ECOLOGIA BALKANICA
2021, Vol. 13, Issue 1 June 2021 pp. 27-33

Data on the Stomach Content Analysis and Fat-body Morphology in the Agile Frog (Rana dalmatina Fitzinger in Bonaparte, 1838) during the Mating Season in Bulgaria

Teodora V. Koynova[1*], Simona I. Valkova[1], Nikolay P. Simov[2], Mario S. Langourov[2], Nikolay D. Natchev[1,3]

1 - Konstantin Preslavski University of Shumen, Faculty of Natural Sciences, Department of Biology, Universitetska 115, Shumen 9700, BULGARIA
2 - National Museum of Natural History, Section Invertebrates, Tzar Osvoboditel 1, Sofia 1000, BULGARIA
3 - Vienna University, Department of Integrative Zoology, Althanstrasse 14, Vienna 1090, AUSTRIA
*Corresponding author: t.koynova@shu.bg

Abstract. We present data on the stomach contents and the gross morphology of the fat body in Agile Frogs (*Rana dalmatina*) from a pond which is an object of a long-termed monitoring. We also provide information on the diet of one female of *Lissotriton vulgaris*. All of the specimens were found dead during the mating season, when sudden snowfalls and depression in the temperature covered the ponds with ice and snow layer for several days. The stomach content of the frogs was represented by some chitin rests, shed-skin, and plant material. The fat bodies of the frogs were dramatically reduced in size - an indicator of low level of energetic reserves. We conclude that on the basis of our findings, massive investigations by using stomach flushing methods during the mating season in the Agile Frogs have to be avoided.

Key words: Amphibia, diet, energetic reserves, reproduction success.

Introduction

The Agile Frog (*Rana dalmatina* Fitzinger *in* Bonaparte, 1838) is an explosive breeder (Wells, 1977). During the initial days of the breeding season, the frogs potentially expose themselves to risks, because of the low temperatures (see Hartel et al., 2007). The males may enter the water in large numbers - the sex ratio is strongly male-biased (Stojanov et al., 2011) and the sexual selection is a result of the direct competition. According to Hartel (2005), the pre-productive period of calling and 3-D positioning of the males is rather short. For the Agile Frog was described a unique behavior of "egg clutch piracy", where some males inspect the freshly laid eggs and clasp them in the same way they would clasp a female. By doing so, these males may successfully fertilise the eggs, actually avoiding sexual selection, but contributing to the genetic variation (Vieites et al., 2004).

Many aspects of the biology of *R. dalmatina* are well studied, however to date data on the diet of the Agile Frogs during the mating period are scarce (see Guidali et al., 2000; Mollov & Delev, 2020), with only one study from Bulgaria so far (Angelov & Batschwarov, 1972). In the present study we provide new data on the stomach content of the frogs during the reproduction time and also data on the gross morphology of their fat body – an indicator for the energetical status in frogs (Guidali et al., 2000).

The manuscript represents collateral findings made during a long-term monitoring on the habitat specifications of the Agile Frog larvae. Since February of 2014 we investigate the water quality of two water bodies in the Protected Site "Shumensko plato" BG0000382 (NE Bulgaria), because the larvae of this species are reported to be highly sensitive to certain changes in water chemistry - the mortality rate may reach 100% (see Andren et al., 1988). The region of the Protected site is rather dry, so we monitored the only two water basins which maintain permanent water volumes during the whole year. These two ponds are crucial for the survivability of the population of *R. dalmatina* in the region and are in the vicinity of a road with moderate traffic. The identified local threats are related to an increase in the acidity and conductivity of the water.

