A MORPHOLOGICAL AND BIOLOGICAL ASSESSMENT OF *CRENOBIA ALPINA* AND *CRENOBIA MONTENIGRINA* (PLATYHELMINTHES, TRICLADIDA, PLANARIIDAE) IN THE PARÂNG MOUNTAINS (ROMANIA)

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Abstract

The paper analyses the morphology of the copulatory apparatus and the reproductive isolation of the monopharyngeal and polypharyngeal *Crenobia* (Dana, 1766) in sympatric populations of the Parâng Mountains, Romania. The copulatory apparatus of the two forms shows a non-simultaneous development, indicating a seasonal reproductive isolation: the monopharyngeal *Crenobia alpina* (Dana, 1766) becomes able to mate during the warm season while the polypharyngeal *Crenobia montenigrina* (Mrázek, 1904) becomes able to mate during the cold season. The mature copulatory apparatus of the monopharyngeals and the polypharyngeals shows all the characters typical for *C. alpina*, respectively for *C. montenigrina* as presented in the literature. In both species, some specimens reveal a complex system of fine ducts / sclerotized lines into the wall of the genital atrium, which are attributed to biological structures of unknown function rather than histological artefacts.

Keywords: *Crenobia*, monopharyngeal, polypharyngeal, reproductive isolation

Introduction

Crenobia is a genus of freshwater flatworm with a limited dispersal ability which lives almost exclusively in cold springs and headwaters of mountainous areas in Europe and Turkey (Brändle et al. 2007, Brändle et al. 2017, Sluys 2022).

Taxonomically, *Crenobia* is a group of species (*Crenobia alpina* sensu lato) with different morphology, reproductive biology, karyology, ecological requirements, habitat, geographic distribution (Sluys 2022). The various forms of this complex were regarded either as distinct species within the genus *Crenobia* or as subspecies of *Crenobia alpina* (see the synthetic review of Sluys, 2022).

Regardless of whether they are species or subspecies, the genus *Crenobia* comprises two distinct morphological types in terms of the number of pharynges:
a) the monopharyngeal type includes *Crenobia alpina septentrionalis* (Thienemann, 1938), *Crenobia alpina meridionalis* [= *Crenobia alpina alpina*], *Crenobia alpina alba* (van Oye, 1935), *Crenobia alpina bathycola* (Steinmann, 1911), *Crenobia alpina corsica* (Arndt, 1922)
b) the polypharyngeal type includes *Crenobia alpina montenigrina* with 10 -15 pharynxes, *Crenobia alpina anophthalma* (Mrázek, 1907) with 3 pharynxes, *Crenobia alpina teratophila* (Steinmann, 1908) very similar with *C. montenigrina* and a form with 24 – 35 pharynxes from Bulgaria, described by Chichkoff as *Phagocata cornuta* (Codreanu 1956, Sluys 2022).

The forms present in the Romanian fauna are the monopharyngeal *C. alpina* and the polypharyngeal *C. montenigrina* (Babalean 2020, Codreanu 1956, Sluys 2022). In the Parâng Mountains the two forms occur sympatrically in mixed populations (Babalean 2020). Codreanu

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(1956) indicates the seasonal reproductive isolation of *C. alpina* and *C. montenigrina*. The copulatory apparatus of the two forms is very similar; the minor differences between them should not prevent mating. The two morphological types are discussed from the biological species concept, of reproductive isolation, according to literature (Bănărescu 1973, De Queiroz 2007).

The aim of the paper is to assess and verify the seasonal reproductive isolation of the monopharyngeals and polypharyngeals, on morphological grounds.

Materials and Methods

The study is based on more than 300 specimens sampled between 1.07.2016 and 7.11.2022 from Rânca Resort – the Parâng Mountains. There were chosen several locations (Fig. 1) with different habitats: three reocrene springs (CB-Romanul, 45°17′51.83″N / 23°40′27.30″E), springs with sandy reservoirs and their collecting streams (Tidvele area, 45°19′52.32″N / 23°42′ 00.62″E, Paltinul Mountain area, 45°18′45.98″N / 23°40′01.08″E), springs modified by human activity – spring with collecting pipe and reservoir Păpușa Peak, (45°19′19.82″N / 23°41′14.22″E), mountain stream (ski lift area 45°18′48.75″N / 23°41′08.32″E). The sampling was done during summer (June, July) and during the cold season (November). To avoid defaunation, only a 1/4th to maximum a half of the visible specimens were collected from most identified populations; in only one location, all the visible specimens (1-3-5) of the population were collected.

Part of the worms was fixed in Beauchamp solution for 24 hours, thereafter, removed and stored in ethanol 75º; another part of the worms was fixed and stored in 96º ethanol. The maturity of the copulatory area was assessed on histological sections and by visual inspection of fixed, uncut worms. In this latter case, the specimens with large copulatory area were considered capable for the sperm transfer during mating. Some specimens were processed by usual histological techniques for Haematoxilin-Eosin staining, for histological frontal and sagittal serial sections at 5 µm thick:

- **monopharyngeal** specimens: CR1, Cm1, CR4, Cm2 – sexually mature, collected in summer: CR1 – (CB-Romanul), sagittal sections on 53 slides; Cm1 – (Paltinul Mountain), sagittal sections on 63 slides; CR4 – (CB-Romanul), frontal sections on 12 slides; Cm2 – (CB-Romanul), transversal sections on 25 slides
- **polypharyngeal** specimens: CR2, CR3, Cp1, Cp2 – immatures, collected in summer; CR8 – sexually mature, collected in November.

