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Artificial Reproduction of *Pachychilon pictum* (Heckel & Kner 1858) from Wild Populations from Lake Ohrid

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Abstract: Artificial reproduction of *Pachychilon pictum* (Heckel & Kner 1858) was investigated, using gametes obtained from spawners, collected from wild populations from Lake Ohrid. Standard methods for artificial reproduction in a modified form were applied, resulting in high fertilization and survival rates, confirming the success of the procedure. This is the first study of this kind for cyprinid fish from Lake Ohrid, highlighting the importance of the resulting data, which could serve as a foundation for further research on additional cyprinid fish species from the lake, and if needed for future conservation purposes.

Key words: Aquaculture, conservation, cyprinids, Lake Ohrid

Introduction

Fishes populate most of the world's aquatic habitats and are represented by diverse groups of species (Mayar & Psenicka 2024). The constant rise of anthropogenic pressures is threatening fish populations, which could result in a declining fish abundance and loss of biodiversity (Aral et al. 2011, Targonska et al. 2011, Mayar & Psenicka 2024). This highlights the need for the development of solutions for the future conservation of fish populations (Mayar & Psenicka, 2024). Reproduction is fundamental to the continued existence and success of a species, so it plays a critical role in the field of conservation biology and the preservation of biodiversity (Wildt et al. 1993). Breeding fish in captivity and reintroduction into the wild, are among the most widely used techniques for conservation and restoration of vulnerable populations and endangered fish species (Philippart 1995, Gazsi et al. 2021). The rearing of any fish species depends on understanding and implementing control on its reproduction, either by collecting gametes from wild individuals at spawning time in the natural environment or by inducing sexual maturation in the individuals held in captivity (Philippart 1995). Understanding the reproductive strategy of different fish species is essential for creating protocols to control their repro-

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duction (Mananos et al. 2008). The most commonly used methodology for artificial reproduction of fish, is in vitro fertilization of gametes obtained through hormone stimulation (Muller et al. 2018). In some cases, these hormonal treatments are used only as a tool to enhance the efficiency of egg production, but in others, hormones are the only way for the reproductive material to be acquired (Mylonas et al. 2010). Obtaining good quality gametes is one of the most important factors that could influence the fertilization rate, and the further production and development of the offspring (Kucharczyk et al. 2007, Kucharczyk et al. 2016). The process of artificial fertilization of the gametes is normally carried out by using the "dry" method (Billard et al. 1995), after which several steps for inducing the fertilization process and removal of the adhesive properties of the eggs are applied (Billard et al. 1995, Philippart, 1995, Al Hazzaa & Hussein 2003, Ljubobratović et al. 2017). The fertilized eggs need to be kept in favorable conditions that are optimal for embryo development, and are usually species-specific (Muller et al. 2018, Piech & Kujawa 2021).

Lake Ohrid is an oligotrophic water body located on the Balkan Peninsula, shared between two countries, North Macedonia and Albania. As the oldest natural lake in Europe, it has a high level of endemism and unique ichthyofauna (Albrecht & Wilke 2008). Artificial reproduction and stocking on Lake Ohrid are routinely done only for the endemic salmonid *Salmo letnica* (Spirkovski et al. 2017). Lake Ohrid in terms of fish species composition is predominantly a cyprinid lake, with most of the species being endemic to the lake itself, or to the Southwestern Balkan region (Kottelat & Freyhof 2007, Talevski et al. 2024). But still, there is no research done on the artificial reproduction of any of the cyprinid fish species. With the increasing anthropogenic pressures on the lake (Matzinger et al. 2006, Kostoski et al. 2010, Lorenschat et al. 2014), having reproduction methodologies available for the cyprinid fish species, could be of great importance for conservation and preservation purposes.

