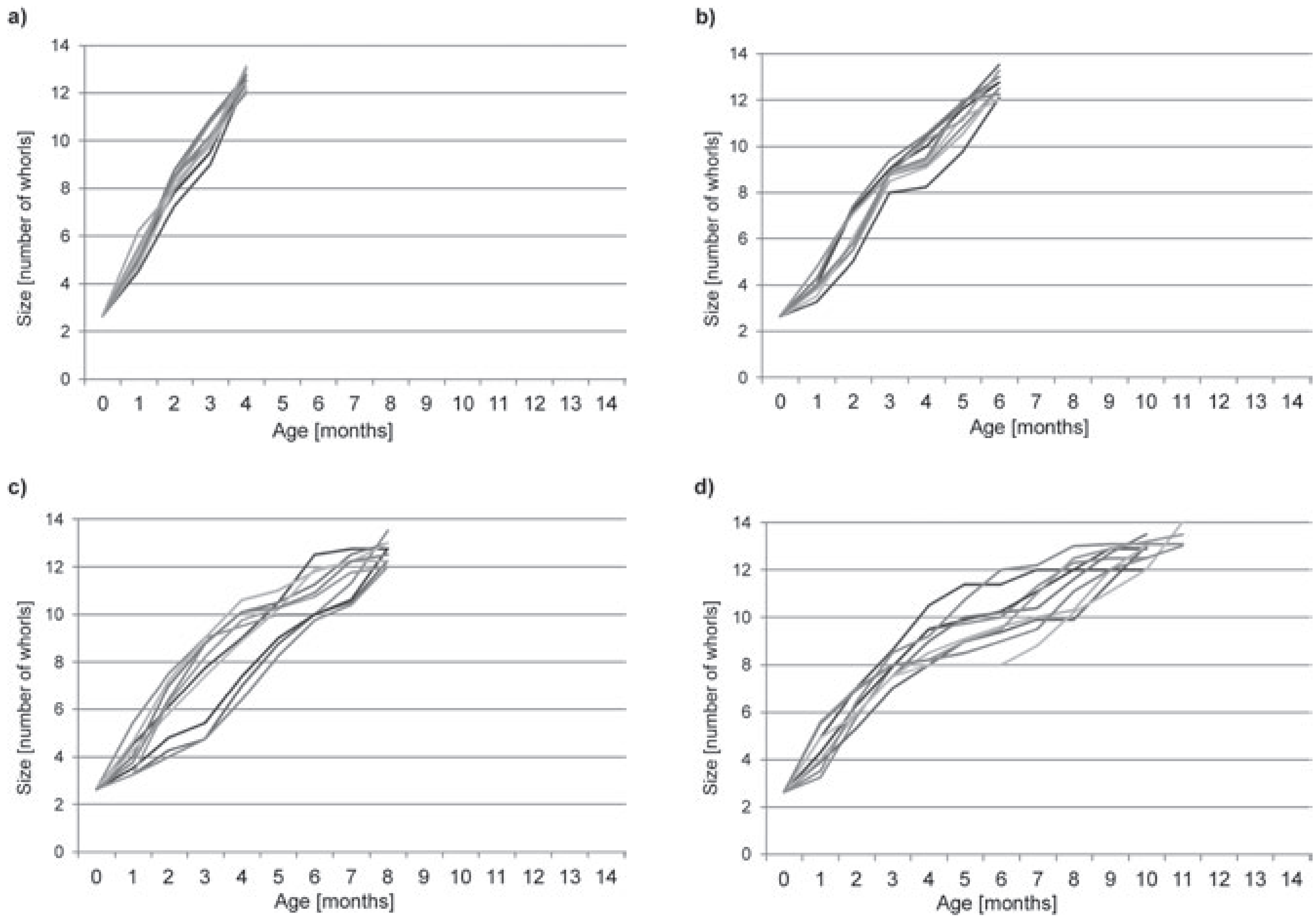


Figure 5 *B. cana* shell size (number of whorls) in subsequent months of life (quarter spread) and percentage of specimens which completed their shell growth in age classes; n – number of juveniles.

the growth period and the final size of the shell (expressed as the number of whorls): individuals with larger ultimate size took longer to grow (r=0.4 p<0.0001; n=182) (Fig. 9). Juvenile individuals kept in artificial winter conditions showed a slight growth (Table 3).

Growth interruption was observed in 33% of juveniles at various growth stages (between 8 and 12.5 whorls). The majority of such individuals (76.7%) were subadults; their shells reached the final size, and after the break, they finished the formation of the apertural part, i.e. folds, lamellae and the lip. The remaining juveniles continued shell building after the break. The growth interruptions lasted for 1–4 months. Such snails, as expected, needed more time to complete their shells (r=0.5 p<0.0001; n=182) (Fig. 10).

Various shell deformations occurred in 12.8% of the individuals for which the growth period was observed (Fig. 11). The most common deformation (12 specimens) was the lack of differentiation of the last whorl, with an unusual aperture shape. The shell reached more than 12 whorls, but still looked like a juvenile's shell. A very thin thickening of the outer edge of the aperture appeared with time, which probably was a poorly developed lip. Individuals with such deformation did not lay eggs. Another deformation (nine individuals) was an unusual form of the penultimate whorl of the shell: it was larger, and its surface appeared to be corroded, old and damaged; the aperture was unusually large, shapeless, without typical folds and lamellae.



