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Removal of the adhesive gum layer surrounding naturally fertilised ballan wrasse (*Labrus bergylta*) eggs

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ABSTRACT

Commercial production of ballan wrasse (*Labrus bergylta*) as a cleaner fish for the removal of sea lice (*Lepeophtheirus salmonis*) from farmed salmonids (*Salmo salar*) has increased due to its proven efficiency. One bottleneck in commercial hatchery production is working with the benthic adhesive eggs, which makes disinfection and incubation of eggs challenging; therefore, this study aimed to find a chemical or enzymatic treatment and process to remove the adhesive gum layer. Naturally spawned eggs were collected from artificial spawning substrates up to 24 h post spawning from wild caught broodstock kept in captivity at the Marine Harvest, Machrihanish facility. Four treatments were tested: tannic acid (0.2, 0.1, and 0.05%), sodium sulfite (2, 1, and 0.5%), L-cysteine (2, and 1%), and enzyme alcalase® (4.0, 3.0, 2.0, 1.0, and 0.5%) *in vitro*. Eggs were exposed for 25 min while being continually agitated, and the proportion of "degummed" eggs was counted at the end of each time period. Enzyme alcalase® was the only treatment that proved successful in degumming eggs, with the time to complete degumming (≥96%) inversely related to enzyme concentration. Complete degumming occurred between 15 and 30 min for all enzyme alcalase® dose rates. Mean hatch rates for eggs treated with enzyme alcalase ® were not compromised by the treatment and in the highest dose tested were actually found to be higher in treated eggs (78.9 ± 2.4%) than controls (71.3 ± 3.3%). The use of enzyme alcalase® has proven effective in degumming ballan wrasse eggs without affecting hatch rates. However, translation of this method to *in situ* degumming and thus removal of eggs from spawning substrate on farm remains to be standardised.

Statement of relevance: Increasing efficiency for commercial production of ballan wrasse.

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1. Introduction

Commercial interest in the farming of ballan wrasse (*Labrus bergylta*), for the biological control of sea lice (*Lepeophtheirus salmonis*) on farmed Atlantic salmon (*Salmo salar*), has increased in recent years. This is due to the proven delousing efficiency of farmed ballan wrasse (Skiftesvik et al., 2013; Leclercq et al., 2014a) alongside practical difficulties and sustainability concerns associated with the catching and stocking of wild wrasse.

Ballan wrasse are protogynous hermaphrodites that spawn benthic adhesive eggs coated with a gelatinous gum layer (D'Arcy et al., 2012). Commercial hatcheries currently rely on spontaneous natural spawning with artificial spawning substrates being placed on the tank floor to focus spawning effort and aid the subsequent recovery of the adhesive eggs. Spawning substrates are then placed into aerated incubators and larvae hatch directly from the substrate after a period of 6 days (approximately 72 °C days post fertilisation, Ottesen et al., 2012).

The incubation of ballan wrasse eggs, while still adhered to the spawning substrate, makes egg disinfection more challenging which could compromise egg survival, but equally it represents a heightened biosecurity risk for vertical transmittance of pathogens from broodstock to larval rearing systems. Furthermore, adhered eggs may experience suboptimal aeration during incubation again reducing survival and equally general husbandry data collection is challenging or impracticable e.g. the volumetric estimation of batch fecundity. Common practise in other commercially farmed teleost species that spawn adhesive eggs is to remove the adhesive layer from eggs prior to incubation (Linhart et al., 2003a). If a method to eliminate ballan wrasse egg adhesiveness and/or remove the gum layer prior to incubation was demonstrated, this would allow incubation in more traditional upwelling systems as is typically the case in marine species including Atlantic halibut (*Hippoglossus hippoglossus*) (Mangor-Jensen et al., 1998) and Atlantic cod (*Gadus morhua*) (Brown et al., 2003).