Pachychilon pictum (Heckel & Kner 1858) is a cyprinid fish species, endemic to the freshwater bodies of southwestern Balkan (Lakes Ohrid and Skadar, river Black Drin and its watershed) (Kottelat & Freyhof 2007, Talevski et al. 2024). The spawning period of this fish begins in May when the water temperature is above 12 ºC, and lasts until the second half of August when the water temperature is 18-20ºC or more, in the littoral area (Točko 1987, Talevski et al. 2024). The spawners come at a depth of 0-4 m in areas with submerged vegetation or large stones. During the spawning period the fish group in shoals of 3-10 out of which 1-2 are females, who are larger than the males (Točko 1987). Although this fish is not of commercial interest, it plays a vital role in the local aquatic biodiversity. Its localized distribution and specific habitat requirements make it vulnerable to environmental changes and human activities (Talevski et al. 2024).

Therefore, the aim of this study was to develop a model methodology for artificial reproduction of *P. pictum* from wild populations, as a representative of the cyprinid fish fauna from Lake Ohrid.

Materials and Methods

Two experiments were conducted for the artificial reproduction of *P. pictum*, during two separate spawning seasons, in years 2022 and 2023. Male and female specimens were sampled during their spawning period in late June. The sampling took place in the littoral area of Lake Ohrid, at 0 to 5 meters depth. Gill nets with mesh sizes of 16 mm, 18 mm, 20 mm, 22 mm, and 24 mm were used as sampling gear. The nets were planted at dusk and lifted after 2 hours in the water. After lifting the nets, the specimens of the targeted species were carefully released from the fishing gear and were placed in a water tank with an oxygen supply, to keep the fish alive. The fish were anesthetized in order to minimize the stress, using clove oil with 10 drops / 10 L water (Muller et al. 2018). In the first experiment one female and 3 males, and in the second experiment 3 females and 7 males provided the reproductive material. The fish were wiped dry, after which eggs and milt were collected by stripping, with gentle pressure on the abdomen of the spawners (Targonska et al. 2011). After stripping, the fish were put in a container with clean water, and after regaining composure from the anesthesia, were released into the lake. Hormonal stimulation was not used in the process. The artificial reproduction was carried out by using the "dry" method (Billard et al. 1995). The collected reproductive material was mixed in dry conditions, and the subsequent mixture was left to rest for 5 minutes. After the rest period a solution for activation of fertilization and stickiness removal of the eggs, in ratio 3:1 was added. For this purpose, a solution of milk was used in the concentration of 200 ml/L (mixed with water), and the mixture was left to rest for 30 minutes with few stirrings as proposed by Billard et al. (1995). Afterward, the eggs were placed in an aquaria tank, modified as an incubator, with a temperature regulator and constant aeration of the water. The temperature, oxygen concentration, and pH of the water in the incubator, were measured daily using Bante 903P multiparameter instrument. The eggs were checked every 12 hours for fertilization rate, mortality and hatching. After finishing the hatching process, the larvae were kept until they had completed the endogenous feeding period, and afterward were released in Lake Ohrid, at the area from where the spawners for the artificial reproduction were sampled. The eggs were observed under stereo microscope Zeiss Stemi 305, and Zeiss PrimoVert inverted microscope. The pictures were taken with Zeiss ZEN Software using the Zeiss Axiocam 105 camera.

For comparing the fertilization and hatching rates between the two experiments chi-square test was used, and for comparing the water parameters between the two experiments, a Shapiro – Wilk normality test was performed followed by a t-test. The statistical analysis was performed at a significance level of 0.05, using statistical software (SPSS 23.0).

Table 1. Artificial reproduction parameters and the incubator water parameters in both experiments (dO_2 - dissolved oxygen; std - standard deviation; ns - not significant)

Parameter	Experiment 1	Experiment 2	p - value
Egg number	591	3855	
Reproduction parameters			
Fertilization rate (%)	86.63	99.01	0.038
Hatching rate (%)	81.64	76.19	ns
Water parameters (mean ± std)			
dO_2 (mg/L)	7.13 ± 0.26	6.72 ± 0.07	0.005
T(°C)	23.84 ± 0.87	23.92 ± 0.74	ns
pH	8.84 ± 0.08	8.80 ± 0.20	ns

