Master's thesis

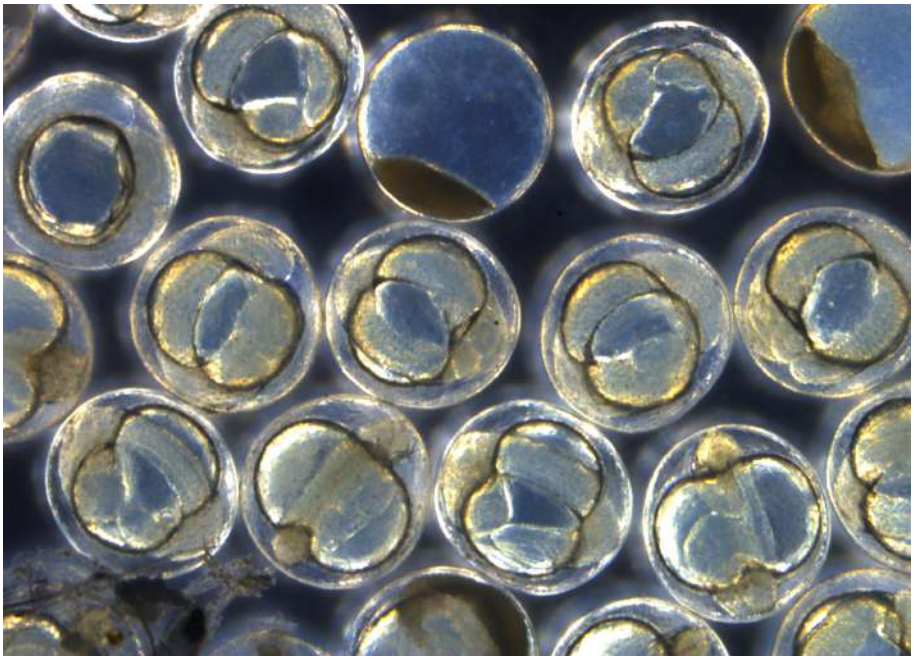
NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Fahad Shabbir

Degumming of Ballan wrasse (*Labrus bergylta*) eggs

Master's thesis in MSc Ocean Resources
Supervisor: Elin Kjørsvik
Co-supervisor: Andreas Hagemann, Bjørn Henrik Hansen, Antonio Sarno, Arne Malzahn

February 2022



NTNU
Norwegian University of
Science and Technology

Fahad Shabbir

Degumming of Ballan wrasse (*Labrus bergylta*) eggs

Master's thesis in MSc Ocean Resources
Supervisor: Elin Kjørsvik
Co-supervisor: Andreas Hagemann, Bjørn Henrik Hansen, Antonio Sarno, Arne Malzahn
February 2022

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

NTNU
Norwegian University of
Science and Technology

Acknowledgement

This M.Sc. thesis was part of the research project “STARTRENS (901561)”, funded by the Fishery and Aquaculture Industry Research Fund (FHF) in collaboration with NTNU, SINTEF Ocean and MOWI. The experimental phase was conducted at NTNU Centre of Fisheries and Aquaculture, Trondheim.

The thesis was written under the supervision of Elin Kjørsvik (Professor, NTNU), Bjørn Henrik Hansen (Senior Research Scientist, SINTEF), Antonio Sarno (Research Scientist, SINTEF), Andreas Hagemann (Senior Research Scientist, SINTEF), and Arne Malzahn (Senior Research Scientist, SINTEF) at the Department of Biology at NTNU

First of all, I would like to thank all my supervisors for their motivation and guidance. I will miss the round table discussions during the initial planning phase of the experiment where biology met chemistry, and all the puzzles were assembled. Thank you, Elin, for always caring and understanding me, your kindness was not less than any blessing.

Also, a special thank to Iurgi Imanol Salaverria-Zabalegui, Dag Altin, Tora Bardal, Frank Thomas Mlingi, Luciana Alves Musialak, Essa Ahmed or providing me with academic help and motivation during my master's journey. I appreciate the extra help from all of you because it wouldn’t be possible for me to get through my masters without you all.

Words fail to express my heartfelt thanks to my family for their love, understanding, and belief in my efforts and their constant support and prayers.

Fahad Mohammad Shabbir

Trondheim, 10th February 2022

Abstract

The demand for Ballan wrasse (*Labrus bergylta*) has increased over the years due to its proven efficiency as a cleaner fish to remove sea lice (*Lepeophtheirus salmonis*) from farmed salmon (*Salmo salar*) in sea cages. One of the hurdles in commercial hatcheries is working with benthic adhesive eggs. The current techniques result in low survival and high mortality of the incubated eggs due to the challenges with dirt clinging, ineffective disinfection, biosecurity risk, anoxia and more. A few attempts were carried out to eliminate the stickiness of the Ballan wrasse eggs using tannic acid, sodium sulfite, L-cysteine, clay suspension, milk, and enzyme alcalase with and without ringer solution, out of which only enzyme alcalase was found effective. This study was the first of its kind to test more potential protease enzymes that could effectively degum Ballan wrasse eggs. Six treatments were tested: enzyme alcalase (2%), trypsin (2000, 200, 40, 20 U/ml), proteinase K (900, 700, 400 µAnsonU/ml), pineapple juice (16, 8, 4, 1%), papain from *Carica papaya* (2, 4%), papain from papaya latex (50, 10, 5 U/ml) *in vitro*. Eggs were received while attached to spawning mats from MOWI on the following day of fertilization. A mat fragment of 2.5x5cm containing eggs was attached to a flocculator arm dipped into the enzymatic solution and agitated at 50rpm. The detached eggs from the mats were considered non-sticky, categorising the fallen eggs in four stages. (a) degummed, (b) swelled, (c) partly degummed or (d) not degummed. The study was divided into two phases; 1st phase was a screening phase, which focused on the optimization of enzyme working conditions by changing parameters such as pH, time and EDTA addition. The 2nd phase was the main experiment, which focused on applying proteases at optimized conditions for a 30min duration. Consistent with the previous studies, alcalase liberated 68 ± 8.2% of the eggs from the mats while placing 58.6 ± 10.9 % of eggs in the degummed stage. Besides alcalase, papain from papaya latex was the only effective treatment that liberated 88 ± 5.7%, 78 ± 9.9% and 84 ± 6.6% of eggs from the mat at 50, 10, 5 U/ml concentrations, respectively. Further, gum layer classification revealed that papain (50, 5 U/ml) had degummed >80% of the fallen eggs while reaching >90% for the papain 10 U/ml treatment. The hatching rate revealed no significant differences among the enzymatic treatments. However, the papain treatments (50, 10, 5 U/ml) had low hatch rate 55 ± 6.5%, 58 ± 19.7% and 62 ± 14.9% respectively, when compared to control (seawater) 77 ± 5.6% and alcalase

67 ± 4.7%. Morphological measurements of newly hatched larvae showed no significant difference among the treatments, except for alcalase treatments that displayed significant lower myotome height and larger eye-to-body ratio compared to controls. Spinal deformities including tail flexures, breaks and curved spine showed no significant difference among the treatments. Yolk sac/pericardial edema was rated from 0 to 2 depending on severity, where the mild edema deformity was present in all the treatments, >50% in the papain treatments with a significant difference. Alcalase was the only treatment that caused the break deformity >22%. The use of papain from papaya latex has proven to be effective in detaching the eggs from the substrate with no effects significant effects on hatching rate but with the occurrence of mild edema deformity. However, these degumming protocols are *in situ*, and achieving degumming of the Ballan wrasse eggs at a commercial scale still needs to be standardized.

Statement of relevance: Improving the commercial production of Ballan wrasse (*Labrus bergylta*) by optimizing the hatchery phase.

Keywords: Adhesive eggs, Enzymatic treatments, Elimination of eggs stickiness, Cleaner fish, Hatching, deformities.

Sammendrag

Etterspørselen etter berggylt (*Labrus bergylta*) har økt gjennom årene på grunn av dens effektivitet som rensefisk for å fjerne lakselus (*Lepeophtheirus salmonis*) fra oppdrettslaks (*Salmo salar)* i merder. En av utfordringene i kommersielle klekkerier er å jobbe med bentiskeklebende egg. Dagens metoder resulterer i lav overlevelse og høy dødelighet for eggene på grunn av utfordringene med smuss som klamrer seg fast, ineffektiv desinfeksjon, biologisk sikkerhet, anoksi og mer. Noen få forsøk ble utført for å eliminere klebrigheten til berggylt-eggene ved bruk av garvesyre, natriumsulfitt, L-cystein, leiresuspensjon, melk, og enzymalkalase med og uten ringers løsning, hvorav kun var effektiv. Denne studien var den første i sitt slag som testet flere potensielle proteaseenzymer som effektivt kunne løsne de klebrige bergylteggene. Seks behandlinger ble testet: enzym alkalase (2%), trypsin (2000, 200, 40, 20 U/ml), proteinase K (900, 700, 400 µAnsonU/ml), ananasjuice (16, 8, 4, 1%), papain fra *Carica papaya* (2, 4 %), papain fra papaya lateks (50, 10, 5 U/ml) in vitro. Egg ble mottatt mens de var festet til gytematter fra MOWI påfølgende befruktningsdag. Et mattefragment på 2,5 x 5 cm som inneholdt egg ble festet til en flokkulatorarm dyppet i den enzymatiske løsningen og rørt om ved 50 rpm. De eggene som løsnet fra mattene ble ansett som ikke-klebrige, og kategoriserte de falne eggene i fire trinn. (a) avgummet, (b) svellet, (c) delvis avgummet eller (d) ikke avgummet. Studien ble delt inn i to faser; 1. fase var en screeningsfase, som satte søkelys på optimalisering av enzymarbeidsforhold ved å endre parametere som pH, tid og EDTA-tilsetning. Den andre fasen var hoved-eksperimentet, som fokuserte på å påføre proteaser ved optimaliserte forhold i en varighet på 30 minutter. I samsvar med de tidligere studiene frigjorde alkalase 68 ± 8,2 % av eggene fra mattene mens de plasserte 58,6 ± 10,9% av eggene i avgummet kategori. Foruten alkalase var papain fra Papaya-latex den eneste effektive behandlingen som frigjorde henholdsvis 88 ± 5,7%, 78 ± 9,9% og 84 ± 6,6% av eggene fra matten ved konsentrasjoner på 50, 10, 5 U/ml. Videre avslørte gummilagsklassifiseringen at papain (50, 5 U/ml) hadde avgummet >80% av de fallne eggene mens de nådde >90% for papain 10 U/ml-behandlingen. Klekkehastigheten viste ingen signifikante forskjeller mellom de enzymatiske behandlingene. Imidlertid hadde papainbehandlingene (50, 10, 5 U/ml) lav klekkehastighet på henholdsvis 55 ± 6,5%, 58 ± 19,7% og 62 ± 14,9% sammenlignet med kontroll (sjøvann) 77 ± 5,6 % og alkalase 67 ± 4,7 %.

Morfologiske målinger av nyklekkede larver viste ingen signifikant forskjell mellom behandlingene, bortsett fra alkalasebehandlinger som viste signifikant lavere myotom høyde og større øye-til-kropp-forhold sammenlignet med kontrollene. Spinaldeformiteter inkludert halebøyninger, brudd og buet ryggrad viste ingen signifikant forskjell mellom behandlingene. Plommesekk/perikardieødem ble vurdert fra 0 til 2 avhengig av alvorlighetsgrad, hvor den milde ødemdeformiteten var til stede i alle behandlingene, >50 % i papainbehandlingene med en signifikant forskjell. Alcalase var den eneste behandlingen som forårsaket brudd-deformiteten >22%. Bruken av papain fra papaya lateks har vist seg å være effektiv for å løsne eggene fra underlaget uten noen signifikant effekt på klekkehastigheten, men med forekomst av mild ødemdeformitet. Imidlertid er disse avgumming-protokollene in situ, og oppnåelse av avgumming av berggylt-egg i kommersiell skala må fortsatt standardiseres.

Erklæring om relevans: Forbedring av kommersiell produksjon av berggylt (Labrus bergylta) ved å optimalisere klekkefasen.

Nøkkelord: Klebrige egg, Enzymatiske behandlinger, Eliminering av eggs klebrighet, Rensefisk, Klekking, Misdannelser.

Contents

List of figures

List of tables

1. Introduction

One of the biggest issues in salmonid production are sea lice infestations. Salmon lice (*Lepeophtheirus salmonis*) are caligid copepods naturally occurring in the northern hemisphere. This marine parasite feeds on the salmon's mucus, blood, and skin (Mordue and Birkett, 2009). The infestation rate could vary from low to high, resulting in skin erosions, physical damage, osmoregulation failures, immunosuppression, secondary infections, and chronic stress (Hamre et al., 2009). Lice infestation damages the epidermis layer of salmon, making it more prone to other diseases where severe accretion of infections could result in mortality (Hjeltnes et al., 2018). An aquaculture site favours high numbers of salmon to feast on and, the lice is also considered a threat to the wild salmon population due to high infestation of lice on the migrating smolts from rivers (Grefsrud et al., 2019, Torrissen et al., 2013, Vollset et al., 2016).

To accomplish sustainable growth in the salmon aquaculture division, a “traffic light system” was initiated by the Norwegian government on 30th October 2017 (Myksvoll et al., 2018). The concept connects the sea lice pressure to the growth of the fish farming sector. This implies that the sea lice effect on native salmon population mortality acts as an indicator regarding possible production growth. A green light means that in regions of less than 10% mortality of native salmon smolt population caused by sea lice gets a green signal for expanding production by 6%. A yellow light is set when the triggered mortality is between 10-30%. A yellow signal means constant production. If a region is set to red light, the production should be lowered, as the triggered mortality reaches more than 30%. However, this implication does not apply until the subsequent periodic evaluation (Olaussen, 2018).

1.1 Biological control

A biological control refers to the exploitation of living agents to combat pestilential organisms (e.g. pathogens) for various reasons to support human benefits (Stenberg et al., 2021). Currently, to remove the sea lice from salmonids, four species of wrasse from the Labridae family and lumpfish (*Cyclopterus lumpus*) are utilized in aquaculture. The wrasse species include Ballan wrasse (*Labrus bergylta*), Rock cook (*Centrolabrus exoletus*), Corkwing wrasse (*Symphodus*

melops), and Goldsinny wrasse (*Ctenolabrus rupestris*). These carnivore wrasses are opportunistic and inhabit shallow rocky and algal habitats in the coastal region of northern Europe (Halvorsen et al., 2017) and are mainly caught by traps in shallow waters (Blanco Gonzalez and de Boer, 2017). On the other hand, the lumpfish are opportunistic omnivores and spend their early stages in coastal habitats either floating or attached to algae and after one year of hatching they are found in pelagic habitats (Ingólfsson et al., 2002).

The benefits of cleaner fish (wrasses) over other methods include (1) co-existence with salmon (2) non-toxic to the environment (3) continuous feeding on sea lice even at low densities (4) sea lice cannot develop resistance against cleaner fish (Deady et al., 1995, Groner et al., 2013, Treasurer, 2012).

1.2 Ballan wrasse as a cleaner fish

Ballan wrasse is one of the largest wrasse species found in northern Europe and is known as bergylt in Norwegian (Costello, 1991, Quignard et al., 1986). It can live up to 29 years and grow up to 60 cm in total length (Darwall et al., 1992; Dipper et al., 1977). Ballan wrasse is a protogynous hermaphrodite, that can change when sex when they attain a certain size (Muncaster et al., 2013). Plus, they form harems in localized areas where they spawn demersal, sticky, spherical (0.7-1.15 mm in diameter) eggs in the algae nest (D'Arcy et al., 2012, Darwall et al., 1992, Fives, 1976). Ballan wrasse is the fastest-growing wrasse and third most abundant in the Norwegian waters, with a label of being the most effective cleaner fish due to its robust adaptability (Espeland, 2010). However, wrasse displays winter inactivity and do not feed below 6.0°C (Kelly et al., 2014), limiting their deployment in the colder region and winter seasons (Treasurer, 2013). The Ballan wrasse is considered sufficient for efficient salmon delousing with only 2-5 % (number of individuals to number of farmed fish per sea cage), while the other wrasse species are required in a larger number (Kvenseth et al., 2003). Moreover, Ballan wrasse prefers feeding on adult sea lice, which controls the next generation's population (Kvenseth et al., 2003). In addition, Ballan wrasse eats lice from larger salmon (3-6 kg) (Kvenseth et al., 1996), which led to the initiatives of dedicated breeding programs for the species (Ottesen et al., 2011). Rapid growth towards a sustainable future has shifted the focus from wild capture to farmed Ballan

wrasse (Fig.1). In 2020, the farmed Ballan wrasse exceeded the wild-caught in numbers (Norwegian Directorate of Fisheries, 2020).