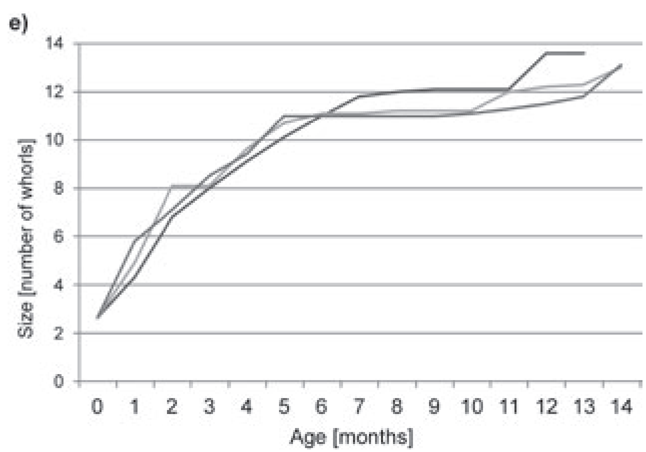


Figure 6 *B. cana* growth in the following months of life under laboratory conditions. Stories of selected individuals whose growth took: a) 4 months (n=10); b) 6 months (n=10); c) 8 months (n=10); and all individuals whose growth took: d) 10 and 11 months (n=10); e) 13 and 14 months (n=3).

Such individuals laid normal eggs and juveniles hatched. Another deformation (eight individuals) was represented by unfinished aperture forming – incomplete lip, folds and lamellae poorly developed. Such individuals laid normal eggs and juveniles hatched. The last and rarest deformation (two individuals) was a scalar form of the last whorl. One individual laid a single egg, but no juvenile hatched. In other cases, the offspring of individuals with deformations (only adults with the same deformation type were crossbred) developed normally, without any deformation.

The growth of individuals with various types of deformation took longer compared to that of typical individuals, i.e. from 11 to 36 months. All individuals with shell deformations experienced growth interruptions, which were sometimes extremely long, i.e. from 6 to 12 months.

Juvenile mortality in the laboratory was 32.1%; mortality occurred in all sizes and ages. Until

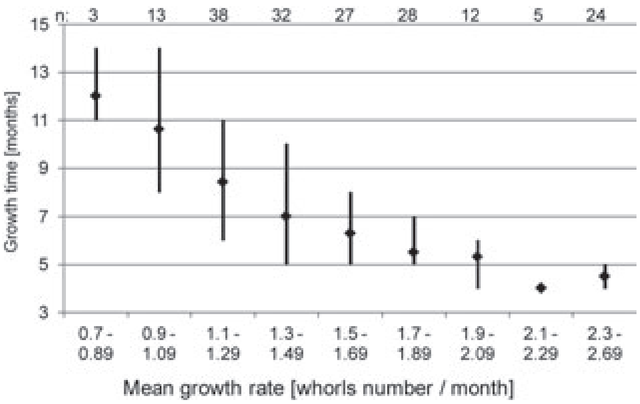


Figure 7 *B. cana* growth period (mean and range) related to the average growth rate of each individual (growth interruptions were excluded); n=182 individuals

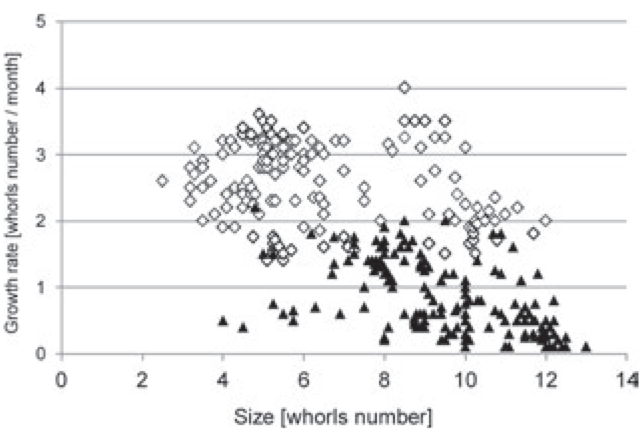


Figure 8 Minimum (triangles) and maximum (diamonds) growth rate of *B. cana* juveniles (n=182).

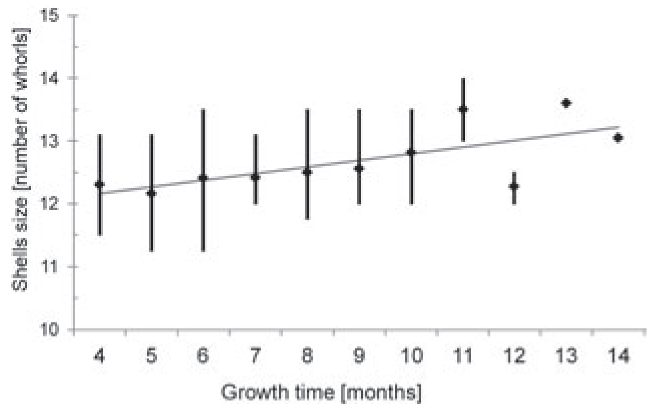


Figure 9 Comparison of shell size (mean and range) of *B. cana* adults with different growth periods; regression line is marked in grey; n=182 individuals.

Table 3 Growth of *B. cana* juveniles under conditions imitating the natural winter period.

Individuals	Number of whorls before wintering	Number of whorls after wintering	whorls increase	wintering period [days]
1	3.2	3.5	0.3	120
2	3.3	3.3	0	120
3	4.1	4.2	0.1	162
4	4.1	4.6	0.5	120
5	4.3	4.3	0	162
6	4.3	4.3	0	162
7	4.5	4.5	0	162
8	4.5	4.8	0.3	162
9	4.5	4.9	0.4	162
10	4.8	5	0.2	162
11	4.8	5	0.2	162
12	5	5.1	0.1	162
13	5.2	5.2	0	162
14	5.5	5.8	0.3	162
15	6.2	6.3	0.1	162
16	6.3	6.6	0.3	162
17	6.5	7	0.5	162
18	7.1	7.1	0	162
mean:	4.9	5.1	0.2	155

death, snails grew at the same rate as the other individuals kept in the same container (r=0.94, p<0.0001; n=115). The density of juveniles in the containers did not affect mortality rates (r=0.1 p=0.7 n=23 groups with different densities). Adult mortality in consecutive years of life ranged from 7 to 32%.

