Knowledge and Management of Aquatic Ecosystems (2011) 401, 13 http://www.kmae-journal.org

DOI: 10.1051/kmae/2011027

Sodium chloride as effective antifungal treatment for artificial egg incubation in *Austropotamobius pallipes*

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Received January 2, 2011
Revised March 6, 2011
Accepted March 22, 2011

ABSTRACT

Key-words: *formaldehyde, fungicide treatment, hatching, sodium chloride, white-clawed crayfish*

In this study, sodium chloride at three different concentrations, 30 000 ppm (S30), 60 000 ppm (S60) and 90 000 ppm (S90), and formaldehyde at one concentration, 3000 ppm (F), were tested as antifungal chemicals during artificial incubation (AI) of *Austropotamobius pallipes* eggs. Two treatments were tested without chemicals as control groups with (R) and without (C) the removal of dead eggs. After AI, formaldehyde treatment ensured high survival of stage 1 (89.7 ± 2.3%) and stage 2 (85.5 ± 2.4%) of juveniles. However, comparable survival rate to stage 1 and stage 2 (85.5 ± 5.5% and 80.6 ± 3.2%) were also found in the treatment with the highest sodium chloride concentration (S90). Significantly lower survival rate of juveniles (stage 1: 60.6–70.3% and stage 2: 56.1–67.3%) were evident in groups S60, S30 and R. However, group R demanded high labor and related costs. The lowest juvenile survival levels to stage 1 (46.4 ± 8.2%) and stage 2 (45.2 ± 6.8%) were observed in treatments without fungicide chemicals and removal of dead eggs (C).

RÉSUMÉ

Le chlorure de sodium comme traitement antifongique efficace dans l'incubation artificielle des œufs d'*Austropotamobius pallipes*

Mots-clés : *formaldéhyde, traitement antifongique, éclosion, chlorure de sodium, écrevisse à pieds blancs*

Dans cette étude, le chlorure de sodium à trois différentes concentrations, 30 000 ppm (S30), 60 000 ppm (S60) et 90 000 ppm (S90), et le formol à une concentration de 3000 ppm (F) ont été testés comme produits antifongiques pendant l'incubation artificielle (AI) d'œufs d'*Austropotamobius pallipes*. Deux traitements ont été testés sans produits antifongiques comme témoins avec (R) et sans (C) retrait des œufs morts. Après incubation artificielle, le traitement au formol assure une survie élevée au stade 1 (89,7 ± 2,3 %) et au stade 2 (85,5 ± 2,4 %) des juvéniles. Toutefois, un taux de survie comparable au stade 1 et 2 (85,5 ± 5,5 % et 80,6 ± 3,2 %) a été trouvé avec le traitement à la plus forte concentration en chlorure de sodium (S90). Un taux de survie significativement plus faible des juvéniles (stade 1 : 60,6–70,3 % et stade 2 : 56,1– 67,3 %) est observé pour les groupes S60, S30 et R. Mais le groupe R demande un gros travail coûteux. Les survies les plus faibles des juvéniles au stade 1 (46,4 ± 8,2 %) et au stade 2 (45,2 ± 6,8 %) sont observées en l'absence de traitement chimique et de retrait des œufs (C).

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Article published by EDP Sciences

INTRODUCTION

Captive breeding of white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet) can be considered such as an effective method for conservation and restoration of this endangered crayfish species (Reynolds, 1998; Reynolds *et al*., 2002; Policar *et al*., 2010). Effective production of juveniles in *A. pallipes* for reintroduction has used the artificial egg incubation (AI) of eggs (Policar *et al*., 2008).

Advances of the artificial incubation in European native crayfish were described by many authors (Cukerzis *et al*., 1978; Rhodes, 1981; Matthews and Reynolds, 1995; Pérez *et al*., 1998a, 1998b, 1999; Carral *et al*., 2003, 2004; Policar *et al*., 2006; Kouba *et al*., 2010b). The duration of the period of AI is generally dependent on the timing of the egg stripping and applied water temperature. We can distinguish a short-term (several days) and a long-term (several weeks) AI. The short-term AI is less demanding for energy, time, space and hatched juveniles are protected against female's cannibalism in the same way compared to long-term AI (Policar *et al*., 2006). The protecting hatched juveniles against females was published by Keller (1987) who used special containers with perforated bottom for the separation of juveniles from their females during juvenile detaching.

