Egyptian Journal of Aquatic Research xxx (2018) xxx–xxx

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Contents lists available at ScienceDirect

Egyptian Journal of Aquatic Research

journal homepage: www.sciencedirect.com/locate/ejar

Full length article

Artificial propagation of the endangered Rumanian endemic warm water rudd (*Scardinius racovitzai* Müller 1958, Cyprinidae, Cypriniformes) for conservation needs

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ARTICLE INFO

Article history:
Received 27 October 2017
Revised 23 July 2018
Accepted 26 July 2018
Available online xxxx

Keywords:
Critically endangered fish
Lake Petea
Propagation
Larvae rearing
Conservation biology

ABSTRACT

Scardinius racovitzai appeared to be locally adapted to an extreme high temperature environment (26–35 °C). As a species of conservation concern, due to declining ecological condition of the solitary thermal lake it could be found in (Lake Petea, Oradea, Romania) and eventual near complete abstraction of this lake, 60 adults were saved in 2014. In this study we investigated methods for induced spawning and sperm cryopreservation to aid recovery and reintroduction to restored habitats. Induced spawning was introduced successfully by intraperitoneal induction of dried carp pituitary extract and human chorion gonadotropin. Larvae and juveniles were successfully reared to age 3 months using standard methods for cyprinids. Our experiments on the cryopreservation of *S. racovitzai* sperm show that the extender consisting 40 mM KCl 200 mM glucose, 30 mM Tris buffered with HCL pH 8.0 is suitable for freezing warm water rudd sperm together with methanol as a cryoprotectant. By rescuing the remaining wild stock into captivity and undertaking propagation research we have prevented extinction of this rare species – more so *S. racovitzai* will be important in the study of freshwater fishes and how they can adapt to significant warming.

Introduction

Peţea Lake is situated in the north-western part of Romania, near to the city of Oradea. The lake is located above a rich geothermal aquifer, which has been known since earlier period (Sîrbu et al., 2013). Peţea Lake was the only natural habitat in Europe of Egyptian white water-lily (*Nymphaea lotus thermalis*) other two local endemic species; Parreys snail *Melanopsis parreyssii* and Thermal rudd *Scardinius racovitzai*. The snail and fish classified as critically endangered on the IUCN Red List of Threatened Species. *S. racovitzai* as a new species was described from this mineral thermal spring by Müller (1958) but the taxonomic status of the fish is still obscure, and it may be subspecies of Common rudd, *S. erythrophthalmus* or local ecophenotype (Berinkey 1960, Bănărescu 2002, Grigoraş et al. 2015). In 2011–2012, the aquatic community of the lake was disturbed by the strong reduction of the discharge of thermal underwater springs which was caused by the overexploitation of geothermal waters in the region (Telcean et al., 2013). The droughts of subsequent years exacerbated existing water scarcity. During winter illegal water abstraction increased between the end of 2012 and beginning of 2013 which resulted in the lake almost drying out (Figs. 1 and 2) and natural scientists of Museum of the Three Rivers Land (Muzeul Ţării Crişurilor), Oradea saved fish (n = 60) of unknown age and gender and also snails (*M. parreyssii,* n = 40) in aquaria and also pumped thermal water back into the lake in order to rescue the landlocked populations. Despite the efforts the lake dried out completely in September 2014. In 2015 *M. parreyssii* was searched for in the remnants of the lake, as well as the whole surrounding area during July, August

Peer review under responsibility of National Institute of Oceanography and Fisheries.

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https://doi.org/10.1016/j.ejar.2018.07.005
1687-4285/

Fig. 1. Changes in the Peţea Lake water level and general view of the habitat: above: November 2013 (photo: Tamás Müller), below February 2015 (Daniel Man, Ioana Mateaş).

