Accepted refereed manuscript of:

Grant B, Picchi N, Davie A, Leclercq E & Migaud H (2016) Removal of the adhesive gum layer surrounding naturally fertilised ballan wrasse (Labrus bergylta) eggs, *Aquaculture*, 456, pp. 44-49.

DOI: 10.1016/j.aquaculture.2016.01.016

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 hours 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 minutes 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 ($\geq$ 96 %) inversely related to enzyme concentration. Complete degumming occurred between 15 and 30 minutes 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.

Key words: cleaner fish, adhesive eggs, gum layer, hatching, enzyme alcalase®

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 practice 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-Jenson *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 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 & 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 $mg.L^{-1}$ (0.05 - 0.2 %) for 2 - 5 minutes 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 $mg.L^{-1}$ for 10 - 12 minutes immediately post fertilisation, Rottmann *et al*. 1991). Both sodium sulfite 15.0 -30.0 $g.L^{-1}$ (1.5 - 3.0 %) and L-cysteine-HCL 5.0 - 30.0 $ml.L^{-1}$ (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 hours 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.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,b). In addition, enzyme treatment has been used to eliminate egg adhesiveness in European catfish (*Silurus glanis* L., 20 ml.L^{-1}, 3 minutes post fertilisation) (Linhart *et al*. 2003a) and common carp (*Cyprinus carpio* L., 2 - 20 ml.L^{-1}, 8 - 20 minutes post fertilisation) (Linhart *et al*. 2003a,c).

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, 70x40 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 %; 126741-500; 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 minute; 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 minutes. After 25 minutes, 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.

2.4 Effect of enzyme alcalase® treatment on hatching rate

Based on results from the enzyme optimisation, hatching rate of eggs exposed to the three lowest enzyme dose-durations (0.5 %, 30 min; 1.0 %, 20 min; and 2.0 %, 10 min) were assessed following three discrete (independent egg batches) challenges against a control and in triplicate according to the standardised methodology (Section 2.2.1). For each challenge, a total of 6 petri-dishes ($n \approx 60$ eggs each) were exposed to a single enzyme dose-duration (Treatment, $n = 3$) or seawater (Control, $n = 3$). Upon completion of exposure time, all eggs were rinsed in hatchery water and placed into a 24-well microplate (5 eggs per well) pre-filled with 500 µl / well of hatchery water. Egg viability and stage of development were assessed under a stereomicroscope (GX Stereo microscope; XTL3T, GT Vision, Suffolk,

UK). Microplates were covered, sealed, and incubated in darkness within a temperature controlled incubator (12 ± 1 °C; LMS Cooled Incubator, Model 305, Series 1, 60 L capacity, LMS Ltd, Kent, UK). The number of hatched larvae was counted (expressed as the proportion of stocked eggs per replicate to define the hatching rate) under a stereomicroscope at 9 days (108 °C days) post egg-collection to allow sufficient time for hatching to occur (Ottesen *et al*. 2012).

2.5 Progression of gum layer removal under enzyme alcalase® treatment

In order to look at the progression of gum layer removal over the course of enzyme exposure, replicate treatments of $n \approx 60$ eggs were exposed to 1 % enzyme alcalase® and seawater controls ($n = 3$ replicates per time point; 0, 5, 10, 15, 20, and 25 minutes) following the previously described methodology (Section 2.2.1). At each designated time interval the appropriate replicates for the treatment and control treatments were removed for the orbital shaker and then solutions were removed, eggs were rinsed in sea water, and immediately photographed using a computer controlled digital microscope camera (1x magnification, GXCam3, GT Vision, Suffolk, UK) fitted onto a stereo microscope (GX Stereo microscope; XTL3T, GT Vision, Suffolk, UK). The breakdown of the adhesive gum layer was described using a 4 point subjective scale in $n = 30$ eggs per replicate explained further below to characterise the progression of the enzymatic treatment.