Material and Methods

We started the inspections of the ponds in the second half of February each year because these are the days when the initial seasonal activity of *R. dalmatina* can be detected in Bulgaria (Naumov, 1999, pers. comm.). In the range of our study we investigated 20 specimens of the Agile Frog. The frogs were found dead in the water basins they use as breeding spots at 43.2488°N, 26.8933°E, at about 480 a.s.l. (Datum: WGS84). The findings were performed in three different days in the period 2018-2020. All the frogs except one were males. On 29.03.2018 we found also one dead female Smooth newt (*Lissotriton vulgaris* Linnaeus, 1758) - for comparative analysis of the stomach content we included that specimen in our study. All dead animals were immediately fixed in Bouin solution and then conserved in Formol. The samples were transported to the laboratory for dissections, stomach contents analysis and observation of the fat body (only for the frogs). All frogs are now preserved in alcohol and are a part of the collection of the "Center for Natural Sciences" at the Shumen University.

We checked the stomach contents in all specimens and photographed the fat bodies of the frogs by using Sony RX 10 III. The contents of the stomachs which were not completely empty were preserved in fixative (Bouin solution) and investigated under a light microscope "Carl Zeiss Jena Amplival" microscope and "Carl Zeiss Stemi 2000" stereomicroscope. All particles were documented and we analysed the origin and the conditions of the materials. The photographs were made using a digital camera "Canon EOS 2000D" and "AxioCam ERc 5s". The preparations of the epithelium tissues were stained using "Methylene blue".

Results and Discussion

During our investigations, we detected a total of 48 nights in which the ponds were covered fully or partly with ice layer during the period the frogs were into the water. The ice coverage indicates that the water temperatures may drop dramatically even in the end of March. However, we were able to detect dead frogs only on three occasions: on 29.03.2018, on 31.03.2018 and on 30.03.2020. On 29.03.2018 were found 12 specimens (including the dead female *Lissotriton vulgaris*); on 31.03.2018 – seven and on 30.03.2020 – one specimen. In all three cases, the occurrence of dead specimens was not related to dramatic changes in the water chemistry (water pH was 6.25, 6.27, 6.84 and conductivity was 50, 64, 66 μs/cm), but presumably because of the ice and snow-covered the total surface of the basins.

In the stomach of the female *L. vulgaris* were obtained six specimens of *Asellus aquaticus* Linnaeus, 1758 (Fig. 1a) and six larvae of Chironomidae (Diptera) - most of them in an advanced stage of digestion. In this season of

the year, *L. vulgaris* is in its aquatic stage (Stojanov et al., 2011; Heiss et al., 2016) and its feeding apparatus is in the "aquatic mode" (see Heiss et al., 2017). Obviously, there is plenty of potential prey in the water basins, however, the ranid frogs are constrained by their feeding morphology and may feed almost exclusively on land (see Nishikawa, 2000). The Agile Frog is reported to feed only terrestrially and preys on a variety of invertebrates (Aszalos et al., 2000; Guidali et al., 2000; Hodisan et al., 2010; Cicort-Lucaciu et al., 2011). Aszalos et al. (2000) reported an aquatic origin of some prey items found in the stomach content of Agile Frogs, but stressed also that the frogs have consumed them on land.

Fig. 1. Microscopical photographs of the stomach content of the investigated specimens: a. rests of *Asellus aquaticus* extracted from the gaster of a female *Lissotriton vulgaris* found dead together with 12 of the dead Agile Frogs on 29.03.2018; b. chitin remains found in frog specimen coded 07; c. chitin remains found in the stomach of frog specimen coded 08; d. plant material from the stomach of frog specimen coded 02; e. plant material from the stomach of frog specimen coded 07.

