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The methodology for rearing the Fire-bellied Toad (*Bombina bombina*) from protected, small, isolated (but degraded) habitats in its northern distribution range

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Abstract.—The Fire-bellied Toad (*Bombina bombina* Linnaeus, 1761) is a vulnerable and protected species in Europe, where it is suppressed in small, isolated populations in its northern distribution range. The main cause of *B. bombina* population declines in this region is the loss of suitable habitats due to either anthropogenic factors or natural succession. Recently, very hot summers with prolonged dry and very heated periods have contributed to the declines of *B. bombina* populations on a very large scale. Therefore, it is important to preserve the natural, although small, populations of *B. bombina* to save the gene pool of the rare northern populations for the future, which is essential for conservation breeding, research, and outreach with this species. This study provides the rearing methodology, growth rates, and sexual dimorphism of protected *B. bombina* individuals in their first year.

Keywords. Anura, endangered species, conservation, sexual dimorphism, morphometrics, Natura 2000

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Introduction

Interest in species conservation is growing each year, and amphibian species are one of the most endangered groups of vertebrates worldwide (Kurnaz and Kutrup 2019; Stuart et al. 2008; Wake and Vredenburg 2008). The Fire-bellied Toad (*Bombina bombina* Linnaeus, 1761) is one of these vulnerable amphibian species that is distributed mainly in Central and Eastern Europe (Nicoara and Nicoara 2007; Sillero et al. 2014).

Bombina bombina is a tailless amphibian. The body of this frog is flat, and its length varies from approximately 40 to 55 mm. The back of *B. bombina* is dark brown or greyish, and the underbelly is mottled with wide red and orange spots that they use to scare away predators (Rimšaitė 2021). The most suitable habitats for *B. bombina* are in lowlands such as shallow fish-free ponds with plenty of sunlight, or shallow edges of larger water bodies (Rimšaitė 2021; Schröder et al. 2012). This species can also inhabit swamps, flooded ditches, quarries, and peat bogs (Chikhlyaev and Ruchin 2021). *Bombina bombina* breeds in stagnant, good-quality water with natural eutrophic conditions (Kinne et al. 2006). According to research conducted in Latvia, *B. bombina* calling males were found in small (<0.5 ha) and medium (0.5–10.0 ha) sized lentic waterbodies and ditches (Čeirans et al. 2020).

The northern range of the distribution of *B. bombina* in Europe is within northern Lithuania and the south-eastern parts of Latvia (Kuzmin et al. 2008; Pupina and Pupins 2008, 2009). This species is protected in both countries (Berzins 2003; Rimšaitė 2021). Although the range of *B. bombina* covers all Lithuanian territory, it has a very fragmented distribution. In the territory of Lithuania, approximately 20,000–50,000 *B. bombina* individuals can be found, but there are only a few suppressed populations in the northeastern and western parts of Lithuania. Most of these populations are small, consisting of 10–20 adult individuals (Ivinskis and Rimšaitė 2011; Rimšaitė 2021).

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At the beginning of the 20th century, *B. bombina* was not classified as a rare species in Lithuania (Fedorowicz 1918). Today it is classified as a Near Threatened species, and it is included in the Red Data Book of Lithuania (Rimšaitė 2021). A similar situation is seen in Latvia, where *B. bombina* populations are very small and isolated (Pupins and Pupina 2012). In most of these populations, no more than 20 vocalizing males were recorded (Pupina 2011; Pupina and Pupins 2007). In Latvia, this species is included in the Red Book of Latvia and assigned to the first endangered species category (Berzins 2003). This is the most recent information described in the literature, but the current situation could be even worse. Furthermore, it is also listed as a protected species in Appendix II of the Bern Convention and Annexes II and IV of the EU Habitats Directive. *Bombina bombina* is included in the IUCN Red List of Threatened Species, where its most recent assessment was conducted in 2008 and it was listed as Least Concern (IUCN 2023).