Fig. 2. *Scardinius racovitzai* broodstock (photo: Csaba Posztós).

and December, but none were found (Sîrbu and Benedek, 2016). Ex situ conservation activities is one of the possible solutions for survival of threatened and endangered species. The ration of fish reintroduction projects worldwide is low amount (Seddon et al. 2005). Part of the reason for lack of captive fish conservation measures is the difficulty in maintaining a captive stock on any timescale, successful captive breeding and the complementary procedures to restore the natural habitat - due to the importance of in situ and ex situ conservation measures being conducted in an integrated manner (Grigoraş et al. 2014). Successful artificial reproduction in captivity is one such way to overcome some of the limitations of ex situ conservation, and this can include for immediate rearing of juveniles for re-introduction or instead for cryopreservation storage of the sperm, ova or embryos of endangered fish species until such a time that reintroduction is feasible. The aim of this paper was to investigate the possibility of the *ex situ* conservation of this species through spawning induction of males/females, in vitro fertilisation, rearing of offspring to viable size and cryopreservation of gametes starting with methods appropriate for use with other cyprinid species.

Material and methods

The sixty fish that were caught from the lake were kept in a 700 L aquarium in Muzeul Ţării Crişurilor, Oradea, Romania (from 2012 to 2013). A randomly selected 20 fish from this broodstock were transported to the Department of Aquaculture, Szent István University on the 17th February 2014 (standard length 92.13 ± 7.2 mm; female sex ratio 18:2). Fish were treated by a 0.05% EMS (formalin and malachite green) solution bath for 10 min as a general treatment against external infection. The water temperature was 23 °C at introduction and it was increased to 26 °C during 12 h and kept it during the spawning time. The photoperiod was close to the natural rhythm in all experimental cycles (light:dark 11:13 h).

Induced spawning

Before handling fish were anaesthetised in a plastic tank (40 L) by clove oil (*Syzygium aromaticum*) with10 drops/10 L water. Two tanks were used. One of them contained the anaesthetic solution and the other just fresh aerated water. Treated females (n = 8) and male (n = 1) were rinsed in this clear aerated water in order to remove the residues of anaesthetics from their gills. 200 International Unit (IU) Human Chorionic Gonadotropin (hCG)/fish was used for induced spawning. The injection was given intraperitoneally (16.00–16.30 h p.m.) using a 1 mL syringe (OMNICAR®). Fish were then introduced to a 700 L clean water aquarium with plastic plants (n = 7, Tetra Anacharis Dekor S 13 × 6 cm) as a potential spawning substrate. Chemical investigation of water was done with a JBL Testlab® test kits at (PO_4 < 0.02 mg/L, NO^{2-} 0.05 mg/L, NH^{4+} 0.15 mg/L, NO_3 4 mg/L, general hardness 6° d, pH 7.5).

Juvenile fish rearing from eggs

After spawning eggs were removed from the spawning aquarium attached to the aquatic plants, incubated and aerated in a smaller 110 L aquarium. It was unknown at this stage whether eggs were successfully fertilised. The water temperature in the rearing aquarium was controlled by a heater at 26 °C. Eggs were disinfected by using Black alder cones (*Alnus glutinosa*) solution (10 cones/10 L water for one hour) at 24 and 48 h after the ovulation to prevent infection by *Saprolegnia sp*. After a time allowed for successful hatching to occur, the plastic plants were removed from the aquarium. Larvae were fed newly hatched *Artemia* sp. nauplii six times per day *ad libitum*. The first 3 weeks larvae were fed by only artemia and after that artemia (two times per day) and different kind of commercial foods; one time/day; Classic C22 (Skretting), Nutra HP 0.3 (Skretting) and SDS 100, 200, 300 (Special Diets Services Limited International Dietex GB).