CR2 – (CB-Romanul), sagittal sections on 43 slides; CR3 – (Tidvele area), frontal sections on 10 slides; Cp1 – (Tidvele area), sagittal sections on 40 slides; Cp2 – (Tidvele at the highest altitude – 1841 m), frontal sections on 9 slides; CR8 – (Păpușa spring), sagittal sections on 71 slides.

Abbreviations used in the figures: af – atrial fold, bc – bursal canal, cb – copulatory bursa, cov – common oviduct, da – distal atrium, fa – fibrous atrium (fibrous part of the atrium), fd – fine ducts, go – gonopore, ma – muscular part of the atrium, mo – mouth, ov – oviduct, ph – pharynx, pp – penis papilla, vd – vas deferens.

Results and discussions

Results

1. Types of populations

In the studied area, *C. alpina* and *C. montenigrina* formed the following types of populations with respect to the pharyngeal condition (mono- / polypharyngeal) and the number of collected specimens:

A) non-mixed populations
a) exclusively monopharyngeals, with a small number of specimens – only one monopharyngeal population (ski lift area)
b) large populations, exclusively polypharyngeals – springs and some collecting streams
B) mixed populations:
a) predominant monopharyngeals – collecting stream at the Paltinul Mountain base
b) predominant polypharyngeals – some collecting streams
c) nearly equal proportions of mono- and polypahryngeals – reocrene no. 3 (CB-Romanul)

Some non-mixed populations were identified as mixed at the next collection (next years) and in the different seasons (warm season – cold season) - reocrene no. 2 (CB-Romanul). However, the existence of non-mixed populations exclusively monopharyngeals or exclusively polypharingeals is uncertain because no population was sampled completely.

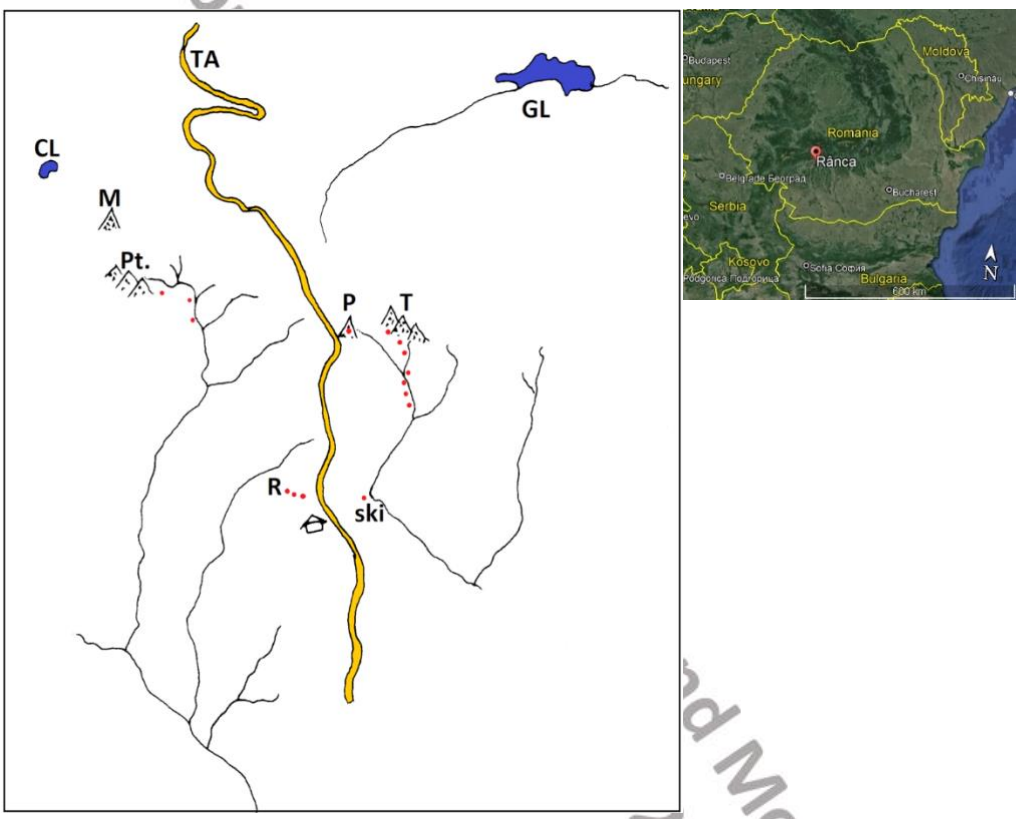

Figure 1. Location of the collecting site: Rânca, the Parâng Mountains, Romania (inset - Google Earth Pro map of Romania showing the location of the Parang Mountains); sampling points are indicated as red dots, CL – Câlcescu Lake, TA – Transalpina road, GL – Galbenul Lake, M – Mohorul Mountain, Pt. – Paltinul area, Păpușa peak, T – Tidvele area, R – CB-Romanul area with reocrene springs, ski - ski lift area (redrawn and adapted from Popescu (1986))

2. Morphology

The monopharyngeal *Crenobia alpina* (specimens CR1, Cm1, Cr4, Cm2)

These four specimens were collected in summer and are sexually mature.
The mouth opening is located at the posterior end of the pharyngeal pocket.
With very few exceptions these specimens reveal ovaries, few prepharyngeal ventral follicular testes, ducts and fully developed copulatory apparatus. The exceptions are attributed to a physiological state (the lack of ovaries and oviducts in specimen CR1 is considered a physiological state / a stage of the reproductive cycle).
The copulatory apparatus (Figs. 2-6).

