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First Report on Natural Reproduction of Rainbow Trout *Oncorhynchus mykiss* (Walbaum, 1792) (Actinopterygii: Salmonidae) in Bulgaria

Stefan A. Kazakov, Nina V. Vancheva & Luchezar Z. Pehlivanov

Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 2 Gagarin Street, 1113 Sofia, Bulgaria;
E-mail: skazakov.iber@gmail.com

Abstract: The rainbow trout *Oncorhynchus mykiss* is non-indigenous species for Europe, with numerous records for occurrence, spawning and self-sustaining populations across the continent. The species has been found in surface water bodies in Bulgaria but naturally-reproducing have not been yet detected. In 2020, 2021 and 2022, we observed spawning behaviour of mature fish in the Ogosta River, downstream of Ogosta Reservoir, north-western Bulgaria. In order to verify whether the rainbow trout reproduces naturally in Bulgaria, complete process of spawning was tracked. Through the winter-spring period of 2022, fish material was sampled from the trout redds and identified via DNA analysis. The results showed that rainbow trout reproduce successfully in the sampled river stretch. Vital specimens at stages of egg and fry development were found through the whole period, from February to April. The registered water temperature and recent flow management occurred as beneficial for the periods of rainbow trout spawning, egg development as well as fry emergence.

Key words: Salmonid fish, trout spawning, redd sampling, European tailwater fishery, alien species

Introduction

Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) is native to streams along the Pacific coasts of North America and North-Eastern Siberia (Behnke 1979, Page & Burr 1991, 2011). The species is non-native to Europe; however, for the last century, there are numerous reports of its occurrence, introduction, stocking, natural reproduction or self-sustaining populations across the continent (Stanković et al. 2015 and bibliography therein). The species is a desired gamefish as well as aquaculture object (Frimodt 1995), with high popularity among anglers (Gartner et al. 2002); often, it has higher recreational values (Hickley 2018) compared to native fish species (Cambray 2003, Quist & Hubert 2004). The species has been introduced in numerous tailwater fisheries (Bettinger & Bettoli 2002) due to altered thermal regime and as supportive measure for recreational angling (Bettinger & Bettoli 2002, Runge et al. 2008) but often experiences limited survival, low growth rates, delayed maturation and low rates of recruitment (Meerbeek & Bettoli 2005, Runge et al. 2008, Korman & Kennedy 2017, Cunningham et al. 2022). Maintaining a desirable size structure of the trout population often needed regular stocking (Pender & Kwak 2002, Williams et al. 2004). Regime of the water temperature was considered as crucial factor determining dispersal, growth, feeding, maturation, spawning, egg development and survival of this cold-water species (Myrick & Cech 2000, 2001, 2004, Bear et al. 2007, Runge et al. 2008). Natural flow events due to snowmelt or heavy rains as well as flow management (hydropeaking, flow retention, water abstraction, etc.), were considered essential factors for determining the successful establishment and recruitment of rainbow trout populations (Quinn & Kwak 2000, Dibble et al. 2015, Hasegawa 2020, Brain et al. 2020). Hydropeaking events impact negatively the spawning behaviour (Vollset et al. 2016), egg development (Casas-Mulet et al. 2015) as well as fry emergence (Moreira et al. 2019).

The rainbow trout is considered among the most invasive fish species (Cambray 2003, Copp et al. 2009), with moderately high risk of invasiveness for Bulgaria (Simonović et al. 2013). It was introduced to Bulgaria from Germany during 1920's for aquaculture purposes (Lever 1996, Welcomme 1988) and randomly occurs in surface water bodies in Bulgaria (personal observations), especially downstream fish farms indicating its presence being caused by fish farm escapes. However, data about its natural reproduction or self-sustaining populations in Bulgaria are not available.

The river section downstream Ogosta Reservoir was stocked with brown trout *Salmo trutta* and declared as "Catch and Release" (C&R) area in 2018. Although the river section was severely modified (straightened watercourse, disrupted lateral connectivity with floodplains, decreased river continuity and passability due to weirs and sluices, annually regulated flow regime), it was found that the brown trout spawns and reproduces successfully (Kazakov et al. 2022). As beneficial factors for the successful trout reproduction, several water-body traits were recognised: favourable oxygen saturation, low turbidity, mitigated water temperature, relatively stable flow regime as well as abundant food recourses (Kazakov et al. 2022). Surprisingly, rainbow trout consistently occurred among the angling catches, although it had never stocked intentionally (personal communications with anglers). In the winter and spring seasons of 2020, 2021 and 2022, we observed different age classes of rainbow trout, including newly hatched fry and fingerlings among the bank vegetation, parr and yearlings among the angling catches as well as matured spawning individuals that constructed redds and deposited eggs. Few kilometres downstream the area, a local fish-farm regularly raising rainbow trout witnessed that the species population in this river section was maintained by fish-farm escapes.