2.6 Statistical analysis

All figures were presented as mean ± standard deviation (SD). Statistical analysis was carried out with Minitab 16. All data sets were checked for normality using the Anderson Darling test and arc-sine transformation of percentage data was carried out before statistical

analysis. Hatch rate and degumming data were analysed with a one way analysis of variance (ANOVA) and post-hoc analysis was carried out using Tukey's Multiple Comparison tests ($P \leq 0.05$).

3. Results

3.1 Treatment efficacy screening

Results of the screening tests indicated that enzyme alcalase® was the only treatment that liberated adhered eggs effectively (≥69%) (One-way ANOVA: DF = 5, F = 62.91, P = 0.000) (Table 1). The highest enzyme concentrations (3.0 - 4.0 %) resulted in 100 % liberation of eggs after 25 minute exposure time which will be referred to as "degumming rate" hereafter. In the same time period, even the lowest enzyme concentration (0.5 %) showed a 69.3 ± 5.5 % degumming rate. Visual counts at intermediary time points revealed that by 10 minutes the mean degumming rate in each of the enzyme alcalase® treatments was significantly higher than control treatments, which did not vary over the course of the exposure period (Fig. 1). Furthermore, eggs exposed to higher enzyme doses (3.0 and 4.0 %) were degummed faster than the lower doses (0.5, 1.0 and 2.0 %). Given the success of the enzyme treatment in removing the adhesive gum layer of ballan wrasse eggs, it was chosen for further investigation in subsequent experiments.

With respect to the other treatments; sodium sulfite was unsuccessful in freeing adhered eggs, with the highest degumming rate (11.1 ± 10.8 %) obtained with the 2 % sodium sulfite seawater solution (all freshwater based treatments showed no higher than 0.3 ± 0.5 % degumming rate, data not shown). Similarly, tannic acid and L-cysteine solutions were both ineffective in degumming eggs during the 25 minute exposure time with the highest degumming rate of 0.7 ± 0.6 and 13.5 ± 7.2 % for tannic acid and L-Cysteine, respectively.

Furthermore, the mean degumming rates for all concentrations of sodium sulfite, tannic acid, and L-Cysteine were not significantly higher than control treatments (One-way ANOVA: Sodium sulfite, DF = 6, F = 2.82, P = 0.052; tannic acid, DF = 3, F = 3.77, P = 0.059; L-Cysteine, DF = 2, F = 3.59, P = 0.108).

3.2 Optimisation of enzyme alcalase® treatment

The mean degumming rate was found to be inversely related to concentration of enzyme alcalase®, i.e. the higher the enzyme dose the shorter period of time for degumming. Complete degumming occurred between 25 - 30 minutes for the lowest enzyme dose rate (0.5 %), between 15 - 20 minutes for 1.0 - 2.0 %, between 15 – 17 minutes for 3.0 % and between 12 - 15 minutes for the highest enzyme dose (4.0 %) (Table 2).

3.3 Impact of enzyme alcalase® treatment on hatching rate

Overall, mean hatch rate were statistically comparable in eggs treated with enzyme alcalase with respect to controls for 0.5 and 1.0 % treatments, while hatch rate was higher for the 2.0% treatment (One-way ANOVA: DF = 1, F = 8.79, P = 0.052) (Table 3).

3.4 Progression of gum layer removal under enzyme alcalase® treatment

Observation of the gum layer under enzyme alcalase® treatment revealed that the mode of action was not to erode the gum evenly from the outside, rather the gum layer was observed to swell and detach from the egg chorion before finally breaking open and then ultimately completely detaching from the egg. Therefore a subjective four point scoring criteria was created as follows: 1.Gum layer fully intact; 2. Gum layer starting to swell and break down; 3. Gum layer swollen and partially removed from egg, although still attached; and 4. Gum

later fully detached (clean egg). This was then used to characterise the progression of the gum layer removal (Fig. 2, 1-4).