The copulatory apparatus match the patterns of the genus *Crenobia* and *C. alpina*:
- an oversized (well developed) atrium consisting of 2 distinct parts: (i) the external muscular part, very thick, composed by muscular plates (bundles) in radial disposition, surrounded by an outer zone of myoblasts (Sluys 2022) and (ii) the internal fibrous layer
- the penis has an indistinct bulb and a long papilla, extending beyond the atrial fold – Fig. 3
- the genital atrium is divided by an atrial fold in 2 regions: (i) the proximal region which houses the most part of the penis papilla; it also receives the common oviduct and the bursal canal (Sluys 2022) and (ii) a distal region which opens by the gonopore (the genital orifice).
- the two vasa deferentia (spermiducts) with large spermiducal vesicles in their terminal part, at a certain distance before opening into the penis bulb.

Besides the above general characters, the histological slides of the four analysed specimens reveal some peculiarities: the course of the two vasa deferentia is symmetric in specimens CR1 and CR4 and asymmetric in specimen Cm1; the presence of a complex system of apparently fine ducts or sclerotized lines in the wall of the genital atrium (Figs. 2-4). These structures seem to be arranged between the atrial space housing the penis papilla, the distal atrium, and the penis bulb, interconnecting them. Orifices corresponding to the fine ducts are visible in the penis bulb and in the wall of the distal atrium.

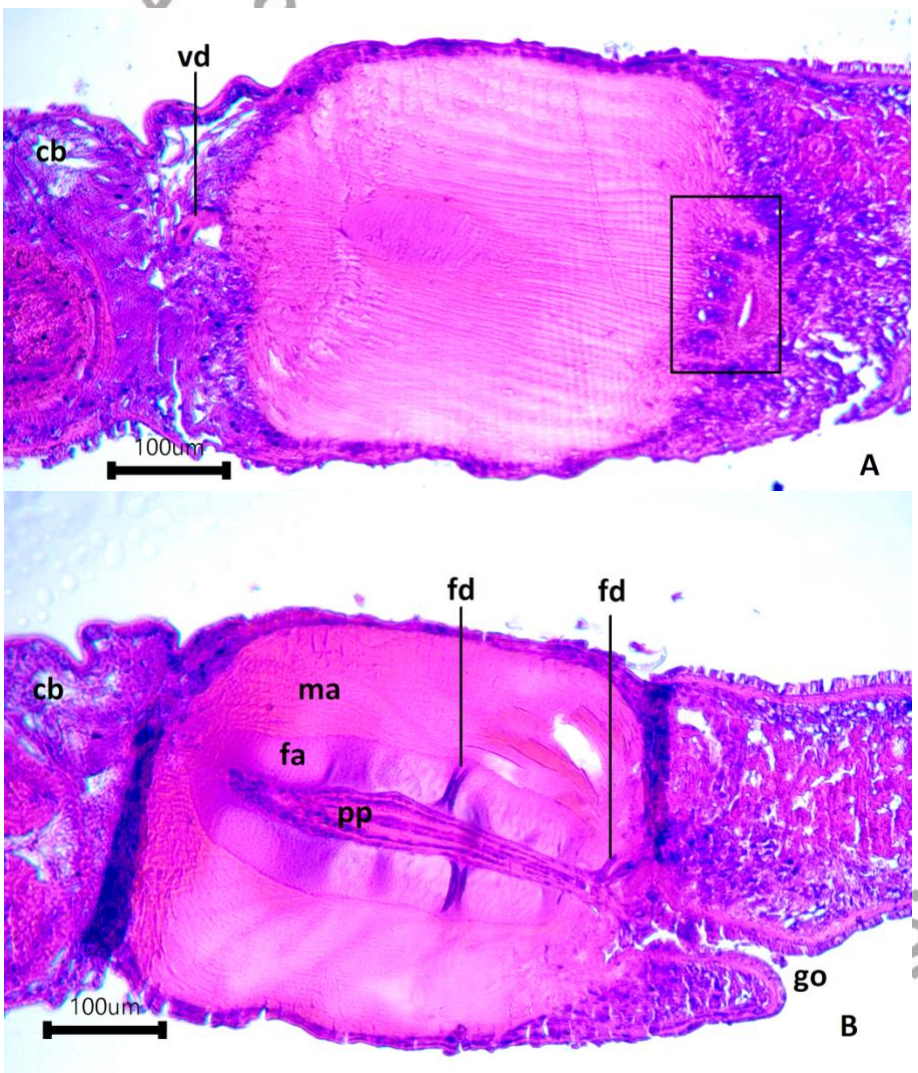

Figure 2. Microphotographs of the copulatory apparatus of specimen CR1 (monopharyngeal), sagittal sections: A – slide CR1-22-2 showing the orifices corresponding to the fine ducts (in square); B – slide CR1-25-1

