

The use of formaldehyde for the disinfection of maternally incubated eggs of noble crayfish (*Astacus astacus*)

Benedetto Sicuro 

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Abstract The high mortality of maternally incubated eggs represents a serious problem that prevents the development of noble crayfish (*Astacus astacus*) farming in Italy. In this experiment, formaldehyde bath technique has been tested with maternally incubating females. The survival rate of maternally incubated eggs, exposed to progressively reduced concentrations of formaldehyde (4000, 3000, 2000, 1000, 1500 and 500 mg l⁻¹), has been compared with that of untreated controls for two consecutive years (2010 and 2011). The formaldehyde treatments were administered as disinfection baths, for a period of 15 min, twice a week. A concentration of formaldehyde of 500 mg l⁻¹, or greater, was found to be effective in controlling egg mortality, without apparent harming the female broods.

Keywords Crayfish farming · Disinfection · Antifungal treatment · Oomycetes · Branchiobdellid

Introduction

In Italy, crayfish farming is principally based on a few extensive family managed farms (Quaglio et al. 2008). At the moment, crayfish production is somewhat limited, with a production of 4.1 tons in 2009, which was principally represented by the red swamp crayfish (*Procambarus clarkii*) (AA.VV 2011). One of the main problems for European native crayfish farming is the spread of alien crayfish species originating from North America (Kozubikova et al. 2008; Kouba et al. 2014), which often carries crayfish plague pathogens, oomycete *Aphanomyces astaci* (Adelrman 1996; Hirsch et al. 2008; Aquiloni et al. 2010; Coignet et al. 2012). The most valuable crayfish species for European aquaculture is the noble crayfish (*Astacus astacus*), which can reach prices of 60–80 € kg⁻¹ (Bohman and Edsman 2011; Gherardi 2011) with a European production of 41 tons in 2011 (FAOSTAT 2013). The main crayfish producing countries in Europe are Spain and Italy, where 1509 tons of red swamp crayfish were produced in 2010 (Vedia and Miranda 2013). The main problem of crayfish rearing in Italy is the high incidence of aquatic mycosis during egg incubation, which has two main consequences: the hampering of the diffusion of crayfish farming and the increasing environmental impact of crayfish farms. Dead eggs can be invaded by aquatic fungi, e.g., *Saprolegnia* sp. and other oomycetes, which often spread to the surrounding eggs, and may result in 100% mortality in the worst cases (Edgerton et al. 2002; Kouba et al. 2013). The diffusion of this mycosis takes on an important ecological relevance as the disinfectants used in rearing conditions can contaminate output waters and the farms can also become direct vectors of disease in natural ecosystems. This fungal disease is usually controlled by manually removing dead

B. Sicuro (✉)
Department of Veterinary Sciences, University of Torino, L. go Braccini, 2, 10095 Grugliasco, TO, Italy
e-mail: benedetto.sicuro@unito.it



eggs, the application of antifungal treatments, or a combination of both (Lilley and Inglis 1997; Policar et al. 2006, 2011; Kouba et al. 2010). Many disinfectants have already been tested for the treatment of artificially incubated crayfish eggs in experimental conditions (González et al. 2009). Formaldehyde, an alternative to malachite green, has been found to be the most effective for signal (*Pacifastacus leniusculus*) and noble crayfish (Sáez-Royuela et al. 2009; Kouba et al. 2010). Considering that maternal incubation is usually adopted in Italy, the aim of this work was to evaluate the disinfection efficacy of a formaldehyde bath on maternally incubated noble crayfish eggs.

Materials and methods

Experimentation site

This research was carried out on a crayfish farm in Northwest Italy, in Crava (Cuneo province). The farm utilizes a freshwater spring source (conductivity $5.0 \pm 0.7 \mu\text{S cm}^{-1}$; pH 7.1 ± 1.0 ; nitrites $0.01 \pm 0.0 \text{ mg l}^{-1}$; nitrates $12.5 \pm 10.6 \text{ mg l}^{-1}$) situated near the farm in an area where white-clawed crayfish (*Austropotamobius pallipes*) are naturally present. Based on previous observations on the farm, this study was focused on noble crayfish (*Astacus astacus*) egg incubation and disinfection, from December to February, as this is the most critical phase in two consecutive years.