At the 0 and 5 minute time intervals for 1 % enzyme treatments and at all time points for control treatments 100 ± 0.0 % of eggs had a gum layer score of 1 (fully intact gum layer) (Fig. 3). Thereafter, the proportion of eggs with fully intact gum layers decreased as enzyme exposure time progressed. By the 25 minute time point, 93.3 ± 3.3 % of eggs exposed to the enzyme treatment had fully detached gum layers (Score 4). However, mean degumming rate started to increase from the 5 minute time point (5.1 ± 5.8 %) until complete degumming (100 ± 0.0 %) i.e. all eggs detached from petri dish, at the 25 minute time point, suggesting that the gum layer does not have to be fully removed before an egg is detached from the substrate.

4. Discussion

The issue of egg adhesiveness in ballan wrasse has been initially addressed by adapting egg collection and incubation protocols. However, the development of techniques for the removal of the adhesive layer surrounding the eggs and thereby removal of eggs from artificial spawning substrates would benefit commercial production by allowing for more thorough egg disinfection, accurate fecundity estimates and for easier egg incubation and hatching techniques. Therefore, this study aimed to find an effective method for eliminating egg adhesiveness by testing a range of candidate treatments. Through this investigation an *in vitro* method for eliminating egg adhesiveness was developed whereby the proteolytic enzyme, alcalase®, was found to successfully eliminate egg the adhesive gum layer without negatively impacting hatch rate.

During the initial chemical screening trial it was found that sodium sulfite, tannic acid, and

L-cysteine solutions (at all concentrations) were unsuccessful at degumming eggs during the 25 minutes exposure time with no greater than 13.5 ± 7.2 % degumming rate for any of the tested chemicals. This was an unexpected result, particularly for sodium sulfite, as 1 - 1.5 % sodium sulfite applied for only 1-3 minutes has been successfully used to separate the glycoprotein matrix of channel catfish egg masses up to 24 hour post spawning (Issac & Fries, 1991; Rottmann *et al*. 1991). However, the noted effects on salinity that lead us to test sodium sulfite both in fresh and seawater and the ability of the chemical to drastically reduce dissolved oxygen (Ringle *et al*. 1992) may be potential reasons behind its ineffectiveness. Furthermore, L-cysteine has been successful in dissolving the egg masses of channel catfish up to 48 hours post spawning (Ringle *et al*. 1992) as well as the gelatinous coating surrounding amphibian eggs (Dawid, 1965). This suggests that the effects of these degumming chemicals are highly variable between species and that the specific adhesive properties of the eggs, in addition to the timing of chemical application, must be taken into account. Moreover, the fact that these chemicals were applied to ballan wrasse eggs in full strength sea water as opposed to fresh or brackish water in the case of catfish species may have implications towards its ineffectiveness. Tannic acid, on the other hand, may have been more successful in removing the gum layer of ballan wrasse eggs when applied closer to the time of fertilisation. Due to hatchery limitations, it was not possible to test this, however this could be further tested on manually stripped eggs.

In this study, positive results were obtained using enzyme alcalase® for the removal of the adhesive gum layer around ballan wrasse eggs. During the initial screening, all dose rates tested were successful at degumming 69 - 100 % of eggs after the 25 minute exposure period. Subsequent trials demonstrated that the exposure time required for complete degumming was inversely related to the dose, with time to complete

degumming ranging between 10 and 30 minutes. Subsequently, as all enzyme doses rates were effective, the lowest enzyme concentrations were then selected for further testing as using a lower enzyme concentration would be favourable to limit the costs of commercial upscaling.

