Survival of Early Stripped Eggs of the Noble Crayfish, *Astacus astacus,* and Effects of Saline Solution During Artificial Incubation

ULI B. SEEMANN,[1,2,*] KAI LORKOWSKI,[1,2] MELANIE SCHIFFER,[1,2] CHRISTINA HÖRTERER,[1,2] MATTHEW J. SLATER [1,2] AND BELA H. BUCK [2,3]

[1] Institute for Marine Resources GmbH, Bussestraße 27, 27570 Bremerhaven, Germany.
 Corresponding Author.— uli.seemann@awi.de

[2] Alfred Wegener Institute, Helmholtz Center for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany.

[3] University of Applied Sciences Bremerhaven, An der Karlstadt 8, 27568 Bremerhaven, Germany.

ABSTRACT

There is growing interest in using recirculating aquaculture systems (RAS) to produce juvenile noble crayfish, *Astacus astacus* (Linnaeus), a valuable and once plentiful food species in Europe, now a highly endangered species. The survival rates of early stripped eggs of *A. astacus* were compared across anti-fungal treatments in an artificial RAS incubation system based on a saline bath of approx. 20 – 22 mS cm⁻¹ or 15 – 16 PSU over two different durations. Time from fertilisation to hatching was 82 days or 1191 degree-days. Low survival rates (live successfully hatched juveniles at end of experimental period) between 11.0 ± 9.5% and 26.6 ± 3.7% were obtained. Survival was significantly affected by the duration of egg bath within the saline solution with twice as high survival rates when eggs were saline-bathed once every two days for five minutes in comparison to eggs without treatment. Fungal rates were significantly affected by treating the eggs with a saline solution with infection rates decreasing from 3.7 ± 1.0% to 0.8 ± 0.5% in the ten minute saline bath treatment. Appropriately applied saline bathing can markedly improve survival of early-stripped noble crayfish eggs. However, further research is required to determine whether viable levels of hatching success can be obtained using early stripping and saline treatment.

INTRODUCTION

The native European crayfish *Astacus astacus* (Linnaeus) is a critically endangered species, which is classified as "vulnerable" in the international Red List and is included in the Bern Convention and in Annex V of the EU Habitat Directive (Reynolds and Souty-Grosset 2011). The proceeding expansion of introduced North American crayfish species like *Pacifastacus leniusculus* (Dana) and *Orconectes limosus* (Rafinesque), and concomitant replacement of native crayfish species, is a further threat to this species (Vorburger and Ribi 1999). However, aquaculture of freshwater crayfish is not only of conservation, but also of commercial interest. Freshwater crayfish can reach high market prices and are a valuable food source in some European countries (Reynolds and Souty-Grosset 2011).

Successful breeding techniques are a crucial prerequisite in obtaining appropriate know-how to rejuvenate the natural resources of *A. astacus*. While the importance of temperature for the reproduction cycle of *A. astacus*, as well as survival rates of stage two juveniles, are well known (Hager 2003; González et al. 2008; Füreder 2009), information about a reliable method for artificial incubation and especially the time of egg stripping and egg quality are still under investigation (Policar et al. 2004; González et al. 2008). Research findings indicate artificial incubation of freshwater crayfish eggs results in higher survival rates (68%) of stage two juveniles when compared to maternal incubation (56%) (Strempel 1974; Pérez et al. 1999). Furthermore, artificial incubation reduces maternal egg brooding problems, allows for controlled rearing conditions and could prevent transmission of pathogens from brood stock to offspring (Pérez et al. 1999; Policar et al. 2006).