Experimental plan

During first year, the experimentation was started on December 10th 2009 and finished on February 29th 2010. During the first week, the crayfish were acclimatized to the experimental conditions and then the disinfection protocol was started. The experimental plan was monofactorial and balanced, the experimental factor tested was the disinfection, and four levels and four replicates (4×4) were adopted. Eighty similar-sized adult females (average body weight $46.8 \pm 2.5 \text{ g}$ and length $11.2 \pm 1.9 \text{ cm}$) with approximately 80–120 eggs were selected from the farm, and five females were randomly allotted to each of the 16 tanks (volume 250 l; water flow 6.5 l min^{-1}). This density is commonly applied in rearing conditions ($3\text{--}4 \text{ crayfish m}^{-2}$). Sand, stones, aquatic plants and natural material were introduced into the bottom of the tanks to provide comparable farming conditions to natural ones. Similar formaldehyde (utilized as Sigma/Aldrich 37% formalin) concentrations to those previously recommended by Kouba et al. (2010), that is, 2000, 3000 and 4000 mg l^{-1} , were compared with a chemically untreated control. The disinfection process consisted in transferring gravid females to separate containers (4 l of volume) for a 15 min disinfection bath, two times per week. Before the disinfection bath, the crayfish were all checked weekly for any eventual dead eggs. During the second year, the experiment was started on November 10th 2010 and finished on February 24th 2011. During the first week, all the tanks were stocked with crayfish, which were allowed to adapt to the experimental conditions. The experimental design was similar to the previous year. Ninety-six females (body weight $72.0 \pm 5.3 \text{ g}$, total length $13.0 \pm 2.1 \text{ cm}$) were selected from the farm and randomly placed into 16 tanks (water flow 5.5 l min^{-1}), six crayfish per tank. Considering the results obtained from the previous year, three concentrations of formaldehyde (500, 1000, and 1500 mg l^{-1}), were compared against an untreated control group. All the females were checked weekly and any dead eggs or infested by fungal infection were removed from the experimental groups. The water temperature and dissolved oxygen levels were monitored weekly ($7.0 \pm 1.5 \text{ }^\circ\text{C}$ and $10.0 \pm 1.1 \text{ mg l}^{-1}$ in 2010 and $8.1 \pm 2 \text{ }^\circ\text{C}$ and $8.0 \pm 2.3 \text{ mg l}^{-1}$ in 2011), respectively. The presence of ectoparasites on crayfish carapace was already noticed in the first weeks of the experimentation in 2009, and their numbers were subsequently counted twice a week in the final part of the experiment in the 2009–2010 season. In the 2010–2011 season, branchiobdellids were counted weekly on all the crayfish, and their presence was compared with that of the experimental groups. The presence of ectoparasites was also monitored at the end of both experiments, by means of a visual inspection.



Statistical analysis

For the statistical analysis of the egg mortality data, normality of data was tested with the “Theoretical quantiles” (Venables and Ripley 2002) test. The data were then analyzed by means of the Kruskal–Wallis test and the Tukey’s test for multiple comparisons of the means, using R software (R version 2.5.0, 2007-04-23). The response variable was the number of dead eggs in each experimental treatment.