Enzyme treatment showed no negative effects on egg quality, with respect to hatching rate and, in fact at the highest dose, treated eggs showed higher hatching rates compared to control groups as previously reported in common carp (Linhart *et al*. 2003c) and tench (Linhart *et al*. 2000; 2003a). In this study, it was observed that the enzyme treatment liberated the eggs by actually breaking down and removing the outer gum layer surrounding the eggs suggesting that higher hatch rates may arise from treated eggs that have less of a barrier surrounding the chorion to break through. The breakdown of the adhesive gum layer was an expected result because the general chemical function of proteolytic enzymes is to digest or breakdown the long chain molecules into amino acids (Neurath & Walsh, 1976). However, the mechanism by which the gum layer removal occurred was both interesting and unexpected; as enzyme exposure progressed, the gum layer started to swell and become less structured and instead of breaking down or dissolving into pieces the gum layer swelled until it was completely bloated, then came off as a single casing, leaving a clean non-adhesive egg. The adhesiveness of teleost eggs is due to the specific composition of the outer layer of eggs (Hazzaa & Hussein, 2003) which, in some species, has been found to be rich in glycoproteins and other polysaccharides that are accountable for the interactions between the egg and the aquatic environment (Rizzo *et al*. 2002). However, the specific composition of the adhesive gum layer in ballan wrasse eggs is yet to be determined.

Removal of the adhesive gum layer is not only important for egg handling purposes, but

as these benthic eggs are laid on artificial substrates on the bottom of broodstock tanks they are in close proximity to waste feed and faecal matter that, in turn, causes excess bacterial and fungal loading in egg incubation tanks. Krise *et al*. (1986) pointed out that the adhesive layer surrounding the chorion is particularly susceptible to viral, fungal, and bacterial pathogens and that removal of this can significantly reduce the development of such pathogens. Furthermore, routine disinfection and removal of dead eggs during egg incubation are important for improved biosecurity, both of which can be made easier and more effective by incubating eggs in suspension.

Future research into the adhesive properties of ballan wrasse eggs is required as well as assessing the potential effects of applying enzyme treatments closer to the time of fertilisation in manually stripped vs. naturally spawned eggs. Furthermore, full scale testing of the methodology of enzyme treatment for direct removal of eggs from artificial spawning substrates in a commercial setting is still to be optimised.

Acknowledgments

This research was co-funded by Innovate UK (Project ref. 81199), Marine Harvest (Scotland)^{Ltd} and Scottish Sea Farms. The authors would like to thank the supporting staff at the Machrihanish Marine Farm and Machrihanish Marine Environmental Research Laboratory (University of Stirling) for support and the use of facilities.

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Tables

Table 1. Proportion of ballan wrasse eggs degummed following 25 minutes of exposure to candidate degumming solutions, each tested at varying concentrations (%). Control treatments for each concentration are pooled (*n* = X replicates). Values are expressed as mean ± SD (*n* = 3 replicates). Superscripts indicate significant differences between treatments and pooled controls.

Solution	Concentration (%)	Degumming rate (%)
Sodium Sulfite	2.0	11.1 ± 10.8^{a}
	1.0	0.9 ± 0.1^{a}
	0.5	2.1 ± 0.2^{a}
Control (*n* = 3)	0.0	1.6 ± 1.1^{a}
L-Cysteine	2.0	13.5 ± 7.2^{a}
	1.0	6.0 ± 5.0^{a}
Control (*n* = 2)	0.0	0.0 ± 0.0^{a}
Tannic acid	0.2	0.0 ± 0.0^{a}
	0.1	0.0 ± 0.0^{a}
	0.1	0.7 ± 0.6^{a}
Control (*n* = 3)	0.0	0.0 ± 0.0^{a}
Enzyme Alcalase	4.0	100.0 ± 0.0^{a}
	3.0	100.0 ± 0.0^{a}
	2.0	90.2 ± 7.1^{ab}
	1.0	72.9 ± 24.3^{b}
	0.5	69.3 ± 5.5^{b}
Control (*n* = 5)	0.0	1.0 ± 0.0^{c}

Table 2. Degumming rate (Mean % ± SD (*n* = 3; control *n* = 1)) for each enzyme alcalase® concentration assessed at varying time points until complete degumming was achieved.