However, organic water contamination, and damaged or dead eggs are suitable substrates for fungi during AI (Vey, 1977; Policar *et al*., 2006). Therefore AI need to use the prevention of incubated live eggs against fungi invasion (Mason 1977; Rhodes 1981; Carral *et al*., 2004; Celada *et al*., 2004; Policar *et al*., 2006). Kouba *et al*. (2010b) summarized three techniques of fungi elimination during AI as antifungal treatments, removal of dead eggs or a combination of both.

Up to now, sodium chloride was not found to be a suitable antifungal bath during AI of crayfish eggs (Celada *et al*., 2004). However, the positive antifungal effect of this environmentally friendly agent has been described in freshwater finfish aquaculture during egg incubation by many authors (Marking *et al*., 1994; Froelich and Engelhardt, 1996; Schreier *et al*., 1996; Weirich and Tiersch, 1997; Khodabandeh and Abtahi, 2006; Rasowo *et al*., 2007).

The aim of this study was to compare the survival to stage 1 and stage 2 in *A. pallipes* eggs treated by three different concentrations of sodium chloride during AI as antifungal treatments.

MATERIAL AND METHODS

> BERRIED FEMALES AND STRIPPING OF EGGS

In total, 45 berried females (mean ± SD; total length (TL) = 85.9 ± 7.1 mm; carapace length (CL) = 38.2 ± 3.3 mm; body weight (W) = 17.5 ± 4.1 g) were used as a source of eggs for this study. All these females mated with 15 males (TL = 88.5 ± 6.2 mm; CL = 39.6 ± 4.1 mm; W = 18.2 ± 3.1 g) under controlled conditions of one square tanks (100 × 100 × 50 cm; 1 m^2 surface area) in the Moneycarragh crayfish hatchery (Policar *et al.*, 2009). After mating and egg laying, all males were removed from the tank. Berried females were kept in the tank under controlled water temperature what was decreased after egg laying from 8.0 ± 0.3 °C to 4.5 ± 0.3 °C over five days. After this initial period, females were kept under lowered temperature for 90 days, at 9.0 ± 0.5 °C for 50 days, and 18.0 ± 0.5 °C for 5 days. In total, 3300 experimental eggs at embryonic phase XII, described by Celada *et al*. (1991), were stripped gently by tweezers from females after 150 days of maternal egg incubation.

> ARTIFICIAL INCUBATION WITH ANTIFUNGAL TREATMENTS

Immediately after stripping, all eggs were pooled and stocked into 18 one-liter bottles described by Policar *et al*. (2006). Each bottle was stocked with 110 eggs at an egg density of 6.9 eggs·cm^{-2}. Sodium chloride (NaCl ⩾ 99.5%; Sigma-Aldrich, Czech Republic) and

formaldehyde (35.2% formaldehyde; Dr Kulich Pharma, Czech Republic) were tested such as chemical antifungal treatments at three concentrations: S30 (30 000 ppm), S60 (60 000 ppm) and S90 (90 000 ppm) and one concentration (as control to sodium chloride): F3 (3000 ppm), respectively. Other two groups were tested without fungicide chemicals with (R) or without (C) the removal of dead eggs as controls to chemical treatments. All treatments were tested in triplicates. Each fungicide chemical treatment was applied to the eggs by a static bath once a three days with exposure of 15 min up to hatching (stage 1). The removal of dead eggs in group R was carried out with the same frequency as the application of chemical baths. After the hatching and the first juvenile moult, the number of stage 1 and stage 2 juveniles were recorded. During the first moulting period, juveniles in stage 2 were removed daily from each bottle according to recommendations to reduce juvenile cannibalism during AI (Melendre *et al*., 2007).