The time of crayfish egg stripping and CTU (Celsius Temperature Units = degrees Celsius x days) is a crucial element
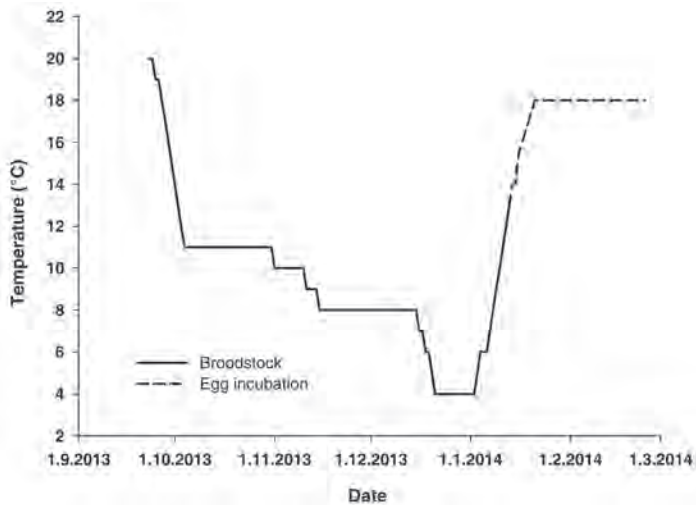
Figure 1. Temperature profile during broodstock maintenance and artificial egg incubation of *Astacus astacus*.
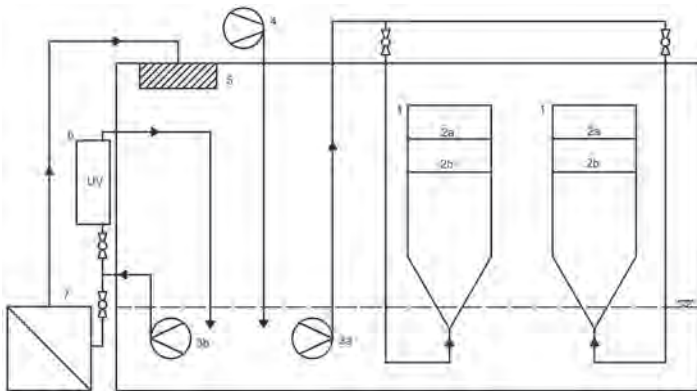
Figure 2. System design for artificial incubation of noble crayfish eggs. 1, Zougg jars; 2, Sieves with egg batches; 3, Pumps; 4, Air compressor; 5, mat and fine filter; 6, UV-filtration unit; 7, Cooling aggregate.

for obtaining pre-grower juveniles in a shorter incubation period. Early egg stripping of various crayfish was previously investigated (Cukerzis 1973; Strempel 1974; Mason 1977; Celada et al. 1987; Hessen et al. 1987; Carral et al. 1992; King 1993; Pérez et al. 1999). In summary, early egg stripping and artificial incubation are suitable for gaining both higher survival rates and a source of pre-grown juveniles, and allow for better breeding control in high production systems. Shortening the time before egg-stripping post-fertilization is a crucial parameter to multiply these advantages. Survival rates of 51% up to juvenile stage-2 have already been achieved for other crayfish species when using artificial incubation for more than three-quarters of the total embryonic development period, reducing disadvantages of maternal breeding in recirculating aquaculture systems like food, energy and space expenses (Carral et al. 1992; Gonzáles et al. 1993) as well as proving methods for incubation of eggs at high densities (Järvenpää and Ilmarinen 1995).

During artificial incubation, dead eggs are usually infected by fungi, further spreading to viable eggs if no treatment is applied. Consequently, removal of dead eggs leads to a higher hatching rate and higher juvenile survival. A variety of antifungal agents

(formaldehyde, malachite green, hydrogen peroxide, isopropyl alcohol, copper sulphate, potassium permanganate and iodine) have already been tested on astacid crayfish eggs, but proved inefficient for regular use due do toxic characteristics for humans and/or eggs (formaldehyde, potassium permanganate, malachite green, iodine) (Meinertz et al. 1995; Celada et al. 2004; Sáez-Royuela et al. 2009) or showed no antifungal effects (isopropyl alcohol, copper sulphate, hydrogen peroxide) (Celada et al. 2004; Melendre et al. 2006). Formaldehyde showed good effectiveness at high concentrations (Sáez-Royuela et al. 2009) but would not be an option due to its reported harmful reproductive and developmental toxicities, including chromosome and DNA damage (Duong et al. 2011) especially under eight degrees Celsius. In comparison, the survival and hatching rate of several freshwater fish was improved by treating the eggs with a salt solution (Edgell et al. 1993; Phelps and Walser 1993; Froelich and Engelhardt 1996; Rasowo et al. 2007).