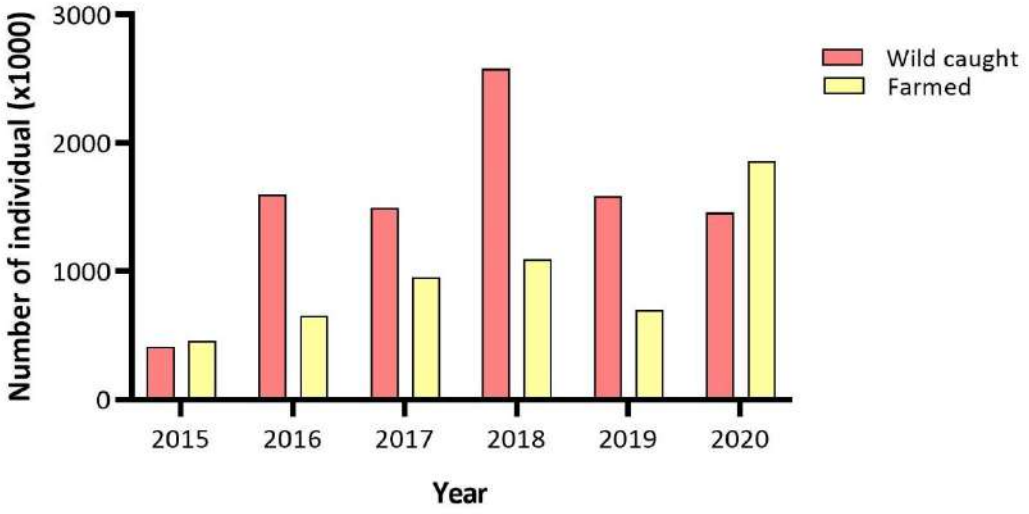


Figure 1: Number of Ballan wrasse deployed in the year 2015-2020 (Norwegian Directorate of Fisheries, 2020)

1.3 State of Art: Ballan wrasse aquaculture techniques

Commercial hatcheries for Ballan wrasse are currently dependent on the natural spawning of wild-caught broodstock, where fish are maintained in controlled photo-thermal conditions. A standard setup is to hold wild-caught broodstock in large communal tanks with low stock densities (5-10 kg m^{-3}) for most of the year. The tanks are occupied with so-called "furniture", for example, artificial kelp, plastic pipes, etc., in which the wrasse will actively seek shelter. Before the spawning period, the wild-caught broodstock will be individually examined and placed into spawning tanks (7-10 m^3 volume tanks) in smaller groups of 20-30 individuals. Gender distribution is around 10-20% male. The area near tank wall is kept open (30-50% of tank surface), providing a spawning ground within the tank (Fig.2) (Treasurer, 2018).

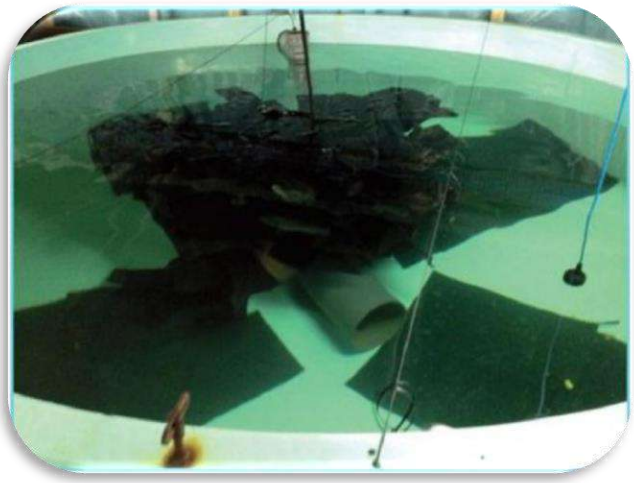


Figure 2: A usual wild-caught broodstock tanks setup for natural spawning of Ballan wrasse. The artificial kelp and pipe are for refuge, whereas the square-shaped carpets (0.28m^2 area per carpet) act as spawning substrates. (Treasurer, 2018)

Similar behaviour to wild habitats is observed in these artificial tanks, where a dominant male pays regular visits to females as a sign of interest in mating. Just before the copulation, the female lay down on her side, quivering caudal and pectoral fins afterwards, a male aligns, eggs and milt are discharged on the chosen spawning site. The spawning season under controlled photo-thermal conditions is stretched over two to three months, generally early April, and end of June, with the utmost spawning activity recorded in May. In a single spawning season, there are four to six spawning periods which will last on average of 13-15 days (Grant et al., 2016a). The substrates (carpets) containing eggs are taken out of the spawning tanks and inspected for egg quantity and viability. The carpets/mats are then transferred to the incubation tanks, where the mats are disinfected daily with bronopol as Pyceze® (Novartis)(Treasurer, 2018). Due to the stickiness of the eggs, disinfection is more difficult compared to non-sticky pelagic eggs such as cod. Survival of larvae and hatchability of the eggs improves with better disinfection routines (Bone et al., 2021, Treasurer, 2018), as it prevents the bacterial and fungal loads in the incubation tanks.

1.4 Eggs stickiness: Challenges and potential remedies.

Ballan wrasse spawn eggs that stick together after getting in contact with seawater (Helland et al., 2014). The egg stickiness is not desirable in the hatcheries as it possesses many challenges

during the incubation phase of the Ballan wrasse eggs which lead to low egg survival and high mortality. Some of the challenges are dirt clinging to the adhesive layer, ineffective disinfection, biosecurity risk for vertical transmittance of pathogens from wild-caught broodstock to incubation systems and the possibility of non-optimal aeration during incubation (Grant at al., 2016b, Helland et al., 2014). A demo of dirt clinging to some sticky eggs after being scraped off from the spawning mats can be observed in Figure.3.

Figure 3: Ballan wrasse eggs scrapped off the spawning mats with an intact gum layer. Credits: Fahad Shabbir

A common practice in other commercially farmed teleost species that spawn adhesive eggs is to remove the adhesive layer from eggs before incubation (Linhart et al., 2003a). If a technique could be developed for eliminating the egg's stickiness in Ballan wrasse eggs before incubation, this will allow the hatcheries to adopt a more traditional upwelling incubation system as used for other marine species. Moreover, other benefits such as improved aeration, reduced risk of fungal diseases, more effective disinfection, and less effort associated with egg count and husbandry (Ringle et al., 1992). A similar approach to eradicate the gum layer before incubation has been practised for commercially farmed Common Carp (*Cyprinus carpio* L.) (Thai and Ngo, 2004). Developing such a method to eliminate the stickiness of the Ballan wrasse eggs will be a breakpoint that could positively impact the cleaner fish farming industry.

The gelatinous layer responsible for the egg adhesion is made of various proteins (Mansour et al., 2008) and the application of serine protease could potentially break down the protein layer surrounding the eggs chorion making them non-sticky. Various techniques to eliminate the stickiness of naturally spawned eggs have been developed. For example, using trypsin to get eggs un-sticky in Siluroidei (*Triplophysa siluroides*) (Legendre et al., 1996) and wels catfish (*Silurus glanis*) (Linhart et al., 2002). Moreover, trypsin at 6 U ml^{-1} concentration eliminated the eggs stickiness in Persian sturgeon in 11 minutes with 89.6% of survival rate (Neitali et al., 2014). On the other hand, papain is another degumming agent with a degree of success in eliminating egg stickiness for channel catfish (Isaac and Fries, 1992, Ringle et al., 1992). Moreover, FDA (U.S. Food and Drug Administration) included papain (0.2% solution) in LRP drugs (Low regulatory priority) due to its ability to eliminate the gelatinous layer of egg masses for better hatchability and reduced transferability of diseases (Treves-Brown, 2000), for example in Tilapia (*Oreochromis niloticus*), 0.15 micromole papain dosage led to 73.33% hatching success (Fani and Sri, 2019).

The raw pineapple juice contains many proteolytic enzymes, mainly bromelain enzymes, which can digest protein (Dickson and Bickerstaff, 1991). This feature has been applied successfully in eliminating the adhesive egg layer in common carp (*Cyprinus carpio* L.) with a fertilization rate of 89.3% ± 0.7 and hatching rate of 86.6% ± 1.4, by using 1% of pineapple juice (Thai and Ngo, 2004). Also, mudfish (*Heterobranchus bidorsalis*) eggs were degummed in only 3 minutes using 1% pineapple juice with a hatching rate of 88% (Paterson and Nwachi, 2014). Furthermore, alcalase is also known for the elimination of eggs stickiness, in common carp (*Cyprinus carpio* L., 2–20 $ml \cdot L^{-1}$, 8–20 min post-fertilization) (Linhart et al., 2003a; Linhart et al., 2003b) and European catfish (*Silurus glanis* L., 20 $ml \cdot L^{-1}$, 3 min post-fertilization) (Linhart et al., 2003a), ss well as in tench (*Tinca tinca* L.), a dose of 10–20 $ml \cdot L^{-1}$ (1.0–2.0%) removed egg stickiness with improved hatching rates and reduced egg handling time (Gela et al., 2003, Linhart et al., 2000, Linhart et al., 2003a; Linhart et al., 2003b). Further, Helland et al. (2014) confirmed no development of adhesive layer in Ballan wrasse eggs when treated with enzyme alcalase and replacing the ovarian fluid with ringer solution before fertilization. Also, exposure to 1-2% of alcalase on fertilized Ballan wrasse eggs for 20 minutes achieved 100% degumming without compromising the hatching rate (Grant et al., 2016b). Another serine endoproteinase is proteinase K, it is also

known for its broad cleavage specificity that can break down the protein into very short peptides. For example, in the removal of the outer membrane protein of bacterium (*Staphylococcus aureus*) (Khan et al., 2016), or to digest the plasma membrane-associated proteins (Stoorvogel et al., 1989).

The addition of EDTA (Ethylenediaminetetraacetic acid) as cofactors could influence the enzymatic activity in a solution, as it binds strongly to metal ions such as Mn (II), Cu (II), Fe (III), and Co (III) (Mohammadi et al., 2013). A study conducted by Lönning et al. (1984) revealed that the presence of calcium and/or magnesium is necessary for hardening the chorion in Cod (*Gadus morhua*) and lumpsucker (*Cyclopterus lumpus*). Thereby an addition of EDTA and protease enzyme could potentially aid in removing the adhesive layer of the eggs.

2. Aim of the study

The sustainable and prosperous cultivation of Ballan wrasse (*Labrus bergylta*) needs to produce good quality fish with a high survival and growth rate. The current rearing technique of Ballan wrasse poses many challenges due to egg adhesion. With the interest of sustainable growth, this study will explore the effectiveness of various enzymatic treatments (Trypsin, Papain, Proteinase K, Pineapple, and Alcalase) to eliminate the stickiness of the Ballan wrasse eggs. The study was divided into two parts: screening and the main experiment. The screening was to test the enzymatic treatments at varying concentrations and exposure times with and without EDTA and pH adjustments. In which, alcalase treatment was assigned as a positive control with the fixed concentration of 2%, also as at this concentration Grant et al. (2016b) obtained the best degumming and hatching results. Negative control was also introduced in correspondence to the highest concentration 16% of the pineapple treatment as it dropped the pH to 3.3. Thereby, quantifying the effect of acidic environment on degumming and larval morphology. The morphological measurements of larvae were set as an indicator of larvae quality. An optimized main experiment was carried out in terms of dose rate, time, and pH. All treatments, whether successful or not in eliminating egg stickiness, were followed to observe the effect of enzymatic treatments on larvae's hatch rate, quality, and occurrence of deformities.

2.1 Objectives:

- To find a possible degumming method without compromising the hatching rate and larval quality.
- Analyze the post-treatment effect of alcalase on larval deformation and hatching rate.

3. Materials and methods

This study consisted of two phases: Screenings from (17-11-2020) to (20-11-2020) and the main experiment from (26-11-2020) to (8-12-2020).

3.1 Management and collection of eggs

This experiment was conducted in SeaLab, NTNU's Centre of Fisheries and Aquaculture, in Trondheim, Norway. The whole investigation was conducted at a controlled room temperature of 10 ± 0.5 ℃. The eggs were delivered by MOWI hatchery in two batches. The first batch was supposed to last the whole experiment (screening and main experiment), but due to its bad quality, meaning lots of dead eggs, second batch was ordered for the main experiment (Table.1). The eggs were received on mats in plastic bags filled with seawater in a sealed Styrofoam box. Upon receiving the mats, the ambient water temperature and oxygen level were measured (Table 1) using a handheld digital water quality meter (ProSolo DIGITAL (20D102055) and probe ODO Cond/Temp (19C101381), YSI, OH 45387 USA). The naturally spawned eggs on two mats were then hung vertically in each tank (Fig.4). The circular tanks (200 L) were flow-through systems with 10 L/min water flow. The intake water in tanks was pumped in from Trondheim fjord at 70 m sea level depth and sand filtered. The temperature of the tanks was set to 9.5 ^{o}C ± 0.5 with low aeration (20%). The abundance and homogeneous spread of naturally spawned eggs on the mats were visually inspected.

Table 1: Temperature and oxygen measurement for egg batches received.

Receiving date	Batch	Temperature ^{o}C	Dissolved oxygen
17-11-2020	1st	12.8	92.4%, 9.78 mg/l
26-11-2020	2nd	12.3	95.4%, 10.36 mg/l

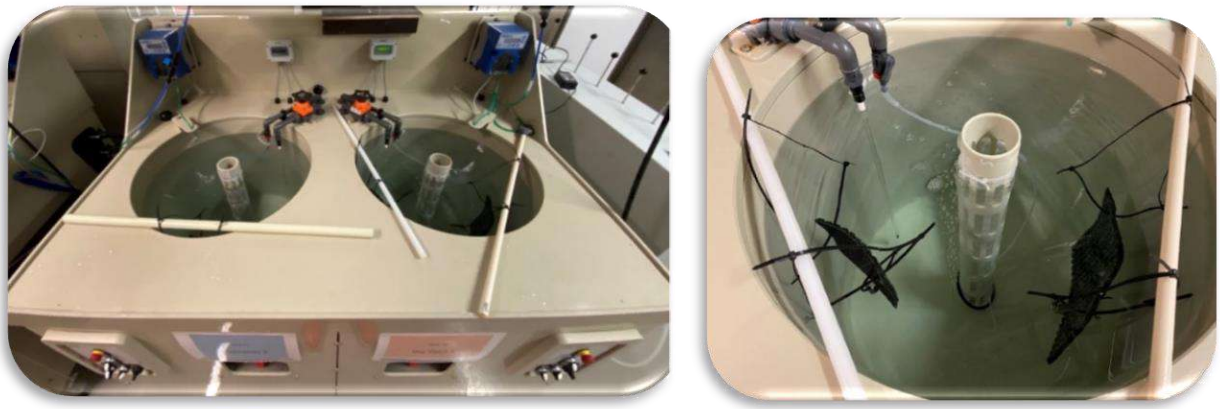


Figure 4: Naturally spawned eggs on mats hanging underwater in a flow-through system.

3.2 Solution preparation

All the enzymes were purchased from Sigma-Aldrich, except pineapple juice and EDTA. SINTEF supplied EDTA (10% solution) while the pineapple juice was prepared in the lab. Ripe pineapples were brought from REMA 1000, peeled, chopped into smaller pieces, and then blended for 3-5 minutes. The juice was then stored in the fridge overnight to settle down. The upper layer of fibreless pineapple juice was then extracted using a 50ml syringe, centrifuged for 10 min at 4000rpm and 4^{o}C (Megafuge™ 16R (75004271), Thermo Scientific™, Germany). The collected supernatant was then used for making solutions. The water used to prepare all the samples was from the same source that of the facility which was sand filtered along with 0.22µm filtration. The screening test had no replication, so the solutions were made in a 600ml beaker to a final volume of 300ml (Table.2). But for the main experiment, each treatment had four replicates, a large volume of 1200ml solution were made to avoid variation among the replicates and then transferred to a 600ml beaker to a final volume of 300ml (Table.3).

Each solution was prepared one day prior to use and stored in the fridge before testing; however, they were acclimated to room temperature (10 ^{o}C) before use. The formula below was used to achieve the desired concentrations of the enzymes (Appendix.1),

$$\mathbf{M_1V_1 = M_2V_2}$$

Where M_1= Stock concentration

V_1=Volume from stock

M_2= Final concentration

V_2= Final solution volume

3.3 Treatments efficacy screening

Both the screening and the main experiment followed similar experimental procedures and setup elaborated in (3.3.1). Further explanation of enzyme concentration, duration of exposure and pH for screening and main experiment is mentioned in Tables 2 and 3. The purpose of the setup (3.3.1) was to collect and photograph the eggs that detach from the mats upon exposure to the enzymatic solution to quantify the percentage of the eggs detaching and classify the gum layer status of detached eggs.

3.3.1 Standardize experimental design

The mat was taken out of the circular tank (Fig.4) into a deep rectangular tub prefilled with seawater from the same source as the tanks. Then, using scissors, 12 pieces of the mat of ca 5x2.5cm dimension were cut and labelled (Fig.5). Each mat piece was then taken out from the tub into a deep petri dish prefilled with seawater to photograph the mat piece using iPhone 11 Pro max under LED ring light (MEB123, Leica Microsystems) for egg count and then returned. The eggs on the mats were counted before and after the treatment (Appendix.2). The water in the petri dish was changed frequently to achieve optimal conditions for maintaining egg quality.

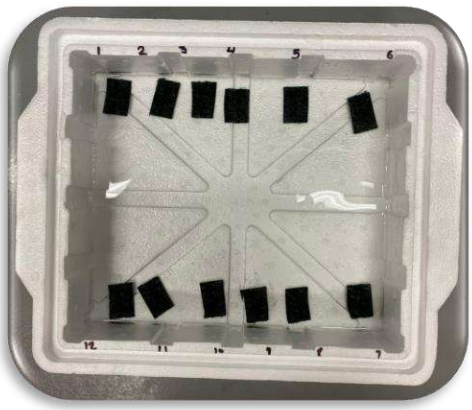


Figure 5: Pieces of spawning mats with eggs cut to ca 5x2.5cm.

The enzymatic solutions were then poured into labelled 600ml beakers filling up to 300ml (Fig.6). Each mat piece was then attached to the flocculator arm (JLT6, F105A0109, VELP Scientifica, ITALY) using a clamp (Fig.6) and dipped into the different enzymatic solutions (Fig.6). The flocculator arm was set to rotate at 50 rpm for the desired time (30 min or 1hr), and upon time termination, a movement in an up-down direction was applied to the flocculator arm. This movement was applied because the detached adhesive layer of eggs post-treatment remains in the solution and attached to the mats, and the mat's fragments act as a trap for the eggs even after the separation of the eggs from the gum layer. The fallen eggs from the mats were then poured into a micro sieve (corning® cell strainer 100 µm, REF 431752), in addition, seawater was poured 2-3 times into the micro sieve to wash the enzyme off the eggs.

Figure 6: Mat piece ca 5x2.5 cm dipped into enzymatic solutions attached with a clamp to the flocculator arm.