The aim of the present study was to test the hypothesis that the rainbow trout was reproducing naturally in the river section downstream the Ogosta Reservoir as well as to assess the auspiciousness of crucial environmental factors like water temperature and water flow in relation to the process of natural reproduction.

Materials and Methods

Study area

The investigation has been carried out within river section from 0.8 up to 3.8 km, downstream the Ogosta Reservoir (Fig. 1). As the rainbow trout is a winter-spring spawner, usually from February to May, for the northern hemisphere, the collection of redd material was conducted between 17th February 2022 and 25th April 2022 within four sampling fieldtrips. Overall, twenty redds were sampled. In order to found spawning material from rainbow trout, a redd was selected for sampling if rainbow trout was observed to spawn on it. In order of obtain egg stages, younger redds (Gallagher et al. 2007) were selected. In order to found alevin or fry stages, older redds (Gallagher et al. 2007) were sampled. Most of the constructed redds were concentrated between 0.8 and 1.8 km downstream the reservoir, which determined the majority of the selected sampling sites. Each location of a sampled redd was recorded on GPS Garmin eTrex 30x. Fish material was collected from the most probable location of the egg chambers (Burner 1951) using a hand-size shovel. Excavation was performed while sequential digging at depth up to 30 cm if possible, considering the majority of the trout eggs distribution. The excavated material, dominantly gravel and sand was settled immediately behind the excavated hole, on the top of the redd, in front of a hand net, with mesh size 2 mm, small enough to catch the lighter organic material drifted by the water current, including fish eggs, alevins and fry. Digging was re-established until at least one vital specimen from redd was found and collected. In order not to cause too much damage on the redds, no more than four shovels per redd were applied. Eggs damaged due to fungi development were considered as non-vital. At the early stages of egg and fry development, morphological differentiation between brown trout and rainbow trout is hardly possible; therefore, a DNA analysis was conducted in order to identify the species.

DNA analysis

Specimens were stored in test tubes filled with 90 % ethanol at -25^{o}C prior to isolation. Samples were prepared by cutting a small piece of tissue from the