Growth in natural conditions

Under natural conditions, juveniles grew (built their shells) only during the four warmest months of the year, from June to September. Mean growth rate in summer was 0.71 whorls/month (Fig. 12). The highest observed growth rate was 1.3 whorls/2 weeks (which means 2.6 whorls/month, but the average growth of this individual during the entire growing season was 0.72 whorls/month). No growth was observed between October and May (Table 4). The *B. cana* growth model in natural conditions is shown in Fig. 13. The growth rate over 1 year or longer was 0.21 whorls/month (Table 5). At this rate, shell growth completion to 11.0–12.4 whorls (range of sizes of adults in the local population) took at least 40 months, i.e. more than 3 years.

Adulthood, fecundity in laboratory conditions

None of the observed individuals started breeding at the time of shell building. Sexual maturation was delayed in relation to shell growth

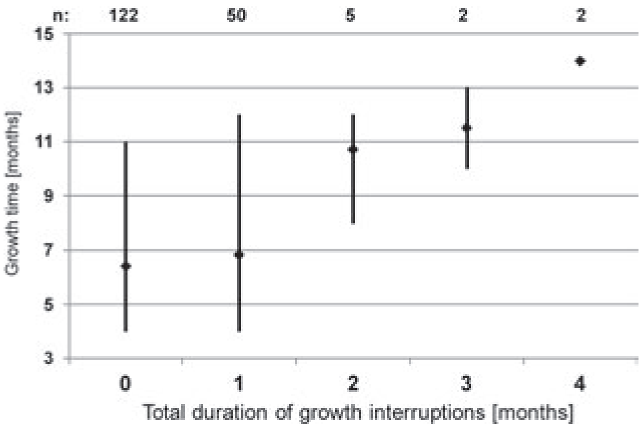


Figure 10 Influence of growth interruptions on growth period (mean and range); n=182 individuals.

completion by at least 106 days (3.5 months); the mean delay was 325.9 days (11 months) (SD=261.1, range 106–1316 days, n=30 pairs). Due to the difficulty in capturing exact signs of maturity (e.g. copulation), both individuals kept together were considered to be mature at the time when one of them laid eggs.

No mating dance or other pre-copulatory behaviour was observed in this species. During copulation, the snails almost completely retracted into the shells, only fragments of their feet were visible. Such a position made by two individuals did not always mean copulation – to confirm this, an attempt was made to separate the pair. After separating the copulating pair, the penis of one or of both of the snails remained left out and was retracted after a while. Under natural conditions, copulation of *B. cana* was observed several times in July, August and September. Cases of potential copulation (i.e. not confirmed by separation) were observed throughout the period of snail activity, namely from May to October.

The duration of reproduction varied widely among the pairs (Fig. 14). Some pairs laid eggs throughout their adult life, while others only laid eggs during a short period of time. The mean reproductive period (i.e. the time between the first and last batch of eggs laid by one pair) in the laboratory was 429 days (1.2 years) (SD=441.6, range 1–1,661 days, n=30 pairs). The longest observed reproductive period was more than 4 years (1,661 days); during this period, a pair of snails laid 216 eggs. On average, the pairs produced 60.74 eggs per year (n=16 pairs, whose reproduction lasted not less than one year). There were no differences in the number of eggs laid by one pair in two consecutive years (Student's t-test for pairs t=-0.309, p=0.764, n=9 pairs whose reproduction lasted for 2 years or longer) (Fig. 15).

The seasonality of reproduction differed between the snails kept in the laboratory