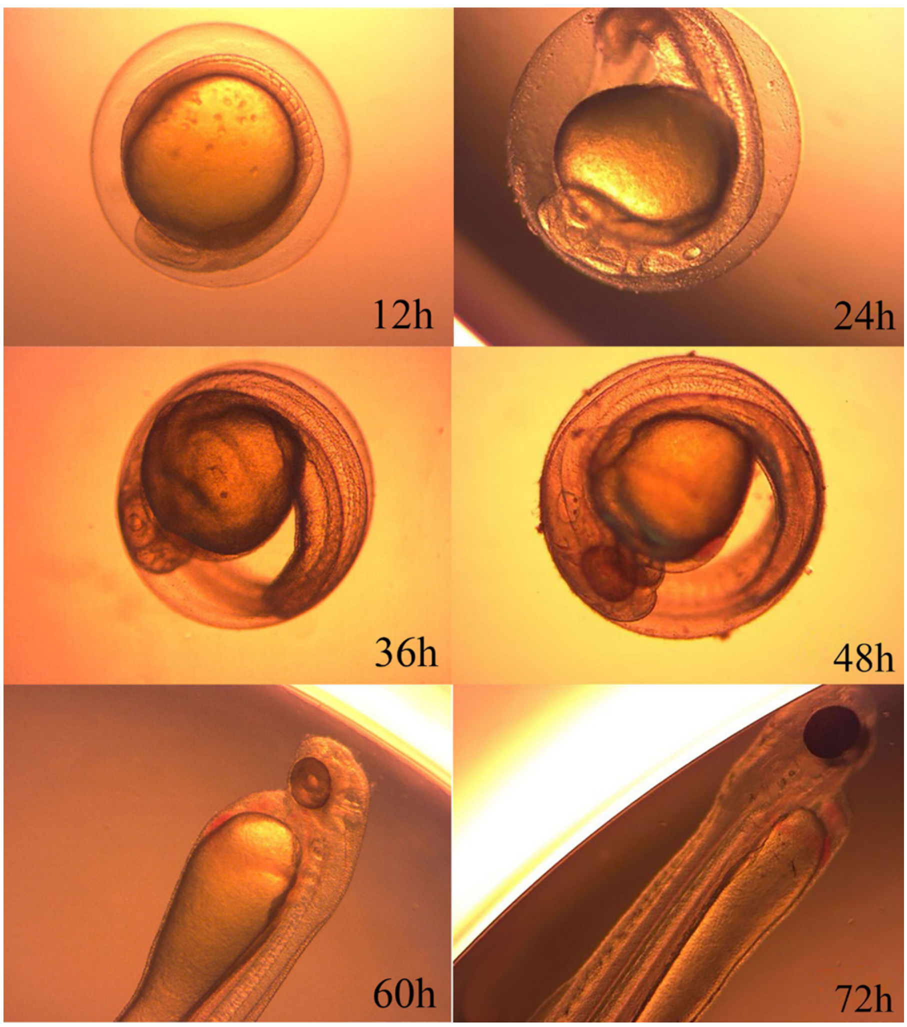

Fig. 1. Stages of embryo development of *Pachychilon pictum* in different time periods

Results

The described method for artificial reproduction was successfully applied with high fertilization and hatching rate in both experiments (Table 1). Statistically significant difference was observed for the fertilization rate between the experiments ($p < 0.05$). The hatching of the embryos in both experiments started at 48 hours post fertilization and the highest levels were observed at 60 hpf with all the embryos hatched until 72 hpf. The highest mortality was observed at 36 hpf and 24 hpf for the first and second experiment respectively. The adhesive properties of the eggs were successfully removed, and the eggs were easy to manipulate lasts, and the transparent chorion made it easy to observe the embryo development. The stages of embryo development of *P. pictum* for every 12 hours until the completion of the hatching are presented on Fig. 1. The values for the water parameters in the incubator for both experiments are presented in Table 1. Statistically significant difference was only observed for the level of oxygen concentration in the incubator water ($p < 0.05$).