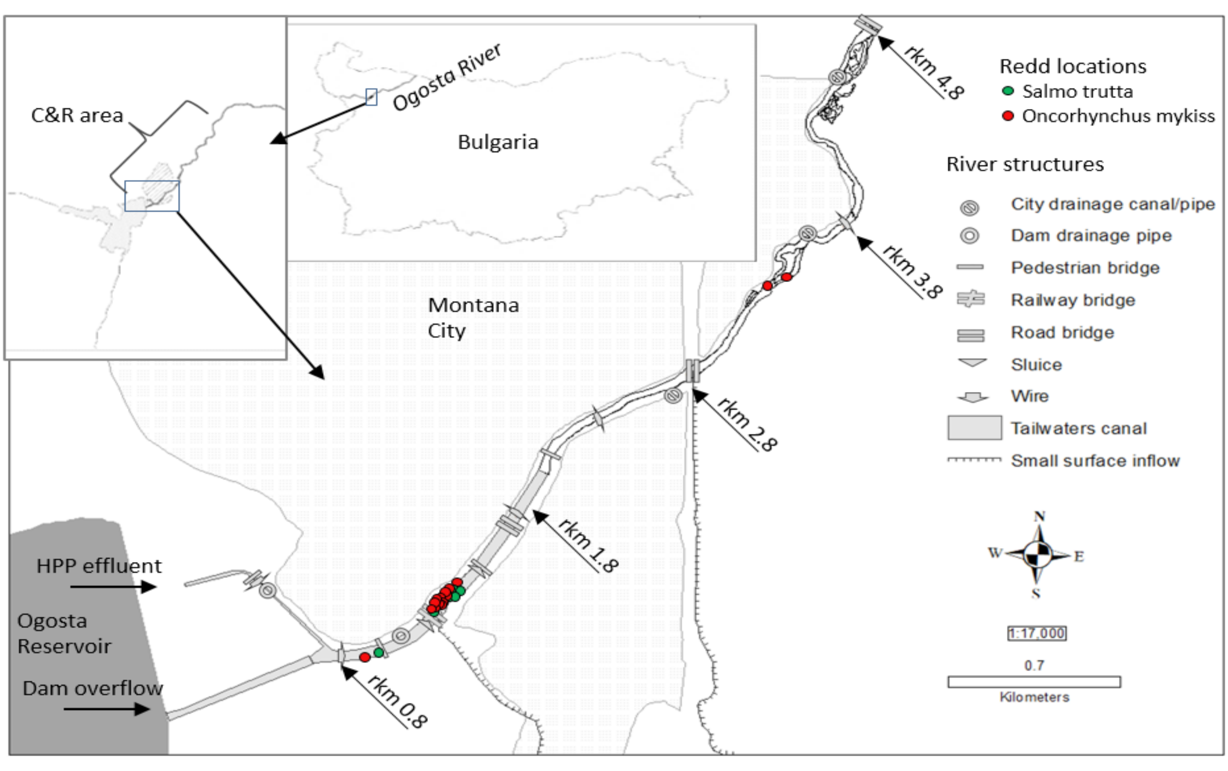

Fig. 1. Sampled redds downstream Ogosta Reservoir. Red coloured spots indicate redds with genetic material from *O. mykiss,* while green coloured spots indicate redds with identified *S. trutta* material. Twelve kilometres Catch & Release area (C & R area).

caudal fin of the fry or, in case the sample consisted solely of eggs, a whole egg was used. DNA was extracted using GeneMATRIX Tissue & Bacterial DNA Purification Kit (EURx) in accordance with manufacturer's instructions.

Primers L14841 (5`-AAAAAGCTTCCATC-CAACATCTCAGCATGATGAAA-3`) and H15149 (5`-AAACTGCAGCCCCTCAGAATGATATTT-GTCCTCA-3`) (Kocher et al. 1989) were used to amplify a 397 bp long fragment of the cytochrome b mitochondrial gene. Each PCR test tube contained 12.5 µl Thermo Scientific™ DreamTaq™ Hot Start Green PCR Master Mix (Thermo Fisher Scientific), 9.5 µl water, nuclease-free (Thermo Fisher Scientific), 1 µl of each primer (10 µM) and 1 µl target DNA to a final volume of 25 µl. The samples were amplified under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min; and final elongation at 72 °C for 10 min.

Four µl of each sample were used for electrophoresis. The PCR products were visualised on 2 % agarose gel in 1x TBE buffer with GelRed® (Biotium). Samples were sequenced by Macrogen Europe B.V. (Amsterdam Zuidoost, the Netherlands). Sequences were aligned, edited and compared with other sequences in GenBank using CodonCode Aligner 8.0.2. (CodonCode Corporation, MA, USA).

Water temperature, considered as leading environmental factor for the trout spawning and eggs development, was traced from 9th January to 1st April 2022. Two temperature sensors with data loggers were set at the bottom of constantly flowing pools in the river, at 0.5 m and 0.3 m depth, at 0.8 km and 2.8 km downstream the reservoir (Fig. 1). The sensors recorded temperature at 10 min intervals (Fig. 3). Data for daily water discharge of the reservoir from 1st January to 30st April 2022 was collected from the bulletin of the Ministry of Environment and Waters (MOEW) of the Republic of Bulgaria.

Results

Overall, 215 specimens were collected; most of them (c. 200) were considered as vital. Twenty specimens were selected for DNA analysis and species identification. Four stages of ontogenesis were observed visually, like two stages of egg development followed by two stages of fish larvae development (Fig 2): a) fertilized egg at different stages of cell division; b) advanced stage of egg development like embryo outline formation; c) yolk-sac fry, also called alevin; d) fry with resorbed yolk sac. Stages