The main causes of declining *B. bombina* populations in its northern distribution range are the loss of suitable habitats due to either anthropogenic factors or natural succession (Pupina et al. 2018; Pupins and Pupina 2012; Tytar et al. 2018). In some water bodies, populations of *B. bombina* are reduced by the appearance of the highly invasive, predatory fish species *Perccottus glenii* Dybowski, 1877 (Pupina and Pupins 2008). In Lithuania, the main drivers of *B. bombina* decline are changes in land use within the habitats of the species, and climate change leading to prolonged heated periods in the spring and summer (Rimšaitė 2021). When small ponds dry up, the tadpoles or even adult individuals often die. This can represent a crucial loss for the small, isolated *B. bombina* populations that are at a very high risk for the loss of genetic variation through genetic drift and inbreeding (Frankham and Ralls 1998). Furthermore, strong shifts in the sex ratios in several isolated populations have been noted, as they have become more isolated during such habitat degradation. Some of these populations became male dominated, while others became female dominated, possibly resulting from the very small numbers of *B. bombina* individuals in these populations. Therefore, it is important to save these natural, although small, populations of *B. bombina* in order to stabilize their sex ratio and re-establish them in more suitable habitats for their continuing occurrence within their northern distribution range. To achieve this, it is important to use *ex situ* methods such as rearing *B. bombina* individuals in the laboratory and releasing juveniles back into their natural habitats, as they will have a higher chance of survival compared to other stages of development. It is also important to choose suitable ponds for the release of *B. bombina* individuals, where their survival and breeding *in situ* in their natural environment would be ensured. Releasing individuals into nature in an equal sex ratio contributes to better survival of the population.

The aim of the study was to develop an effective methodology for the rearing of collected *B. bombina* spawn from degraded habitats of the small, isolated populations. Several spawn of *B. bombina* individuals were collected from degraded habitats in three locations in Lithuania between 2017 and 2021. Collected spawn was incubated, and the hatched tadpoles were grown and released into more suitable, protected habitats near the initial collection sites. In this article, we provide the rearing methodology, growth rates, and sexual dimorphism of reared *B. bombina* individuals. This methodology of *B. bombina* egg-rearing until the juvenile stage may contribute to saving the small, isolated gene pools of *B. bombina* populations for the future at the northern edge of its species distribution.

Materials and Methods

Collection of *Bombina bombina* Spawn

Spawn of *B. bombina* was collected from naturally degraded ponds in four Natura 2000 sites in Lithuania: Juodabalė Zoological Reserve (LTLAZ0010); Drapalai village surroundings (LTDRU0004); Kučiuliskė village surroundings (LTLAZ0001); and Margiai village surroundings (LTLAZ0035) (Table 1). Natura 2000 is an ecological network of rare and threatened species protection sites, which stretches across all 27 European Union countries. Spawn was collected during the end of spring and beginning of summer (May–June), when water temperatures reached 13–14 °C, in 2017, 2018, 2020, and 2021. The year 2019 was not included in the study, because that spring was very hot and dry which caused the total drying out of the *B. bombina* spawn. The spawn of eggs was carefully collected from flooded plants at the earliest stage of their development and placed in a 10 L bucket half-filled with pond water. Water from the ponds was taken along with the resident microfauna so that the tadpoles could feed in the early stages. The buckets with eggs were kept outdoors to protect them from strong temperature fluctuations, and at 1–2 days after collection, they were delivered to the Lithuanian Zoological Gardens (Kaunas, Lithuania).

Incubation of Spawn

The delivered spawn with water was very carefully transferred to 60 L plastic containers (Fig. 1A) that were filled with water to three-fourths capacity. The water pH was 7.6–7.8, and total water hardness was 2.6–2.8 mmol/L. In the laboratory, the initial water temperature was maintained similar to outdoors (13–14 °C), and within 5 days the temperature was gradually increased to 24–25 ºC and maintained at that level for the remainder of the study. The temperature was increased gradually to avoid thermal shock to the eggs. Overall, the average water temperature was maintained