The washed eggs were then placed in deep Petri dishes containing prefilled seawater, where images of detached eggs were captured by a CMOS camera (MC170HD, Leica Microsystems) attached to a macroscope (Z16 APO with 0.5x PlanAPO objective, Leica Microsystems) using a remote (Fig.7). A glass stage micrometer (Leica Microsystems) was also imaged at all magnification to calibrate with the measurements captured at any magnification. Consistent illuminations were provided by attaching a LED ring light (MEB123, Leica Microsystems) to the front objective of the lens, giving a shadow-free illumination.

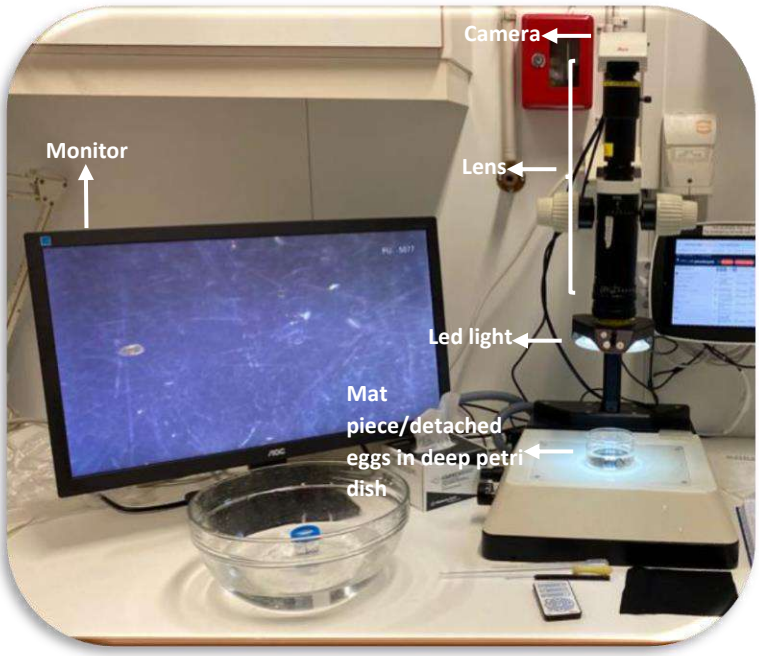


Figure 7: Setup for capturing the images of the eggs attached or detached from the mats.

The attached eggs on the mats or remaining mat pieces on the flocculator arm were removed and placed into 96% ethanol for egg count. This step made the eggs appear more visible, increasing the feasibility of counting the eggs attached to the mat pieces. The pH of the treatment solutions was measured using a pH meter (Sension+ PH31 451155, HACH, Spain) along with the daily measurement of the tank's water temperature using a thermometer (VWR® Stem Thermometer (98000-156), VWR Life Science, China). The images of the fallen or gently scraped eggs by effective and non-effective treatments respectively were analysed using image processing software ImageJ (Java 1.8.0_172(64-bit), Wayne Rasband and contributors National Institutes of Health, USA). The status of the adhesive layer of the eggs post-treatment was classified into four categories (Fig.8) (Grant et al., 2016b)

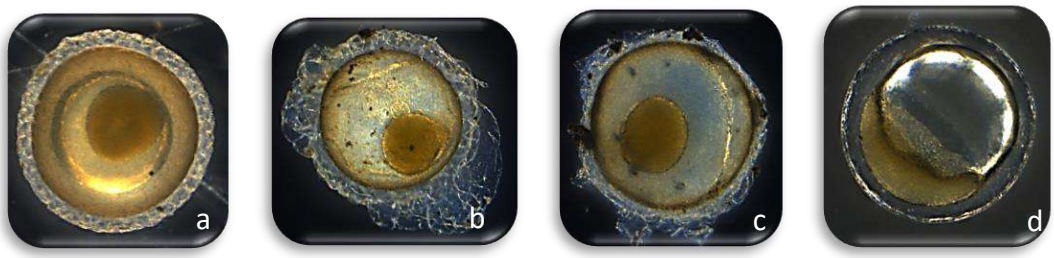


Figure 8: Gum layer classified based on the scoring criteria (Grant et al., 2016b). (a) Not degummed (gum layer fully intact), (b) Swelled (gum layer started to swell and break down), (c) partly degummed (gum layer swollen and partially removed from the egg), (d) degummed (gum layer fully detached- clean egg).

3.3.2 Screenings

For the first screening, the setup was tested to check the feasibility of the experimental design and draw some conclusions to optimize the time and concentration to achieve degumming of the eggs. The test execution followed the steps mentioned in section 3.3.1 with the collection of detached eggs from the mats at 30min and 1hr for imaging and counting. Each enzyme was tested with and without EDTA with zero replicates (Table.2). Enzyme alcalase was selected as the positive control because of its proven effects on degumming on Ballan wrasse eggs (Grant et al., 2016b), while the rest of the enzymes were tested for the first time to eliminate the egg stickiness on Ballan wrasse eggs.

Following the first screening, some amendments were performed in the second screening for further testing the enzymes for their efficacy in degumming the eggs (Table.2). Firstly, the concentration of each enzyme was doubled to observe the effect of increased concentration in the elimination of eggs stickiness. Alcalase concentration was set to 2% to achieve consistency by the positive control. Secondly, the pH was adjusted to be around 7.5-8.0 (using 2M-NaoH and 3M-HCL), as the addition of EDTA dropped the pH level which could affect the activity of the enzyme (Sarker et al., 2013). Moreover, the pH level 7.5-8.0 corresponds to the pH of seawater (approximately 8.0) (Marion et al., 2011), where Ballan wrasse eggs are naturally found. The test execution followed the steps mentioned in section 3.3.1 with the collection of detached eggs from the mats at 30min and 1hr for imaging and counting. Each enzyme was tested with and without EDTA with no replication (Table.2).

Table 2: Solution prepared for the screening trails with no replication.

Treatments		First screening			Second screening			Exposure time
		Concentration	pH		Concentration	pH (adjusted)		
			- EDTA	+ EDTA		- EDTA	+ EDTA	
Alcalase® Enzyme, *Bacillus licheniformis*	± 5mM EDTA	2%	6.8	4.9	2%	7.5	7.6	30 min & 1 hr
Trypsin, bovine pancreas	± 5mM EDTA	20 U/ml	7.5	4.9	40 U/ml	7.5	7.8	
Pineapple juice	± 5mM EDTA	1%	7.4	4.9	8%	7.6	7.8	
		4%	5.2	4.6				
Papain, *Carica papaya*	± 5mM EDTA	2 U/ml	7.7	5.0	4 U/ml	7.6	7.5	
Proteinase K, *Tritirachium album*	± 5mM EDTA	-	-	-	700 µAnsonU/ml	7.9	7.8	
Control (Seawater)	± 5mM EDTA	-	7.9	5.1	-	7.7	7.5	

3.4 Main experiment

Critical assessments from the screening trials lead to further testing of the enzymes in terms of concentrations (Table.3). Screening tests revealed that EDTA did not have any significant role in aiding the detachment of the eggs from the mats and therefore was ejected from further testing. The ejection of EDTA also led to no decrease in the pH level and therefore no adjustments were made to the pH levels, further supporting the fact that the finest function of an enzyme is at its optimal pH (Robinson, 2015). In addition, the readily available papain from papaya latex (latex is thixotropic fluid with a milky appearance (Macalood et al., 2013)) was used instead of papain from *Carica papaya*, due to the time restriction in ordering a new papain enzyme from sigma Aldrich. An additional treatment (acidified seawater) was introduced as a negative control due to the drastic drop of pH with the highest concentration (16%) of pineapple juice (Table.3). Additionally, for unsuccessful treatments, the eggs were gently scraped off the mats using a glass

pipette to further quantify the effects of enzymes on the gum layer, hatch rate and larval quality. The test execution followed the steps mentioned in section 3.3.1 with the collection of detached eggs from the mats at 30min for imaging and counting. Each enzyme was tested with four replicates (Table.2).

Furthermore, the papain solution at the desired concentrations became highly opaque, as it had high amounts of insoluble materials (Appendix.3). In order not to clog the micro sieve during the separation of detached eggs from the solution. The papain solution was diluted six times along with the frequent cleaning of the micro sieve. Also, the opacity of the papain solution 50U/ml led to the wastage of two replicates, as the eggs were not visible in the solution and the micro sieve got clogged.

Table 3: Solution prepared for the main experiment with four replicates for each treatment.

Treatments	Concentration	pH	Exposure time
Alcalase® Enzyme, *Bacillus licheniformis*	2%	7.13	30 min
Trypsin, Bovine pancreas	2000 U/ml	7.8	
	200 U/ml	7.97	
	20 U/ml	7.96	
Papain, Papaya latex	50 U/ml	6.33	
	10 U/ml	7.00	
	5 U/ml	7.10	
Pineapple juice (Bromelain)	16%	3.32	
	8%	3.46	
	4%	3.75	
Proteinase K, *Tritirachium album*	900 µAu/ml	8.04	
	700 µAu/ml	8.04	
	400 µAu/ml	8.03	
Acidic seawater	pH 3.38	3.38	
Control (Seawater)	-	7.95	

3.4.1 Incubation

A custom-made flow-through incubation system (AUER, Germany) was set up with an approximate volume of 70 litres with a water temperature 9.5 ^{o}C ± 0.1 (AUER, Germany) (Fig.9) to incubate the detached eggs. The incubation system consisted of a sealed plastic plate with 112 openings to fit the incubation tubes (50ml - with 200µm mesh on both sides). There were two

water inlets at the bottom (1.8 L/min each) of the incubation system pushing the water through the incubation tubes providing a consistent water flow throughout the system. The consistent water flow eliminated the factor of randomisation in the placement of the incubation tubes (Table.4). Plus, this approach was opted to avoid any unnecessary confusion or error during the experiment. The detached eggs post-treatment was then transferred to the labelled incubation tubes using a glass pipette and followed until hatching.

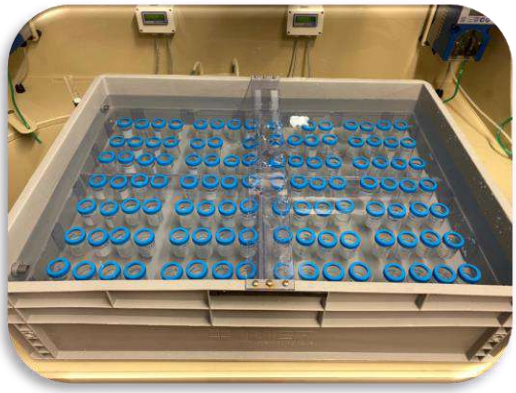


Figure 9: Incubation setup for following up the eggs until hatching post-treatment. The eggs were placed in the incubation tubes with mesh (200µm) on both sides for optimal water supply.

Table 4: Incubation tubes setup in the flow-through incubation system. (Box details- treatment name, their concentration and replicate number)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
H	**Alcalase** (2%) R1	**Alcalase** (2%) R2	**Alcalase** (2%) R3	**Alcalase** (2%) R4	**Trypsin** (2000U/ml) R1	**Trypsin** (2000U/ml) R2	**Trypsin** (2000U/ml) R3	**Trypsin** (2000U/ml) R4	**Trypsin** (200U/ml) R1	**Trypsin** (200U/ml) R2	**Trypsin** (200U/ml) R3	**Trypsin** (200U/ml) R4	**Trypsin** (20U/ml) R1	**Trypsin** (20U/ml) R2	**Trypsin** (20U/ml) R3	**Trypsin** (20U/ml) R4
I	**Papain** (50U/ml) R1	**Papain** (50U/ml) R2	**Papain** (50U/ml) R3	**Papain** (50U/ml) R4	**Papain** (10U/ml) R1	**Papain** (10U/ml) R2	**Papain** (10U/ml) R3	**Papain** (10U/ml) R4	**Papain** (5U/ml) R1	**Papain** (5U/ml) R2	**Papain** (5U/ml) R3	**Papain** (5U/ml) R4	**Proteinase K** (900U/ml) R1	**Proteinase K** (900U/ml) R2	**Proteinase K** (900U/ml) R3	**Proteinase K** (900U/ml) R4
J	**Proteinase K** (700U/ml) R1	**Proteinase K** (700U/ml) R2	**Proteinase K** (700U/ml) R3	**Proteinase K** (700U/ml) R4	**Proteinase K** (400U/ml) R1	**Proteinase K** (400U/ml) R2	**Proteinase K** (400U/ml) R3	**Proteinase K** (400U/ml) R4	**Pineapple** (16%) R1	**Pineapple** (16%) R2	**Pineapple** (16%) R3	**Pineapple** (16%) R4	**Pineapple** (8%) R1	**Pineapple** (8%) R2	**Pineapple** (8%) R3	**Pineapple** (8%) R4
K	**Pineapple** (4%) R1	**Pineapple** (4%) R2	**Pineapple** (4%) R3	**Pineapple** (4%) R4	**Control** (Acidic) R1	**Control** (Acidic) R2	**Control** (Acidic) R3	**Control** (Acidic) R4	**Control** (Seawater) R1	**Control** (Seawater) R2	**Control** (Seawater) R3	**Control** (Seawater) R4				
L																
M																
N																

3.4.2 Follow up and hatching percentage

Each incubation tube was monitored daily until 100% of eggs hatched (12days/114 degree days). In sequential order, each incubation tube was carried in a big bowl prefilled with seawater from the same source as the incubation system to the imaging setup. The incubation tubes were then inspected for a hatchling, if found then the hatchlings were transferred to the designated glass containers prefilled with seawater from the same source as the incubation system placed in a controlled room temperature 10 ^{o}C ± 0.5 (Fig.10). Otherwise, the unhatched eggs were placed into a deep petri dish and imaged using the imaging setup as described in the standardized experimental setup (3.3.1) and returned to the incubation centre (Fig.9). The formula mentioned below was used to calculate the hatch rate.

Hatching success = (Number of larvae hatched/Number of eggs incubated) *100

Figure 10: Designated containers for placing the hatched larvae prefilled with seawater at a controlled room temperature of 10 ^{o}C.

3.4.3 Measurements of larvae morphology

Measurements of larval morphology were undertaken upon reaching 100% hatching of the incubated eggs at 0dph (days post-hatch). The measurements recorded were to quantify the larval quality in regard to eye diameter, myotome height, total length, standard length, total area and body area (Fig. 11). Single larva from the designated container was taken out using a glass pipette and placed onto the gel (3% methylcellulose solution) to limit the larva's mobility and ideal photography (Cocchiaro and Rawls, 2013). The captured larvae images were then processed using the same setup as described in the standardized experimental setup (3.3.1). For the eye

diameter, a mean of three measurements was recorded, and the myotome height was measured perpendicular to the axial skeleton, right behind the anus (Fig.11).

For eye diameter: πr^2

Eye to Body ratio: Eye area/body area

Fin fold area: 100-((Body area/total area) *100)

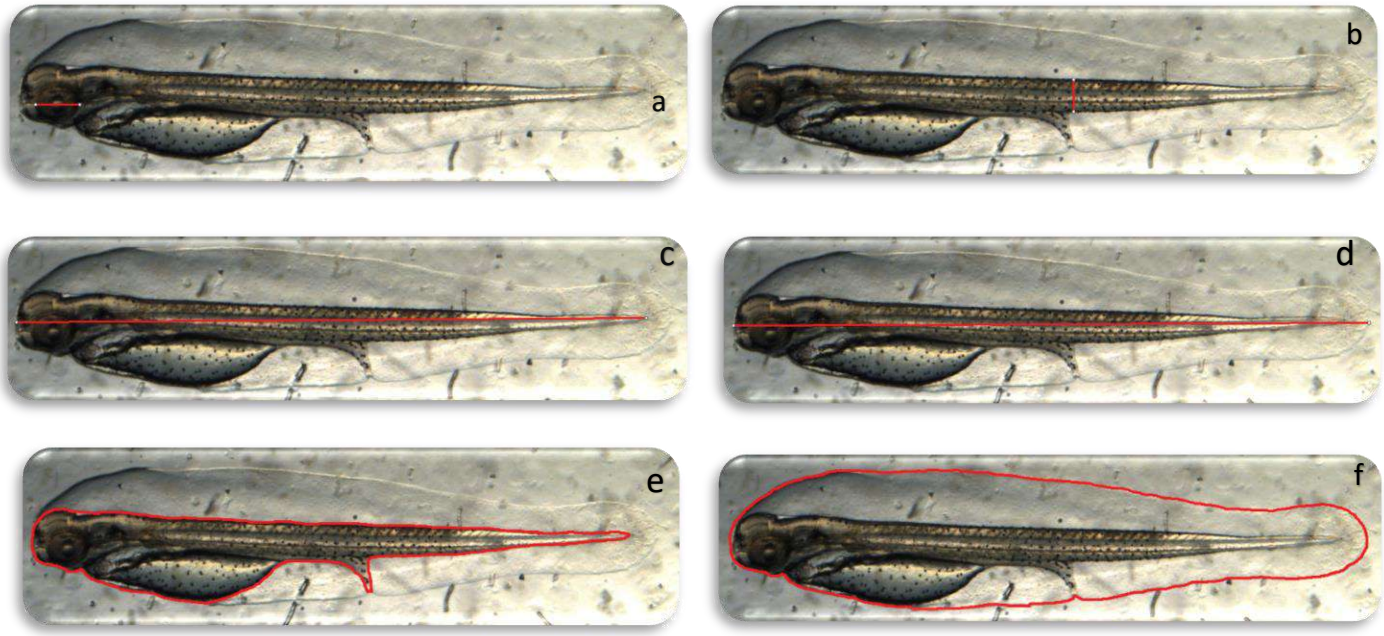


Figure 11: Measurements captured using ImageJ (a) Eye diameter (b) Myotome height (perpendicular to the axial skeleton, right behind the anus) (c) Standard length (distance from the tip of the snout to the end of notochord) (d) Total length (distance from the tip of the snout to the caudal–fin end), (e) Body area (outlined by red line), (d) Total area (outlined by red line).