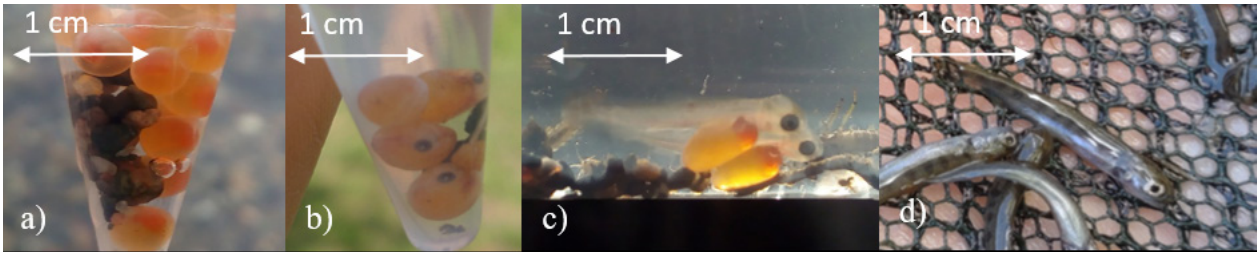

Fig. 2. Four stages of ontogenesis were visually differentiated: a) fertilized egg at different stages of cell division, sample No 2; b) advanced stage of egg development like embryo outline formation – eyes (black dots) and spinal cord (white line), sample No 5; c) yolk-sac fry (alevins), sample No 9; d) fry with resorbed yolk sac, found near the red surface, sample No 17.

Table 1. Sample number, date of sampling, number of specimens found in the sample, stage of development, number of vital specimens and identified trout species.

Sample No	Sampling date	Total number of specimens	Number of vital specimens	Stage of devel-opment	Species
1	22.02.17	3	1	Egg	*S. trutta*
2	22.02.17	16	8	Egg	*O. mykiss*
3	22.03.06	4	4	Alevin	*S. trutta*
4	22.03.06	10	8	Embryo	*O. mykiss*
5	22.03.06	7	7	Embryo	*O. mykiss*
6	22.03.06	15	15	Egg	*O. mykiss*
7	22.03.06	11	11	Fry	*S. trutta*
8	22.03.06	6	6	Fry	*S. trutta*
9	22.03.06	2	2	Alevin	*S. trutta*
10	22.03.06	14	14	Alevin	*S. trutta*
11	22.03.06	17	17	Egg	*O. mykiss*
12	22.04.01	8	8	Egg	*O. mykiss*
13	22.04.01	12	12	Egg	*O. mykiss*
14	22.04.01	25	22	Egg	*O. mykiss*
15	22.04.01	10	10	Embryo	*O. mykiss*
16	22.04.01	17	17	Embryo	*O. mykiss*
17	22.04.25	2	2	Fry	*O. mykiss*
18	22.04.25	5	5	Egg	*O. mykiss*
19	22.04.25	30	30	Fry	*O. mykiss*
20	22.04.25	1	1	Fry	*O. mykiss*

of fertilized egg, embryo and alevin were found in the redd pockets between 10 and 20 cm depth. Developed fry, with resorbed yolk sac, were found closer to the surface of the redds.

Between one and thirty-eight specimens were found in each sampled redd (Table 1). Twelve redds contained specimens at egg stage, including eight redds with fertilised eggs and four ones with outlined embryos. Five out of eight of the remaining redds contained fully-developed fry, and the remaining three redds contained alevins with more or less advanced yolk sac resorption (Table 1).

The DNA analysis showed that the genetic material from six of the investigated redds belonged to brown trout while the remaining 14 redds contained material from rainbow trout (Table 1). One of the samples taken in 17th February contained eggs from brown trout, while the other sample contained eggs from rainbow trout. Five out of nine redds sampled on 06th of March contained fry of brown trout while the rest four redds contained eggs from rainbow trout. All samples from April contained material from rainbow trout only. At 1st of April, only eggs were found; on 25th of April, one out of four redds