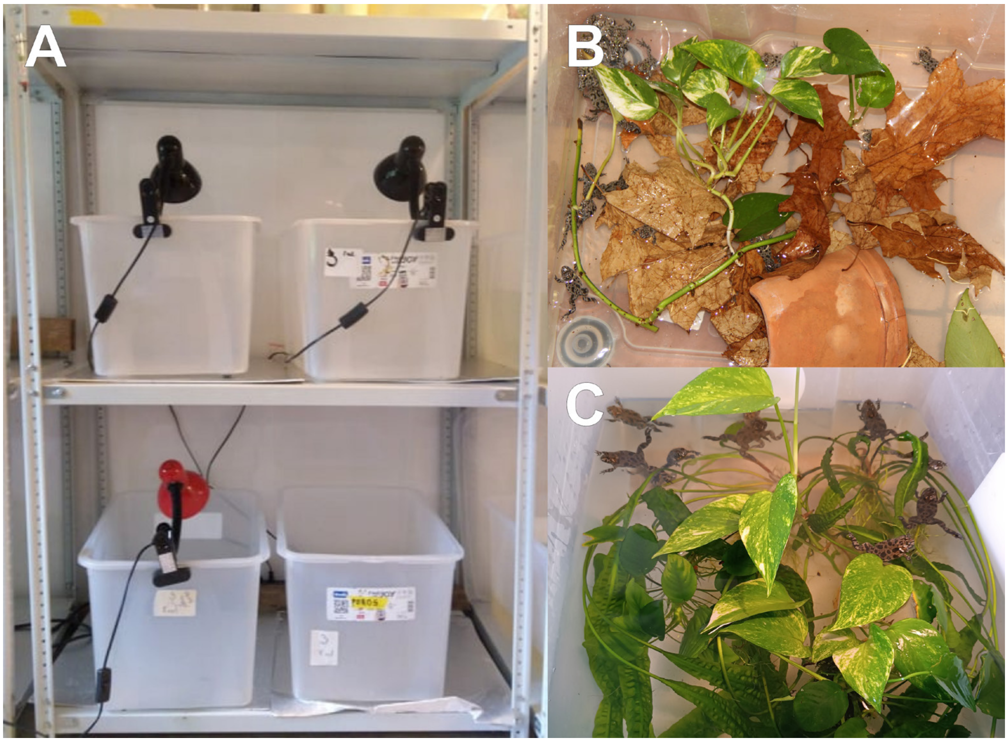

Fig. 1. *Bombina bombina* rearing system. **(A)** Plastic rearing tanks. **(B)** Three-month-old *B. bombina* individuals. **(C)** Eight-month-old *B. bombina* individuals.

at 21.0 ± 2.3 ºC during all incubation periods. Before filling the containers, they were washed with antiseptic and rinsed thoroughly with hot water. The lids of containers were with perforated with holes. The larvae hatched in 5–7 days.

Rearing during the Larval Period

After hatching, the *B. bombina* tadpoles were kept in 60 L plastic containers with dimensions of 58 x 35 x 30 cm. Every day, dirt was removed from the bottom with a sieve, and one-fifth of the water was drained, and fresh tap water was added slowly. The water with larvae was moved as little as possible. The water pH was 7.6–7.8, and total water hardness was 2.6–2.8 mmol/L. No water aeration or filtration was used. The average water temperature in the containers was maintained at 23.8 ± 1.1 ºC during all metamorphosis stages. We evaluated the embryonic development according to the stages described by Gosner (1960), and the 23–25 stage of *B. bombina* development was reached in 3–5 days. At weekly intervals up to 3 months of age, the juveniles were grouped by their stage of development because their rate of development varied. Before metamorphosis was complete, when the tadpoles entered the 4-leg stage, they needed a place to climb, rest, and put their head above the water (i.e., land). They were transferred to 35 L plastic tanks with a water level of about 5–7 cm and parts of land without water. The tanks were placed at an angle of about 35–45º. Dried oak leaves, live plants, stones, and other materials were placed in the water to make it easier for the juveniles to reach the land. The tanks were covered with dense gauze of 2 x 2 mm mesh size held in place with a rubber band because the juveniles could escape. Lighting was provided by 28W incandescent lamps and REPTI PLANET Repti UVB 2.0 13W (Czech Republic) lamps installed 20 cm above the tanks. The UVB-containing lamps were turned on every day for about 4 hours. UVB rays stimulate the production of vitamin D and calcium uptake (Michaels et al. 2015). The length of the photoperiod was similar to the natural rhythm of day and night, and it was adjusted according to the season.

During metamorphosis, it was important to keep enough microfauna in the water for the tadpoles to feed upon. Microfauna was collected along with the water during egg collection. Tree leaves that began to decay were added to the water so that microorganisms (such as *Paramecium* genus) could begin to proliferate. Dry oak leaves were added to the water due to their disinfectant properties and soft leaves of oak, willow, apple, and pear trees, along with blanched nettles, provided more food for the tadpoles. At the beginning, they were also fed daily with dry feed for fish fry Vipan Baby (Sera, Germany), and later with Bio-vit (Tropical, Poland).

The full larval development period of *B. bombina* lasted for about 45–65 days. Metamorphosis was completed when the juveniles completely lost their tail and had four fully grown legs.

Table 1. The locations (as the codes for LT Habitat Protection Important Territories) of *B. bombina* spawn collection and where reared individuals were released during the study period (2017−2022).