Concentration (%)	Exposure Time (Mins)	Mean Degumming rate (%)	
		Treated (n=3)	Control (n=1)
0.5	25	69.1 ± 6.8	0.0
	30	100.0 ± 0.0	0.0
	35	100.0 ± 0.0	5.3
1	15	59.5 ± 13.2	0.6
	20	100.0 ± 0.0	3.7
	25	100.0 ± 0.0	0.0
2	10	98.4 ± 1.4	0.0
	15	98.7 ± 2.2	1.0
	20	100.0 ± 0.0	0.0
3	12	68.0 ± 6.7	0.0
	15	96.9 ± 3.9	0.0
	17	100.0 ± 0.0	2.1
4	10	10.9 ± 4.4	0.9
	12	16.1 ± 2.8	0.0
	15	100.0 ± 0.0	0.0

Table 3. Hatching rate (%) of ballan wrasse eggs after exposure to varying dose/durations of enzyme alcalase treatment vs. control treatment. Values are expressed as mean ± SD (*n* = 3 replicates). Superscripts indicate significant differences between treatment and control.

Concentration (%)	Exposure time (min)	Hatch rate (%)	Control hatch rate (%)
0.5	30	77.5 ± 4.9[a]	75.5 ± 15.1 [a]
1.0	20	85.6 ± 8.2 [a]	73.3 ± 7.6 [a]
2.0	10	73.7 ± 2.6[a]	65.1 ± 4.4[b]

FIGURE CAPTIONS

Figure 1. Proportion of ballan wrasse eggs degummed following exposure to enzyme alcalase (4.0, 3.0, 2.0, 1.0, and 0.5 %) compared to seawater control for 2, 5, 10, 15, 20 and 25 minutes. Superscripts indicate significant differences between treatments at each time point. Values are expressed as mean ± SD with n = 3 replicates for treatments and n = 5 replicates for control.

Figure 2. Ballan wrasse eggs at varying levels of gum layer removal during exposure to 1 % enzyme alcalase over a 25 minute time period: (1) Score 1; fully intact gum layer prior to enzyme exposure. (2) Score 2; gum layer starting to swell and break down after 15 minutes of enzyme exposure. (3) Score 3; gum layer swollen and partially removed from egg although still attached after 20 minutes of enzyme exposure. (4) Score 4; gum layer fully detached from egg (clean egg).

Figure 3. Proportion (Mean % ± SD (n = 3)) of ballan wrasse eggs classed with a gum layer score of 1-4 at 5 minute time intervals following exposure to 1% enzyme alcalase. Degumming rate (Mean % ± SD (n = 3)) at each time point was: 0 minutes = 0.0 ± 0.0; 5 minutes = 5.1 ± 5.8; 10 minutes = 46.2 ± 21.0; 15 minutes = 66.1 ± 14.0; 20 minutes = 89.7 ± 13.8; and 25 minutes = 100.0 ± 0.0.