Egg adhesiveness has been encountered in other fish species, mainly freshwater teleosts, where various chemical or enzymatic treatments and modes of application have been successfully applied to either prevent eggs becoming adhesive following artificial fertilisation or, in few studies, to remove the existing adhesive gum layer in naturally spawned eggs. However, the timing and treatments used for removal of egg adhesiveness differ between species. Substances such as urea and salt solutions, powdered milk and clay have been traditionally used for the

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prevention of egg adhesion or to coat the egg surface when applied at various time intervals immediately post artificial fertilisation of manually stripped gametes (Kowtal et al., 1986; Rottmann et al., 1991; Ringle et al., 1992; Linhart et al., 2000; El-Gamal and El-Greisy, 2008). Alternative methods are applied to eggs for the removal of adhesive gum that has already been formed either immediately post artificial fertilisation or with naturally spawned eggs. For example, tannic acid, applied at 500–1000 $mg \cdot L^{-1}$ (0.05–0.2%) for 2–5 min shortly after dry fertilisation, has been successfully used to remove the egg adhesiveness in pikeperch (*Sander lucioperca*) (Demska-Zakes et al., 2005), white bass (*Morone chrysops*), sturgeon (*Acipenseridae sp.*), and paddlefish (*Polyodon spathula*) (~150 $mg \cdot L^{-1}$ for 10–12 min immediately post fertilisation, Rottmann et al., 1991). Both sodium sulfite 15.0–30.0 $g \cdot L^{-1}$ (1.5–3.0%) and L-cysteine-HCL 5.0–30.0 $ml \cdot L^{-1}$ (0.5–3.0%) (Ringle et al., 1992) have been used to dissolve the naturally spawned egg masses of the channel catfish (*Ictalurus punctatus*) up to 24 h post fertilisation without any negative impact on hatching rate (Rottmann et al., 1991). Furthermore, proteolytic enzymes, such as enzyme alcalase® have been used in many cases to separate adhered egg masses. For example, in tench (*Tinca tinca* L.) enzyme alcalase® applied shortly after fertilisation at a dose of 10–20 $ml \cdot L^{-1}$ (1.0–2.0%) successfully removed egg adhesiveness, improved hatch rates, and decreased overall egg handling time (Linhart et al., 2000; Gela et al., 2003; Linhart et al., 2003a, 2003b). In addition, enzyme treatment has been used to eliminate egg adhesiveness in European catfish (*Silurus glanis* L., 20 $ml \cdot L^{-1}$, 3 min post fertilisation) (Linhart et al., 2003a) and common carp (*Cyprinus carpio* L., 2–20 $ml \cdot L^{-1}$, 8–20 min post fertilisation) (Linhart et al., 2003a, 2003c).

The aim of this study was to find an effective method for eliminating the adhesiveness of naturally spawned ballan wrasse eggs by testing a range of candidate chemical treatments as well as an enzymatic treatment at varying concentrations and exposure times. Thereafter, the most efficacious treatment was further optimised in terms of dose rate, its impact on larvae hatch rate characterised and its mode of action described.

2. Materials and methods

2.1. Broodstock management and egg collection

Wild caught ballan wrasse broodstock were obtained from the Mull of Kintyre (55°17′ N/5° 47′ W; Scotland, UK) and Dorset (50° 44′ N/2° 20′ W; England, UK) in 2010 and 2011 and maintained indoor under a simulated natural photoperiod (SNP) at the Machrihanish Marine Farm hatchery for commercial breeding purposes. Prior to the spawning period, fish were separated into 10 spawning tanks holding an average of 17 presumed females and 2 presumed males, as determined by morphometric assessment (Leclercq et al., 2014b) [~1:10 male:female sex ratio; Mean body-weight (BW) = 1075.5 ± 64.1 g and 765.7 ± 28.9 g for male and female, respectively]. Spawning tanks were housed indoors under SNP each within a 7 m^3 circular tank all connected to a recirculating system using 10% daily exchange of pre-treated pumped ashore natural seawater and a targeted constant 12 °C water temperature. A daily ration of fresh langoustine (*Nephrops norvegicus*) tails and mussels (*Mytilus edulis*) was provided and bottom waste was syphoned daily. Water quality parameters were checked routinely and averaged over the length of the study: temperature, 12.2 ± 0.6 °C; salinity, 33.3 ± 0.3 ppt dissolved oxygen (DO), 94.3 ± 4.3% saturation and pH, 8.0 ± 0.1.