Collection date	Locations of collected spawns	Release date	Locations of release
2017 06 29	LTDRU0004	2018 06 22	LTLAZ0010
2018 05 04 2018 05 15	LTLAZ0010	2019 06 14	LTLAZ0010
2020 05 30 2020 06 02	LTLAZ0010	2021 06 11	LTLAZ0010 LTLAZ0001 LTDRU0004
2021 05 13	LTLAZ0010 LTLAZ0001 LTDRU0004	2022 06 17	LTLAZ0035

Post-metamorphosis Rearing

After they had completed metamorphosis, the 3-month-old juveniles were transferred to 60 L plastic tanks (Fig. 1B). The juveniles were grouped into three tanks according to size, in order to reduce interspecific competition, with 10 to 15 juveniles in each tank. The smallest juveniles were transferred to 10 L tanks, with a lot of vegetation (*Epipremnum* spp., *Anubias* spp., and *Cryptocoryne* spp.) to allow the juveniles to get out of the water. In these tanks, the water depth was about 2–4 cm. All the water in the tanks was changed at least twice a week, or more often if necessary. The average water temperature in the containers was maintained at 23.8 ± 0.6 ºC during all post-metamorphosis periods. During post-metamorphosis rearing in the tanks, UVB-containing lamps were turned on every day for about 4 hours.

The juveniles were fed daily with an amount of food that corresponded to about 10–12% of their body weight. The first food source given to juveniles was live fruit flies (*Drosophilidae*), which were added to the rearing tank. House Crickets (1- to 3-day-old *Acheta domesticus*) and Two Spotted Crickets (1- to 5-day-old *Gryllus bimaculatus*) were given to very small and weak individuals. From 5 to 7 flies were provided for each juvenile, and the uneaten fruit flies were fed on carrot slices, cabbage leaves, etc. Vitamins and minerals were dusted on the fruit flies twice a week. Small crickets (*Gryllus* sp.) were added to the diet of one-month-old juveniles. Live mosquito larvae (*Chironomus* sp.) were added to the diet of juveniles at 6–7 weeks old. From the age of 2 months, juveniles were fed Turkestan Cockroaches (*Shelfordella tartara*). All feed was provided live.

Wintering Period of Rearing Juveniles

The overwintering period was carried out in the lab because the seasonal temperature variation is important for the development and phenology of temperate amphibians, especially their reproductive cycles. Five-month-old *B. bombina* juveniles were prepared for wintering. Over a period of two weeks, the water temperature was gradually reduced to 10 ºC and the photoperiod was reduced to 5 hours. The amount and caloric content of the food was also reduced gradually by 20% per day for five days in a row, and starting at two days before the wintering period, juveniles were no longer fed at all. This was done to ensure that the guts of overwintering toads were empty, otherwise the food inside would rot. Plastic tanks (15 L) with moist 5.5–6.5 pH neutralized peat (DURPETA, Lithuania) and softened dried oak leaves were prepared for the wintering of *B. bombina* juveniles. This substrate was placed in tanks at a depth of 5–7 cm. Ten to 15 juveniles were placed in the tanks for wintering. The wintering tanks were transferred to a wintering laboratory where the water temperature was maintained at 6–8 ºC and checked daily. The wintering laboratory was locked, and the results of the temperature sensors were observed without entering the laboratory, in order to avoid causing any additional sounds. The laboratory was lit by a faint blue light. The condition of the juveniles was checked once a month and the *B. bombina* juveniles wintered for 12 weeks.

Post-wintering Period of Rearing Juveniles

At the end of wintering, the juveniles (now six months old) were transferred to the laboratory, where the temperature was 10 ºC. After wintering, when the temperature reached at least 15 °C, the *B. bombina* juveniles were fed minimally with fruit flies. Later, the amount and caloric content of the food was gradually increased. Over one week, the water temperature was gradually raised to 17 ºC. The photoperiod was extended to 8 hours. The juveniles were kept in plastic tanks for two more months until they reached 8 months of age (Fig. 1C), and then 20 individuals were placed into each 100-liter aquarium with a 2–3 cm thick layer of gravel (2–5 mm diameter). The water level in the aquariums was about 10 cm. On the bottom of the aquariums, the substrate consisted of stones and other items, and artificial or live plants were added. The aquariums were equipped with automatic thermostats and filters with air compressors. Above the tanks were lighting fixtures with 28 W incandescent lamps and UVB 2.0 lamps. After full recovery, the average water temperature in the containers was maintained at 23.7 ± 0.5 ºC during all post-wintering periods. Each time the water was changed, its level was raised by 1–2 cm to a final depth of 17–22 cm.