Results

The effect of disinfection in 2010 was clearly positive, and the difference between the disinfected groups and the control was significant (Fig. 1), although there were no differences between the three formaldehyde concentration treatments at the end of the experiment (Table 1). In general, egg mortality was very low with some tanks containing disinfected females with no apparent egg mortality. During the second year, the disinfectant doses were reduced, and positive results were again obtained, regardless of the formaldehyde concentration (Fig. 2; Table 2), thus showing the positive effect of the disinfectant. Moreover, considering the time trend of the egg mortality (Figs. 1, 2), a maximum egg mortality peak was clearly visible during the third week of the experimentation, after which the eggs reached a steady state condition without any substantial mortalities. This peak was more evident in the control group in both experiments. At the end of this experimentation, the effect of disinfection was confirmed by the almost complete absence of branchiobdellids on the crayfish and disinfected egg. During the 2010–2011 season, these annelids were classified as *Branchiobdella parasita*, belonging to the Branchiobdellidae family. A significant reduction in the number of branchiobdellid parasites was observed between the controls and females treated with the formaldehyde bath treatments. The number of parasites did not differ significantly among the formaldehyde treated groups, but tended to be lower at higher concentrations (Fig. 3).

Discussion

Artificial incubation has been suggested as a possible method for the intensification of crayfish farming (Carral et al. 1992; Henryon and Purvis 2000; Leonard et al. 2001), but the level of this aquaculture activity is rather underdeveloped in Italy, and maternal incubation is still commonly used. Until now, the best results among the disinfectants that are normally used have been obtained with formaldehyde concentrations of between 2500 and 4500 mg l⁻¹, for treatments of 15 min on alternate days or three times a week (Celada et al. 2004; Melendre et al. 2006; Sáez-Royuela et al. 2009; Kouba et al. 2010). Moreover, a great egg survival was only

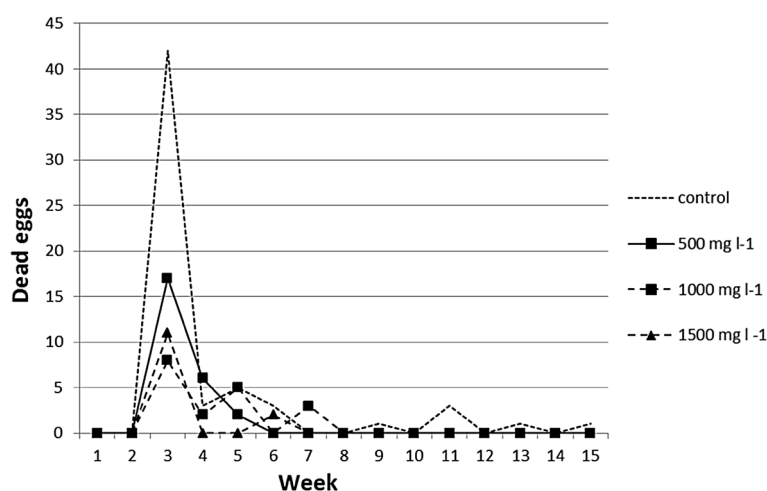


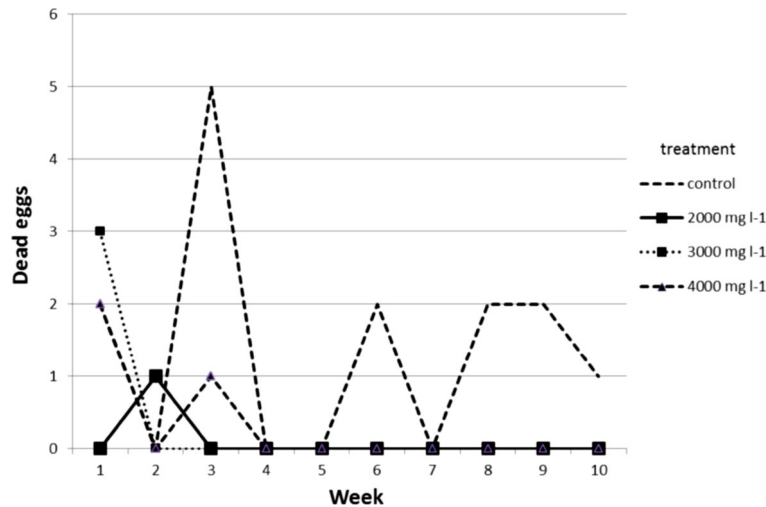
Fig. 1 Time trend of the noble crayfish (*Astacus astacus*) egg mortality, in the 2009–2010 season