3.4.4 Deformities

The deformities were also analysed from the larvae images captured in the previous section (3.4.3). The larval deformities were mainly divided into two: the spinal deformities including tail flexures, curved spines and breaks, and the occurrence of edema (abnormal swelling of yolk sac) (Fig.12) (Quilang et al., 2009, Chao et al., 2017). The edema was further given a rating depending upon its severity: (0) none, (1) mild (yolk sac pushed either back or forth), (2) severe.

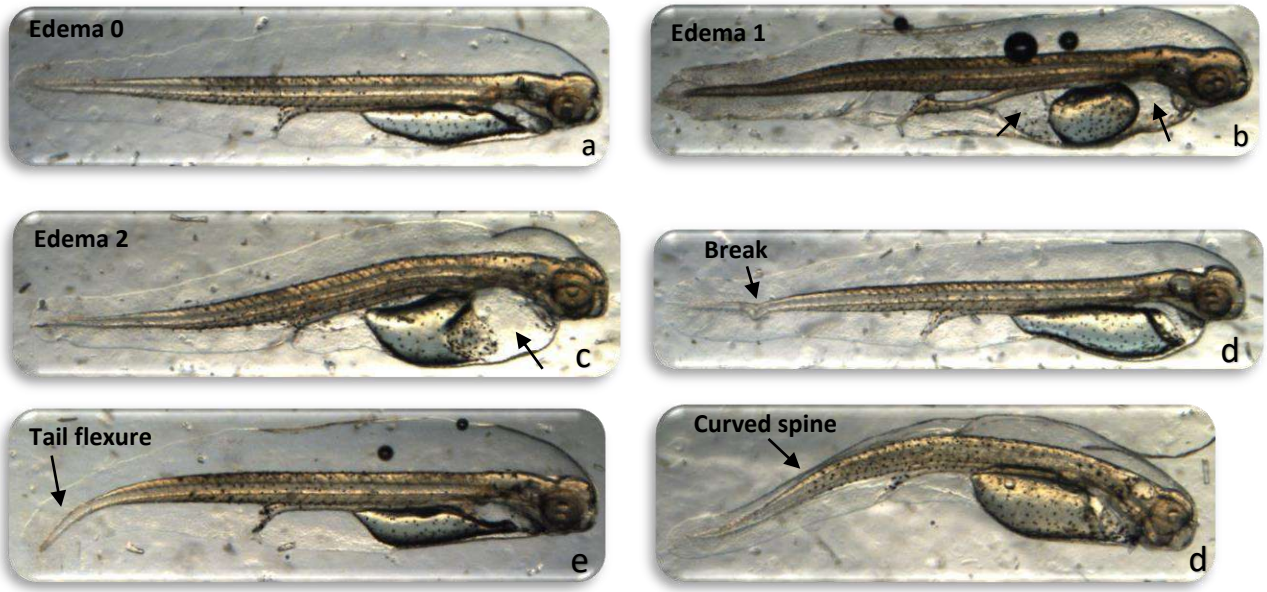


Figure 12: Larval deformities divided into two categories; edema (a) 0- none, (b) 1- mild (yolk sac either push forward or backwards or both), (c) 2 severe (excess swelling), and Malformation of the spine (d) breaks in spine, (e) tail flexures, (f) curved spine.

3.5 Statistical analysis

Preliminary data were processed using Microsoft Excel (2019 version), while all the statistical analyses of the data along with its graphical representation was performed in GraphPad Prism (Version 9.2.0 (GraphPad Software, Inc).

All the data sets (% detached eggs, gum layer classification, hatching success, larval measurements, and deformities) were checked for normality using the Shapiro-Wilk test

(GraphPad), and arc-sine transformation of the percentage data (Excel) was carried out before statistical analysis. For the screening tests, there was no statistical analysis performed due to no replication (n=1) and a low number of fallen eggs for ineffective treatments. For the main experiment, if the data did pass the normality test, a one-way analysis of variance (ANOVA) ($P \leq 0.05$) was performed, followed by Tukey's multiple comparisons test ($P \leq 0.05$). And if the data did not pass the normality test, a non-parametric Kruskal-Wallis test and post hoc analysis was carried out using Dunn's multiple comparisons test ($P \leq 0.05$). Means with a different letter in the superscripts indicate the significant differences between treatments.

4. Results

4.1 Screening

4.1.1 Detached eggs from the mats

For the first screening, during the preliminary investigation before the test it was observed that most of the eggs were dead on the mats, but the adhesive mechanism was still functional. Also, no statistical tests were performed on the screening data due to no replication. Exposure to enzymatic treatments revealed that enzyme alcalase was the only treatment effective in detaching the eggs from the mat with 52% after 30 minutes of exposure and 71% after 1 hour of exposure (Fig. 13). The addition of EDTA in enzyme alcalase acted as an inhibitor reducing the detached eggs to 14% for 30 minutes and 20% for 1 hour of exposure. The inhibition of the enzyme alcalase could be due to the drop in the pH with the addition of EDTA (Table.2). All the other treatments had <15% of the eggs detached by the enzymatic exposure. Trypsin treatment was the most ineffective treatment detaching almost no eggs from the mats. However, for pineapple treatment, some potency of degumming was observed, where 4% pineapple treatment with EDTA liberated 14% of eggs from the mat, followed by 1% pineapple + EDTA treatment (13%) and 4% pineapple treatment (12%) (Fig.13) For the pineapple treatment addition of EDTA did not act as an inhibitor but with a minor aid in detaching the eggs for 1% pineapple treatment, while for pineapple 4% no such difference was observed. Moreover, the

pineapple enzyme is acidic in nature, thereby no negative effects of pH drop by addition of EDTA were observed.

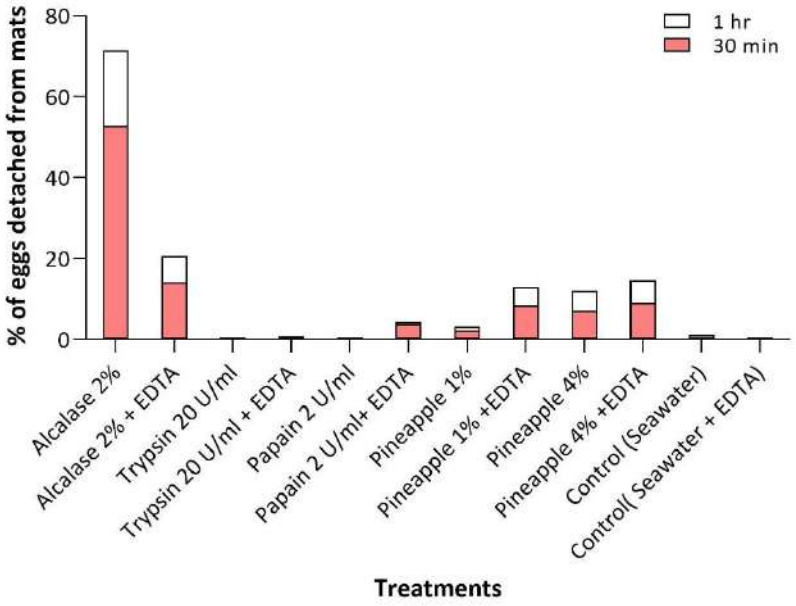


Figure 13: Percentage of eggs detached from the mats for 30 minutes and 1 hour of treatment exposure. The sample size (n=199-430 eggs mats^{-1}) with no replication.

For the second screening, results might have been affected by the dead eggs on mats, as the eggs started to lose their adhesive feature. Therefore, some eggs were falling off the mats with any applied movement. However, comparing the effect of treatments with the control reflects that the detaching of eggs from the mats was enzyme induced (Fig.14). The highest percentage of eggs detaching with the adjusted pH (7.5-8.0) was for alcalase with EDTA treatment 81% but with not much difference from the alcalase without EDTA 79% for 1 hour of exposure (Fig.14). All the other treatments had detached eggs <21%, except for papain 4U/ml with EDTA treatment with 40% eggs detached from mats at 1-hour exposure (Fig.14). Seawater with EDTA was also observed to have 13% of eggs detached from the mats and 5% without EDTA for 1 hour of exposure (Fig.14). A pattern of higher detached eggs with EDTA treatment had been observed with alcalase, papain, and control treatments.

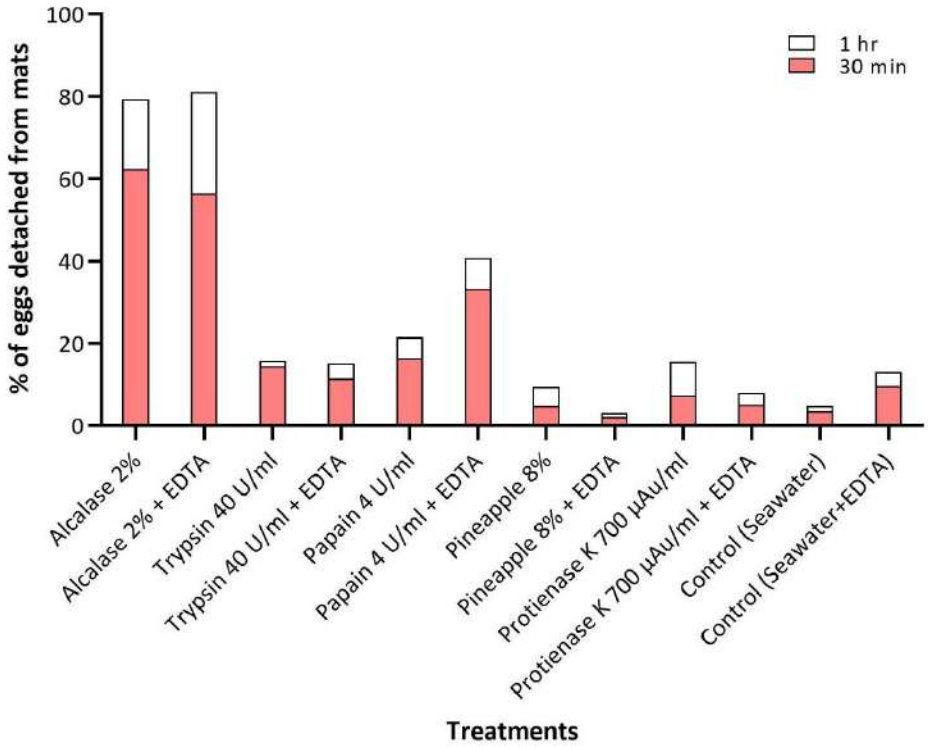


Figure 14: Percentage of eggs detached from the mats for 30 minutes and 1 hour of treatments exposure. The sample size was (n=238-353 eggs mat^{-1}) with no replication.

Overall, for screening trails at 1hr enzyme exposure, it was observed that the first half-hour yields a higher proportion of un-sticky eggs than the last half hour. Moreover, the alcalase with and without EDTA show similar efficiency in detaching the eggs from the mats given that the pH should be optimum. Increasing the concentration of the enzymes, in this case, trypsin and papain resulted in a higher percentage of detached eggs (Fig. 13-14).

4.1.2 Gum layer classification for the detached eggs

The detached eggs from the enzymatic treatments were analysed for the status of their gum layer at 30 min and 1 hr of exposure and classified into four categories; degummed, partly degummed, swollen and not degummed (Fig.8). The mean diameter of eggs was measured to be 1.08 ± 0.1mm (Mean±SD) with a mean gym layer thickness of 0.09 ± 0.01mm (n=30).

For the first screening, except alcalase, all the other treatments had <15% of the eggs detached by the 1 hr of enzymatic exposure, thus very few eggs (0-30) in the first 30 min of exposure and between 0-16 eggs for 1 hr exposure (Fig.13). Therefore, the graphical presentation of gum layer classification (Fig.15) only illustrates the alcalase treatment with and without EDTA. For 30 min of alcalase exposure, the gum layer status of eggs was classified as 60% degummed, 32% swollen, 8% partly degummed, and 0% not degummed. Moreover, it can be observed that approximately 30% higher degummed eggs were observed at 1 hr exposure compared to 30 min, with 90% degummed and 10% partly degummed eggs. The addition of EDTA drops the proportion of degummed eggs to 14%, leaving the remaining gum layer of eggs at 14% partly degummed, 42% swollen, and 28% not degummed for 30 min exposure. Also, at 1hr exposure of alcalase with EDTA, 81% of the eggs had gum layer in the swollen stage. Further, strengthens the inhibition of alcalase enzyme upon the addition of EDTA.

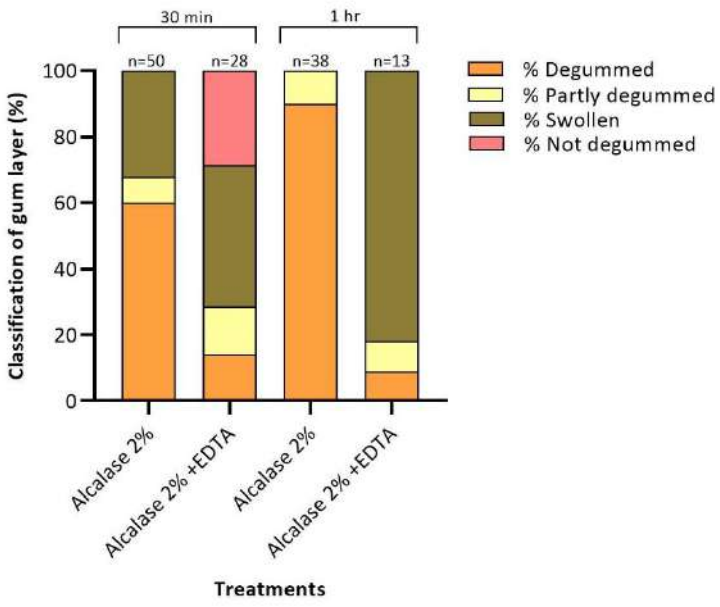


Figure 15: Classification of the gum layer of detached eggs from the mats post-treatment at 30 min and 1 hr exposure with no replication.

For the second screening, except alcalase and papain, all the other treatments had <16% of the eggs detached by the 1 hr of enzymatic exposure, thus very few eggs (6-40) in the first 30 min of

exposure and between 3-23 eggs for 1 hr exposure (Fig.14). Therefore, the graphical presentation of gum layer classification (Fig.16) only illustrates the alcalase and papain treatment with and without EDTA. For 30 min of alcalase exposure, the gum layer status of eggs was classified as 70% degummed, 16% swollen, 14% partly degummed, and 0% not degummed. However, alcalase with the addition of EDTA lowered the degumming percentage of the detached eggs by 12%, where the overall degumming was achieved by 57% of the eggs while 37% of the eggs were in the partly degummed stage. At 1 hr exposure time, alcalase had no eggs in the not degummed and swollen stage meanwhile achieved 87% degumming of eggs without EDTA and 67% with EDTA. The papain on the other hand had degummed 20% and 21% of the detached eggs with and without EDTA, respectively at 30 min exposure. Also, a higher number of eggs were observed in the partly degummed stage 46% without EDTA whereas with the addition of EDTA the eggs in the partly degummed stage were reduced to 19%. However, holding 51% of the eggs in the swollen stage at 30 min of exposure. Furthermore, at 1hr exposure of papain, there were no eggs with intact gum layer and a degumming of 33% and 35% was observed among the eggs for papain with and without EDTA, respectively.

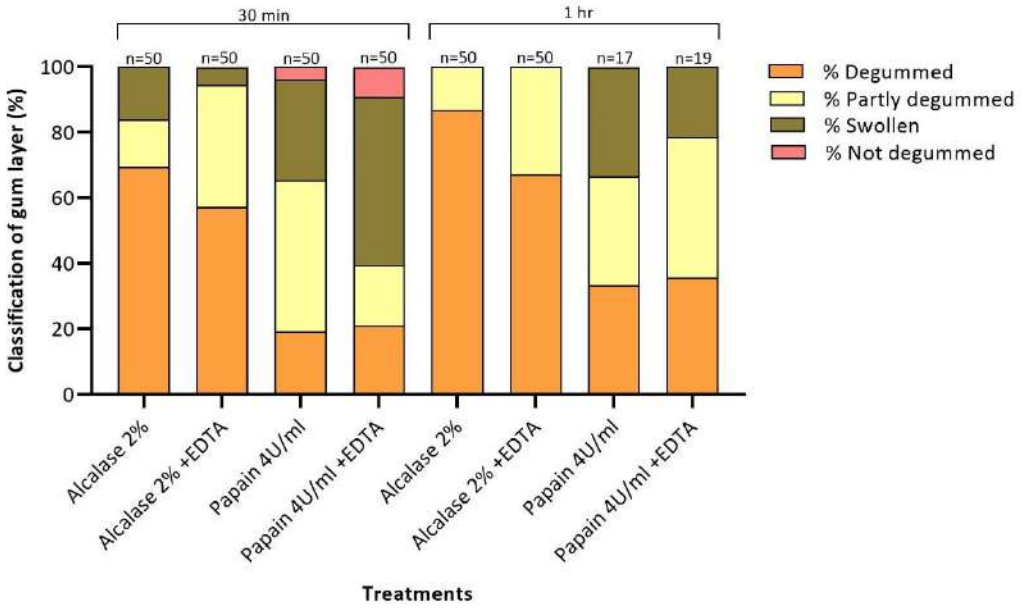


Figure 16: Classification of the gum layer of detached eggs from the mats post-treatment at 30 min and 1 hr exposure with no replication.

Overall, 1 hr of enzymatic exposure provided a higher number of degummed eggs compared to 30 min exposure (Fig.13-14). Further, the classification of the gum layer revealed that the majority of the eggs that detached from the substate were mostly in the degummed stage while followed by the other stages, swelled or partly degummed and minorly in not degummed (Fig.15-16).