After recovery from wintering, all juveniles were divided into three equal groups according to weight to avoid interspecific competition. The separate groups were not mixed until their sex was determined, and they were released back into the wild.

Juvenile Rearing in Outdoor Tanks

When the maximum outdoor air temperature reached approximately 21–23 °C during the day, *B. bombina* individuals were moved outside. They were placed into 1.5 m^2 tanks covered with a fine net. In rainy weather, the tanks were covered with lids that had air vents. The bottoms of the tanks were covered with pebbles, and water plants were planted under the water. On cold nights (when the outside temperature dropped below 15–18 °C), the thermostats were switched on. In general, animals that will be reintroduced into nature must have as little contact with humans as possible. For this reason, the *B. bombina* individuals were not exhibited to visitors of the Lithuanian Zoological Gardens where they were reared.

Environmental Conditions and Promotion of Natural Behavior during Post-metamorphosis

After metamorphosis, the *B. bombina* juveniles lived in a more sterile environment than they would have experienced in nature. However, the soil, stones, oak leaves, live aquatic plants, and other natural materials provided hiding places and created an environment similar to nature. The juveniles would try to hide when they felt threatened.

Another even stronger way to promote the natural behaviour of the juveniles was to let them search for live food, i.e., to hunt for it. As mentioned above, the diet included live fruit flies, mosquito larvae, various cockroaches, and crickets. Competitive relations emerged between the *B. bombina* juveniles. In adaptation tanks, the juveniles were placed in a small area similar to their natural environment. There *B. bombina* juveniles could swim freely, and climb over the branches, rocks, or plants which were above the water.

Sex Determination and Morphometric Measurements

The sex of *B. bombina* individuals was determined as they matured (at 9 months old, after the full metamorphosis). In addition to sex identification by nuptial pads, croaking males were noted to confirm their sex. Moreover, some of the females spawned before their sex had been determined.

Total body weight and the two morphometric parameters of body (snout-vent) length (SVL) and sacral width (SW) were measured for the reared *B. bombina* individuals before they were released into the wild. Morphometric measurements were taken using a digital calliper (Carbon Fiber Composites, model CTCF1506, China; resolution 0.1 mm, accuracy ± 0.1mm). The 1 mm margin of error in length and width measurements was permissible in order to avoid traumatizing the animals. The weight of juveniles was measured using scales (Romansas, model KB, Lithuania; accuracy 0.01 g and error ± 0.1 g).

Transporting the Animals and their Release into Nature

At 9 months of age, depending on the weather conditions, the *B. bombina* juveniles were handed over to the workers responsible for the protected areas for release into the wild. Juveniles were not fed for one day prior to being released into the wild. In the evening before release, juveniles were brought from the adaptation tanks and kept 2 degrees cooler. It was very difficult to predict the outdoor air temperature on the following day, and if the juveniles are abundantly fed and the air temperature cools suddenly, the feed can begin to spoil and kill the juveniles. In the morning, 10–15 *B. bombina* juveniles were placed in pre-made 15 L plastic boxes. The bottoms of the plastic boxes were covered with a 2 cm thick layer of moss (*Sphagnum* sp.) and a small amount of water to minimize the stress of the juveniles during transportation. Juveniles were released in pre-arranged Habitat Protection Important Territories (HPIT) of the Natura 2000 network territories in Lithuania (Table 1). The number of *B. bombina* juveniles to be released into each water body was arranged with the responsible personnel of the protected areas before the date of release.

Statistical Analyses

For collating the data, all measured juveniles were divided into two groups according to their gender, which was derived from their sexual behavior. The gender effects on the weight and measurements of snout-vent length (SVL) and sacral width (SW) of juveniles were estimated by two-way ANOVA, also including a year factor and their interaction. In all three cases, the model residuals did not significantly deviate from a normal distribution (Shapiro-Wilk's tests: $p > 0.05$). The analyses were performed using STATISTICA 12.0 software. The significance level of $p < 0.05$ was specified for all statistical analyses *a priori*.