The use of a salt solution might be a suitable alternative for controling fungal infestations of crayfish eggs. Salt is already mentioned as a suitable antifungal treatment for fish culture and, like formalin and hydrogen peroxide, is apparently suited for registration for therapeutic use (Taylor and Bailey 1979; Marking et al. 1994). Prior studies indicated appropriate saline treatments on crayfish eggs of approximately ten minutes (Pasini, personal observation). In addition, salt is easy to handle and relatively cheap in comparison to other agents (e.g., marine salt costs approx. 4 EUR kg^{-1} while formaldehyde costs approx. 37 EUR L^{-1}).

The aim of the present study was to test the survival rates of early stripped eggs of the noble crayfish, *A. astacus,* and the effectiveness of a salt solution as an easy to handle agent for controlling fungal infections during artificial incubation. We investigated the effects of two different durations (five and ten minutes) of treatment with a salt solution of 15 – 16 PSU (practical salinity units) on fungal infection and survival rates of *A. astacus* eggs.

MATERIALS AND METHODS

Animal Maintenance

The broodstock of two-year-old adult males and females (4:11) of *A. astacus* were collected from a noble crayfish aquaculture operation in Poggenhagen (Göckemeyer, Germany). They were maintained in a recirculating aquaculture system (RAS) in the Centre for Aquaculture Research at the Institute for Marine Resources in Bremerhaven, where experiments were conducted from September 2013 to March 2014. In order to trigger reproduction (Huner and Lindqvist 1985; Westin and Gydemo 1986), the temperature of the RAS was decreased stepwise (max. 1°C day^{-1}) from 20°C to 11°C (Figure 1) starting on 25th of September. To induce the mating process, the animals were maintained from the fourth of October in the recirculating system for 23 days at 11.4 ± 0.6°C. Every month some crayfish were randomly netted out and checked for sperm packages and eggs. From 11th November onward, the temperature was reduced to 4°C over 18 days, according to a natural temperature gradient. In order to trigger egg development, the temperature was slowly increased after 13 days from 4°C to

Table 1. Rate of fungal infection, fungal infections (after three days) and survival rate during artificial incubation *of Astacus astacus* eggs for each treatment, respectively. Different letters indicate significant differences ($P < 0.05$) between treatments.

Treatment	Fungal infection (%)	Fungal infection (n)	Fungal infection (n after 3 d)	Survival rate (%)
control	3.7 ± 1.0 (a)	9	7	11.6 ± 9.5 (a)
5 min	1.2 ± 0.4 (ab)	3	1	26.6 ± 3.7 (b)
10 min	0.8 ± 0.5 (b)	2	2	22.9 ± 2.8 (ab)

Table 2. Water parameters measured during maintenance of adults and egg incubation of the crayfish *Astacus astacus* in broodstock (BS) and artificial incubation (AI).

	pH	oxygen (%)	total hardness (°)	carbonate hardness (°)	ammonium (mg L^{-1})	nitrite (mg L^{-1})	nitrate (mg L^{-1})
BS	–	–	11.9 ± 0.7	7.5 ± 0.8	0.06 ± 0.06	0.06 ± 0.05	5.2 ± 2.9
AI	7.8 ± 0.3	101.6 ± 1.3	16 ± 0.6	7.2 ± 0.8	0.01 ± 0.00	0.01 ± 1.02	4.3 ± 3.3

13°C (Westin and Gydemo 1986) and eggs were stripped on the 14th day of temperature increase. A constant diurnal rhythm of 10 hr day^{-1} that simulated the natural light duration and intensity of October and November (Bremerhaven, Germany, 10 hr 08 min day^{-1}) was applied (Gerding 2013). Illumination was provided by one neon tube 150 cm in length (OSRAM, 58W) which was positioned upside down in the middle of the tank. The case of the neon tube was a waterproofed IP65 case (Type F 7158, Far East Lightning Co. Ltd, China). Crayfish were fed with commercial shrimp feed ((SHR-A 42/09) Ex; vitafeed, Spezialfuttermittelwerk Beeskow, Germany) at low rates of approx. 0.5% bw d^{-1}. Feed and faeces were removed daily. In addition, *Elodea* sp. (waterweed) was offered *ad libitum* as a natural diet.