The male frogs start their activity in the ponds immediately after hibernation at the end of the winter and remain in the water for a prolonged time (Naumov, 1999 and pers. comm.; Guidali et al., 2000). It can be expected that the Agile Frogs (or at least the males) do not feed in the mating period, however, some data indicate on feeding activity during this time (Guidali et al., 2000). These authors report the presence of insects, insect eggs, diplopods, araneans, and oligohets in the diet of the frogs. In our investigation, we found that the stomachs of most specimens are completely empty (Fig. 2a). In five of the males we were able to detect some content in the stomach (Fig.1b,c,d,e): in one of the stomachs we found chitin remains and also some materials in a very late stage of digestion; in two we found material of plant origins (in one of them also chitin remains); in two there was shed-skin. This food particles indicate that the frogs attempt to feed during the mating period, but obviously the prey is scarce, because of the weather conditions. Similar situation is observed with another anuran species from Bulgaria – the Green Toad, *Bufotes viridis* (Laurenti, 1768), which is also an explosive breeder and breeding takes place in February-March. Mollov et al. (2020) registered a significant portion of empty stomachs in the toads captured, during the breeding season, which indicates, that most of the individuals do not feed during that time. The plants have apparently no food value for the frogs and the plant remains found within the stomachs can be regarded as material that may be ingested by a fail capture attempt or as collateral debris taken up during the predation (Whitaker et al., 1977; Mollov & Delev, 2020).

The shed-skin consumption in frogs was an object of some discussions. According to some authors, it has a random character (Ferenți & Covaciu-Marcov, 2009) and does not have any nutritious value, but Weldon et al. (1993) stated that this act is important for the recycling of the epidermal proteins. The rate of dermophagy is detected to increase in periods with low number of prey (Covaciu-Marcov et al., 2010).

The fat body is closely associated (both embryologically and anatomically) with the gonads and plays a very important role in the production of the sperms and the eggs (Chieffi et al., 1975). This is also the main deposit of fat in amphibians and the main energy deposit of the body for supporting the general metabolism and reproduction. As such, the fat body of the frogs may change dramatically in size during the year cycles and in animals which had starved for prolonged periods. That organ may undergo an almost full regression of the fat tissue to mesenchyme (Zancanaro et al., 1996). Girish & Saidapur (2000) report that even in frogs which are fed once a week, the fat body may be reduced to filiform shape, so the form and the volume of the fat body is a good indicator for the energy status of the particular individuals. In anurans, the annual fat body cycle and the cycle of the ovaries are in inverse correlation (Hoque & Saidapur, 1994). This is well supported by our finding represented in Fig. 2a - the female Agile Frog had developed eggs, but the fat body was reduced completely. Our sections revealed that the fat body in all males was dramatically reduced in size - the most prominent fat body we were able to found is represented in Fig. 2b. In three of all frogs we were not able to detect visually any fat body, in other three frogs the fat body was reduced in size, but had retained the typical star-formed shape of the organ and in all other frogs the fat body was filiform. The gross anatomy of the fat bodies indicates, that the frogs are on the limit of their energetic reserves. This is normal for the Agile Frogs and plenty of frog species are able to handle prolonged periods with low energetic income and high energetic demands (for an overview see Zancanaro et al., 1996), however the Agile Frogs do attempt to feed during the mating season and the potential income of food may be critical for the fitness of the animals prior to post-mating period in which more intense

feeding begins (see Guidali et al., 2000). In our opinion, mass investigations by the use of stomach flushing techniques during the mating season are inappropriate for that species - potential loss of energy cannot be recovered by simply "eating again", because prey could be scarce during the late winter and the early spring.