Results

Survival

In total, 229 tadpoles developed from the collected spawn during the study period. The proportion surviving to successful metamorphosis and the overall percentage of specimens that reached the adult stages were very high at 93.2 and 92.4%, respectively (Table 2). However, there were some considerable differences in the metamorphosis success of tadpoles between the years. The first two years showed the lowest survival rate, with very low numbers in 2018. That year tadpoles were reared in tanks with water filters, which made an effect of running water, while in later years the hatched tadpoles were grown in still water. After methodical changes in tadpole rearing, the success rate increased remarkably (Table 2).

Growth and Sexual Dimorphism

In nine months, the reared *B. bombina* individuals reached 38.9 ± 2.5 mm in body length, 22.7 ± 3.3 mm in body width, and they weighed 7.4 ± 1.3 g. The total body weight, length, and width indicating the growth of juveniles varied between males and females in the different years (Table 3). The available data allow for statistical testing of a simple hypothesis. Significant differences were observed in all three measurements between males and females, and females were bigger (Table 4). However, only the SVL and SW measurements varied significantly between the different years, while the comparison of different study years did not reveal any difference in juvenile weight (Table 4).

A retrospective comparison of the reared *B. bombina* juveniles revealed gender differences in their weight. Each year, the reared juveniles were grouped into three equal groups according to their total body weight after six months (Table 5). The sexing of the adults showed that the first juvenile weight group included almost 95% of the females. While the third group included almost entirely (90%) males. Gender was distributed almost equally in the middle group, at 43% male and 57% female. Overall, the results showed that the gender of six-month-old juveniles could be ascertained by separating them into three groups according to size even before they had truly matured. In this case, the gender could be assigned with more than 90% accuracy within the first (largest) and last (smallest) groups of separated juveniles (Table 5).

Discussion

Bombina bombina is considered to be of Least Concern globally (IUCN 2023), but it is also important to prevent local extinction. This species is distinguished as the targeted protected species in 31 Habitat Protection Important Territory (HPIT) areas of the Natura 2000 network territories in Lithuania (SRIS 2023). However, the significant decline of small, highly fragmented *B. bombina* populations was recently observed within the territory of Meteliai HPIT of the Natura 2000 network. In recent years, *B. bombina* individuals, and especially their spawn, have been dying due to prolonged late spring or summer droughts, during which the spawn laid in their usual reproduction habitats have dried up. *Bombina bombina* females usually spawn in shallow ponds on the stems of vertical water plants (Rimšaitė 2021). Each year, natural ponds are full of water at the end of spring, but then the water level drops suddenly, and the eggs die before they can hatch; and even if some of the tadpoles do hatch, they die as the water level drops.

Furthermore, fragmented *B. bombina* populations are declining because of accelerated natural succession, after remarkable changes in surrounding land use have occurred. In recent years, small water ponds that were once suitable for *B. bombina* breeding have become overgrown with grass, ponds are becoming shady, or they are being deepened for the development of extensive fisheries which cause dramatic habitat changes and fragmentation (Rimšaitė 2021). Moreover, the rapidly decreasing number of small farms at the country scale is leading to the destruction of many small ponds, which had been used as the drinking water source for farm animals and were also very suitable habitats for *B. bombina* individuals to thrive (Rimšaitė 2021). As a result, many *B. bombina* habitats with previously known populations are disappearing. Therefore, to protect the remaining small and protected *B. bombina* populations, our aim was to create a methodology for the collection and incubation of spawn, and post-metamorphosis tadpole rearing based on the environmental conditions where their development is highly threatened.

Spawn of *B. bombina* was collected and the subsequently reared individuals were released in the four HPITs (Table 1). *Bombina bombina* spawn was collected from specific water ponds where they had no chance for survival, while the reared specimens were released to either a different HPIT or the same HPIT, but in different watercourses with a good state for maintaining viable *B. bombina* populations. These watercourses are deep enough for *B. bombina* to successfully survive and reproduce, they do not dry up during hot spring or summer months, and do not become overgrown with grass. The release of *B. bombina* individuals in suitable water ponds will increase natural local populations of the species in the Natura 2000 protected sites. By increasing the *B. bombina* populations in these areas, the species has

Table 2. Numbers of developed *B. bombina* tadpoles, individuals after metamorphosis, and individuals released to water ponds from 2017−2022.