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Semen collection, cryopreservation and induced propagation

In parallel with the artificial propagation study described above (i.e. March 2014). Two males and 5 female fish were both injected with a single dose of 10 mg per kg of body weight of acetone-dried carp pituitary extract 18 h before attempting stripping them of gonads (pituitary extract, Hortobágy Fish Farm Co., Hungary (CPE)). This treatment has been found to be effective on other cyprinids. Male and females were anaesthetized in a solution of clove oil (10 drops/10 L water), then they were removed from the water onto wet towels and their genital aperture was dried with dry paper towels. Semen was collected by applying abdominal pressure with an automatic pipette and emptied into a 1.5 mL Eppendorf 1.5 mL tube and was kept on crushed ice at 4 °C. Sperm was diluted in an extender at a ratio 1:9: 200 mM glucose, 30 mM Tris, 40 mM KCl, pH 8.0 (Horváth et al., 2012). Ten percent of methanol (in v/v final concentration) was added to the prediluted sperm as cryoprotectant. The prediluted sample was loaded into 0.5 mL straws (Minitube GmbH, Tiefenbach, Germany). Liquid nitrogen was poured into a styrofoam box. The straws were placed on a floating styrofoam frame at 3 cm above the level of liquid nitrogen. Straws were cooled in liquid nitrogen vapours for 3 min and then plunged directly into liquid nitrogen. The straws were stored in canister storage dewars for one hour before being used for fertilization trials.

Sperm quality assessment

Sperm motility parameters (WHO, 2010) were analysed both before (for control quality) and after cryopreservation using a computer-assisted sperm analysis system (CASA, Sperm VisionTM v. 3.7.4., Minitube of America, Venture Court Verona, USA). Samples were activated in an activating solution used with other cyprinids (45 mM NaCl, 5 mM KCl, 30 mM Tris, pH 8, Horváth et al., 2003) to which 0.01 g/mL Bovine Serum Albumin (BSA) was added. Using CASA we measured the samples for % of progressive motility, VCL (velocity curved line, μm/s), STR (straightness, VSL/VAP, %).

Egg stripping, fertilisation

As we did not know the ovulation time in *S. racovitzai* the release of the first eggs was regularly monitored in females by gentle pressing of their abdomens until two females were ready to spawn (standard length 58 and 72 mm). Eggs from these two females were then used in post sperm cryopreservation fertilisation tests. Individuals that were releasing eggs were anaesthetized as described above. They were removed from the anaesthetic solution and their genital areas were wiped dry. Eggs were collected into a dry plastic bowl by applying gentle abdominal pressure. Straws were thawed for 13 s in a waterbath at 40 °C. Eggs were mixed with half a straw (500 μl) of thawed sperm. Sperm were added to the eggs and then 1 mL of water was used to activate the gametes. Following mixing, the eggs were allowed to stick to the bottom of a weighing boat spread in a monolayer (polystyrene, rectangular). Eggs were disinfected similarly to the previous experiment by using Black alder cones solution (10 cones/10 L water for one hour) at 24 and 48 h after the spawning. Fertilization rates were calculated at 48 h after the mixing of eggs and defrosted sperm and after that dead eggs were removed. After hatching the larvae were introduced to another 110 L aquarium for rearing.

Statistical analysis

Statistical analyses of differences in parameters between fresh and cryopreserved sperm were carried out by independent sample *t*-test in SPSS v22 for Windows and also checked by a non-parametric alternative due to the small sample sizes and non-normal data, Mann-U-Whitney. The results did not differ so we present the results from the t-tests here. Treatment means were compared using $\alpha = 0.05$ for significance. For fertilisation and hatching success using fresh or frozen sperm only two batch samples has been obtained so there will be no formal statistical testing.

Results

Induced ex-situ spawning

The fish spawned in early morning (6.00–8.00 a.m, water temperature 26 °C) 14–16 h after exposure to hCG. This was confirmed by eggs being observed on the leaves of plastic plants that were checked every hour. The size of the swollen eggs suggested that fertilisation had been successful and developed embryos could be seen of approximately 1.5–1.7 mm. Embryo development to hatch took 3–4 days over which period freshly hatched larvae (total length 4.5–4.7 mm) could be seen hanging on leaves and on the wall of aquarium. On the 5th day larvae entered the exogenous feeding stage and started to feed (total length 5.4–5.7 mm). Juvenile thermal rudd, reared on standard diets at 22 °C, reached 30 mm standard length in three months. From our single induced spawning event of 8 females and one male about 5000 juveniles have been reared in total.