Experimental Treatment

In January 2014, 20 days after triggering egg development (159 degree-days) and at 13°C, 732 eggs were stripped from seven females as per Policar et al. (2006). The eggs were gently mixed and distributed equally into 12 egg batches with 61 eggs each with a density of 3.5 eggs cm^{-2}. Each partition was placed on a sieve with 1000 µm mesh size and two partitions were randomly placed in two levels in a zougg glas jar from a fish egg incubator system (AquaTech, Kitzbuehl, Austria) after Cukerzis (1973). The temperature was increased approximately 1°C/day until reaching 18°C. The system (Figure 2) was built in a 1 m³ crate (polyethylene, Engels Behältertechnik GmbH, Germany), with six zougg jars that were 15 cm in diameter, a cooling aggregate (Titan 2000; Aqua Medic, Bissendorf, Germany) with a water flow of 2500 L hr^{-1}, a downstream mat and fine filter and a UV-filter (Helix Max 11 W; Aqua Medic, Bissendorf, Germany) which operated in a bypass after the cooling aggregate with a low water flow (approx. 500 L hr^{-1}). The water was pumped from below into the jars and flowed over the edges of the jars into the crate (1000 L hr^{-1}). The water was aerated with an air compressor (Medo 28B, Nitto Kohki, Germany) and a bubble diffusor (11 cm bubble diffusor disk, REBIE, Germany) on the tank bottom. Three treatments were applied:

1. four egg batches were left in the incubation jars as a control treatment

2. four egg batches were bathed in a 15 – 16 PSU saline solution (artificial sea salt, Seequasal, Muenster, Germany) with a conductivity of 20 – 22 mS cm^{-1} for five minutes once every two days

3. four egg batches were bathed in the saline solution as above for ten minutes once every two days

The eggs were macroscopically checked twice a day for fungus growth and dead eggs. Fungal infected and dead eggs were removed and counted in order to calculate infection and survival rates. The infection and survival rates were expressed as the percentage of total number of eggs at the start of artificial incubation. Survival rate is defined by hatched juveniles + 24 h survival. Every second day, the eggs were bathed according to their treatment in a freshly prepared and temperate 15 – 16 PSU saline solution for five or ten minutes. After the bath, the eggs were placed in their original position in the incubation jar. Survival was recorded daily in order to calculate survival rates, expressed as the percentage of the total number of eggs at the start of the artificial incubation. The degree-days for egg incubation were estimated according to Hessen et al. (1987) at approximatley 1300 degree-days.

Water Parameters

Temperature of the RAS system was controlled by a cooling aggregate (Titan 4000: broodstock, Titan 2000: egg incubation; Aqua Medic, Bissendorf, Germany) and measured every second day. In the incubation system, temperature, pH and oxygen saturation were measured daily between January 13th and February 24th 2014. Weekly measurements of the water parameters, including total hardness, carbonate hardness, ammonium, nitrite and nitrate concentrations (mg L^{-1}) took place in all systems throughout the experimental time. The total and carbonate hardness were measured with a commercial colourmetric droplet test (Test GH/KH; Tetra, Melle, Germany). The concentrations of ammonia, nitrite and nitrate were measured with commercial HACH Permachem test kits (Hach Lange, Düsseldorf, Germany). The intensities of the
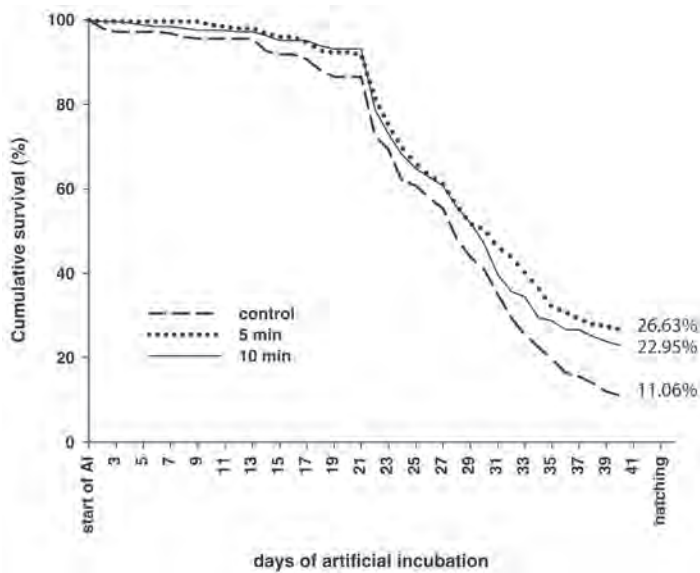
Figure 3. Cumulative survival (%) of *Astacus astacus* eggs treated with salt solution for 5min (dotted line) or 10 min (solid line) during artificial incubation. The dashed line represents the control treatment. AI = artificial incubation.