Each tank was furnished with artificial kelp and PVC pipes as shelters in addition to polypropylene carpets ($n = 16$, 70 × 40 cm; Miami Gel carpet, MDC, Glasgow, Scotland) as spawning substrate for collection of benthic eggs. Over the study duration (20th April to 3rd July 2013) which spanned the natural spawning season, spawning substrates were checked daily at 9 am for presence of eggs from natural spawning events. Random daily egg batches were selected from spawning pairs within the 10 spawning tanks; eggs used for each given trial (with replicates) were taken from single egg batches. Eggs were removed from spawning substrates using a metal spatula and placed into petri dishes prefilled with 20 ml of hatchery water (UV treated and filtered to 0.2 μm, hereafter referred to as hatchery water).

2.2. Treatment efficacy screening

Three different chemical compounds and one enzymatic treatment were tested at varying concentrations as preselected from the literature. Solutions of tannic acid (0.05, 0.1 and 0.2% by weight; $C_{76}H_{52}O_{46}$; W304204; Sigma-Aldrich, UK), L-Cysteine (1.0 and 2.0% by weight; $C_3H_7NO_2S$; W326205; Sigma-Aldrich, UK), sodium sulfite (0.5, 1.0 and 2.0%; Na_2O_3S; S0505, Sigma-Aldrich, UK) and the proteolytic enzyme, Alcalase® Bacillus Licheniformis (4.0, 3.0, 2.0, 1.0 and 0.5%; 126,741–500; VWR, UK) were prepared using hatchery water. Sodium sulfite had a measurable effect on salinity (35 and 48 ppt at 0.5 and 2.0%, respectively) such that each concentration was also prepared and tested in distilled freshwater (1, 3, and 21 ppt at 0.5, 1.0 and 2.0%, respectively). The pH of each solution was adjusted to that of the hatchery water (pH 8.0) with 5 M HCl or 5 M NaOH using a calibrated pH-meter (Mettler Toledo, MP220/225), and salinity was measured using a hand-held refractometer.

2.2.1. Standardised experimental design

Each treatment and concentration was tested in triplicate against a control ($n = 4$ petri-dishes per challenge). Experimental eggs previously separated from randomly selected egg batches were allocated into one of four petri dishes ($n \approx 100$ eggs per petri dish) pre-filled with 20 ml of hatchery water. Eggs were left to settle and adhere to the petri-dish for 1 min; those that did not adhere were discarded. Water from each petri dish was then removed and replaced by 20 ml of test solution (or hatchery water for controls) when exposure time started. Petri dishes were immediately placed onto a horizontal orbital mixer (Denley Orbital Mixer; OM501) rotating at 240 rpm to provide a constant and consistent physical force across the experiment. Solution temperature was measured before and after exposure time 14.6 ± 1.4 °C. The cumulative number of non-adhering eggs was counted using a hand held 4-digit manual counting clicker (without removing solution or eggs from petri dishes) at 2, 5, 10, 15 and 20 min. After 25 min, the contents of each petri dish was discharged into a sieve and the number of eggs within the sieve and those that remained adhered to the petri-dish were counted constituting the percent of "degummed" eggs from the total stocked. The same challenge protocol was repeated for each treatment and concentration tested such that control treatments were replicated within each chemical compound.

2.3. Optimisation of enzyme alcalase® treatment

Following initial screening, the proteolytic enzyme (Alcalase®) treatment was selected for further investigation to define the time required for complete degumming at each enzyme concentration. Each enzyme concentration previously tested (4.0, 3.0, 2.0, 1.0 and 0.5%) was retested in triplicate and assessed on at least three intermediary time points against a non-treated control until maximum (100%) degumming was reached. Time points were initially selected based on observations of highest % degumming during the previous experiment; however later time points were adjusted accordingly after degumming rate was assessed at the first time point, based on how fast degumming was occurring so as to not miss the point at which 100% degumming was reached. A total of 12 petri-dishes per treatment were prepared according to the standardised methodology (Section 2.2.1); 9 of which were filled with enzyme solution at a single concentration and 3 with hatchery water prior to placing onto the horizontal orbital mixer at 240 rpm. At each time-point, 3 replicate and 1 control petri-dishes were measured. The same challenge protocol was repeated for each enzyme concentration tested.

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