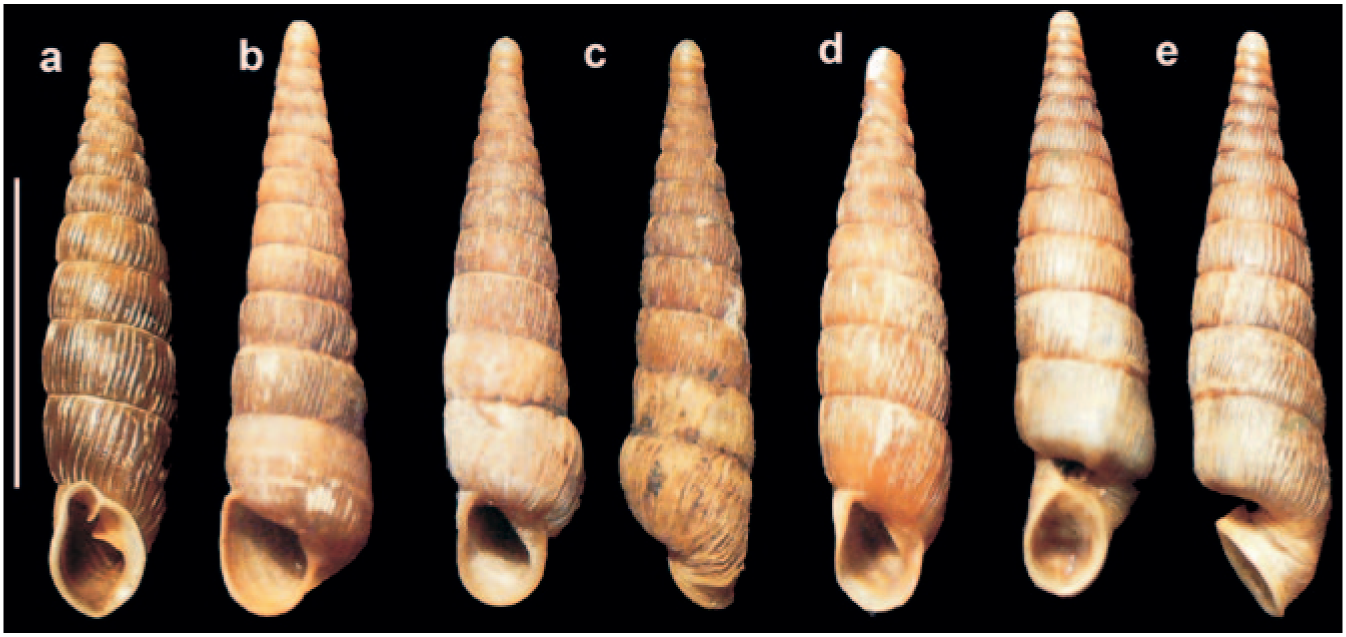


Figure 11 Deformations of *B. cana* shells growing under laboratory conditions; a) typical shell without deformations; b) lack of differentiation of the last whorl; c) unusual form of the penultimate whorl; d) unfinished aperture forming; e) scalar form of the last whorl; scale bar=10mm. Photo: Magdalena Marzec.

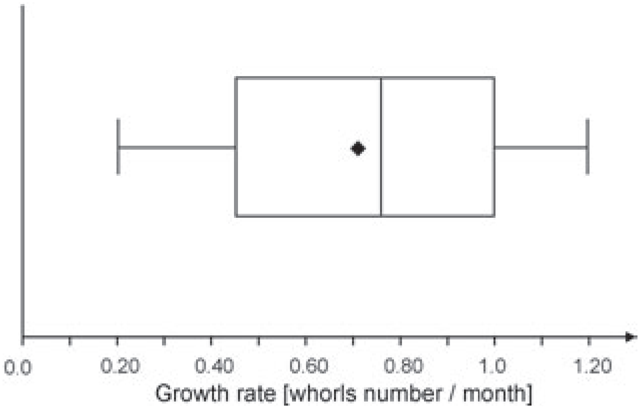


Figure 12 Growth rate of *B. cana* under natural conditions between May and October; quarter spread; diamond – mean value; n=23 juveniles observed for 1–4 months.

throughout the year (room temperature) and those kept at low temperatures during a few winter months. The wintering snails displayed a pronounced seasonality (Fig. 16) – most eggs (59%) were laid in April, i.e. the first month after being transferred from winter to laboratory conditions. The minimum period between the placement of individuals at room temperature and egg-laying was 8 days. Seasonality was much less marked in the individuals kept at room temperature throughout the year. (Fig. 16). The number of eggs laid annually by pairs kept only at room temperatures and by pairs overwintering at low temperatures was different, although these differences were not statistically significant: Student's t-test; t=-1.7078; p=0.11 (not wintered individuals: mean=46.6 eggs/year, n=8 pairs, wintered individuals: mean=74.9 eggs/year, n=8 pairs). Under natural conditions, *B. cana* eggs were found twice in June (the species was confirmed by growing adults from eggs in the laboratory).

No uniparental reproduction was observed. None of the five individuals kept individually laid any eggs. Snails, however, have the ability to reproduce alone (without contact with other snails) if they had an earlier opportunity to mate with another individual. Two of the three individuals kept first in pairs and then individually after the death of their partners laid eggs from which juveniles hatched. Sperm retention lasted over 2 years (from the partner's death until the last egg was laid) (Table 6). The fecundity of such individuals was smaller than that of snails kept in pairs. The mean number of eggs per batch was lower (Mann-Whitney's test Ub=566.5 p<0.0001; Me=4.5 for individuals kept individually, n=13 batches; Me=8 for pairs of individuals, n=260 batches), as well as the total number of eggs laid over the period of 1 year.

Longevity

The maximum lifespan of *B. cana* under laboratory conditions was over 9 years (3,416 days), with

Table 4 All observations of *B. cana* juveniles in the winter period, i.e. between October and May, under natural conditions.

Individual number	Period of observation [days]	Period without growth [days]	Capturing: Date	Capturing: Juvenile size [number of whorls]	Recapturing: Date	Recapturing: Juvenile size [number of whorls]
11	8	8	2007-05-16	10	2007-05-24	10
14	51	21	2007-05-16	8	2007-06-06	8
15	84	8	2007-05-16	9.5	2007-05-24	9.5
16	21	8	2007-05-16	8.5	2007-05-24	8.5
31	391	11	2007-09-26	10.1	2007-10-07	10.1
41	25	11	2007-09-26	8.3	2007-10-07	8.3
43	648	196	2007-09-26	11.2	2008-04-09	11.2
49	341	220	2007-10-07	9.1	2008-05-14	9.1
53	262	172	2007-10-07	8.5	2008-03-27	8.5
56	313	205	2007-10-22	9.4	2008-05-14	9.4
57	205	205	2007-10-22	7.5	2008-05-14	7.5
59	326	247	2007-10-22	10.1	2008-06-25	10.1
67	35	35	2008-04-09	10.1	2008-05-14	10.1
92	417	54	2008-08-30	9.5	2008-10-23	9.5