colourmetric reactions were measured with the photometer (Lange DR2800, Hach-Lange).

Statistical Analysis

The data for survival rate and fungal infection were analysed with one-way ANOVA using Sigma Plot (Version 12, Systat Software, Inc., San Jose, California). Differences in survival curves between treatments were tested by a log-rank test using SigmaPlot. Mean developmental times for eggs incubated for different durations in the saline solution were determined using SigmaPlot. Survival (S) was calculated according formula S = a / b * 100% with live successfully hatched juveniles at end of experimental period (a) and total number of eggs at outset (b) as variables.

RESULTS

Fungal Infection

Fungal infection of *A. astacus* eggs occurred in the first week. Rates of infection were significantly lower among eggs treated with a 15 – 16 PSU saline solution (one-way ANOVA, F = 4.962, $P = 0.035$). The highest fungal infection rate ($3.7 \pm 1.0\%$) was recorded within the control treatment with the highest levels of infection (n = 7) during the first three days (Table 1). While a five minute saline bath did not reduce the fungal infection rate, a significantly lower rate of $0.8 \pm 0.5\%$ was observed after a ten minute saline bath (Table 1). The water conditions were stable for the duration of the experiment (Table 2).

Survival Rates

The artificial incubation period lasted for 54 days, from January 13th to March 9th 2014, at which point, all crayfish hatched. In total, egg development took 82 days (1191 degree-days) which was measured starting with the crucial 4°C that was used to trigger egg development on December 21th 2013. Low final survival rates

between $11.0 \pm 9.5\%$ (Control) and $26.6 \pm 3.7\%$ (5 minute saline treatment) were obtained (Table 1). A survival rate of $22.9 \pm 2.8\%$ was achieved when eggs were bathed in a saline solution for ten minutes. Highest pro rata mortality over all groups was measured in the 2nd and 3rd week (Figure 3) before hatching with $79.7 \pm 2.2\%$ of total mortality, resulting in non-significant differences (one-way ANOVA, F = 1.225, $P = 0.338$) between the groups. Mortality per batch group during this period ranged from $27.9 \pm 7.8\%$ for the control group, $25.0 \pm 3.0\%$ for five minute group, and $26.8 \pm 4.7\%$ for ten minute group.

Survival was significantly affected by the duration in the egg bath within the saline solution (Kruskal-Wallis One Way Analysis of Variance on, $P = 0.015$). A five-minute egg bath led to survival rates twice as high as control values, while there were no significant differences between the control and ten minute treatments.

Mean survival time of control eggs was 28.8 ± 0.6 days, compared to 30.0 ± 0.6 days in eggs treated with saline solution for ten minutes every second day. A higher mean survival time of 32.8 ± 0.6 days was recorded for eggs incubated in saline solution for five minutes every second day. There was a significant difference between survival curves between the five minute treatment and the control and the ten minute treatment, respectively (log-rank test, DF = 2, $P = < 0.001$). Survival rate of all hatched juveniles was 100% until juveniles completed their 2nd moult.