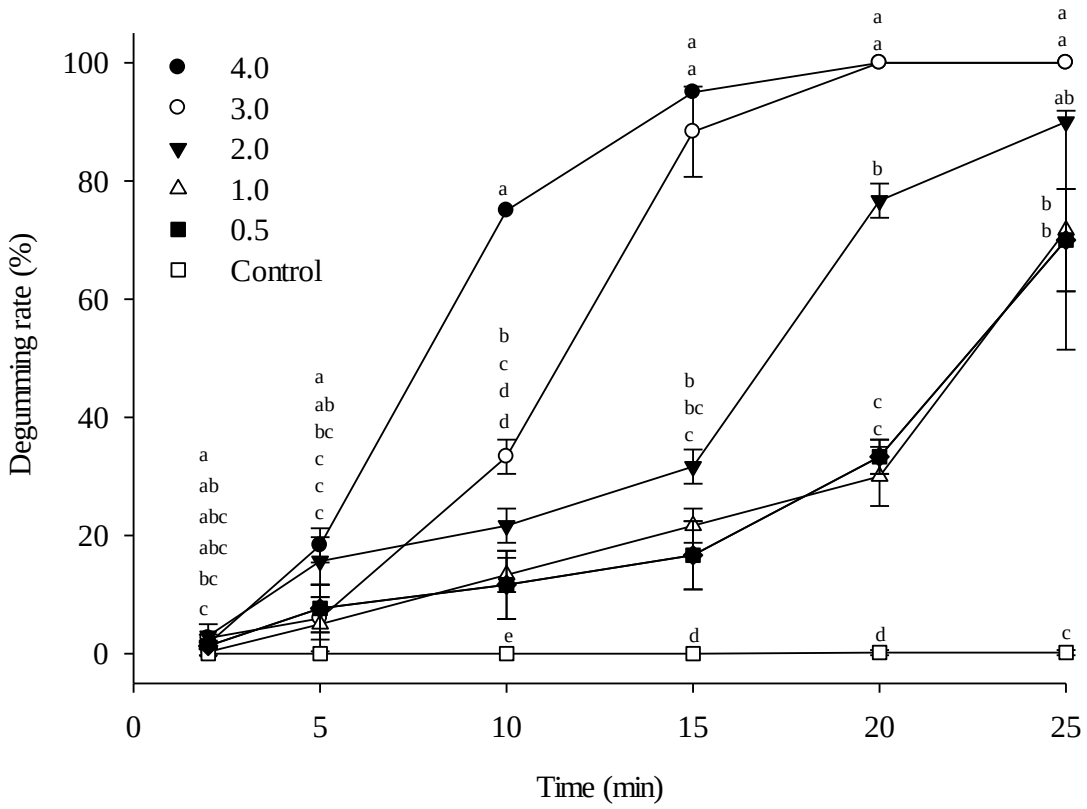
4.0
3.0
2.0
1.0
0.5
Control
Degumming rate (%)
Time (min)
0
20
40
60
80
100
0
5
10
15
20
25