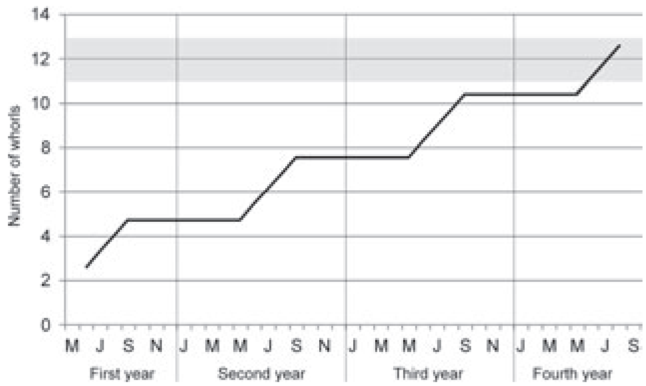


Figure 13 *B. cana* growth model under natural conditions. The range of adult size in this population (11–12.4 whorls, mean 11.77 whorls) is shown in grey.

a growth time of 5 months. The average lifespan of individuals kept in the laboratory from hatching to death (n=51) was 916 days (2.5 years) (Fig. 17). The longest confirmed life period of an adult in natural conditions was 6 years (Table 7). This individual was marked as an adult, so the actual length of its life would have been greater than 6 years.

Discussion

Eggs, incubation period, hatching success, fecundity under laboratory conditions

Eggs of terrestrial gastropods, due to the construction of outer covers, can be divided into three main types: uncalcified (with a transparent, gelatinous cover), partially calcified (with calcium carbonate crystals densely packed in a gelatinous suspension) and strongly calcified (aggregated calcium carbonate crystals form a hard and rigid outer shell) (Tompa, 1984). Like most European clausiliids (Maltz & Sulikowska-Drozd, 2008), *Bulgarica cana* eggs are partially calcified with numerous calcium carbonate crystals, densely packed in a gelatinous suspension, which ensures durability and flexibility for the eggs.

Oviparity is the most common reproductive mode among clausiliids (Maltz & Sulikowska-Drozd, 2008), and *B. cana* is a typical oviparous species. Terrestrial snails do not lay eggs in random places. High humidity is important during the incubation of eggs to protect them from drying out (Tompa, 1984; Whitney, 1987; Maltz, 2003; Maltz & Sulikowska-Drozd, 2008; Kuźnik-Kowalska & Roksela, 2009; Sulikowska-Drozd & Maltz 2012a; b) and to guarantee newly hatched individuals can easily access food (Pokryszko, 1990; Kuźnik-Kowalska, 1999). *Bulgarica cana* spends almost its entire life on trees. Under both natural and laboratory conditions, eggs were laid exclusively in moist bark crevices. A similar strong preference for this substrate was observed in *Helicodonta obvoluta*, associated with dead wood (Maltz, 2003), or in arboreal tropical species e.g. *Pseudachatina downesi* (Heller, 2001). Other European clausiliids lay eggs on bark or dead wood (*Cochlodina laminata, Macrogastra tumida*), moss (*Macrogastra latestriata, Clausilia parvula, Charpentieria ornata*) or show no preference for any substrate (*Laciniaria plicata*) (Maltz & Sulikowska –Drozd, 2008). Laying eggs in batches also offers some protection against drying because it reduces water loss compared to a single laid egg (Tompa, 1984).

Most of the reproduction parameters of *B. cana* are similar to such parameters of other oviparous clausiliid species; its eggs (Table 1) are similar in size to the eggs of those Clausiliidae species whose adults have shells of similar size

Table 5 All-year growth rate of individuals observed for 1 year or longer under natural conditions.

					first capture		last capture	
Individual number	Number of captures	Total observation time [months]	Total growth [number of whorls]	Growth rate [number of whorls/ month]	date	size of individual [number of whorls]	data	size of individual [number of whorls]
54	3	18.8	2.5	0.13	2007-10-07	9.2	2009-04-23	11.7
92	4	13.9	4.5	0.32	2008-05-14	7	2009-07-05	11.5
31	5	13	3	0.23	2007-07-20	9.5	2008-08-14	12.5
35	3	12.4	2	0.16	2007-08-23	9.2	2008-08-30	11.2
49	3	11.4	2.3	0.20	2007-10-07	9.1	2008-09-12	11.4
Mean:				0.21				

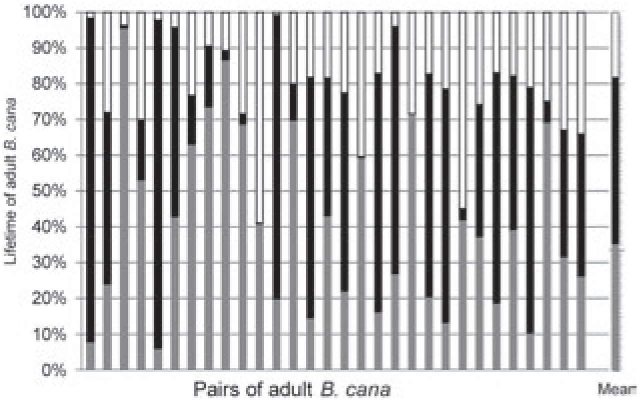


Figure 14 Proportions of pre-breeding period (grey bars), breeding period (black bars) and senile, post-breeding period (white bars) in the adult life of *B. cana* kept in pairs