Table 1 Comparison of the number of dead eggs of noble crayfish (*Astacus astacus*) at the end of the experiment in the 2009–2010 season

Experimental treatment	Dead eggs
Control	3.5 ± 3.0 ^a
2000 mg l ⁻¹	0.25 ± 0.5 ^b
3000 mg l ⁻¹	0.75 ± 1.5 ^b
4000 mg l ⁻¹	0.75 ± 1.0 ^b

Mean ± SD, $n = 4$

Values with differing letters are significantly different ($P < 0.05$)

**Fig. 2** Time trend of noble crayfish (*Astacus astacus*) egg mortality, in the 2010–2011 season**Table 2** Comparison of the number of dead eggs of noble crayfish (*Astacus astacus*) at the end of the experiment in the 2010–2011 season

Experimental treatment	Dead eggs
Control	2.3 ± 4.7 ^a
500 mg l ⁻¹	1.0 ± 2.4 ^b
1000 mg l ⁻¹	0.7 ± 1.4 ^b
1500 mg l ⁻¹	0.5 ± 1.7 ^b

Mean ± SD, $n = 4$

Values with differing letters are significantly different ($P < 0.05$)

obtained only by removing any dead eggs during incubation (Polcar et al. 2006; 2011) or when a microplate was used in a Japanese crayfish *Cambaroides japonicus* culture (Nakata et al. 2004). However, considering that these methods are labor intensive, the use of a disinfectant can be considered beneficial in terms of labor reduction. The concentration of formaldehyde (2000 mg l⁻¹) used in our experiment in the 2009–2010 season was comparable with that of reference studies. In the 2010–2011 season, the concentration of formaldehyde was reduced even further, and it was still effective at 500 mg l⁻¹. This value is 5–6 times lower than that used in the past, and the disinfectant was administered twice a week, not on alternate days or three times a week as in previous studies (Celada et al. 2004; Sáez-Royuela et al. 2009; Kouba et al. 2010). Moreover, the adoption of a disinfection bath, which has already been used successfully in noble crayfish (Polcar et al. 2006; Kouba et al. 2013), has almost completely solved the problem of the environmental impact of the use of disinfectants. In fact, the use of separate tanks for disinfection reduces the quantity of treated water to a great extent (tank



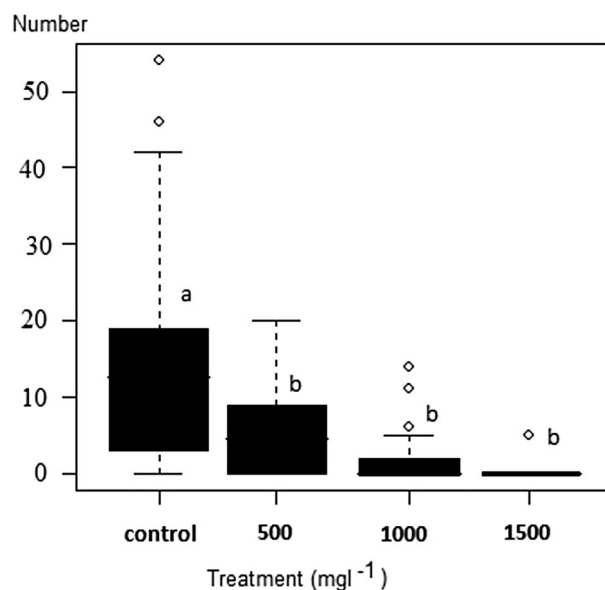


Fig. 3 Branchiobdellids incidence at the end of the experiment in the 2010–2011 season