Sperm cryopreservation and induced propagation

The main differences between the parameters of fresh and cryopreserved sperm samples can be seen in Table 1. The two female fish began to release eggs the morning after inducement (8.00 a.m., latency time 18 h, 23 °C) after which they were stripped (female I. = 585 eggs (SL 58 mm), female II. = 1284 eggs (SL = 72 mm)). There were significant differences among the three sperm motility parameters tested suggested in a significant decline in sperm function following preservation - % progressive motility (fresh sperm: 92.0 ± 3.7%, cryopreserved sperm 31.7 ± 16%, $t_{11} = -9.8$, $P < 0.001$), VCL (fresh sperm: 141.5 ± 11.4%, cryopreserved sperm 49.5 ± 7%, , $t_{11} = -17.1$, $P < 0.001$) and STR (fresh sperm: 0.77 ± 0.09%, cryopreserved sperm 0.89 ± 0.03%, $t_{11} = 3.2$, $P < 0.01$). Cryopreserved and post-thawed sperm samples fertilized egg batches at a lower rate and the eggs hatched at a lower rate than from fresh sperm samples Table 1.

Discussion

Artificial induction of ovulation by using two types of hormones (hCG and CPE) was successful in *S. racovitzai*. Induced spawning of *S. racovitzai* is possible by using the combined method of artificial propagation and captive breeding techniques as in other ornamental and economically important fish species. In the aquarium the spawning substrates with fertilized eggs were removed in order to avoid the egg eating behaviour of adults and rearing of the newly hatched fish to 30 mm size was successful.

Our *S. racovitzai* propagation method was similar to *S. erythrophtalmus*, which was described by Breteler (1977) and Kucharczyk et al. (1997). According to the result of Breteler (1977) *S. erythrophtalmus* females were treated with 0.3 mg CPE per kg body weight as priming dose and 18 h later a resolving dose (3.0 mg CPE/kg) was administrated. Ovulated eggs were stripped between 12 and 18 h after the resolving dose at 22.5 °C. In our experiments the latency time was shorter (14–16 h) in higher water temperature (26 °C). Kucharczyk et al. (1997) tested CPE (4 mg/kg) with or without hCG (5000 IU/kg) and FSH + LH (75 IU/kg) with metoclopramide or pimozide (both of them are dopamine receptor antagonists, 10 mg/kg). Considering on the reproduction parameters the

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Table 1
Table a: Summarised average parameters of native and post-thaw sperm samples of thermal rudd (n = 3–4/measurements); table b: data about the cryopreservation; table c: summarised data about the transported captive bred juveniles.

Table a	Donor ID	Progressive motility (%)	VCL (μm/s)	STR (%)
Fresh sperm	No:1	95.03	81.03	0.67
	No:2	90.01	105.65	0.81
post-thawned sperm	No:1	42.03	39.77	0.86
	No:2	25.22	41.17	0.90
Table b	Egg numbers	Fertilisation (%)	Hatching rate (%)	
Control I.	426	93.7	89.2	
Cryopreserved I.	858	79.0	76.5	
Control II.	176	79.0	68.2	
Cryopreserved II.	409	69.2	66.3	
Table c Target place	Date	No of transported living individuals	Length (cm)	
Lake Petea, Oradea (ROM)	20.03.2014	1805	0.7–1	
Tavirózsa Association, Szada (HUN)	13.06.2014	400	1.5–3	
Tiergarten Schönbrunn, Wien (AUS)	13.09.2014	100	2–3	
Tropicarium and Oceanarium Budapest (HUN)	03.12.2014	100	3–5	
Budapest Zoo, Budapest (HUN)	12.12.2014	200	3–5	

best hormone administrations were HCG + CPE and FSH + LH with pimozide. There were not possibilities to compare the hCG and CPE treated groups in our experiments. Crăciun (1997) and Grigoraş et al. (2014) managed to induce spawning in *S. racovitzai* in captivity by influencing the environmental conditions without hormonal treatment between December-April. The spawning season of thermal rudd in the wild was restricted to February-March (Bănărescu, 1964).