The recirculation system described by Policar *et al*. (2006) comprised storage and filtration tanks (total volume 400 L, Depur Systems, Northern Ireland), a pump (OceanRunner OR6500, AquaMedic, Germany), a heater system (T computer set with a 500 W heater, AquaMedic, Germany), 18 one-liter bottles (each with diameter 4.5 cm and internal surface area 15.9 cm^2) and water distribution piping system (Depur Systems, Northern Ireland) was used for the AI of this study. Water was exchanged daily (10%) with filtrated and tempered water from Moneycarragh River. Water flow through each bottle was stable around 1 $L{\cdot}min^{-1}$. Water temperature was automatically controlled by the heater system and measured by temperature loggers (RT-F53, Qi Analytical, Prague, Czech Republic) with a four hour interval. Average water temperature and oxygen saturation was 18.0 ± 0.5 °C and 90.0 ± 2.5%, respectively during the whole AI. Dissolved oxygen levels were measured daily at 7 a.m. with an oxymeter Pinpoint II (American Marine Inc., USA). Other water quality parameters such as pH (WTW MultiLine P4 probe, WTW GmbH, Germany), NH_3 and NO_2^- (Tetratest AnalySet, Tetra Company, Germany) were measured weekly. Average value of these parameters was: pH = 7.5; $NH_3 < 0.02$ $mg{\cdot}L^{-1}$; $NO_2^- < 0.01$ $mg{\cdot}L^{-1}$ during this study.

> *STATISTICAL ANALYSIS*

All data are presented as means ± SD. Statistical analysis of data was conducted using statistical software “Statistica 6.1” (StatSoft, Inc., Czech Republic). Results were subjected to one-way analysis of variance (ANOVA) after arc-sine transformation and assessing for normality and homoskedasticity with Kolmogorov-Smirnov and Levene’s tests, respectively. Tukey’s multiple comparison was used as a *post hoc* test. For all statistical tests, P values < 0.05 were considered to be significant.

RESULTS

Artificial incubation lasted for 32 days. Hatching began on day 18 and stage 2 was obtained from day 25. The period of the first moult lasted seven days. Juvenile survival rates to stage 1 and stage 2 from all treatments are summarized in Table I.

Formaldehyde treatment (F3 group) ensured high production of stage 1 (89.7 ± 2.3%) and stage 2 (85.5 ± 2.4%). Comparable survival rate to stage 1 (85.5 ± 5.5%) and 2 (80.6 ± 3.2%) were evident in treatment with the highest concentration of sodium chloride (S90). Lower survival rate of juveniles (stage 1: 60.6–70.3% and stage 2: 56.1–67.3%) were evident in groups with lower concentrations of sodium chloride (S60 and S30) and control group with removal of dead eggs (R). The lowest juvenile survival to stage 1 (46.4± 8.2%) and stage 2 (45.2 ± 6.8%) was found in the treatment without fungicide chemicals and removal of dead eggs (C).

Table I
Production of stage 1 and 2 juveniles (% ± SD) after 32-days IA using different antifungal treartments in Austropotamobius pallipes*.*

Tableau I
Production de juvéniles de stades 1 et 2 d'*Austropotamobius pallipes* (% ± SD) après 32 jours d'incubation artificielle et différents traitements antifongiques.

Group	C	R	S30	S60	S90	F3
Stage 1	46.4 ± 8.2 A	68.8 ± 5.2 B	60.6 ± 5.5 AB	70.3 ± 6.2 B	85.5 ± 5.5 C	89.7 ± 2.3 C
Stage 2	45.2 ± 6.8 A	65.2 ± 5.0 B	56.1 ± 3.7 AB	67.3 ± 2.4 B	80.6 ± 3.2 C	85.5 ± 2.4 C

Within a row, values without a letter in common are significantly different ($P < 0.05$) among treatments.

DISCUSSION

Intensive egg incubation in crayfish under controlled conditions needs to use effective antifungal treatment against fungal invasion in incubated eggs (Carral *et al*., 2004; Celada *et al*., 2004; Policar *et al*., 2006). Malachite green was used as the most effective fungicide for many years in aquaculture. However, the use of this chemical agent on food fishes and their eggs was prohibited for aquaculture in USA and EU since 1991 and 1997, respectively, because of its teratogenic and carcinogenic effects (Carral *et al*., 2009).