volume of 4 l) and facilitates the separate management of treated water, thus avoiding its release into natural water bodies. In Italy, a great deal of attention has been paid to the reintroduction of white-clawed crayfish (Nardi et al. 2005) and several LIFE projects, funded by the EU, are focused on this species (e.g., <http://www.lifecrainat.eu/en/>). The disinfection method proposed here shows the potential for efficacious application to this species. In 2009, an event of crayfish plague infection, a disease caused by the fungus-like organism *Aphanomyces astaci*, completely decimated the crayfish on the farm where the experimentation was conducted. This event increased attention toward the risk of future fungal infection. In light of these considerations, the reintroduction of formaldehyde into crayfish farming through the present suggested methods could be hypothesized. Moreover, it should be recalled that formaldehyde can be used as tank disinfectant on trout farms in Italy, and this fact makes the control of its illegal use during incubation difficult. Another promising alternative treatment against both fungal infections of eggs and the transfer of a crayfish plague pathogen is peracetic acid (Jussila et al. 2011, 2014; Kouba et al. 2012) that has been recently found also effective in signal crayfish (Chupani et al. 2016). More research is needed for this chemical compound concerning the time trend of egg mortality, environmental contamination and the occurrence of branchiobdellids. The principal difference between the two successive years was the low number of infected eggs during first year, in all the treatments. This was likely due to the fact that the experimental tanks were utilized for the first time during the 2009–2010 season, while a general increase in aquatic mycetes was evident during the 2010–2011 season, regardless of the experimental treatment. Therefore, it is important not only to disinfect crayfish, but also the tank environment on the farm to reduce and minimize the occurrence and diffusion of mycetes. Considering the egg mortality time trend on the farm, the increased occurrence of fungal infection was only evident in the first 4 weeks of incubation in both seasons, and a small mortality peak was observed for the third week of egg incubation, in particular for the non-disinfected eggs. This is probably related to the immune-competence of the crayfish and to a reduction in the fungal spores and hyphae. Consequently, in the future, the disinfection period could be shortened to the first 4 weeks of incubation, and in this way a further decrease in the environmental impact would be obtained. A small increase in the disinfectant dosage is also advisable during the first week of incubation. However, Kouba et al. (2013) suggested that if a chemical treatment is applied once, it should be followed by regular chemical baths to prevent uncontrollable microbial recolonization. They also pointed out that the nature of the normal microflora on the surface of crayfish eggs is still poorly understood. The effect of the occurrence of branchiobdellids on the biological cycle of crayfish is still not clear. Some authors consider them commensals more than ectoparasites (Gelder 1999; Edgerton et al. 2002; Oberkofler et al. 2002; Quaglio et al. 2006), and their association with crayfish has recently been indicated as a cleaning symbiosis, that is, an intermediate interaction between mutualism and parasitism (Brown et al. 2012;



Farrell et al. 2014). However, the effect of disinfection is much more evident on these invertebrates. The presence of branchiobdellids has already been observed in crayfish in North Italy (Gherardi et al. 2002). During the first year of this study, 15 individuals in the control groups were found with branchiobdellids, but only two were found in the tanks treated with 3000 mg l⁻¹ of formaldehyde. This formaldehyde treatment was able to reduce but not fully eliminate the parasites. As far as the control treatments are concerned, there was a great increase in branchiobdellids in the 2010–2011 season. This fact confirmed previous considerations on the attention that should be paid to farm environmental contamination and disinfection. It is clear that in Italy and in other European countries where crayfish farming is not very well developed and maternal incubation is still used, an environmental friendly method for disinfection could improve this sector. The introduction of artificial incubation is advisable for the future of crayfish farming in Italy, as highlighted by the Spanish experience (Celada et al. 2004; González et al. 2009). Finally, no apparently negative effect of the disinfection method was observed on the female crayfish, and considering that a formaldehyde concentration of 500 mg l⁻¹ was found to be effective, this concentration can be recommended for future utilization in crayfish farming.

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