4.2 Main experiment

4.2.1 Detached eggs from the mats

Papain from papaya latex at the concentration of 50 U/ml had the highest number of detached eggs 88 ± 5.7% (Mean±SD, N=4) among all the other treatments (Fig.17). The lower concentration of papain 5 U/ml and 10 U/ml also had high percentages of detached eggs with 84 ± 6.6% and 78 ± 9.9% respectively with no significant difference between the papain treatments in detaching the eggs from the mats (Tukey's test; $p>0.05$). Alcalase on the other hand, detached 68 ± 8.2% of the eggs with no significant difference with papain 10U/ml (Tukey's test; $p>0.05$), while being significantly different to 50U/ml and 5U/ml concertation of papain in detaching the eggs from the mats (Tukey's test; $p<0.05$) (Fig.17). All the other treatments detached <17% of the eggs from the mats with no significant difference compared to the seawater treatment in detaching the eggs from the substrate (Table.6).

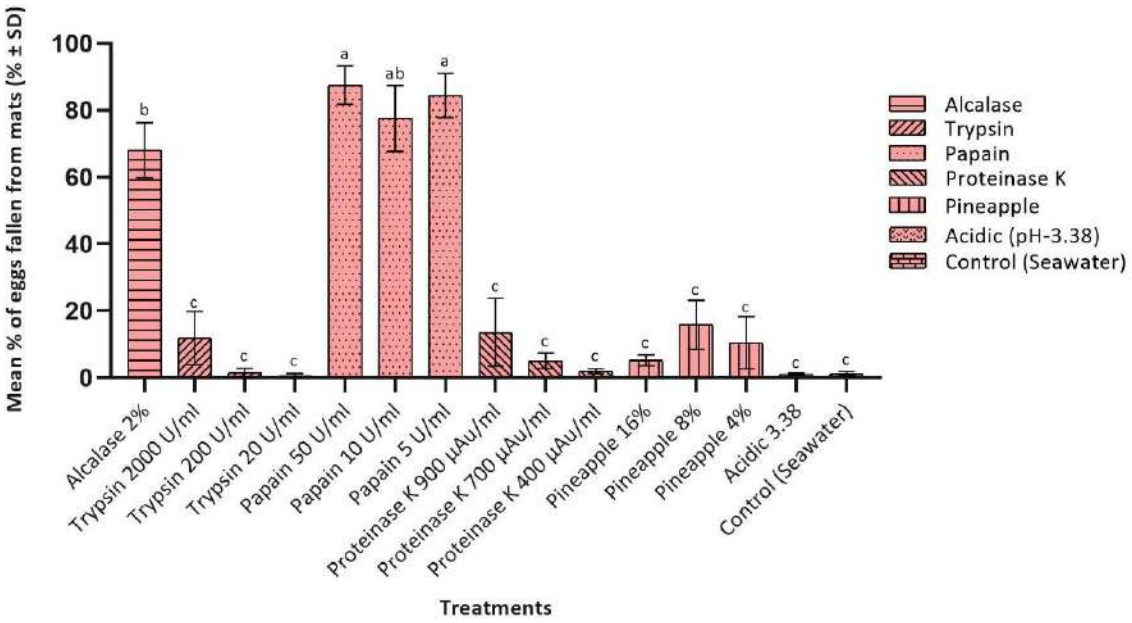


Figure 17: Percentage of Ballan wrasse eggs detached from the mats during 30-minute treatments exposure (Mean ± SD). Each treatment had four replicates (n=4) with (n =57-375 eggs mat^{-1}). Superscript indicates the significant differences between treatments (Tukey's test; $p<0.05$).

Following the detached eggs, Figure.18 represents the proportion of the eggs that were degummed out of eggs that got detached from the mats (Fig.17). Highlighting the correlation between the proportion of detached eggs with the degummed eggs, it was observed that the highest proportion of degummed eggs was observed in papain 10U/ml treatment with 98 ± 2.3%, followed by the papain 50U/ml, 5U/ml and alcalase with 87 ± 4.7%, 82 ± 6.2% and 59 ± 11% respectively (Fig.17). Moreover, in terms of degummed eggs, alcalase was observed to differ significantly from papain 10U/ml treatment by a mean difference of 39% (Tukey's test; $p<0.05$) but not to 50U/ml and 5U/ml of papain treatments (Tukey's test; $p>0.05$). All the other treatments had degummed eggs <1% out of all the detached eggs, except for the proteinase K treatment. For proteinase K, between all the three concentrations no significant difference was observed in regard to degummed eggs (Tukey's test; $p>0.05$), however, the proteinase K 700 µAu/ml treatments had the highest proportion of degummed eggs with 14 ± 8.9%, while 8-9% for the other two proteinase K treatments (Fig.17).

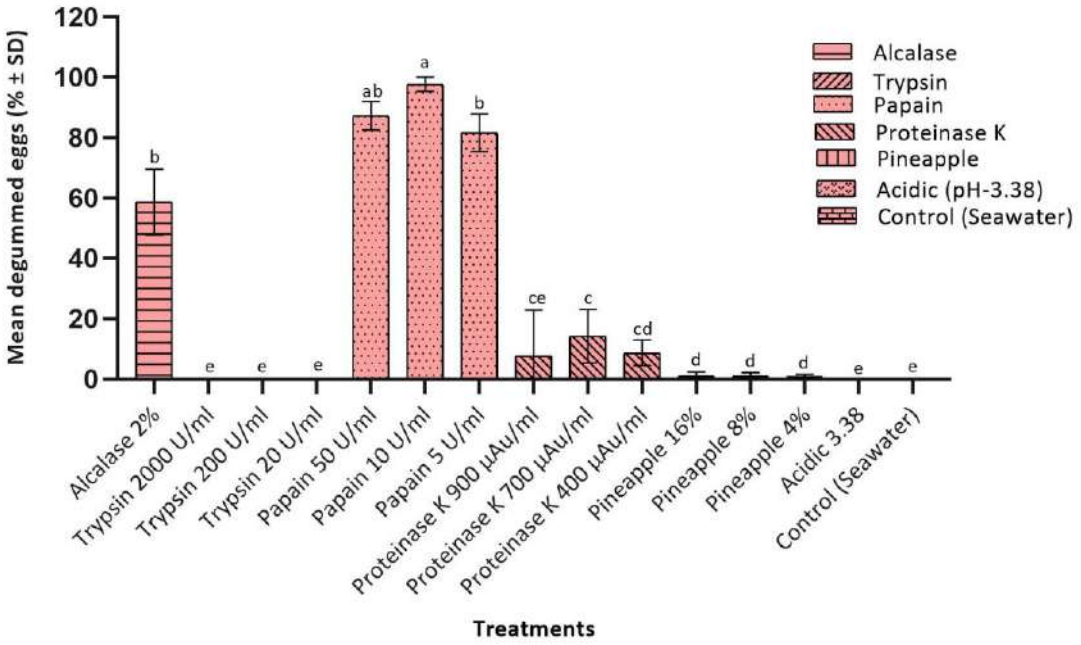


Figure 18: Percentage of degummed eggs out of all the eggs detached from the mats during 30-minute of treatments exposure (Mean ± SD). Each treatment had four replicates (n=4) except for papain 50 U/ml treatment (n=2). The sample size was (n=50 eggs replicate^{-1}) and superscript indicates the significant differences between treatments (Tukey's test; p<0.05)

4.2.2 Gum layer classification for the eggs exposed to enzymatic treatments

The detached eggs by the enzymatic treatments were only observed with papain and alcalase treatment (Fig.17), as these treatments were effective in digesting the gelatinous layer of the eggs resulting in a higher proportion of degummed eggs (Fig.18). In contrast, the other treatments (trypsin, pineapple, and proteinase K) had few eggs detached (Fig.17), as these treatments could not digest the gum layer surrounding the eggs (Fig.18). The control (seawater) and acidic treatment had almost 100% intact gum layer with no significant difference to the trypsin treatments (Table.5) (Tukey's test; p>0.05). The pineapple treatments also had >59% of the eggs in the not degummed stage, with no significant difference between the pineapple treatments (Tukey's test; p>0.05), while the highest concentration 16% had 71 ± 14.5% of eggs in the not degummed stage (Fig.19). This was followed by the proteinase K treatments where >37% of the eggs had a fully intact gum layer, with the highest percentage 57 ± 19% for 900 µAu/ml concentration (Fig.19). Both the pineapple and proteinase K had held 24-45% of the eggs

in the swollen stage with no significant difference among the treatments (Tukey's test; $p>0.05$). Moreover, alcalase treatment also had been observed to hold 33 ± 10.4% of the eggs in the swollen stage with no significant difference with pineapple and proteinase K treatment (one-way ANOVA; $p>0.05$) (Table.5). However, the lowest proportion of eggs held in swelled and partly degum stage was observed for papain, trypsin, and acidic treatment. The exposure of eggs to the acidified seawater showed no action of degumming, meaning the low pH do not cause any damages to the egg's adhesive layer which will aid them to detach. The distribution of eggs in swelled and partly degum stage for pineapple and proteinase K could mean that the enzyme efficacy rate is slower, thus requiring optimization to achieve degumming (Table.5). Overall, from Figures 17 and 19, a higher number of degummed eggs led to an enhanced detachment of eggs from mats, which in this case was by the papain treatments followed by alcalase treatment, as the enzymatic exposure digested the gummy layer surrounding the eggs.

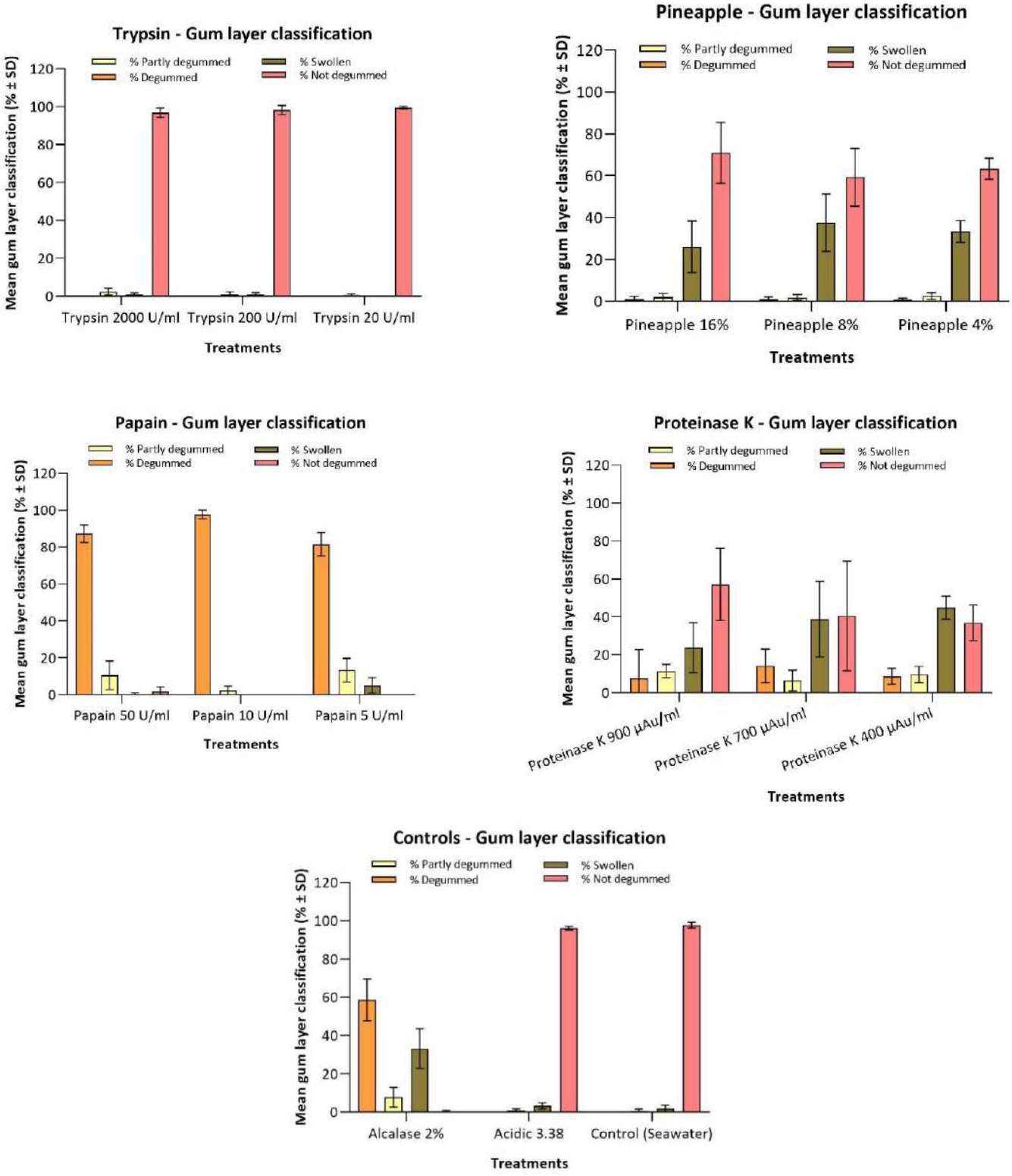


Figure 19: Gum layer classification of the eggs post-treatment at 30-minute (mean% ± SD). Each treatment had four replicates (n=4) except for papain 50 U/ml treatment (n=2) and the sample size was (n≈50 eggs replicate^{-1})

Table 5: Gum layer classification of the eggs post-treatment at 30-minute. Values are expressed as mean ± SD (n=4 replicates, except for degummed eggs for papain 50 U/ml treatment (n=2)).

Treatments	Concentrations	% Detached eggs	% Degummed eggs	% Partly degummed eggs	% Swelled eggs	% Not degummed eggs
Control (Seawater)	-	1 ± 0.6^{c}	0^{e}	1 ± 1.1^{c}	2 ± 1.9^{a}	98 ± 1.5^{a}
Alcalase® Enzyme, *Bacillus licheniformis*	2%	68 ± 8.2^{b}	59 ± 11^{b}	8 ± 5.1^{ab}	33 ± 10.4^{a}	0^{d}
Trypsin, Bovine pancreas	2000 U/ml	12 ± 7.9^{c}	0^{e}	2 ± 1.9^{bc}	1 ± 1^{b}	97 ± 2.5^{a}
	200 U/ml	2 ± 1.3^{c}	0^{e}	1 ± 1.7^{bc}	1 ± 1^{b}	98 ± 2.4^{a}
	20 U/ml	1 ± 0.7^{c}	0^{e}	1 ± 0.7^{bc}	0^{b}	99 ± 1^{a}
Papain, Papaya latex	50 U/ml	88 ± 5.7^{a}	87 ± 4.7^{ab}	11 ± 7.8^{ab}	0^{b}	2 ± 2.4^{d}
	10 U/ml	78 ± 9.9^{ab}	98 ± 2.3^{a}	2 ± 2.3^{bc}	0^{b}	0^{d}
	5 U/ml	84 ± 6.6^{a}	82 ± 6.2^{b}	13 ± 6.4^{a}	5 ± 4.2^{b}	0^{d}
Pineapple juice (Bromelain)	16%	5 ± 1.6^{c}	1 ± 1.3^{d}	2 ± 1.9^{bc}	26 ± 12.3^{a}	71 ± 14.5^{b}
	8%	16 ± 7.3^{c}	1 ± 1.2^{d}	2 ±1.4^{bc}	38 ±13.7^{a}	59 ± 13.9^{bc}
	4%	10 ± 7.8^{c}	1 ± 0.7^{d}	3 ± 1.7^{abc}	33 ± 5.2^{a}	63 ± 5.1^{bc}
Proteinase K, *Tritirachium album*	900 µAu/ml	14 ± 1^{0c}	8 ± 15.2^{ce}	11 ± 3.6^{ab}	24 ± 13.2^{a}	57 ± 19^{bc}
	700 µAu/ml	5 ± 2.3^{c}	14 ± 8.9^{c}	6 ± 5.5^{abc}	39 ± 20^{a}	41 ± 28.9^{c}
	400 µAu/ml	2 ± 0.7^{c}	9 ± 4.3^{cd}	10 ± 4.3^{ab}	45 ± 6.0^{a}	37 ± 9.5^{c}
Acidic seawater	pH 3.38	1 ± 0.3^{c}	0^{e}	1 ± 0.7^{bc}	3 ± 1.6^{a}	96 ± 1^{a}

Means with the different superscript letter are significantly different by Tukey's multiple comparisons test (P<0.05).

The gum layer classification also recorded a few damaged eggshells post-treatment (Fig.20). The damaged eggshells were exclusive to the papain treatments, where the eggshells was seen shrunken or became transparent with no embryo inside.

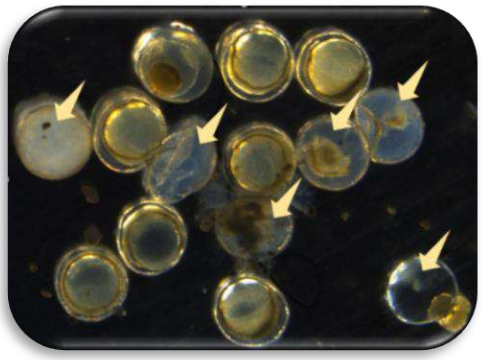


Figure 20: Damaged eggshells induced by the exposure of papain treatments for 30 minutes. (Arrows shows damaged eggshells)

4.3 Hatching success

Most of the eggs hatched between 100-120 degree days with around 76% of the larvae hatched after 110 degree days. The highest mean hatch rate was observed by the control treatment with 77 ± 5.6 % (Fig.21). The degummed eggs from the papain treatment exposure resulted in the lower hatch rates with the lowest by papain 50U/ml treatment 55 ± 6.5%. Statistically, no significant difference was observed in the hatching rate between all the treatments tested (one-way ANOVA; $p>0.05$), but it can be noted that the control hatch rate 77 ± 5.6% has a difference of about 15% with the papain 5 U/ml treatment considering it has the highest hatch rate among all papain treatment. In comparison, the alcalase had a bit higher hatching rate than the papain, however, with no significant difference (one-way ANOVA; $p>0.05$).