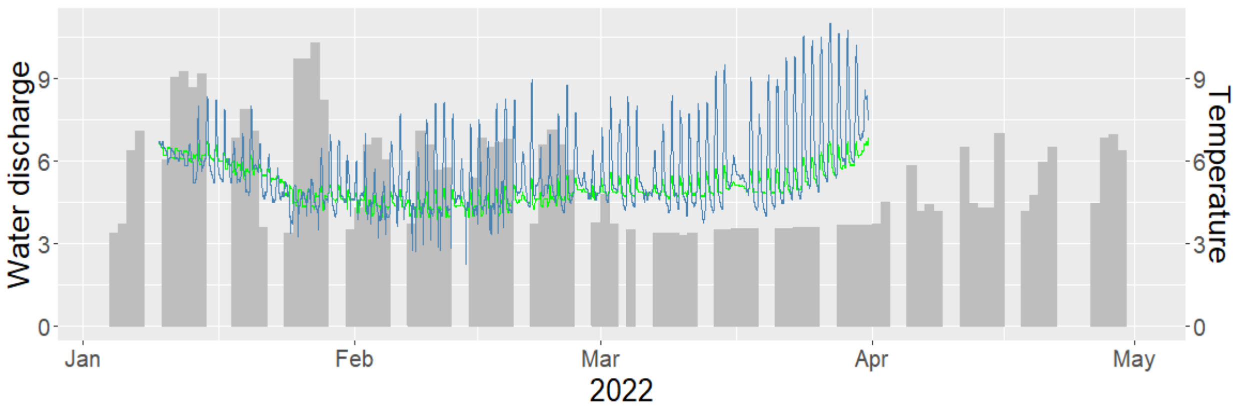

Fig. 3. Daily water discharge (m^3s^{-1}) of Ogosta Reservoir (grey columns) and water temperature (°C) upstream of trout fishery at 0.8 km (green line) and 2.8 km (blue line), downstream Ogosta Dam, from 9 January to 1 April 2022.

contained eggs while the rest three redds were full of fry.

Average water temperature (average ± SD) at the beginning of the spawning area, at 0.8 km downstream the reservoir, was 5.07 ± 0.67 °C and varied between 3.88 °C and 7.69 °C (Fig. 3). Average water temperature at 2.8 km was 5.58 ± 1.33 °C and varied between 2.25 °C and 11.0 °C.

Daily dynamic of the flow regime for the period from January to April ranged from 3.29 $m^3.s^{-1}$ up to 10.30 $m^3.s^{-1}$ (Fig. 3). Highest water discharge was registered in January, with average (± SD) values 6.99 ± 2.44 $m^3.s^{-1}$. The lowest water flow was in March, when it was set to the minimum with values of 3.57 ± 0.3 $m^3.s^{-1}$. In February and April, the water discharge was milder, with 5.9 ±1.16 $m^3.s^{-1}$ and 5.22 ± 1.16 $m^3.s^{-1}$, respectively.

Discussion

The trout spawning process, starting with redd construction, passing through eggs deposition, eggs development and hatching into alevins as well as fry appearance near to emergence on the redd surface were registered. The reproduction of the rainbow trout was considered successful because most of the specimens from different stages were identified as vital. Only 15 eggs were identified as damaged by fungi.

The domination of the rainbow trout specimens among the sampled redds was expected, considering brown trout as earlier spawner than rainbow trout when most of its fry emerged and dispersed from the redd in March. Hereafter in April only rainbow trout material was found and identified from the redd samples. However, it is still unknown to what extend the fishfarm escapes support the rainbow trout population recruitment.

The upper part of the fishery section (at 0.8 km downstream the reservoir) provides more constant temperature conditions compared to 2.8 km downstream, where the daily fluctuations of the water temperature were more than twice as high. Daily drops in water temperature below 3.9 ºC, impairing the spawning behaviour (WDOE 2002) and below 5 ºC, associated with increased egg mortality (Myrick & Cech 2001), more often occurred at 2.8 km downstream the reservoir. Therefore, the upper section of the fishery, closest to the reservoir outflow, was considered as more suitable for spawning and egg development.

In January 2022, the highest water discharge dynamics was registered (Fig 3), with increasing the daily water discharge near three times. However, spawning behaviour was still observed, although considered as impairment by some authors (Vollset et al. 2016). Because of hydropeaking, increased egg mortality often occurred (Casas-Mulet et al. 2015) as reported in February 2022 (Table 1). The luck of flow events, was considered as beneficial for rainbow trout spawning (Dibble et al. 2015, Hasegawa 2020) in March 2022, when the water discharge remained constant (Fig. 3), along with the increasing water temperature.

The hydropeaking events from the Ogosta Reservoir could have dispersal effect on trout population downstream the river Ogosta. Last decade several run-off-the river hydroelectric power plants were built downstream Ogosta Reservoir, with no specific requirement about fish-pass construction. Currently, those fish-passes are hardly passable for fish (personal observations), causing them entrapped in downstream river sections and impairing its returning to the preferred spawning area. As cold-water species, individuals from isolated trout populations could survive through the winter period, although the warmer summer temperatures could be challenging.

Conclusion

This is the first documented natural reproduction of rainbow trout in Bulgaria. The study used DNA analysis to confirm the presence of the species tracking the process of reproduction from egg development up to fry emergence.

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