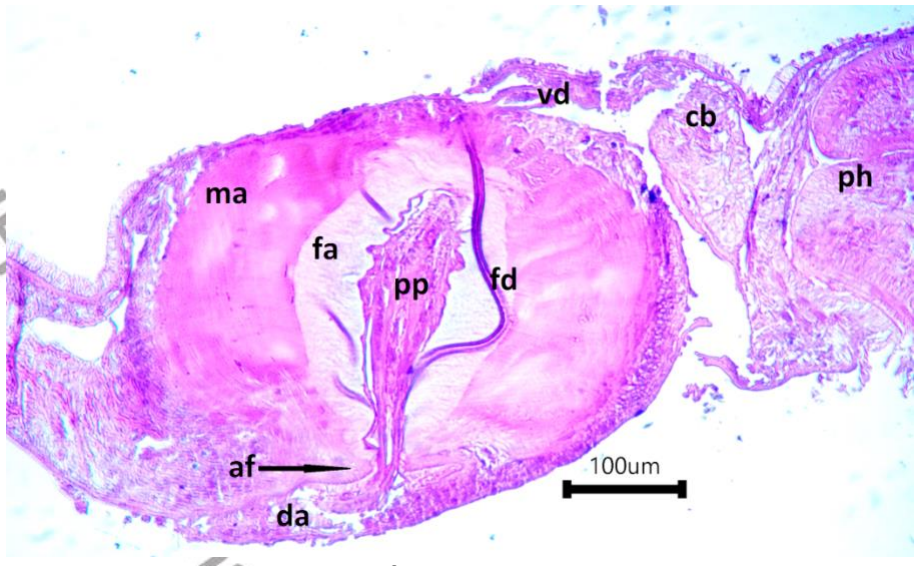

Figure 3. Microphotograph of the copulatory apparatus in specimen Cm1 (monopharyngeal), sagittal sections, slide Cm1-33-3

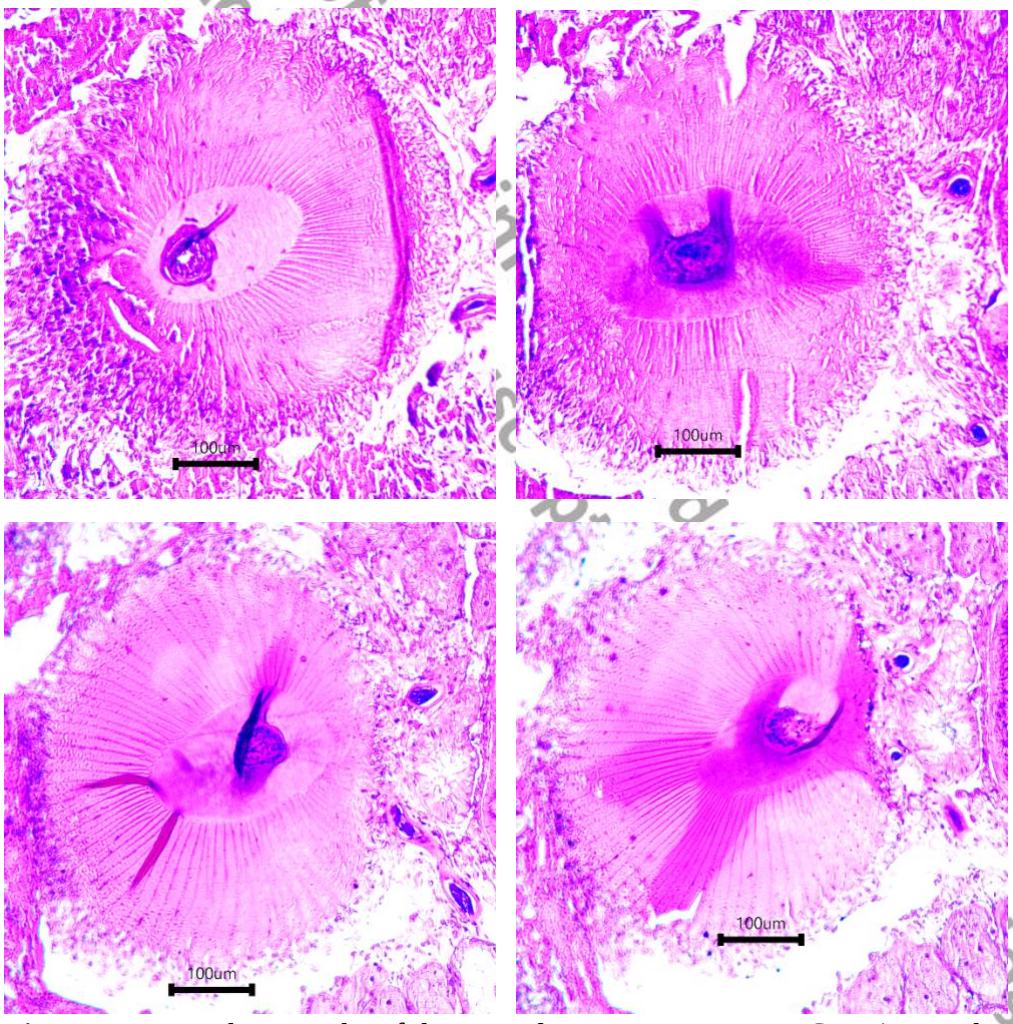

Figure 4. Microphotographs of the genital atrium in specimen CR4 (monopharyngeal), the muscular and fibrous regions showing fine ducts / sclerotized lines