Discussion

This study presents the successful artificial reproduction of *P. pictum* by the use of modified standard methods for the artificial reproduction of fish species. In aquaculture practice, the collection of gametes for artificial reproduction usually is preceded by artificial induction of ovulation, done by hormonal stimulation (Billard et al. 1995, Philippart 1995, Piech & Kujawa 2021). This is especially the case with cyprinid fishes, and previous studies have shown that gametes from cyprinid fish species are difficult to obtain (Mylonas et al. 2010, Piech & Kujawa 2021). In both of the experiments, by timing the peak of the spawning season, mature spawners were sampled from the wild, from which gametes were collected without the use of hormonal stimulation. Kujawa et al. (1999) state that one of the ways to obtain good quality gametes is by catching fish during spawning season on the spawning grounds. This simplifies the procedure, and the spawners don't need to be taken into captivity. Reproductive dysfunctions are reported in fish held in captivity (Mananos et al. 2008), and some studies showed that wild fish had a significantly lower percentage of ovulation compared to cultured fish, when induced with hormonal injection (Targonska et al. 2011). Future studies could show whether the possibility of collecting gametes without hormonal stimulation is applicable to other cyprinid fish species from Lake Ohrid.

In the standard "dry" method procedure, after mixing the male and female reproductive material, the addition of solution is required for activation of the fertilization process, which is changed a few times, after which a solution for removal of the adhesive properties of the eggs is added, a process that last one hour or more (Billard et al. 1995, Philippart 1995, Kucharczyk et al. 2016). In this study, those steps were narrowed down, by directly mixing the eggs and milt, after which a milk solution was added that had the dual role of activating the fertilization and removal of the stickiness of the eggs. Billard et al. (1995) noted that submerging the eggs in a solution of diluted milk, just after fertilization, for 30 min with gentle stirring, is as efficient as the standard methods and is less time-consuming. Although a statistically significant difference in fertilization rates was observed between the two experiments ($p < 0.05$), the rates remained high in both cases (Table 1). This suggests that good quality gametes were collected from the male and female spawners during the study, and that the fertilization process was performed successfully. Multiple studies on artificial reproduction in wild populations of various cyprinid fish species reported different rates of fertilization ranging from low to high (Al Hazzaa & Hussein 2003, Sahinoz et al. 2007, Muller et al. 2018, Kucharczyk et al. 2019). These studies also give different percentages of survival and hatching. Relatively high levels of hatching were observed in each of the experiments, without statistically significant differences between them (Table 1), indicating successful replication of the process. Mortality of the fertilized embryos was observed only before hatching, during the embryo developmental stages, with the highest mortality occurrence in the 12, 24, and 36 hpf, which suggests increased sensitivity of the *P. pictum* embryos during this period (Fig. 1.)

The condition of the water is of great importance for the survival and further development of the fertilized embryos. Temperature is one of the variables that have a great influence, and multiple studies have shown that changes in this parameter can alter the rate of embryo development (Lugowska & Witeska 2018, Kupren et al. 2011, Piech & Kujawa 2021). During the experiments, we maintained the water temperature in the incubator to match the ambient temperature of the littoral area of the lake at the time during the natural spawning period (Table 1). In that way, the fertilized eggs were exposed to the optimal temperature that they would have been exposed to in their native environment. Although the concentration of dissolved oxygen in the water was statistically different between both experiments

($p \leq 0.05$), the oxygen levels in the incubator were in the ranges optimal for cyprinid fishes (6-8 mg/L) (Svobodova et al. 1993) (Table 1).

Conclusion

Our study presents a successful artificial reproduction of *P. pictum*, which is the first study of this kind carried out on a cyprinid fish from Lake Ohrid. High fertilization and hatching rates were observed, and the positive results were replicated in two different spawning seasons. Future research should be directed towards expanding the artificial reproduction investigations on other cyprinid species from Lake Ohrid, to determine whether the methods presented here could be applied to other species, or if species-specific modifications should be implemented. Considering the unique fish biodiversity of Lake Ohrid, and the increasing anthropogenic pressures, having reliable methods for artificial reproduction for different fish species could be of great significance for conservation and preservation plans in the future.

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