The hatching rate of trypsin treatments did not vary much, where the highest concentrations, 2000 U/ml, and the lowest concentrations 20 U/ml, had hatching rates of 61 ± 12.3% and 67 ± 5.1%, respectively. Also, an inverse relation between hatching rate and increasing concentrations was observed for all treatments except for pineapple treatments, where 8% and 4% pineapple treatment had 69 ± 9.9% and 67 ± 5.3%, respectively (Fig.21). The hatch rate for the acidic treatment corresponding to the pineapple 16% treatment was recorded with a hatch rate of 69 ± 7% and 58 ± 8.4%, respectively, indicating that low pH exposure of 30 minutes does not affect the eggs greatly in terms of hatching and the lower hatch rate for pineapple 16% could have been enzyme induced (Fig.21).

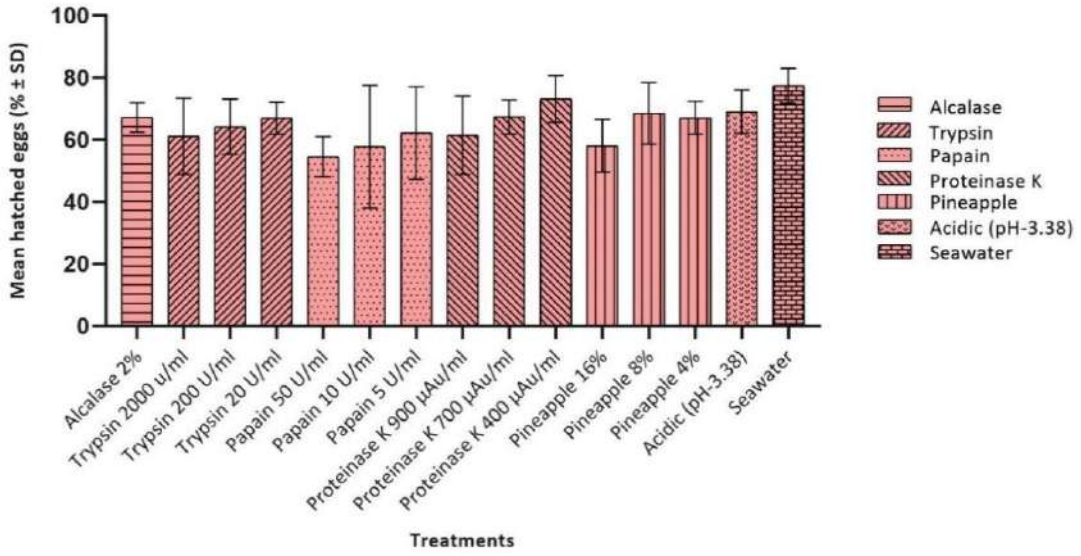


Figure 21: Percentage of the eggs hatched post-treatment with standard error (mean ± SD). Each treatment had four replicates (n=4) except for papain 50 U/ml with (n=2) with each incubation tube containing around (n=40-238 eggs replicate^{-1}). There was no significant difference in hatching success between treatments (one-way ANOVA; p>0.05).

4.4 Measurements for larval quality

4.4.1 Total length, Standard length, and myotome height

Ballan wrasse larvae had a mean total length of 4.35 ± 0.13mm observed by the control (seawater) treatment. The highest mean total length of 4.40 ± 0.07mm was achieved by Proteinase K 900 U/ml while the lowest was 4.16 ± 0.16mm from papain 10 U/ml treatments (Table.6). All the treatments had a total length between 4.16-4.40mm with no significant differences in the mean total length attained by treatment exposure (Dunn's multiple comparisons test; p >0.05).

The mean standard length for the larvae was observed to be 4.14 ± 0.12mm for the control (seawater) treatment, while for all the other treatments the mean standard length was between 3.97-4.21mm with no significant difference between treatments (Dunn's multiple comparisons test; p >0.05) (Table.6).

The highest mean myotome height was observed by the acidic treatment with 0.229 ± 0.002mm (Table.6). Alcalase treatment exposure led to the lowest mean myotome height 0.209 ± 0.005mm

attained by the larvae, which varied significantly to all the other treatments (Tukey's test; $p<0.05$). The larvae subjected to trypsin exposure 200U/ml had the myotome height of 0.219 ± 0.001mm, which was significantly different to alcalase and acidic treatment only (Tukey's test; $p<0.05$). All the other treatments did not vary significantly in regard to the myotome height attained by the larvae post enzymatic exposure (Tukey's test; $p>0.05$) (Table.6).

4.4.2 Eye area, Eye-to-body ratio, and Fin fold area

The largest eye area of the Ballan wrasse larvae post-treatment was observed by the alcalase treatment with 0.053 ± 0.0021mm^2, followed by the control treatment 0.051 ± 0.0012mm^2 (Table.6). A direct relation of the increasing eye area was observed with increasing dosage of trypsin treatments, moreover, the lowest eye area was recorded for the trypsin treatments 20 U/ml treatment with 0.046 ± 0.0022mm^2. The eye area of the larvae was not largely affected by the enzymatic treatments therefore no significant difference was detected between treatments (Dunn's test; $p>0.05$)

The eye area over body area defines the ratio of an eye to the larvae's body. The bigger the eye area, the higher its percentage coverage. The highest eye-to-body ratio was obtained by the alcalase treatment 0.043 ± 0.002 with no significant difference to all the other treatments (Dunn's test; $p>0.05$), except for trypsin 20 U/ml treatment (Table.6). The larvae exposed to trypsin 20 U/ml treatment attained the lowest eye to body ratio 0.037 ± 0.002, which varied significantly from all the other treatments (Dunn's test; $p<0.05$).

All the larvae exposed to the treatments had a mean fin fold area between 55-59%, whereas the alcalase treatment was found to be the lowest with 55 ± 1.4% (Table.6). However, papain treatments 50, 10, 5 U/ml did not vary much from the alcalase treatments with 57 ± 3.4%, 56 ± 3.2% and 56 ± 2.6%, respectively. The eggs incubated for the treatments with the intact gum layer had a slightly higher mean fin fold area 58-59% but statistical analysis revealed no significant differences between treatments that had eggs incubated with and without the gum layer (one-way ANOVA; $p>0.05$)

Table 6: Measurements of larval morphology hatched post-treatment from 30-minute of enzymatic exposure. Each treatment had four replicates with (*n=10* individuals replicate^{-1}) except for papain 50 U/ml treatment (two replicates).

Treatments	Concentration	Mean total length (mm±SD)	Mean standard length (mm±SD)	Mean myotome height (mm±SD)	Mean eye area (mm^2±SD)	Mean eye-to-body ratio (ratio±SD)	% Fin fold area (Mean±SD)
Control (Seawater)	-	4.35 ± 0.13	4.14 ± 0.12	0.23 ± 0.005^{ab}	0.051 ± 0.001	0.041 ± 0.001^{a}	58 ± 2.4
Alcalase® Enzyme, *Bacillus licheniformis*	2%	4.28 ± 0.05	4.08 ± 0.04	0.21 ± 0.005^{c}	0.053 ± 0.002	0.043 ± 0.002^{a}	55 ± 1.4
Trypsin, Bovine pancreas	2000 U/ml	4.33 ± 0.08	4.13 ± 0.07	0.22 ± 0.002^{ab}	0.048 ± 0.003	0.038 ± 0.003^{a}	58 ± 2.2
	200 U/ml	4.29 ± 0.11	4.11 ± 0.08	0.22 ± 0.001^{b}	0.047 ± 0.003	0.038 ± 0.002^{a}	57 ± 3.1
	20 U/ml	4.36 ± 0.05	4.18 ± 0.05	0.22 ± 0.002^{ab}	0.046 ± 0.002	0.037 ± 0.002^{b}	58 ± 1.8
Papain, Papaya latex	50 U/ml	4.23 ± 0.04	4.04 ± 0.04	0.22 ± 0.001^{ab}	0.048 ± 0.003	0.040 ± 0.002^{a}	57 ± 3.4
	10 U/ml	4.16 ± 0.16	3.97 ± 0.15	0.22 ± 0.006^{ab}	0.047 ± 0.003	0.039 ± 0.001^{a}	56 ± 3.2
	5 U/ml	4.27 ± 0.06	4.08 ± 0.06	0.22 ± 0.006^{ab}	0.049 ± 0.002	0.039 ± 0.001^{a}	56 ± 2.6
Pineapple juice (Bromelain)	16%	4.36 ± 0.02	4.16 ± 0.01	0.23 ± 0.003^{ab}	0.049 ± 0.002	0.039 ± 0.002^{a}	58 ± 0.7
	8%	4.37 ± 0.04	4.18 ± 0.02	0.23 ± 0.003^{ab}	0.050 ± 0.003	0.039 ± 0.002^{a}	58 ± 1
	4%	4.37 ± 0.08	4.18 ± 0.08	0.23 ± 0.002^{ab}	0.049 ± 0.001	0.040 ± 0.001^{a}	59 ± 0.3
Proteinase K, *Tritirachium album*	900 µAu/ml	4.40 ± 0.07	4.21 ± 0.07	0.22 ± 0.004^{ab}	0.047 ± 0.002	0.038 ± 0.001^{a}	58 ± 1
	700 µAu/ml	4.25 ± 0.04	4.06 ± 0.02	0.22 ± 0.003^{ab}	0.049 ± 0.001	0.040 ± 0.001^{a}	56 ± 1.1
	400 µAu/ml	4.27 ± 0.13	4.06 ± 0.12	0.22 ± 0.004^{ab}	0.050 ± 0.003	0.040 ± 0.001^{a}	59 ± 0.8
Acidic seawater	pH 3.38	4.34 ± 0.04	4.16 ± 0.03	0.23 ± 0.002^{a}	0.049 ± 0.001	0.039 ± 0.001^{a}	56 ± 2.5

Means with different superscript letter are significantly different by Tukey's multiple comparisons test (P<0.05) for one-way Anova and Dunn's multiple comparisons test (P<0.05) for Kruskal-Wallis test.

4.5 Deformities

The only repetitive deformation was the occurrence of mild edema in almost all the treatments, including the control (seawater), but with a low percentage of 9 ± 6.9% (Table.7). A direct relation of a higher dose with higher deformation has been observed in pineapple treatments 16, 8, 4% with 39 ± 19.4%, 28 ± 9.8%, and 11 ± 8.1%, respectively. The acidic treatment, which shares the same pH with pineapple 16%, had almost a mean difference of 10% in mild edema deformity but with no significant difference (Tukey's test; $p > 0.05$), indicating the possible impact of an acidic environment in causing deformity. The highest proportion of larvae with mild edema deformation was for papain 50 U/ml treatment with 73 ± 8.4%, which was significantly different to all the other treatments (Tukey's test; $p<0.05$), while the second-highest edema mild deformation was shared among papain 5 U/ml and trypsin 200 U/ml with 57 ± 15.9% and 56 ± 16.9% respectively, which also differ significantly from seawater treatment (Tukey's test; $p< 0.05$) (Table.7). Moreover, the high variation in the proteinase K 700U/ml treatment is due to the reason that one replicate was full of deformed larvae with mild edema.

The severe edema was found in smaller proportions that were not repetitive in all the replicates; for instance, papain 5 U/ml had the highest percentage of severe edema, 8 ± 9.8%, but in low numbers in two replicates (Table.7). Moreover, none of the treatments vary significantly in the occurrence of severe edema (Kruskal-Wallis test; $p >0.05$).

Spinal deformities, including tail flexures, curved spine, and breaks, were also registered in low percentages in all the treatments (Table.7). The highest registrations of tails flexure deformities were found in pineapple 8 and 4% treatments with 7 ± 9.5%, and 7 ± 4.9%, respectively (Table.7). However, no significant difference was observed between all the treatments for the tail flexure deformity (Kruskal-Wallis test; $p >0.05$). The highest curved spine deformity was observed in the papain 50 U/ml treatment with 7 ± 10.1%, whereas trypsin 2000 U/ml, proteinase K 700 U/ml and pineapple treatments did not show any sign of curved spines (Table.7). At the same time, the curved spine deformation in all the larvae post-treatment did not differ significantly between treatments (Kruskal-Wallis test; $p>0.05$). The break deformity was absent in all the treatments except for alcalase treatment, where it was present in all the four replicates 22 ± 15.9% and this

exception made alcalase significantly different from the rest of the treatments (Kruskal-Wallis test; p <0.05) (Table.7).

Table 7: Deformities (%) in newly hatched larvae post-treatment including spinal and edema deformities, where (0- None), (1- Mild), (2-Severe) for edema. Values are expressed as mean ± SD. Each treatment had four replicates with (*n=10* individuals replicate^{-1}) except for papain 50 U/ml treatment (two replicates).

Treatments	Concentration	Deformities						
		Spinal				Edema		
		% No spinal deformity	% Tail flexures	% Curved spines	% Breaks	% Edema (0)	% Edema (1)	% Edema (2)
Control (Seawater)	-	93 ± 8.2	5 ± 5.3	2 ± 4.2	0^{b}	89 ± 10.3^{a}	9 ± 6.9^{c}	2 ± 4.2
Alcalase® Enzyme, *Bacillus licheniformis*	2%	75 ± 21.3	0	6 ± 6.4	22 ± 16^{a}	73 ± 7.1^{abc}	27 ± 7.1^{abc}	0
Trypsin, Bovine pancreas	2000 U/ml	100	0	0	0^{b}	48 ± 12.6^{abc}	49 ± 15.7^{abc}	2 ± 4.5
	200 U/ml	92 ± 5.3	6 ± 6.4	2 ± 4.5	0^{b}	44 ± 16.9^{bc}	56 ± 16.9^{ab}	0
	20 U/ml	95 ± 6.1	3 ± 5.6	3 ± 5	0^{b}	59 ± 17.6^{abc}	41 ± 17.6^{abc}	0
Papain, Papaya latex	50 U/ml	89 ± 15.2	4 ± 5	7 ± 10.1	0^{b}	24 ± 13.5^{c}	73 ± 8.4^{a}	4 ± 5
	10 U/ml	90 ± 11.1	5 ± 9.1	5 ± 10	0^{b}	46 ± 13.7^{bc}	52 ± 15.5^{ab}	3 ±5
	5 U/ml	91 ± 6.0	6 ± 6.6	3 ± 6.3	0^{b}	36 ± 16^{c}	56 ± 15.9^{ab}	8 ± 9.9
Pineapple juice (Bromelain)	16%	98 ± 4.5	2 ± 4.5	0	0^{b}	59 ± 21.8^{abc}	39 ± 19.4^{abc}	3 ± 5
	8%	93 ± 9.5	7 ± 9.5	0	0^{b}	69 ± 11.4^{abc}	28 ± 9.8^{abc}	3 ± 5
	4%	93 ± 4.9	7 ± 4.9	0	0^{b}	89 ± 8.1^{a}	11 ± 8.1^{c}	0
Proteinase K, *Tritirachium album*	900 µAu/ml	97 ± 5.6	0	3 ± 5.6	0^{b}	82 ± 9.5^{ab}	18 ± 9.5^{bc}	0
	700 µAu/ml	95 ± 6.1	5 ± 6.1	0	0^{b}	49 ± 34.8^{abc}	48 ± 36.2^{abc}	3 ± 5
	400 µAu/ml	94 ± 11.1	3 ± 5.6	3 ± 5.6	0^{b}	55 ± 9.1^{abc}	45 ± 9.1^{abc}	0
Acidic seawater	pH 3.38	95 ± 10	0	5 ± 10	0^{b}	70 ± 10.5^{abc}	27 ± 11.6^{abc}	3 ± 5

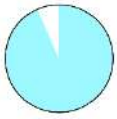
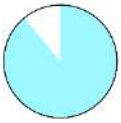
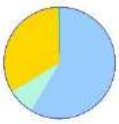
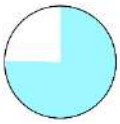
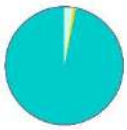
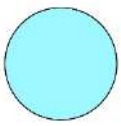
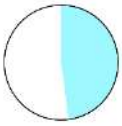
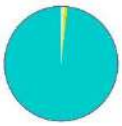
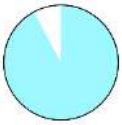
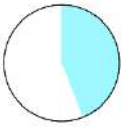
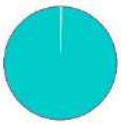
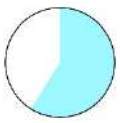
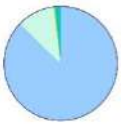
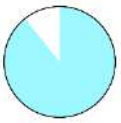
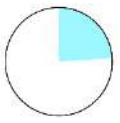
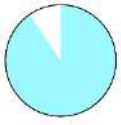
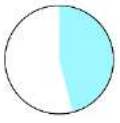
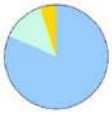
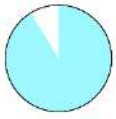
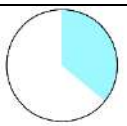
Means with different superscript letter are significantly different by Tukey's multiple comparisons test (P<0.05) for one-way Anova and Dunn's multiple comparisons test (P<0.05) for Kruskal-Wallis test.

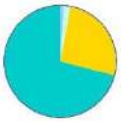
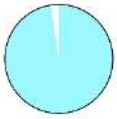
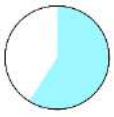
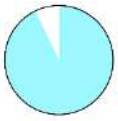
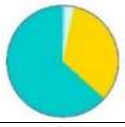
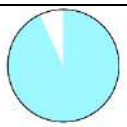
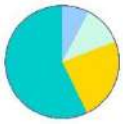
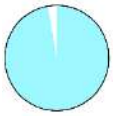
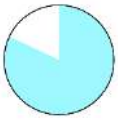
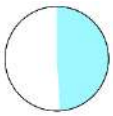
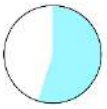
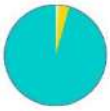
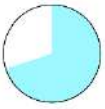
4.6 Summary of the results

In summary, various enzymes were tested to eliminate the stickiness of the Ballan wrasse eggs. Besides the proven degumming action of alcalase, the study discovered that papain from papaya latex also degums Ballan wrasse eggs (Appendix.4). A 30-minute exposure to papain treatments (50, 10, 5U/ml) detached >77% from the mats. Further assessment of the gum layer revealed that >80% of the eggs were without gum layer, reaching >90% for the papain 10 U/ml treatment. In comparison, the alcalase freed >68% of the eggs from the mats out of which approximately 59% of the eggs were degummed. This outcome placed the papain above the alcalase in effectiveness to degum when subjected to 30 minutes of treatment exposure.