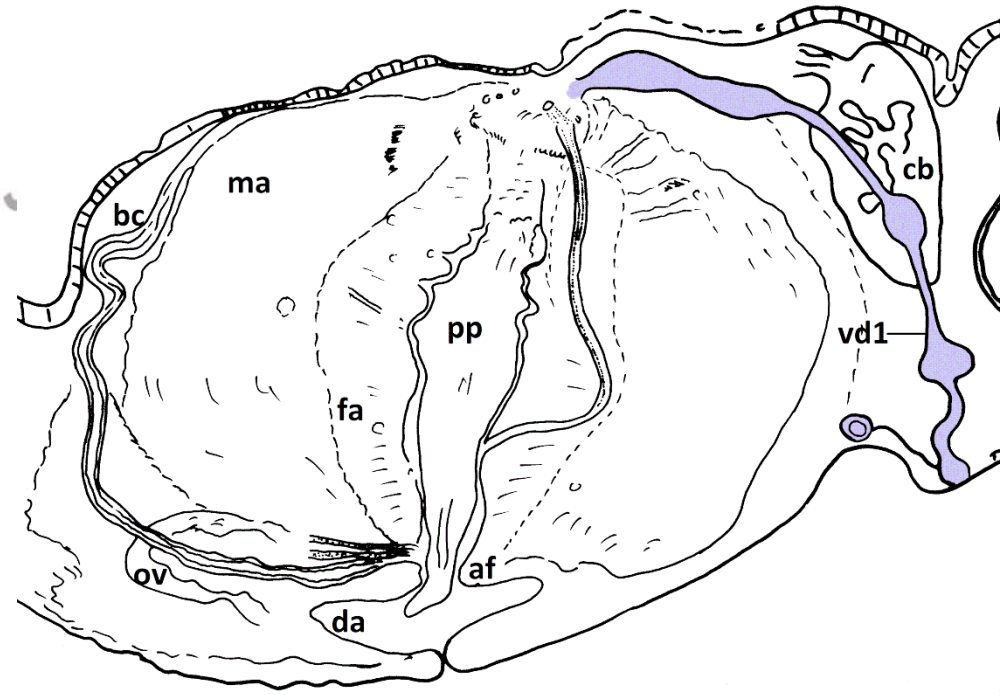

Figure 5. *Crenobia alpina* (monopharingeal, specimen Cm1), sagittal reconstruction of the copulatory apparatus with the course of the right vas deferens, anterior to the right

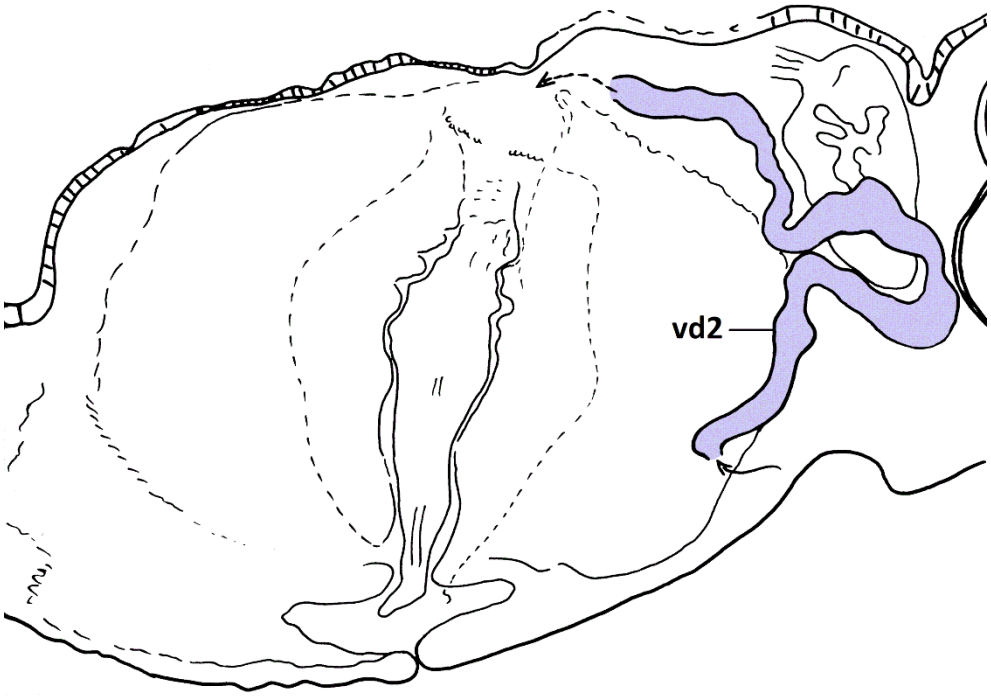

Figure 6. *Crenobia alpina* (monopharingeal, specimen Cm1), sagittal reconstruction of the copulatory apparatus with the course of the left vas deferens, anterior to the right

The polypharyngeal *Crenobia montenigrina* (CR8 – sexually mature, collected in November; CR2, CR3, Cp1, Cp2 – immature individuals, collected in summer)

Besides the pigmented specimens, unpigmented (white) and marbled polypharyngeals were also collected (Fig. S1). Numerous pharynxes, opening into a common space with a posterior mouth, at a certain distance from the genital orifice (shifted anteriad) (Fig. 7).