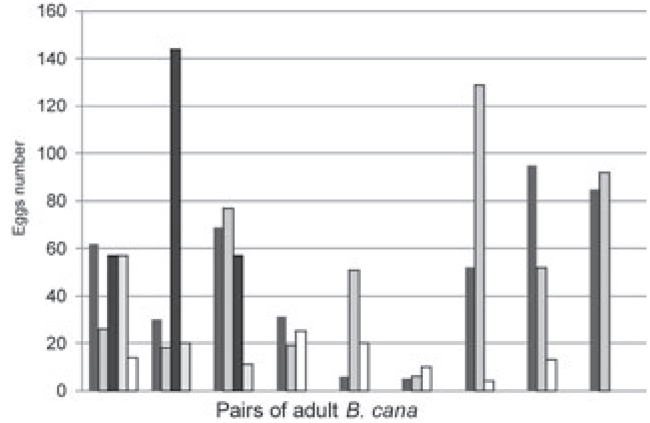


Figure 15 Number of eggs laid by *B. cana* pairs in subsequent years (counted from the first egg); grey bars – eggs laid in the following years of observation; white bar – eggs laid in the last, not finished year of observation.

as *B. cana*, i.e. *L. plicata*, *C. laminata*, *Ch. ornata*, *M. latestriata* and *B. stabilis* (Maltz & Sulikowska-Drozd, 2008). *Bulgarica cana*, together with *M. ventricosa*, *C. laminata* and *Ch. ornata*, belongs to a group of species with the smallest relative egg size in relation to the size of the parent (Maltz & Sulikowska-Drozd, 2008). The incubation period of *B. cana* eggs under laboratory conditions (mean=16.2 days, SD=3.09) is similar or slightly longer than that in other clausiliids (Maltz & Sulikowska-Drozd, 2008; Maltz & Pokryszko, 2009; Sulikowska-Drozd & Maltz, 2012b). Many authors indicate that the incubation period extends with temperature decrease (Kosińska, 1980; Tompa, 1984; Myzyk, 2011). The slightly longer incubation period of *B. cana* eggs may be caused by the unequal "room temperature" of particular laboratories.

The average number of eggs in a batch (8.4; SD=3.97) of *B. cana* is similar or greater to that of other species of clausiliids (Maltz & Sulikowska-Drozd, 2008; Maltz & Pokryszko, 2009; Sulikowska-Drozd & Maltz, 2012a; b). However, the maximum number of eggs found in a *B. cana* batch may raise doubts, as it was higher than that in other clausiliids batches, including species with a larger shell. However, exceptionally large egg batches were rare and did not affect the average value (median) of the number of eggs in the batch. Snails were kept in pairs, and it is therefore possible that two individuals laid their eggs in the same place within a short time. In several cases, two batches of eggs were laid in one place within a few days. As eggs differed in colour, fresher eggs were snow-white and older ones more brownish, there was no doubt that they belonged to separate batches.

Fecundity expressed by the number of eggs laid during the year varied greatly. On average, under laboratory conditions, *B. cana* showed a slightly higher fecundity than other clausiliid species (Baur & Baur, 1992; Sulikowska-Drozd, 2008; 2009; Maltz & Sulikowska-Drozd, 2012; Sulikowska-Drozd & Maltz, 2012a). Such fecundity, expressed both in the large average number of eggs in the batch and the number of eggs laid throughout the year, may be related to the appropriate laboratory conditions. In a laboratory culture, it is important to provide a proper, species-specific substrate for egg laying (Sulikowska-Drozd & Maltz, 2012b). Fecundity may also depend on the density of adults (Baur A., 1990; Baur & Baur, 1992) or on their diet (Dickens, Capinera & Smith, 2018). The size of the individuals may affect their fecundity, too (Dillen, Jordaens, Bruyn & Backeljau, 2010). Larger species of clausiliids lay batches with more eggs (Maltz & Sulikowska-Drozd, 2008). In *Succinea putris* larger individuals laid heavier eggs (Dillen *et al*., 2010). However, in *Balea perversa* and *Helix pomatia*, there was no influence of the size of the parent on fecundity (Baur A., 1990; Gołąb & Lipińska, 2009).

Under laboratory conditions, the hatching success of *B. cana* was relatively high. In contrast to natural conditions, eggs were provided with constant, favourable humidity and temperature and were not exposed to predation. In terrestrial snails, the most common factor causing egg mortality is

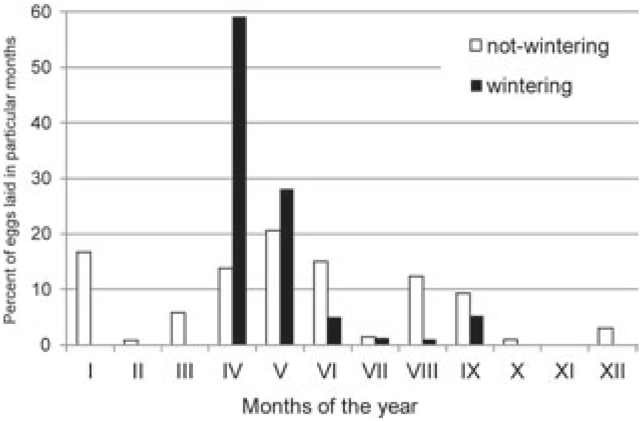


Figure 16 Seasonality of egg-laying by *B. cana* under laboratory conditions; n=8 pairs of wintering snails; n=8 pairs of not-wintering snails (see comments in the text).

desiccation (Tompa, 1984; Heller, 2001). Eggs of particular species show different tolerance levels to drying out: *Vertigo pusilla* eggs are sensitive to water loss (Pokryszko, 1990), while *Deroceras sturanyi* eggs remain viable even with 80% loss of weight due to drying out (Kosińska, 1980). Although the resistance of *B. cana* eggs to desiccation and drowning was observed only in few cases, the results of these observations allow to infer that the eggs of this species tolerate short-term unfavourable conditions during incubation.