DISCUSSION

Hatchery production in recirculating aquaculture systems may allow production of large amounts of juvenile *A. astacus*, if practical and economic limitations are suitably overcome. In the current study, rates of fungal infection were reduced and overall rates of survival of early-stripped *A. astacus* eggs were improved by repeated saline solution bathing. This indicates an initial fungal suppression by the saline treatment, and thereafter, an artificial but positive effect on egg survival during egg development, which enhances the survival rate of early-stripped eggs.

The overall fungal infection rate of eggs was relatively low and occurred only during the first three days of artificial incubation. This time-limited infection rate may be due the UV treatment unit being unable to kill existing fungus on the eggs that were taken from the broodstock at the start of the experiment. The water treatment rate led to an acclimation period due to tank bioturbation (Tidwell 2012).

Some damage to eggs was incurred by the stripping process and may be the main reason for these losses, while the salt treatment on the day of stripping would have reduced existing fungus on the eggs, as well as fungus stress due to shell damage, and showed a significant effect with the ten minute treatment.

Fungal infection of eggs during the first three days of incubation was still significantly reduced by the 15 PSU saline treatment for ten minutes every other day. Assuming an established water treatment using ultraviolet light units over the critical acclimation period, UV treatment of unstressed noble crayfish eggs would be sufficient in an enclosed incubation system and should be favoured over regular antifungal treatments. A salt bath should be included

after stripping, and at the beginning of artificial incubation in order to treat existing fungus that may be present on egg shells or fungus stress caused by damage when stripping eggs.

The overall low survival rate obtained during the present study may be due to the fact that the broodstock was held in an RAS and no industrial crayfish food was available. Therefore, it is questionable if the food composition for females was sufficient for producing high quality eggs (Klavens and Sorgeloos 1991), an effect already observed in the finfish *Hippoglossus hippoglossus* (Linnaeus) (Bromage et al. 1994) and other crayfish like *Cherax quadricarinatus* (von Martens) (Rodríguez-González et al. 2006) and *Astacus leptodactylus* (Eschscholtz) (Harlıoğlu and Barim 2004). Recent experiments showed poor growth performance of *A. astacus* fed with industrial carp feed than animals fed a natural diet (Seemann et al. In Press). Missing essential nutrients can lead to poor egg quality, as Wouters et al. (2001) have shown for shrimp.

Stripping time, temperature, and time of reproduction may have influenced egg quality and survival rate. According to Mason (1977), artificial incubation leads to better survival rates when stripping is delayed for more than six days after fertilization and incubation is done at 13°C. Policar et al. (2006) reached higher survival rates (> 80%) using an identical set-up, but stripped the eggs at the end of egg development, leading to an artificial incubation period of only 16 days. Therefore, the early egg-stripping used in the present study could be partly responsible for the high egg mortality observed.

More likely, and independently of batches, the temperature increase after egg stripping may have been too fast, leading to high mortalities in the last development stages (eyed stage) three weeks before hatching. Even if eggs are considered far more robust when at this developmental stage, and egg-stripping is done during this time, a temperature increase could have caused the dieoff, as Mason (1977) found higher mortalities at temperatures above 13°C. Whether the dieoff was due to the 18°C temperature itself, or the rapid increase (max. 1°C day⁻¹) remains unanswered. The cumulative effects of broodstock feed, time of reproduction, egg-stripping, and temperature gradient cannot be excluded from influencing egg development and survival rate. If a temperature of 18°C proves to be too high for a reliable breeding technique, a commercial production facility has to include conditioning chambers for artificial breeding as well as tank units for hatched crayfish.

CONCLUSION

A saline bath for five minutes every other day can increase the survival rate significantly under artificial conditions and should be considered as a treatment to enhance survival rate. Saline baths of 15 PSU for ten minutes every other day can significantly reduce the amount of fungal infections, especially when UV light treatment is not available or does not work properly. Salt is a viable antifungal agent since it is non-toxic, easy to handle, and has an affordable price. Early egg-stripping may deliver better pre-grown juveniles than assumed so far, if other physical parameters are optimised. Further research is required to identify the earliest time for egg-stripping by excluding or identifying variables like temperature gradient, broodstock feed, reproduction time, egg quality and system functionality in regard to production demands.

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