The mature specimen CR8 show small and rare pre-pharyngeal follicular testes located ventrally. The copulatory apparatus (Figs. 7, 8) presents most of the characteristics of *C. montenigrina* as described in the literature (Sluys 2022), excepting the asymmetry of the vasa deferentia. The fine ducts / sclerotized lines are present but to a much lesser extent than in the monopharyngeals.

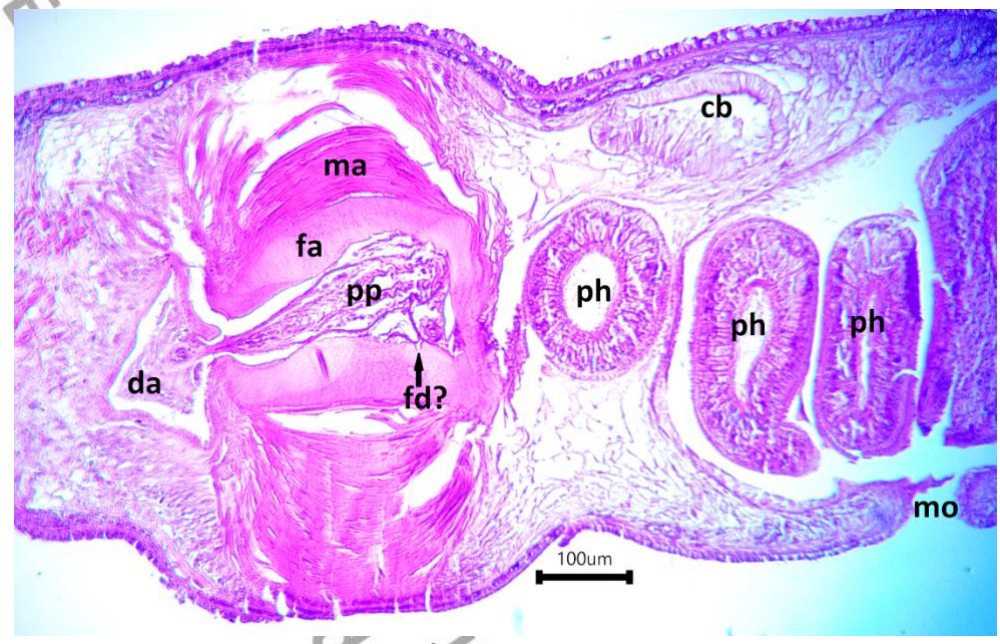

Figure 7. Microphotographs of the copulatory apparatus in specimen CR8 (polypharyngeal), sagittal sections, slide CR8-39-3

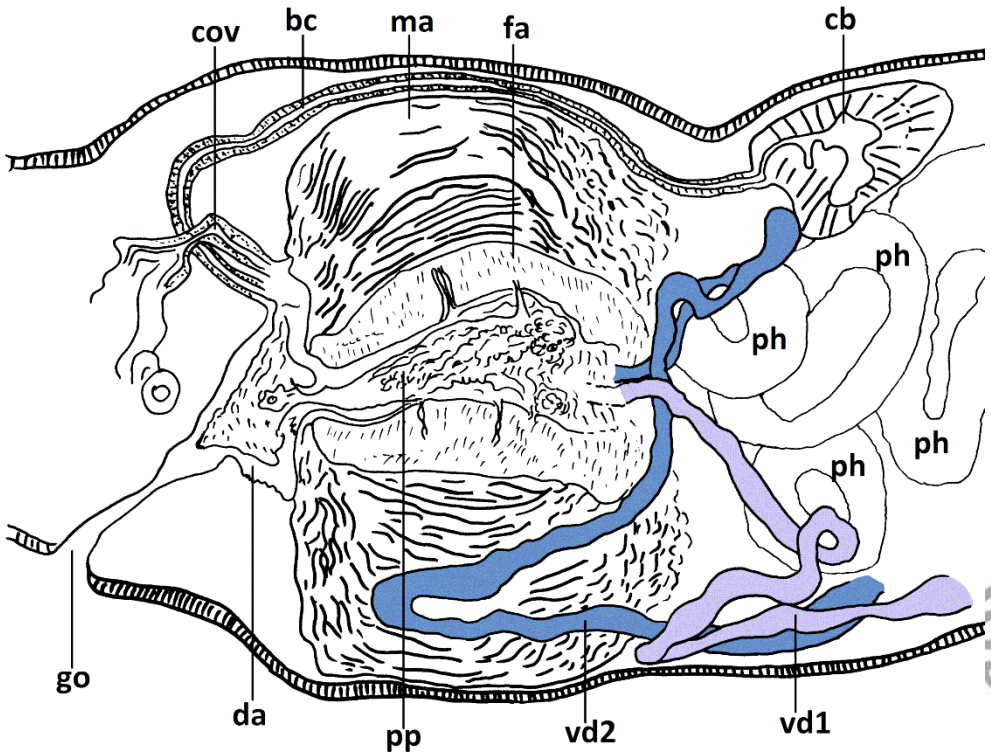

Figure 8. *Crenobia montenigrina* (polypharyngeal, specimen CR8), sagittal reconstruction of the copulatory apparatus, anterior to the right

The immature polypharyngeal specimens (sampled in summer) reveal incompletely developed copulatory apparatus, developing follicular testes and ovaries on histological sections (Fig. S2).