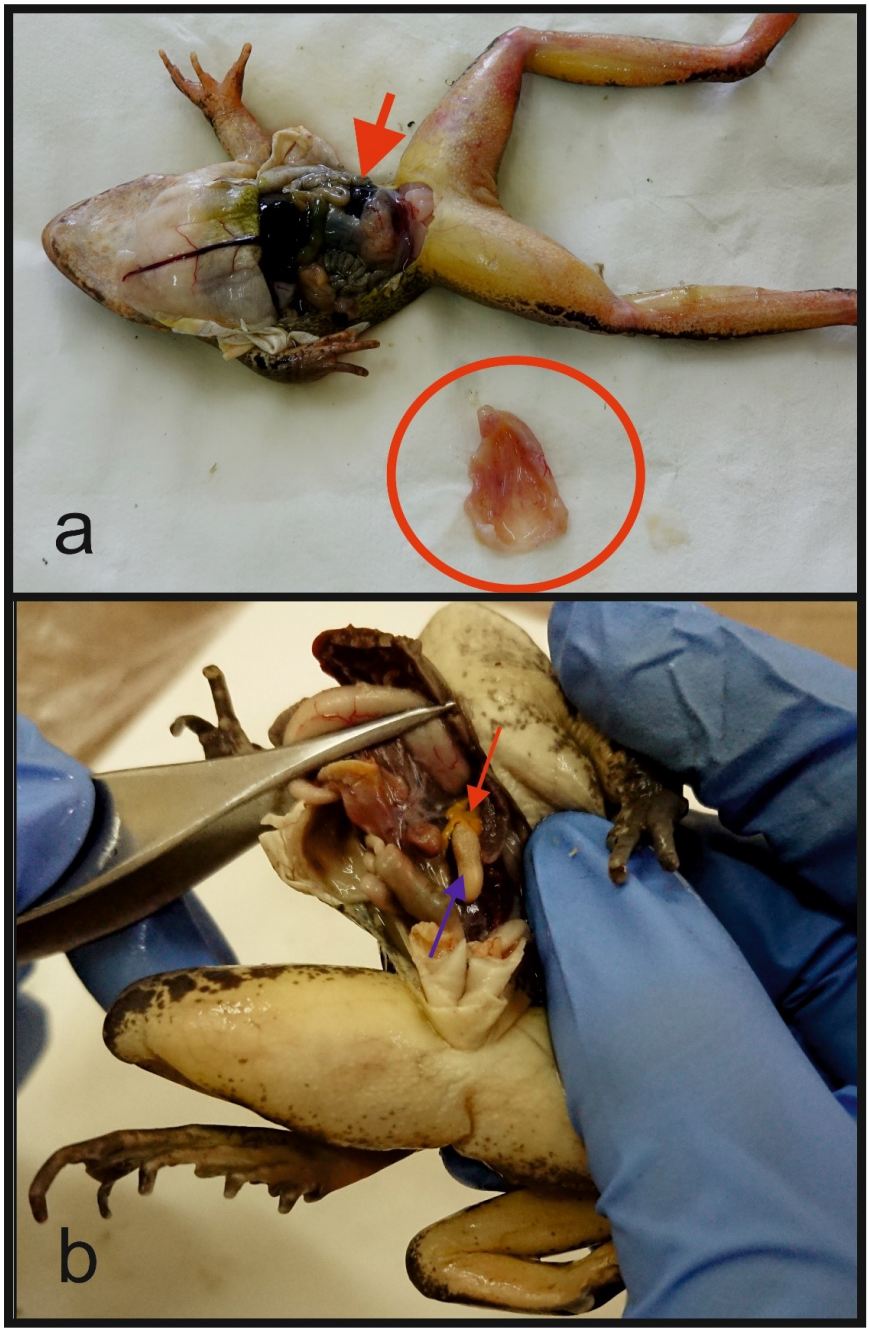

Fig. 2. Dissection of two Agile Frog specimens: a. female found dead on the 30.03.2020, the red arrow indicates the presence of eggs, the red circle is provided around the stomach - the dissection reveals lack of any food particles; b. male Agile Frog revealed the morphological condition of the fat body - the organ is dramatically reduced in size, but still had retained its general form (please note, that this was the largest fat body, which we were able to detect), the red arrow indicates the fat body associated with the left testes (blue indicator).

Acknowledgements

This work was supported by the Research Fund of the Konstantin Preslavsky University of Shumen (Grant No. RD-08-67/25.01.2021) and Bulgarian Ministry of Education and Science under the National Program for Research "Young Scientists and Postdoctoral Students".

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Received: 11.11.2020
Accepted: 13.03.2021