Formaldehyde is currently the most effective antifungal agent approved for using in aquaculture in USA and EU (Celada *et al*., 2004); however, its applying in aquaculture presents potential hazard for aquatic environment as well as farm staff (Arndt *et al*., 2001).

Therefore, new antifungal agents as alternatives to formaldehyde have been searched for AI. Jodisol preparation with povidonum iodinatum as active substance in a static bath and its combination with the removal of dead eggs (Policar *et al*., 2006) might be one possibility. Carral *et al*. (2009) tested three chemicals with copper hydroxide being the most effective one. Copper is ubiquitous in the enviroment. It is essential for all living organisms (among others, Cu is a core part of hemocyanin in crustaceans), and its body concentration is relativelly well regulated in crayfish (Kouba *et al*., 2010a). This chemical seams to be also an acceptable candidate as antifungal treatment for AI.

Our results showed that sodium chloride is also a very good antifungal agent suitable for artificially incubated crayfish eggs. Especially the bath with the highest concentration of sodium chloride (90 000 ppm) provided high survival to stage 1 (85.5%) and stage 2 (80.6%). Thefore we recommend to use this antifungal treatment for effective AI in *A. pallipes* eggs in practice. The benefit of this antifungal treatment is lower demand for labor and related costs and its environmental friendliness and user's safeness.

We tested routinely used static bath with 15 min exposure. Longer exposure of sodium chloride bath can decrease the concentration of sodium chloride such as effective antifungal treatment (Marking *et al*., 1994; Rasowo *et al*., 2007). However this hypothesis should be studied in further research. Longer exposition of sodium chloride will require next testing of peristaltic bath applying increased salinity in incubating systems.

Applying sodium chloride is well established in intensive aquaculture. It has been used as antifungal treatment in egg incubation of different freshwater fish species such as: *Clarias garepinus*, *Cyprinus carpio*, *Oncorhynchus mykiss* and *Ictalurus punctatus* (Marking *et al*., 1994; Froelich and Engelhardt, 1996; Schreier *et al*., 1996; Weirich and Tiersch, 1997; Khodabandeh and Abtahi, 2006; Rasowo *et al*., 2007). Increased salinity was also applied in intensive culture for increased survival and osmoregulatory of percid larvae (Krise *et al*., 1986; Guo *et al*., 1993; Bein and Ribi, 1994) and for control of fish diseases in intensive freshwater finfish culture (Mifsud and Rowland, 2008).

Weirich and Tiersch (1997) found positive effect of increased salinity (application of 1 g $NaCl{\cdot}L^{-1}$) in hatchery water on the hatching rate in *I. punctatus*. Sodium chloride at concentration 30 000 ppm for a 60 min exposure period effectively inhibited fungal infection and

increased the hatching rate of incubated eggs in *Oncorhynchus mykiss* (Marking *et al*., 1994). Rasowo *et al*. (2007) found an effective concentration of sodium chloride such as 1000 ppm with an exposure time 30 min for successful egg incubation of *C*. *gariepinus*. These authors also described the negative effect of higher concentrations of sodium chloride (*i.e.* 2000, 4000 and 10 000 ppm) with the same exposure time for hatching in *C*. *gariepinus*. A similar negative effect of a high concentration of sodium chloride (2500 and 5000 ppm) during 60 min exposure on the hatching rate of *C*. *carpio* was confirmed by Froelich and Engelhardt (1996). However, this study did not observe such problem during AI in *A. pallipes*. This result can support and confirm considerible tolerance of crayfish to higher salinities (Kozák *et al*., 2009). Based on observed findings, the highest concentration of sodium chloride (90 000 ppm) applied once a three days for 15 min is recommended as an effective and safe antifungal treatment during AI of *A*. *pallipes* eggs in practice.

ACKNOWLEDGEMENTS

The authors of this paper gratefully thank Dr Julian Reynolds (Republic of Ireland) for English corrections. This investigation was financially supported by projects: INTERREG IIIA, CENAKVA (CZ.1.05/2.1.00/01.0024), NAZV (QH 71305), KONTAKT (ME 10125) and GAJU (047/2010/Z).

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