Morphologically, the Romanian specimens of both *Crenobia alpina* and *Crenobia montenigrina* fit quite well the description from literature (Sluys 2022). Minor differences such the symmetry of the vasa deferens in specimen CR1 (monopharyngeal) and the asymmetry in specimen CR8 (polypharyngeal) might be individual characters (intrapopulational variability).

A comparison of the morphological characters analysed in this paper for the two species is presented in Table 1.

Character	*C. alpina* s. str.	*C. montenigrina*
Number of pharynxes	One pharynx	Numerous
Mouth opening	At the posterior end of the pharyngeal pocket	Shifted anteriad
Testes position	Prepharyngeal, ventrally	Prepharyngeal, ventrally
Vasa deferens trajectory; the loops	Symmetrical and asymmetrical	Asymmetrical and symmetrical (literature)
Penis papilla	Extends beyond the atrial constriction – Fig. 3	Extends beyond the atrial constriction – Fig. 7

Table 1. A synthetic comparison between *C. alpina* s. str. and *C. montenigrina*

The characters with diagnostic value appear to be the number of pharynxes and the position of the mouth opening; the two species appear to be cryptic at the level of the copulatory apparatus.

The complex system of fine ducts / sclerotized lines are possibly genuine biological elements involved for instance in shedding and releasing cocoons.

3. Aspects regarding the reproductive biology (Figs. 9, 10; Table 2)

The monopharyngeal *Crenobia alpina*

During the warm season (June, July), the following types of monopharyngeals were collected: mostly specimens with large, well-developed copulatory area (Fig. 9Aa); two specimens with a dilated, oversized copulatory area having the aspect of an internal cocoon (Fig. 9 Ab); a few specimens with small copulatory area; a few asexual specimens, without copulatory area. External cocoons were not observed.

During the cold season (November), most part of the monopharyngeals show small copulatory area, a smaller proportion are asexual without copulatory area. The asexuals have a distinct rounded appearance of the posterior end (Fig. 9B**)**. The rounded posterior end is here associated with hatching, such specimens resulted by hatching.

Fission not observed. Mating not observed.

The polypharyngeal *Crenobia montenigrina*

During the warm season (June, July) most specimens have small copulatory area, and some are asexual (they lack the copulatory area).

During the cold season (November), all the specimens in most springs presented large copulatory area; in only one investigated spring, all specimens were asexuals with no copulatory area – reocrene no. 2 (CB-Romanul), November 2022.

Neither external, nor internal cocoons were observed. The polypharyngeals were found undergoing fission during July and November in non-mixed populations (for instance reocrene spring no. 2 – July 2021, Păpușa spring – November 2020 (Fig. 10 A, B). Specimens most

probably resulted from fission were sampled in November (Tidvele area); their rear end has a tapering appearance (Fig. 10C).

The population of the reocrene spring no. 2 (CB-Romanul) consisted of only immatures polypharyngeals on 05 July 2021, and as a mixture of 7 asexual polypharyngeals and one asexual monopharyngeal on 07 November 2022. In this latter case of mixed population, polypharyngeals and monopharyngeals cannot interbreed due to their immaturity. This spring was found almost completely deteriorated in November 2022.

Mating not observed in polypharyngeals.

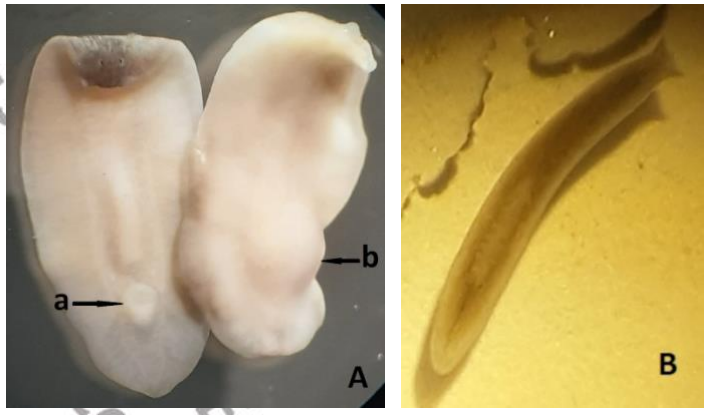

Figure 9. *Crenobia alpina*, general habitus: A – in summer, a – large copulatory area, b – oversized copulatory area; B – in November, small asexual specimen with rounded posterior end, most probably resulted from the late summer /early autumn hatching.

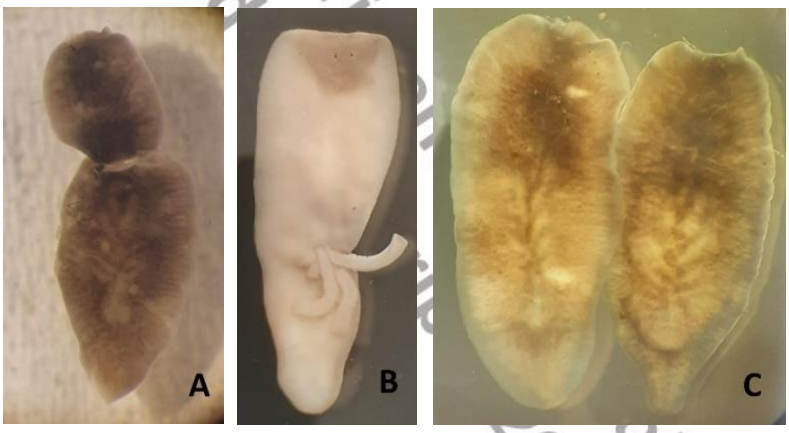

Figure 10. *Crenobia montenigrina* general habitus: A – specimen undergoing fission (5 November 2020), B – specimen undergoing fission (5 July 2021), C – specimen (the right specimen) with the rear end tapered, most probably resulted from fission.