The main causes of *B. cana* hatching failures remained unknown. Such failures could be the result of natural phenomena, such as unfertilised eggs or improper embryo development. Developing abnormalities may be related to the age of parental snails: *Balea fallax* eggs from the oldest specimens were characterised by the smallest hatching success (Sulikowska-Drozd & Maltz, 2012a). An additional factor affecting hatching failures may have been accidental damage suffered during laboratory treatment (e.g. measuring or transferring). Under laboratory conditions, the only recognised cause of mortality of *B. cana* eggs was cannibalism. Egg cannibalism occurs in many land snail species, usually among juveniles in the first days of life (Kosińska, 1980; Baur B., 1990; Desbuquois, Chevalier & Madec, 2000; Speiser, 2001; Maltz & Sulikowska-Drozd, 2008; Sulikowska-Drozd & Maltz, 2012a). In *B. cana*, egg cannibalism was observed in freshly hatched individuals. It was also shown experimentally that the juveniles may treat other eggs as a source of food over a period of 2–3 months. Under laboratory conditions, egg cannibalism did not cause significant losses. On the other hand, the egg cannibalism phenomenon may have a positive effect on the condition of freshly hatched snails (Baur B., 1990; Desbuquois *et al.*, 2000) and most likely increases the chances of survival in the first days after hatching (Baur B., 1990). However, many species from different families, e.g. *Vertigo pusilla, Helicodonta obvoluta, Perforatella bidentata, Ruthenica filograna* and *Balea stabilis*, do not show any signs of cannibalism (Pokryszko, 1990; Maltz, 2003; Kuźnik-Kowalska & Roksela, 2009; Szybiak, 2010; Sulikowska-Drozd & Maltz, 2012b).

Bulgarica cana individuals, as well as other species from the Clausiliidae family, kept at room temperature can reproduce all year round (Maltz & Sulikowska-Drozd, 2008). Similar to *Balea fallax* (Sulikowska-Drozd & Maltz, 2012a), and unlike the majority of other Polish clausiliids (Maltz & Sulikowska-Drozd, 2008), I have observed in *B. cana* only one egg-laying peak in spring. This is in line with the observations for a Russian *B. cana* population (Mamatkulov, 2007). Under natural conditions, one breeding peak is characteristic for snails inhabiting climatic zones with a long and cold winter (Sulikowska-Drozd, Maltz & Kappes, 2013), such as that of the Romincka Forest. In

Table 6 Reproductive abilities of single *B. cana* individuals after the partner's death.

Pair	Observation time of the pair (until the death of one snail)	Number of eggs laid by the pair	Observation time of a single individual		Number of eggs laid by single individual	Mean number of eggs per year	Number of eggs in following years of reproduction			Maximum time of sperm retention	
	days		days	years			1	2	3	days	years
1	242	0	1140	3.1	26	8.3	13	4	9	829	2.3
2	206	22	982	2.7	32	11.9	4	20	8	773	2.1
3	903	22	583	1.6	0						

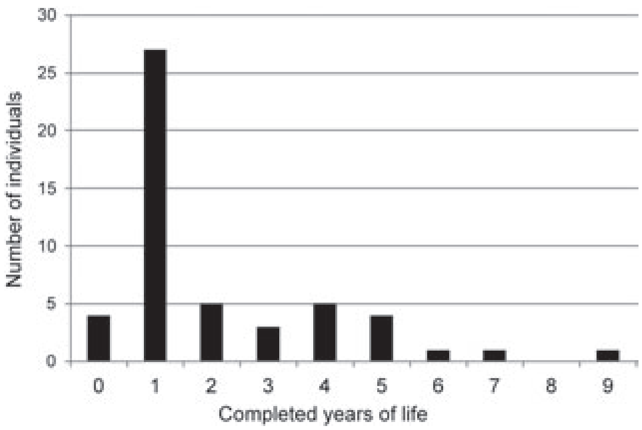


Figure 17 *B. cana* longevity under laboratory conditions; n=51 individuals observed from hatching to death.

eastern European populations, *B. cana* copulates from April to October, with a break in May-June (when laying eggs), which is in contrast to many other clausiliids that also copulate during the laying period (Mamatkulov, 2007). During copulation, *Bulgarica cana, Laciniaria plicata, Cochlodina laminata and C. orthostoma* produce spermatophores, while this was not observed for clausiliids belonging to the genera *Clausilia* and *Macrogastra* (Mamatkulov, 2005; 2007). The spermatophore of *B. cana* has a worm-like shape, and sperm is found only in its distal part (Mamatkulov, 2007). Mamatkulov (2005) states that during *B. cana* copulation, each snail acts once as a functional male and then as a female, changing its role in sequence. In a later publication, the same author (Mamatkulov, 2007) states that snails simultaneously exchange spermatophores during the so-called "reciprocal copulation". After separating the copulating pair, I observed that either the two or only one of the individuals had their genitalia extracted, which indicates that both methods of copulation described above are possible in the studied species.

The main external factors that influence the activity of land snail gonads are temperature, humidity and the length of the day (Tompa, 1984). As *B. cana* pairs stayed all year round at constant temperature and humidity, the intensity of their egg-laying changed throughout the seasons, with changes in the length of the day. There was a low egg-laying intensity in winter, which increased with the lengthening of the day, peaked in May, and showed a slow decline towards winter. The second peak of egg-laying observed in January (Fig. 16) was probably caused by temperature disturbances in December

Table 7 Recaptures of *B. cana* individuals marked in 2005 as adults (n=76);×– recapture of the individual in a given research season.