Figure 1

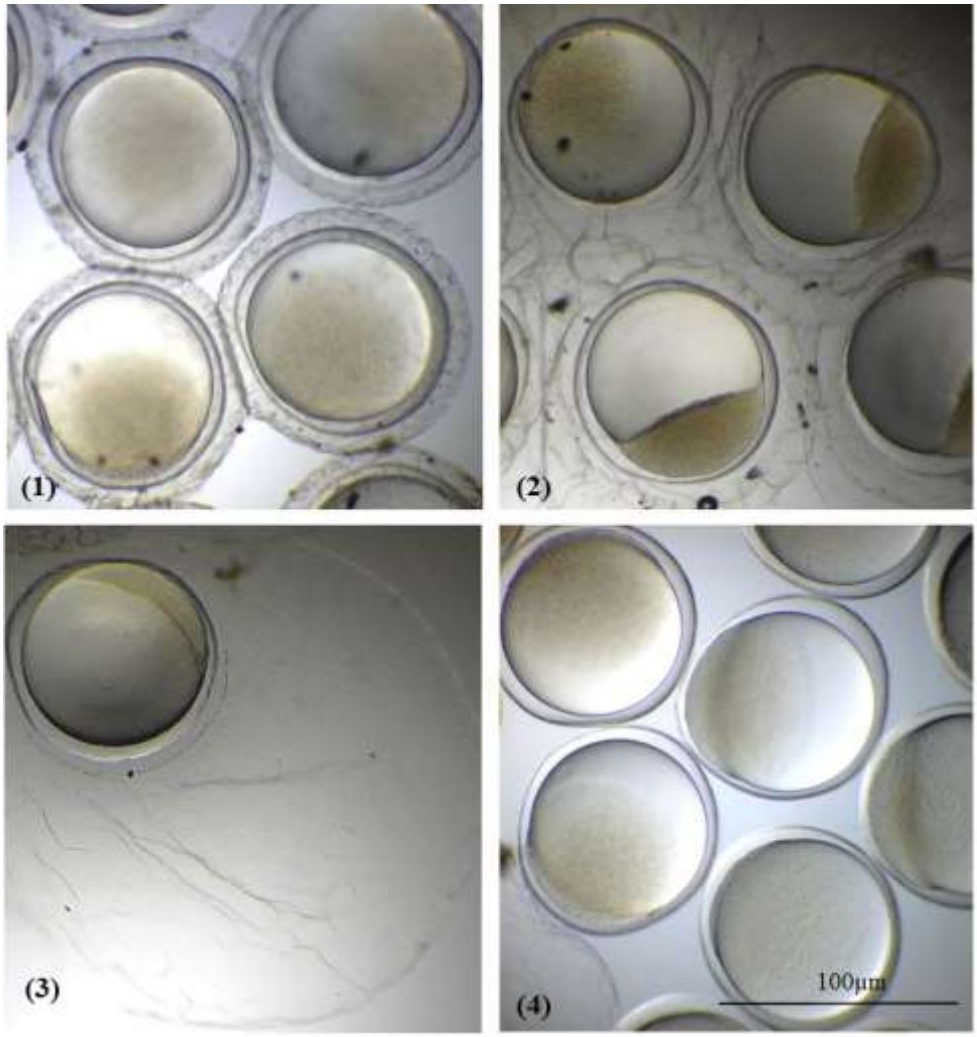

Figure 2

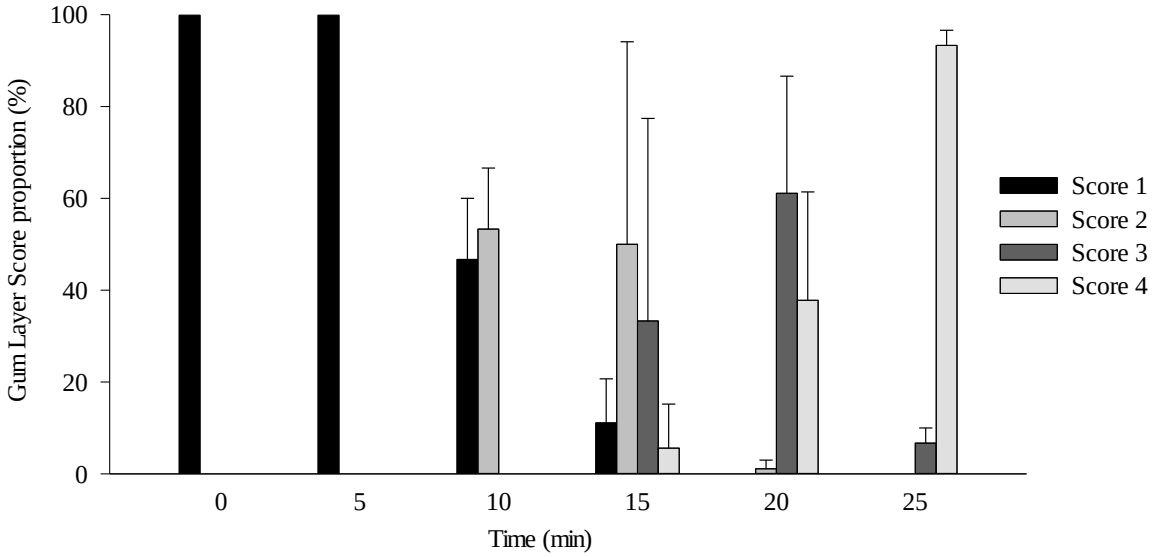
Gum Layer Score proportion (%)
100
80
60
40
20
0
0
5
10
15
20
25
Time (min)
Score 1
Score 2
Score 3
Score 4

Figure 3