The hatching rate revealed no significant differences between the enzymatic treatments. The newly hatched larvae morphological measurements also showed no significant difference among the treatments, except for myotome height and the eye-to-body ratio. The lowest mean myotome height was achieved by the alcalase treatment, while the highest was recorded for the acidic treatment with a significant difference. Alcalase treatment was also observed to have the highest mean eye-to-body ratio which differed significantly from trypsin 20U/ml treatment but not to any other treatment. The elimination of egg stickiness came at the cost of mild edema deformity for all the larvae exposed to the enzymatic treatments, especially for the papain treatments with >52%, where the highest proportion of mild edema deformed larvae was for papain 50 U/ml with >73%, which also differed significantly to all the other treatments. Besides, the mild edema deformity, the treatments did not vary significantly in terms of spinal deformities or the occurrence of severe edema.

Table 8: The table summarizes the finding of the current study without the larval morphological measurements and with the effective treatments to degum the Ballan wrasse eggs marked in bold. Each treatment had four replicates for detached eggs and hatching success, except for papain 50 U/ml treatment which had two replicates for hatching success.

Treatments	**Concentrations**	**% Detached eggs** (Mean±SD)	**Gum layer classification of Detached eggs** (mean) % Degummed % Partly degummed % Swollen % Not degummed	**% Hatch Success** (Mean±SD)	**Deformities** (mean) **Spinal** Normal larvae Abnormal larvae	 **Edema** Normal larvae Abnormal
Control	Seawater	1 ± 0.6[c]		77 ± 5.6		
Alcalase® Enzyme, *Bacillus licheniformis*	**2%**	**68 ± 8.2[b]**		**67 ± 4.7**		
Trypsin, *Bovine pancreas*	2000 U/ml	12 ± 7.9[c]		61 ± 12.3		
	200 U/ml	2 ± 1.3[c]		64 ± 8.9		
	20 U/ml	1 ± 0.7[c]		67 ± 5.1		
Papain, Papaya latex	**50 U/ml**	**88 ± 5.7[a]**		**55 ± 6.5**		
	10 U/ml	**78 ± 9.9[ab]**		**58 ± 19.7**		
	5 U/ml	**84 ± 6.6[a]**		**62 ± 14.9**		

Pineapple juice	16%	5 ± 1.6^{c}		58 ± 8.4		
	8%	16 ± 7.3^{c}		69 ± 9.9		
	4%	10 ± 7.8^{c}		67 ± 5.3		
Proteinase K, *Tritirachium album*	900 µAu/ml	14 ± 10^{c}		61 ± 12.4		
	700 µAu/ml	5 ± 2.3^{c}		67 ± 5.5		
	400 µAu/ml	2 ± 0.7^{c}		73 ± 5.6		
Acidic	pH 3.38	1 ± 0.3^{c}		69 ± 7.0		

Means with different superscript letter are significantly different by Tukey's multiple comparisons test (P<0.05).

5. Discussion

The issue of egg adhesiveness in Ballan wrasse has been initially addressed by adapting egg collection and incubation protocols. However, developing techniques to remove the adhesive layer surrounding the eggs to release eggs from the artificial spawning substrates would benefit commercial production of Ballan wrasse. Removal of the gum layer is expected to reduce mortality rates by allowing for more thorough egg disinfection, easier egg incubation and hatching protocols. Therefore, this study aimed to find an effective method to eliminate egg stickiness by testing various enzymatic treatments. This investigation developed an in vitro methodology to remove the eggs' stickiness. The protease enzymes papain (from papaya latex) and alcalase was found successful in eliminating the eggs adhesiveness with no significant

difference in hatching rate but with the occurrence of mild edema deformity in larvae after hatching.

5.1 Enzymatic treatments and hatching

Alcalase treatment: Enzyme alcalase has been reported by Grant et al. (2016b) and Helland et al. (2014) to successfully eliminate stickiness of Ballan wrasse eggs. Grant et al. (2016b) used a petri dish as a substrate for egg attachment on which the alcalase treatment was applied followed by agitation at 240rpm. This experimental design helped them obtain 100% degumming of the Ballan wrasse eggs in only 15 minutes by 4% alcalase and in 20 minutes by 2% alcalase. Such an experimental setup is, however, not easily implemented in in daily operations in a hatchery. Our study was conducted more in line with realistic operational situations to degum eggs. Therefore, to replicate the operational situation, mats were used instead of Petri dishes and agitation was done at 50 rpm to induce as little harm to the eggs as possible. Our experimental setup led to approximately 68% of the eggs being detached from the mats, of which further approximately 60% were degummed (Table.5). Less detachment compared to those observed by Grant et al. (2016b) is most likely attributed to the different experimental setups used. Compared to the smooth surface of the petri dish (secondary site of attachment), the mats (primary site of attachment) present a rough texture with approximately 2mm pointy rough plastic crest, in which the eggs were stuck. This might require additional mechanical force to achieve maximum detachment together with the enzyme treatment. In Figure 22, it can be observed that the spawning mats themselves possess some challenges due to their manufacturing material or fragments detaching from the mats, which entangle in the bloated gum layer surrounding the eggs and restrict egg detachment.

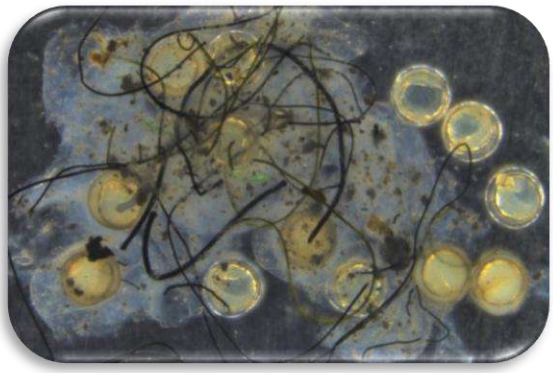


Figure 22: Trapped Ballan wrasse eggs in mat fragments, bloated gum layer and dirt post-treatment of 2% alcalase for 30 minutes.

One very crucial factor affecting the amounts of reagents used to obtain optimum degumming is the time of the spawning period at which the eggs are collected. The gum layer thickness changes throughout the spawning period of the fish. The thickness of the gum layer decreases as the spawning period approaches its end (Grant et al., 2016a). This change in the thickness of the gum layer necessitates optimization of dosage of reagents.

Another approach to obtain the non-adhesive eggs and improve the efficiency of alcalase treatment and fertilization could be through strip spawning and preventing the contact of eggs with seawater before fertilization. Instead, the eggs can be rinsed by ringer solution and then supplemented with a pre-defined amount of alcalase. After keeping the eggs in alcalase solution for a defined time, the eggs can be fertilized and returned to the seawater however, the processed eggs will now produce a partially sticky gum layer and would not aggregate compared to those eggs which have been in contact with the seawater as soon as they were released (Helland et al., 2014). This would also reduce the dependence on the spawning mats employed in the regular procedures for natural spawning. Plus, it will not only decrease the risk of eggs being clumped together but also result in better overall incubation handling. The reason behind this is that after fertilization, the eggshell changes its properties to promote its hardening by taking up more water which brings about ultrastructural and cytochemical changes (Coward et al., 2002; Shibata et al., 2000). Also, the thin film of ovarian fluid covering the egg induces the reaction with ZRE (zona radiata externa) after getting in contact with seawater to form a jelly layer responsible for attachment of the eggs (Mansour et al., 2009). All these reasons further

strengthen the suggestion of treating the eggs with alcalase supplemented ringer solution before fertilization. However, further research is still needed for the proof of concept.

In our study, hatching success of Ballan wrasse eggs treated with 2% alcalase for 30 minutes (Fig.21) was around 67%. Comparable to this, Grant et al. (2016b) observed a mean hatch success of around 74% upon exposure to 2% alcalase for 10 minutes. Differences in exposure duration could have caused the slightly lower hatching success in the current study.

Papain treatment: Our study reports successful application of papain sourced from papaya latex to eliminate the gum layer of Ballan wrasse eggs for the first time. All three tested concentrations of papain resulted in a maximum detachment of around 84-87% (Fig.17). However, among the three concentrations of papain, the 5 U/ml resulted in around 82% successful degumming of the eggs with a mean hatching rate of approximately 62%. Although the present study achieved a different detachment and degumming rate at all concentrations of papain used (Fig.17), the hatching rate was comparable between all concentrations, except at 50U/ml which resulted in the lowest hatching success of around 55% (Fig.21). Consistent with the present study, a similar study was conducted on channel catfish (*Ictalurus punctatus*) eggs by Ringle et al. (1992), where they observed a 20% higher hatching success by using 0.2% papain alone or in combination with Na_2S0_3 and L-cysteine-HCL compared to the traditional incubation treatments.

The FDA (U.S. Food and Drug Administration) has added the use of 0.2% papain solution in low regulatory priority, meaning it can be used commercially but with conditions apply as it increases the hatching rate by removing the gelatinous matrix around the eggs. However, there are some research studies done (Southern Regional Aquaculture Center, 2012, Yamamoto, 1956) where the application of papain concentration up to 10% has not resulted in any effective treatment on goldfish and lamprey eggs. Lack of knowledge about the eggshell matrix of Ballan wrasse does not allow us to dig deeper and discuss the reason for the effectiveness and/or ineffectiveness of the enzymes. However, the action of papain enzyme purified either from *C. papaya* or papaya latex suggests that the egg matrix has basic amino acids, leucine, glycine, lysine, or arginine. Both are cysteine proteases in nature and digest proteins, but the working mechanism varies, as papain from *C. papaya* mainly cleaves peptide bonds containing amino acids such as lysine,

arginine and residues succeeding phenylalanine, whereas papain from papaya latex cleaves peptide bonds of basic amino acids, leucine, or glycine (Florence and Ezekiel, 2012). Besides the cleaving specificity, the time of application, temperature and pH also plays an essential role in achieving successful degumming.

Papain in general is a very potent enzyme in nature. Therefore, to avoid unnecessary protein degradation, papain is generally synthesized as inactive precursors, also known as zymogens (Verma et al., 2016). The zymogens contain a pre-domain that blocks the substrate's access to the active site (Coulombe et al., 1996). Some commercially available papain enzymes as found in *C. papaya* require additional co-activators like cysteine for their optimal activity. This fact is further supported by Homaei et al. (2010), where the maximum specific activity of papain was observed by addition of 200 mM cysteine. Since we have used papain from *C. papaya* in our initial screening trials, missing an activator might have reduced or stopped the purified papain's enzymatic activity. However, the papain source from papaya latex is not dependent on any co-activator such as cysteine for optimum activity, which could be the possible reason to observed egg detachment and degumming without the addition of cysteine in the main experiment of the present study (Fig. 17 and 18).

The elimination of the egg stickiness by the papain treatment came at the cost of disintegration of some eggshells (Fig.20). The eggshell is crucial for the protection of the embryo from external factors, e.g., physical, chemical, and biological hazards (Yamagami et al., 1992, Shibata et al., 2000). Even though the number of damaged eggs were quite low in the present study, the papain dosage and exposure time must be optimized further to avoid adverse effects on eggs. Damaged eggshells were also observed by Linhart et al. (2000) only after 2 minutes of alcalase (20-ml l^{-1}) exposure in an attempt to eliminate the egg stickiness in tench (*Tinca tinca* L.) but with a hatch success of around 80%. Similar damages to eggshells have also been observed upon exposure to trypsin (0.1%), sodium sulfite (0.2N) and sodium borohydrate (0.2-3%) during the chemical separation of Channel Catfish eggs to achieve degumming (Ringle et al., 1992).

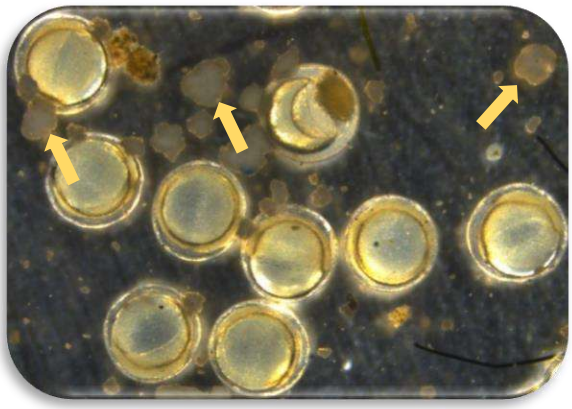


Figure 23: Insoluble content found in the papain 50U/ml solution derived from papaya latex.

The purification of papain from papaya latex has traditionally been achieved by salt precipitation and chromatography. Even after purification, the enzyme remains contaminated with other proteases, as a wide variety of synthetic and natural polyelectrolytes can interact with globular proteins to form stable protein–polyelectrolyte complexes that result in the formation of soluble or insoluble complexes (Braia et al., 2013) (Fig.24). Therefore, the papain solution from papaya latex should be sieved before use to avoid the insoluble content entering the working solution, to prevent extra handling while separating the eggs from the solution.

Trypsin: Trypsin treatment was found ineffective at all concentrations in degumming the Ballan wrasse eggs. In a study conducted by Southern Regional Aquaculture Center (2012), comparable results were found for Ballyhoo eggs where trypsin treatment at concentrations 0.05%, 0.25% and 0.5% for 15 minutes was found ineffective. However, 12 U/ml of trypsin eliminated eggs stickiness of Persian sturgeon after only six minutes exposure, further achieving an embryo survival rate of up to 90% with 11 minutes of exposure to 6 U/ml trypsin (Neitali et al., 2014). Contrary to the present study, the highest number of hatched larvae (approx. 67%) with the lowest trypsin concentration of 20 U/ml in 30 minutes (Fig.21). Trypsin treatment have also been shown to cause thinning of the chorion layer and could prevent fertilization by reducing the sperm entry due to narrowing of micropyle passage (Iwamatsu et al., 1997). In the present study, however, eggs were fertilized before the trypsin treatment. All our observations suggest that trypsin at the concentrations and exposure times used is not the favourable chemical to break down the protein coating of the chorion of Ballan wrasse eggs.

Pineapple and Acidic treatment: All tested concentrations used in the pineapple treatments turned out to be ineffective in the present study. The pineapple juice is an amalgam of different protein enzymes among which the bromelain enzyme is the major consistent for protein digestion that works at an optimum pH and temperature ranges of 3-9 and 45-65 ^{o}C, respectively (Dubey et al., 2012). As the present study was conducted at around 9.5 ^{o}C, this low temperature could have rendered the bromelain enzyme ineffective in digesting the egg's outer protein layer. This observation was further supported by another study conducted by (Thai and Ngo, 2004) where 1% pineapple juice applied at a relatively higher temperature of 26 ^{o}C led to an effective and increased de-sticking of Common Carp eggs (*Cyprinus carpio* L.).

Another important factor is the acidic environment created by the pineapple juice (see table 3). To rule out the effect of pH drop on hatching and larval quality, an acidic treatment (pH 3.3) corresponding to the pH of the highest concentration of 16% of pineapple juice was run in parallel to the main experiment. The eggs in both the negative control and pineapple treatment were subjected to pH drop three days post-fertilization for 30 minutes. The negative control revealed that the pH drop did not affect hatching success compared to the control group (Fig.21). Contrary to these findings, low pH values of 5.6, 4.0 and 2.0 have led to increased mortality in the eggs of *Perca fluviatilus L., P. olivaceus* and *Silurus asotus,* respectively, where the eggs were exposed for a period of 30 minutes to 38 hours (Johansson and Milbrink, 1976, Kim et al., 2020, Gao et al., 2011). The low mortality in our study may be attributed to the fact that the eggs were subjected to the pineapple treatment 3 days post-fertilization. This might have given enough time for the outer membrane of the eggs to get hardened and resistant to low pH. This observation is further supported by research conducted by Cykowska (1972) who reported that soon after fertilization, the egg membrane strengthens by seven-folds in the first 12 hours and by 15 times after 24 hours, forming a solid resistance barrier to protect the developing embryo from the external surrounding.

Proteinase K: Consistent with the results obtained for trypsin and pineapple juice treatment, proteinase K was also found ineffective at all concentrations tested. Proteinase K is a highly active serine protease whose predominant cleavage site is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked α-amino groups (Pähler et al., 1984).

The lack of scientific knowledge on the composition of Ballan wrasse egg's protein layer does not allow this study to speculate on the ineffectiveness of these enzymes further. Also, to the best of our knowledge, the proteinase K has not been reported as an effective agent for eliminating eggs' stickiness. Rather it is found to be utilized in the preparation of biological macromolecules by degrading the constitutive proteins of cells and removing nucleases such as DNases and RNases (Bajorath et al., 1988). Moreover, sodium dodecyl sulfate (SDS) and carbamide or buffer inclusion of 1 mM Ca^{2+} could be added to proteinase K to boost the enzymatic activity. Also, the optimal temperature range for proteinase K is between 25–65°C (Farrell, 2010), while this investigation was conducted at 9.5 °C, which could be a possible explanation for such a strong protease serine to be ineffective in the time and dosage applied.

5.2 Larval quality from the treated eggs

The measurements of hatched larvae did not reveal any major variations among treatments, except for the alcalase treatment and trypsin 20U/ml treatment, achieving the lowest myotome height and eye-to-body area, respectively (Table.6). The master thesis of Stavrakaki (2013) reported a myotome height of 0.24 ± 0.01mm (3dph), which remained in a similar range until 15dph. Similarly, for Dunaevskaya (2010), the myotome height was 0.226 ± 0.011mm (2dph), gradually increasing as the larvae grow. Similar to the myotome height observed in the current study, which is in the range of 0.219-0.229mm (Table 6), except for alcalase. The myotome height observed for the larvae treated with alcalase was significantly lower compared to the control treatment by 0.021mm, indicating the effect of alcalase treatment on the larvae. A reduced eye area (approximately 0.037mm^2) was observed by the trypsin 20U/ml treatment compared to other treatments in the current study, falling in the range of 0.046-0.051mm^2 (Table.6). Thereby, achieving the lowest eye to body ratio, which significantly differed from all the other treatments. The reduced eye size has also been reported for zebrafish larvae upon exposure of various chemical (Baumann et al., 2016).