Number of individuals	Research season						Minimal lifespan (as an adult) [number of years]
	2006	2007	2008	2009	2010	2011	
1	x	x				x	6
1	x	x	x	x			4
1	x	x		x			4
1	x		x	x			4
1				x			4
7	x	x	x				3
1	x		x				3
7		x	x				3
4			x				3
15	x	x					2
10		x					2
27	x						1
Individuals captured last time during the particular season:	27	25	19	4		1	
%	35.5	32.9	25.0	5.3	0.0	1.3	

(in the Christmas and New Year period, the laboratory rooms were less heated). A temperature increase in the laboratory in January could have induced a temporary greater reproductive activity of snails. Individuals wintering at low temperatures showed a relatively high egg-laying intensity immediately after being moved to high (room) temperatures.

Juvenile period, growth

In the Clausiliidae family, the juvenile period is longer than the period of growth. Snails with newly completed shells, including a closed apparatus and lip, do not yet have a fully developed reproductive system (Maltz & Sulikowska-Drozd, 2008). Most land snails are protandric, which means that the male gametes mature earlier (Tompa, 1984); however, clausiliids are simultaneous hermaphrodites: their male and female sex line cells maturate simultaneously (Nordsieck, 2007; Maltz & Sulikowska-Drozd, 2011). Histological examination of *Vestia gulo* and *V. turgida* (Maltz & Sulikowska-Drozd, 2011) showed that the gonad reaches maturity between the third and sixth month after shell completion. In *B. cana*, the maturation period is probably similar, as between the completion of the shells and the first egg-laying, at least 3.5 months passed. Other factors, such as high population density, may also affect the time when snails start to reproduce (Baur & Baur, 1992).

Maltz & Sulikowska-Drozd (2008) distinguished three groups of clausiliids due to the growth time of individuals: fast-growing (14–18 weeks), moderately fast-growing (18–22 weeks) and slowly growing species (26–32 weeks). The growth time of *B. cana* individuals under laboratory conditions varied greatly from 4 to 14 months (16–56 weeks), which means that in the case of this species, only individuals are fast, moderately or slowly growing, and the growth rate is not characteristic for the species as a whole. Such a high variability among individuals kept under the same conditions could be caused by both individual differences and heterogeneous environmental conditions (e.g. differences in the nutritional content of the bark offered). The growth rate of *B. cana* is not constant, similar to that of many other land snail species (Terhivuo, 1978; Kuźnik-Kowalska, 1999; Maltz, 2003; Kuźnik-Kowalska & Roksela, 2009; Myzyk, 2011), including those belonging to the Clausiliidae family (Maltz & Sulikowska-Drozd, 2008). A constant growth rate may occur in some short-lived land snails under laboratory conditions (Whitney, 1987; Myzyk, 2011). According to the model of clausiliid growth (first phase – intense growth; second phase – slightly slower growth; third phase – rapid growth) proposed by Maltz & Sulikowska-Drozd (2008), it was expected that the growth rate of *B. cana* will be the largest at the initial or final growth phases and significantly smaller in the medium phase. However, for most *B. cana* individuals, neither the distribution of minimum and maximum growth rates nor growth curves were consistent with the proposed model.

The growth rate of land snails under natural conditions often depends on the climate. In areas with a shorter growing season and lower temperatures, growth lasts longer (Umiński, 1975; Terhivuo, 1978; Sulikowska-Drozd, 2010; 2011). In the Alpine populations of *Arianta arbustorum*, growth takes up to five years (Baur & Raboud, 1988). An analogous slowdown in growth rate and, as a result, an extension of its period in the Mediterranean region is caused by heat and drought (Kiss, Labaune, Magnin & Aubry, 2005; Lazaridou & Chatziioannou, 2005). In the Romincka Forest, with the harshest climate in the Polish lowland (Matuszkiewicz, 2007), the growth of *B. cana* lasts over three years. In the proposed growth model (Fig. 13), it was assumed that hatching occurs in June. In the field, *B. cana* eggs were only found in June, suggesting that hatching after a minimum of 2–3 weeks (assuming optimal weather conditions) would be at the end of June or in July. Adult individuals of *B. cana* become active in May (Marzec, in prep.) and can therefore potentially lay eggs earlier than June. However, the time required for egg incubating must also be taken into account. Development depends on temperature; at lower temperatures, embryos develop more slowly and hatching occurs later (Tompa, 1984). Therefore, it was assumed that the first young can appear no earlier than in June.

Although under natural conditions, none of the observed *B. cana* juveniles built shells between October and May, some specimens wintering in the cellar showed slight increases during this period. Among the marked animals in the field, there were no extremely small snails (below 6.8 whorls), while individuals wintering in the cellar and growing had 3.2–6 whorls. It is likely that the smallest individuals use even very short warming periods for feeding and growth. As a result, they can grow longer than just four months a year and have the chance to finish growth in the third, not in the fourth year of life.

Longevity

Land snail species can live up to several months or 19 years (Heller, 2001). The lifespan of clausiliids in the laboratory cultures varies from 3 to 10 years (Likharev, 1962; Maltz & Sulikowska-Drozd, 2012), while under natural conditions in a moderate climate, they live from 3 to 8 years (Piechocki, 1982; Maltz & Sulikowska-Drozd, 2008; Szybiak, 2010; Sulikowska –Drozd, 2011) and can therefore be defined as long-lived snails. The longest observed lifetime of *B. cana* is similar to that other European clausiliids. The longest-lived individual lived in the laboratory up to a similar age (9 years) as the longest-observed individual under natural conditions (9 years; at 6 years, it was observed as an adult, and 3 years is the estimated period of growth in this area).

Acknowledgements

This paper is a part of my doctoral dissertation, written under the supervision of Prof. Dr Hab. Beata M. Pokryszko at the Faculty of Biological Sciences, University of Wrocław.

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