Egg collection year	Number of developed tadpoles	Number of individuals surviving after metamorphosis	Percentage of individuals surviving after metamorphosis	Release year	Number of individuals released to ponds	Percentage of overall survival of individuals
2017	69	64	92.8 %	2018	61	88.4 %
2018	56	31	55.4 %	2019	31	55.4 %
2020	70	70	100.0 %	2021	70	100.0 %
2021	73	67	91.8 %	2022	67	91.8 %
Total	268	232	93.2 %	−	229	92.4 %

Table 3. Numbers of male and female *B. bombina* juveniles and their mean weight values, lengths and widths from 2018−2022. Note: n – number of individuals; F – female; M – male; SD – Standard deviation

Year	n		Weight		Length		Width	
	F	M	F	M	F	M	F	M
			Mean ±SD		Mean ±SD		Mean ±SD	
2018	25	36	7.9 ± 0.7	6.3 ± 0.8	38.7 ± 1.9	36.8 ± 2.2	24.2 ± 2.0	19.7 ± 1.0
2019	14	17	7.7 ± 0.8	6.2 ± 0.8	39.2 ± 2.7	36.9 ± 1.9	24.9 ± 2.0	19.4 ± 0.9
2021	39	31	8.2 ± 0.9	6.7 ± 0.5	40.6 ± 2.2	38.3 ± 2.1	26.1 ± 2.4	20.4 ± 1.1
2022	44	23	8.0 ± 1.7	6.6 ± 0.4	40.2 ± 2.0	38.7 ± 1.7	25.0 ± 2.3	19.8 ± 1.8
Mean	122	107	8.0 ± 0.9	6.8 ± 1.6	39.9 ± 2.2	37.8 ± 6.5	25.2 ± 2.3	21.9 ± 4.8

Table 4. Results of two-way ANOVA testing for the effects of gender (male vs. female) and study year on *B. bombina* weight, length, and width measurements.

Measurement	Factor	df	F	*p*
Length (SVL)	Gender	1	45.83	< 0.001
	Year	3	10.62	< 0.001
	Gender × Year	3	0.45	0.72
	Error	221		
Width (SW)	Gender	1	403.47	< 0.001
	Year	3	6.08	< 0.001
	Gender × Year	3	1.25	0.29
	Error	221		
Weight	Gender	1	187.02	< 0.001
	Year	3	2.61	0.05
	Gender × Year	3	0.83	0.48
	Error	221		

Table 5. Separated weight groups of six-month-old *B. bombina* juveniles and their gender distribution within the resulting groups.

Group	n	Weigh mean ± SD	Male number (%)	Female number (%)
I	75	3.8 ± 0.3	4 (5.3 %)	71 (94.7 %)
II	75	3.1 ± 0.2	32 (42.7 %)	43 (57.3 %)
III	79	2.5 ± 0.3	71 (89.9 %)	8 (10.1 %)

its best chance for survival, and this is a way to save this locally endangered species from extinction.

During this study, we considered biosecurity and took all necessary precautions to reduce the risk of accidentally introducing pathogens along with the released *B. bombina*. A separate laboratory was built for *B. bombina* rearing to eliminate the risk of accidental contamination or transport of pathogens from other laboratories. Lab coats were required for working in the laboratory, and only people who worked on this project were allowed to enter the laboratory. Everyone who worked in the laboratory was familiar with the laboratory and biosafety protocols. Separate gloves, aquariums and cleaning equipment were used for each tank in order to avoid any possible contamination of pathogens from other tanks. For tank and laboratory disinfection, a saturated solution of table salt was used because *B. bombina* individuals are very sensitive to disinfectants and cleaning agents. Also, individuals were removed from the tanks during cleaning and disinfection. After disinfection, the tanks and laboratory were carefully washed with water in order to avoid leaving any residues of the disinfectant. Water in the tanks of *B. bombina* juveniles was changed daily because it gets contaminated quickly with leftover food and animal excreta. Tanks were filled only with fresh clean room temperature water.

In total, 229 tadpoles developed from the collected spawn during the study period. The percentage of *B. bombina* individuals which survived after metamorphosis (93.2%) and the overall survival of individuals (92.4%) during this study were very high (Table 2). The observed survival rate of tadpoles in natural habitats is only 6%. The survival percentage in this study was higher than 88% in all years except 2018. That year the tadpoles were grown under different conditions. The tadpoles were transferred to 120-liter aquariums in which the water level depth was about 25–30 cm. The aquariums were filled with live aquatic plants and 5–7 mm sized river pebbles, and water filters produced an effect of running water. The tadpoles began to die a month after they were transferred to these aquariums. The tadpoles were transferred from these unsuitable conditions to 60-liter plastic containers with live aquatic plants and river pebbles, in which water was standing still and its level was maintained at about 30 cm. After the growing conditions were changed the tadpoles stopped dying. During the subsequent years, the survival percentage of the tadpoles increased from 91.8% to 100% (Table 2). These results show that our egg incubation, rearing, and feeding methodology is effective and can be used for rearing this species in artificial conditions and releasing the adults into their natural habitats.