Fertilisation tests of cryopreserved sperm were carried out by using the so called 'dry fertilisation method' and while preservation appears to reduce both fertility and hatching of fertile eggs, over 65% success of both parameters could be achieved. The technology of the whole processes from egg incubation to juvenile rearing did not require additional investments as the facilities used for the induced spawning of other aquatic species are suitable for this purpose as well.

In our study, high fertilization and hatching rates were achieved with cryopreserved sperm despite reductions compared to fresh samples (>66%). The results show the same efficiency of the method which was found in the Adriatic grayling conservation work (Horváth et al., 2012). The success of freezing can be enhanced by the use of smaller straws according to the limited amount of fresh milt. The 0.5 mL straws are also commercially produced and were used efficiently in other conservation and aquaculture projects (Sarder et al., 2012, Yildiz et al., 2013, Dietrich et al. 2014 etc.).

Wolnicki et al. (2009) investigated the growth features of *S. erythrophthalmus* larvae feeding with Artemia from the onset of exogenous feeding. They found that common rudd larvae were among the fastest growing cyprinids. In contrast when larvae were fed with formulated feed exclusively, this species showed very slow growth rate in comparison to other cyprinids in larvae period. Similar observations are found in cyprinids more generally (Wolnicki and Górny, 1995a,b,c; Demény et al. 2012, Kaiser et al., 2003; Żarski et al., 2011). This was the reason that the first food in the first week was only live food and after that commercial food was provided to the juveniles. In the same study by Wolnicki et al. (2009), *S. erythrophthalmus* entered the post larvae exogenous feeding stage on day four at mean body length 5.7 mm, whereas we found *S. racovitzai* to not start until day five at a similar size. Whether this represents a slower growth rate from birth, linked to the reduced body size on thermal rudd, or an effect of size at birth of the individuals in this study is as yet unclear. During the entire rearing period there was no problem with the environmental conditions (i.e. lack of diseases) and the growth rate of fish to 30 mm was suitable.

The quality of developing fish embryos, expressed as a survival of the embryos, were highest in the fresh sperm control. 10 straws were stored into liquid nitrogen and two of them were used for fertilisation experiments and 8 straws were kept as gene bank in Szent István University for further investigations or fertilisation as sperm gene bank.

Sperm parameters were affected by the cryopreservation process which is common in fish sperm. Reduction of motile cells is associated by the damages caused by the process of freezing and thawing as well as solute effects during the first phases of cooling. A reduction of sperm ATP concentrations had been observed in other fish species which results in the reduction of sperm velocity parameters.

There is a concern about a potential population genetic bottleneck effect arising due to using only two males in our study, and this may prevent successful breeding programs in the future. Interestingly, there were only two males in the 40 fish rescued as broodstock. This could indicate a sex ratio shift in response to the declining environmental conditions in the original habitat. The cryopreserved sperm samples we have kept can be used in conjunction with *S. racovitzai* stocks kept in the aquaria of Muzeul Ţării Crişurilor and Galati's Aquarium. The methods we have described can be used to help other endemic fish species. Finally, we have distributed 2605 larvae and juvenile *S. racovitzai to* five new aquatic institutes in order to maintain the species (Table 2).

Acknowledgements

The work was supported by The Mohamed bin Zayed Species Conservation Fund (project no. 13255722; *Captive breeding and maintaining ex situ populations of Scardinius racovitzai and Melanopsis parreyssii*) and the Higher Education Institutional Excellence Program (1783-3/2018/FEKUTSRAT) awarded by the Ministry of Human Capacities within the framework of water related researches of Szent István University as well as the Department for Angling and Fisheries, Ministry of Agriculture and the EFOP-3.6.3-VEKOP-16-2017-00008 project. The protocols of fish sperm cryopreservation and fish propagation and the template informed consent forms contained in Appendix (Scientific Ethics Council for Animal Experimentation; XIV-001-2299-4/2012, XIV-001-2306-4/2012) were reviewed and approved and applicable by National Food Chain Safety Office, Animal Health and Animal Welfare Directorate of Government Office of Pest Country with respect to scientific content and compliance with applicable research subjects regulations.

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