The reproductive characteristics of the monopharyngeals and polypharyngeals are synthesized in Table 2.

	Copulatory area/reproductive biology characters Warm season	Copulatory area/reproductive biology characters Cold season
Monopharyngeal specimens *C. alpina*	- large copulatory area in most specimens - internal cocoons (copulatory areas with the aspect of internal cocoon) - fission not observed	- small copulatory area - asexuals (copulatory area absent) - fission not observed

Polypharyngeal specimens *C. montenigrina*	- small copulatory area - absent copulatory area - undergo fission	- large copulatory area - no cocoons observed - undergo fission

Table 2. Comparative synthetic table of the seasonal reproductive characteristics of the monopharyngeal *C. alpina* and the polypharyngeal *C. montenigrina*

A schematic biological cycle can be sketched based on the observed reproductive characters:

The monopharyngeals mate and produce cocoons (internal, maybe external as well) during the warm season (June, July). The few asexuals or those with small copulatory area are most probably resulted from cocoons hatching. These hatchlings grow and start to develop the copulatory area. They become specimens with small copulatory area, unable for sperm transfer in the cold season (November). The November asexuals (with no copulatory area) result from late summer /early autumn hatching.

The polypharyngeals mate during the cold season when they have completely developed copulatory area. They are unable to mate during the warm season, as they have small copulatory area or lack it.

This study strongly indicates the seasonal reproductive isolation of *C. alpina* and *C. montenigrina*. A deeper and true reproductive isolation should be verified by the comparative study of spermatozoa in the two forms.

The natural history of *C. alpina* and *C. montenigrina*, much discussed in the literature (Codreanu 1956 and reference therein) remains still unsolved and raises many questions: When and how the two species appeared? Why do they form sympatric populations in the Carpathian (Parâng) Mountains? Are they sympatric elsewhere in Europe?

As to their origin, three possibilities can be discussed:

1) *C. alpina* and *C. montenigrina* have a common ancestor which disappeared from the current fauna. In this hypothesis, from a common pre-glacial ancestor (before the Quaternary Glaciation) resulted sympatric *C. alpina* and *C. montenigrina* - with a much northern geographical distribution, later modified by the glaciation. Both species are cryophilic, but regarding the reproductive period, *C. montenegrina* is more cryophilic (sexually mature in the cold season). In the cold periods of the Glaciation (the glaciation stadials) both species would have undergone a southward migration. *C. montenigrina* settled in high mountainous areas, corresponding to their thermal optimum. In warm periods (interstadials), *C. alpina* would have undergone the reverse migration path from south to north while *C. montenigrina* did not.

2) *C. montenigrina* resulted from *C. alpina*. This theory must admit the present geographical range of *C. montenigrina* as speciation center. A question that needs to be answered is: How the pharyngeal number (one / numerous) is related to shifting reproductive season?

3) *C. alpina* resulted from *C. montenigrina* – for the moment this hypothesis is difficult to be accepted.

Conclusions

The present results are consistent with the theory of temporal reproductive isolation of the two species, expressed by Codreanu (1956). *C. alpina* and *C. montenigrina* remain species of great interest for future studies. The factors that influence the reproductive biology of the two species in sympatry are poorly understood.

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Supplementary figures

Figure S1. *Crenobia montenigrina* - black, unpigmented and marbled living specimens

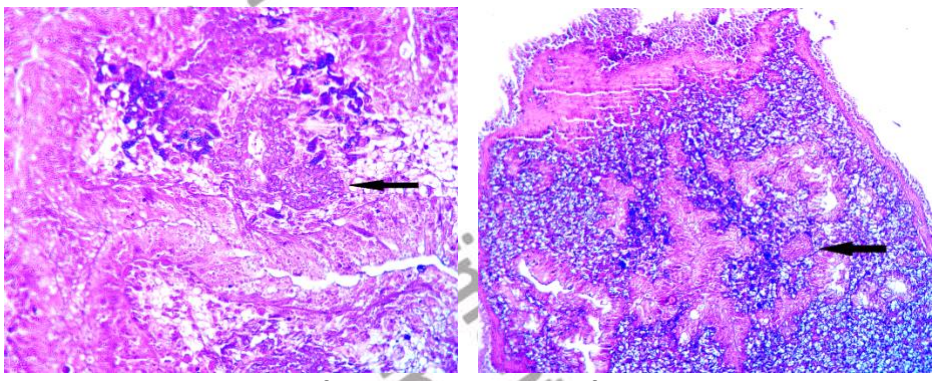

Figure S2. Developing follicular testes (left) and ovary (right) in immature polypharyngeal specimen CR3 (scale bar not available)

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