The survival results of *B. bombina* breeding under the laboratory conditions reported by other authors are consistent with our findings. Earlier studies showed mortality rates of tadpoles from 4 to 7% and juveniles at 8% (Kinne et al. 2006). In this study, the overall tadpole mortality was 6%, while for juveniles, mortality was only 1%. As expected, the survival rate under artificial conditions was much higher than natural conditions because the factors important for the successful development and survival of *B. bombina* were kept stable, such as air and water temperature, water level, and the amount of food. Moreover, the individuals experienced only intraspecific competition for food or habitat, and they did not experience interspecific competition since they were grown without any other species present. There were no predators or species with which they needed to compete for food. In natural habitats, such interspecific competition is unavoidable, and the living conditions are not very stable. Furthermore, the artificially reared *B. bombina* individuals were repeatedly grouped by their size and kept in rather small numbers to reduce the intraspecific competition for food and preferred habitat.

In nature, *B. bombina* individuals reach sexual maturity at 2–4 years (Bülbül et al. 2018). Mature individuals can reach a snout-vent length of 56 mm (Lang 1988), but usually it ranges from 40 to 55 mm (Rimšaitė 2021). The *B. bombina* individuals reached maturity much earlier in the artificial conditions of this study than in their natural habitats. Our findings revealed that *B. bombina* individuals reached maturity at 8–9 months, when the males began vocalizing and some females spawned. The mean snout-vent lengths were 39.9 ± 2.2 mm for females and 37.8 ± 6.5 mm for males (Table 3). By comparison, the observed mean SVL of adult female *B. bombina* individuals was lower in some Romanian populations, e.g., 35.2 mm (Cogalniceanu and Miaud 2004) and 36.8 mm (Cogalniceanu and Miaud 2003). However, it was higher (47.1 mm) in Polish populations (Rafinska 1991). The mean SVL of male *B. bombina* individuals from the Romanian populations was also lower compared to our results, at 34.4 mm (Cogalniceanu and Miaud 2004) and 36.6 mm (Cogalniceanu and Miaud 2003).

Sexual size dimorphism (SSD) is present in over 90% of amphibian species (Nali et al. 2014), although it is weakly expressed in *B. bombina* (Cogalniceanu and Miaud 2004; Bülbül et al. 2018). Males have nuptial pads, which appear only in sexually mature individuals before the breeding season (Bülbül et al. 2018). However, we found a significant difference in measured SVL and SW between *B. bombina* males and females (Table 3). The weight of *B. bombina* juveniles also showed significant differences between the sexes. Female individuals were heavier than males. In contrast to our results, Cogalniceanu and Miaud (2004) in Romania and Bülbül et al. (2018) in Turkey found that adult *B. bombina* individuals in natural habitats did not show any significant differences in body mass or SVL between the sexes. A significant correlation was found between SVL and the age of a *B. bombina* population in Romania (Cogalniceanu and Miaud 2003).

The sex of amphibians is determined genetically, and many studies have shown that high temperatures result in male-biased populations, while female-biased populations develop in low temperatures (Cogalniceanu and Miaud 2003). The number of males per pond is usually less than 10 in the northern distribution countries such as Latvia and Lithuania (Kuzmin et al. 2008). An unbalanced sex ratio affects the size of the population. Our rearing methods allowed us to predict the sex of six-month-old juveniles and individuals could be distributed into groups by their sex before they were mature. The multiyear threshold of *B. bombina* juvenile weight may be used for sex determination with >90% accuracy. This is very useful because this method enables the release of individuals into natural ponds with an equal sex ratio even before they are mature. Such knowledge provides a valuable tool for the management of a declining population by balancing the unbalanced sex ratio in naturally threatened *B. bombina* populations.

Overall, the conservation methods presented here can allow us to save small vulnerable populations of *B. bombina* and prevent them from extinction by maintaining their genetic diversity and releasing individuals into more favorable habitats that are usually unavailable due to their very segregated distribution. Such efforts will help preserve the declining populations of the species at the northern edge of their range for future generations and prevent this species from complete extinction where their habitats are much too fragmented.

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