The larval size of newly hatched larvae had been reported in other research; 2.75-3 mm (Fives, 1976), 2.7 ± 0.2 mm (TL) (Artuz, 2005), 3.72 ± 0.13 mm (TL) (D'Arcy et al., 2012), 3.64 ± 0.05 mm (Ottesen et al., 2012), while Dunaevskaya (2010) reported 3.64 ± 0.1mm (SL). In contrast, the current study attained TL of 4.35 ± 0.13mm and SL of 4.14 ± 0.12mm for the control treatment,

much larger than the other studies mentioned. However, the present finding illustrates no significant variation in standard and total length between the treatments. Moreover, the variations in morphological features (e.g. yolk sac size, length, eye diameter) of the larvae has an ecological importance and is proposed to be a "bet-hedging strategy" by parents to safeguard part of their offspring to survive in a varying food environment, which is by fluctuating capabilities of either feeding immediately at hatch or having sufficient endogenous reserves to endure until food becomes available (Laurel et al., 2008).

Malformation of the larvae body was evident in newly hatched larvae even if the embryonic development was undertaken in unpolluted, well-oxygenated water and at an optimal temperature (Jezierska at al., 2000). A comprehensive study on the malformation in newly hatched Ballan wrasse larvae is lacking. A master's study conducted by Shchepak (2011) summarized some of the deformities observed at different temperatures and salinities, where it was reported up to 90% of the newly hatched larvae at 10^{o}C had deformities with the occurrence of yolk sac mild edema at 4.59% and severe edema 5.74%. In contrast, in the current study, a high occurrence of mild edema was recorded, ranging from <10% in control to >52% in papain treatments (Table.7). The development of yolk sac edema has also been reported on exposure to various chemicals; for example, exposure to valproic acid and nine of its analogues inducing pericardial and yolk sac edema in zebrafish (*Danio rerio*) embryos (Brotzmann et al., 2021). Edema is also caused by suboptimal rearing conditions, especially when the temperature is unfavourable (Ottesen and Bolla, 1998). Deformities might also be caused by disturbance occurring at the early developmental stages or may develop during laborious hatching (Jezierska et al., 2000).

Besides the high occurrence of mild edema by the enzymatic treatments, spinal deformities were also reported but in low proportions, with tail flexures <7.1%, curved spine <7.2% and breaks (only registered in alcalase treatment around 22%) (Table.6). The emergence of break deformity only in alcalase could be due to the exposure time or human error when placing the larvae on the gel for photography. The human error default did not get tracked as a similar procedure was followed for all the treatments. It is probable to induce deformities upon exposing the developing

eggs to chemical or protease enzymes for making them non-adhesive. However, an optimized protocol could lower the side effects significantly. In an attempt to chemically degum the eggs of channel catfish, using 0.2% papain + 1.5% Na2SO3, approximately 13% of deformities were registered, which increased up to 20% when treated with 0.2% papain + 1.5% $_{L}$-cysteine-HCL (Ringle et al., 1992). Deformities do not always appear as a response to any treatment but could naturally prevail in the newly hatched larvae but appear in low proportions (Jezierska et al., 2000). However, a high number of deformities observed in the present study are potentially a response to the application of the enzymatic treatments applied.

5.3 Importance of degumming

Another challenge faced during the incubation of the benthic eggs is the proximity of spawned eggs with the biological material (fecal matter and food waste) which in turn leads to excessive bacterial and fungal loads. In Figure 24b, the enhanced microbial activity can be seen, mainly surrounding the dead eggs. Also, it is reported that the quick growth of opportunistic bacteria and water mould on dead eggs sequentially affects the viable eggs (Davenport, 1983) (Fig.22a). The sticky layer covering the egg chorion is particularly vulnerable to viral, fungal, and bacterial pathogens and removal of this layer diminishes the activity of these pathogens (Krise et al., 1986). Degummed eggs further increase the possibility to adapt to various types of upwelling incubation systems that maintain eggs in suspensions, such as McDonald type jars, pelagic egg jars, and Imhoff cones (Jensen et al., 2008). Having only non-adhesive eggs during incubation increases the opportunity to manually remove non-viable eggs. For that purpose, several techniques are available, e.g., manual siphoning, flotation methods (Leitritz and Lewis, 1976) and automatic egg sorters, which could be highly effective at separating substantial quantities of eggs provided that they are not clumped together.

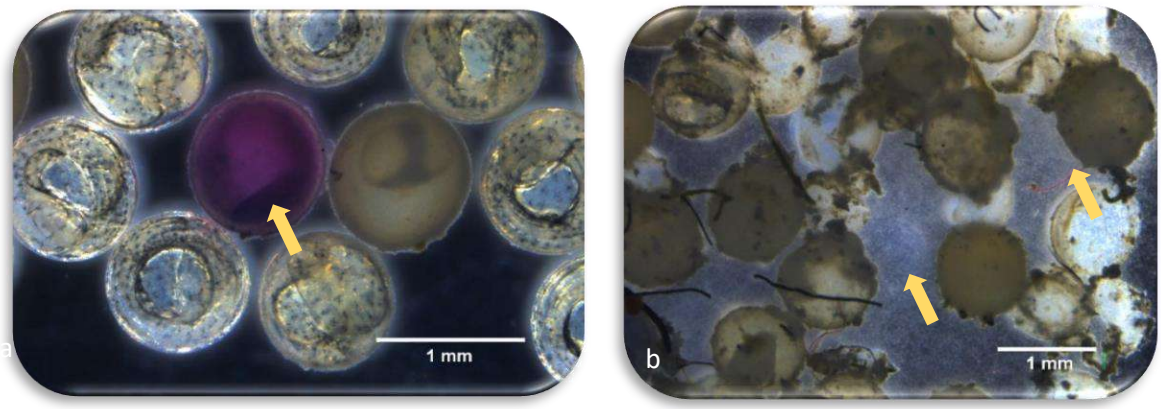


Figure 24: a) Infected egg (pink colour) observed at 7 days post-incubation b) Enhanced microbial activity on eggshell observed at 8 days post-incubation.

6. Conclusion

In summary, we tested various enzymes with a potential to eliminate the stickiness of the Ballan wrasse eggs. Besides the proven degumming action of alcalase, the study discovered that papain from papaya latex also degums Ballan wrasse eggs. A 30-minute exposure to papain treatments (50, 10, 5U/ml) liberated >77% from the mats. Further assessment of the detached eggs revealed that >80% of the eggs were without gum layer, reaching >90% for the papain 10 U/ml treatment. In comparison, the alcalase freed >68% of the eggs from the mats of which >59% were degummed eggs. This outcome placed the papain above the alcalase in effectiveness to degum when subjected to 30 minutes of treatment exposure.

The hatching success revealed no significant differences among the enzymatic treatments. Morphological measurements of newly hatched larvae also showed no significant difference among the treatments, except for alcalase treatments obtaining significant difference by lowest myotome height and the highest eye-to-body ratio. The elimination of egg stickiness came at the cost of mild edema present in all the treatments, especially for the papain treatments, which were significantly different from the control.

Egg degumming can overcome many challenges related to high mortality and low egg survival by giving more control during the incubation, disinfection, and overall handling of the eggs. Moreover, it can facilitate the removal of non-viable and infected eggs by utilizing advanced

incubators that save incubation space and reduce the labour time compared to the current protocols.

7. Recommendation

Based on the present study results, it is recommended to the eliminate the eggs stickiness in Ballan wrasse to improve the incubation phase disinfection and handling. This will not only lower the mortality but also increase the hatching success, by achieving efficient disinfection along with reduced bacterial/parasitic activity on eggs, which in turn is essential to meet the sustainability goals for a growing cleaner fish market. Incubating the Ballan wrasse eggs with papain from papaya latex for 30 minutes led to >77% detachment of the eggs from the mats. Also, the hatching success of the treated eggs was not considerably different from the control group. The papain-treatment caused symptoms of edema in > 52% of the hatched larvae. However, further studies are still needed to observe the efficacy of the papain enzyme and the short and long-term effects of this methodology before taking it to commercial production.

8. Future research

Future research is required to describe the chemical composition of egg gum matrix causing adhesion in Ballan wrasse eggs and assess the possible impacts of applying enzymatic treatments before/after fertilization in naturally spawned eggs versus manually stripped eggs. An investigation of hardening chorion time is also required, as the hardened chorion could better resist the application of enzyme than the soft egg. Besides exploring other potential enzymes, the evident degumming by alcalase and papain (papaya latex) should be further optimized in dosage rate and duration of exposure. Papain from papaya latex should be inspected for insoluble material and should be separated from the working solution via sieving or decantation before use. Papain from *C. papaya* could also be tested in the presence of an activator (cysteine) to quantify the degumming action. A combination of these enzymes could also be tested, where the eggs are exposed to treatments subsequently for a minimum time. Lastly, a cost-benefit analysis

for a commercial scale use of papain from papaya latex needs to be analysed along with the assessment of long-term effects on the development of functional juveniles.

9. References

Artuz, M. 2005. Embryonic and larval development of the ballan wrasse *Labrus bergylta*, Ascanius, 1767. *Hidrobiologica Publications Scientifiques,* 2, 98-101.

Bajorath, J., Hinrichs, W. and Saenger, W. 1988. The enzymatic activity of proteinase K is controlled by calcium. *Eur J Biochem,* 176, 441-447.

Baumann, L., Ros, A., Rehberger, K., Neuhauss, S. C. F. and Segner, H. 2016. Thyroid disruption in zebrafish (*Danio rerio*) larvae: Different molecular response patterns lead to impaired eye development and visual functions. *Aquat. Toxicol,* 172, 44-55.

Blanco Gonzalez, E. and De Boer, F. 2017. The development of the Norwegian wrassefishery and the use of wrasses as cleaner fish in the salmon aquaculture industry. *Fish Sci,* 83, 661-670.

Bone, A., Bekaert, M., Papadopoulou, A., Mcmillan, S., Adams, A., Davie, A. and Desbois, A. P. 2021. Bacterial Communities of Ballan Wrasse (*Labrus bergylta*) eggs at a Commercial Marine Hatchery. *Curr. Microbiol,* 78, 114-124.

Braia, M., Ferrero, M., Rocha, M. V., Loureiro, D., Tubio, G. and Romanini, D. 2013. Bioseparation of papain from Carica papaya latex by precipitation of papain-poly (vinyl sulfonate) complexes. *Protein Expr Purif,* 91, 91-95.

Brotzmann, K., Wolterbeek, A., Kroese, D. and Braunbeck, T. 2021. Neurotoxic effects in zebrafish embryos by valproic acid and nine of its analogues: the fish-mouse connection? *Arch. Toxicol,* 95, 641-657.

Cocchiaro, J. L. and Rawls, J. F. 2013. Microgavage of zebrafish larvae. *J. Vis. Exp : JoVE*, e4434-e4434.

Costello, M. J. 1991. Review of the biology of wrasse (Labridae: Pisces) in Northern Europe. 16, 29-51.

Coulombe, R., Grochulski, P., Sivaraman, J., Ménard, R., Mort, J. S. and Cygler, M. 1996. Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. *EMBO Rep,* 15, 5492-5503.

Coward, K., Bromage, N. R., Hibbitt, O. and Parrington, J. 2002. Gamete physiology, fertilization and egg activation in teleost fish. *Rev. Fish Biol. Fish,* 12, 33-58.

Cykowska, C. 1972. Embryonic development of the Baltic seatrout (*Salmo trutta*) in buffer solutions. *Acta Ichthyol Piscat,* 2, 3-13.

D'Arcy, J., Dunaevskaya, E., Treasurer, J. W., Ottesen, O., Maguire, J., Zhuravleva, N., Karlsen, A., Rebours, C. and Fitzgerald, R. D. 2012. Embryonic development in ballan wrasse *Labrus bergylta*. *J. Fish Biol,* 81, 1101-1110.

Darwall, W. R. T., Costello, M. J., Donnelly, R. and Lysaght, S. 1992. Implications of life-history strategies for a new wrasse fishery. *J. Fish Biol,* 41, 111-123.

Davenport, J. 1983. Oxygen and the developing eggs and larvae of the lumpfish, *Cyclopterus lumpus*. *J. Mar. Biolog. Assoc,* 63, 633-640.

Deady, S., Varian, S. and Fives, J. 1995. The use of cleaner-fish to control sea lice on two Irish salmon (*Salmo salar*) farms with particular reference to wrasse behavior in salmon cages. *Aquac,* 131, 73-90.

Dickson, S. R. and Bickerstaff, G. F. 1991. Pineapple bromelain and protein hydrolysis. *J. Biol. Educ,* 25, 164-166.

Dipper, F. A., Bridges, C. R. and Menz, A. 1977. Age, growth and feeding in the ballan wrasse *Labrus bergylta* Ascanius 1767. *J. Fish Biol,* 11, 105-120.

Dubey, R., Reddy, S. and Murthy, N. Y. S. 2012. Optimization of activity of Bromelain. *Asian J. Chem,* 24, 1429-1431.

Dunaevskaya, E. 2010. Histological investigations of organs and tissues development of ballan wrasse larvae during ontogenesis. Master of science thesis. Faculty of Biosciences and Aquaculture. Norway: Bodo University College.

Espeland, S. H. 2010. *Kunnskapsstatus leppefisk : utfordringer i et økende fiskeri (Knowledge status of wrasse: challenges in increasing fishing),* Bergen: Institute of Marine Research.

Fani, F. and Sri, W. M. 2019. The influence of papain and bromelain enzymes on hatching rate and hatchability of (*Oreochromis niloticus*) eggs. *Russ. j. agric. soc,* 96.

Farrell, R. E. 2010. Chapter 7 - Resilient Ribonucleases. *In:* Farrell, R. E. (ed.) *RNA Methodologies (Fourth Edition).* San Diego: Academic Press.

Fives, J. M. 1976. Labridae of the eastern North Atlantic, Fich. Ident. Zooplancton 149: 7 pp

Florence, M. and Ezekiel, A. 2012. Papain, a plant enzyme of biological importance: A Review. *Am J Biochem Biotechnol,* 8.

Gao, Y., Kim, S. G. and Lee, J. Y. 2011. Effects of pH on Fertilization and the Hatching Rates of Far Eastern Catfish *Silurus asotus*. *Fisheries and aquatic sciences*, 14.

Gela, D., Linhart, O., Flajšhans, M. and Rodina, M. 2003. Egg incubation time and hatching success in tench *Tinca tinca* related to the procedure of egg stickiness elimination. *J. Appl. Ichthyol,* 19, 132-133.

Grant, B., Davie, A., Taggart, J. B., Selly, S. L. C., Picchi, N., Bradley, C., Prodohl, P., Leclercq, E. and Migaud, H. 2016a. Seasonal changes in broodstock spawning performance and egg quality in ballan wrasse (*Labrus bergylta*). *Aquac,* 464, 505-514.

Grant, B., Picchi, N., Davie, A., Leclercq, E. and Migaud, H. 2016b. Removal of the adhesive gum layer surrounding naturally fertilised ballan wrasse (*Labrus bergylta*) eggs. *Aquac,* 456.

Grefsrud, E. S., Karlsen, Ø., Kvamme, B. O., Glover, K., Husa, V., Hansen, P. K., Grøsvik, B. E., Samuelsen, O., Sandlund, N., Stien, L. H. and Svåsand, T. 2019. Risikorapport norsk fiskeoppdrett 2019: miljøeffekter av lakseoppdrett (Risk assessment of Norwegian fin fish aquaculture 2019: Environmental impact of aquaculture).

Groner, M., Cox, R., Gettinby, G. and Revie, C. 2013. Use of agent-based modelling to predict benefits of cleaner fish in controlling sea lice, *Lepeophtheirus salmonis*, infestations on farmed Atlantic salmon, *Salmo salar*. *J. Fish Dis,* 36(3), 195-208.

Halvorsen, K., Larsen, T., Sørdalen, T., Vøllestad, L., Knutsen, H. and Olsen, E. 2017. Impact of harvesting cleaner fish for salmonid aquaculture assessed from replicated coastal marine protected areas. *Mar. Biol. Res,* 13.

Hamre, L., Glover, K. and Nilsen, F. 2009. Establishment and characterisation of salmon louse (*Lepeophtheirus salmonis* (Krøyer 1837)) laboratory strains. *Parasitol. Int,* 58, 451-60.

Helland, S., Dahle, S., Hough, C. and Borthen, J. 2014. *Production of ballan wrasse (Labrus bergylta). Science and Practice.* Eliminating egg stickiness Stripped ballan wrasse eggs. Fiskeri - og havbruksnæringens forskningsfond (FHF)

Hjeltnes, B., Jensen, B. B., Bornø, G., Haukaas, A. and Walde, C. S. 2018. Fiskehelserapporten (Fish health situation in Norwegian aquaculture 2018).

Homaei, A. A., Sajedi, R. H., Sariri, R., Seyfzadeh, S. and Stevanato, R. 2010. Cysteine enhances activity and stability of immobilized papain. *Amino Acids,* 38, 937-942.

Ingólfsson, A., Kristjánsson, B. and Schaefer, S. 2002. Diet of juvenile Lumpsucker *Cyclopterus lumpus* (Cyclopteridae) in floating seaweed: effects of ontogeny and prey availability. *Copeia*, 472-476.

Isaac, J. and Fries, L. T. 1991. Separation of channel catfish eggs in sodium sulfite with and without papain. *Prog. Fish-Cult,* 53, 200-201.

Iwamatsu, T., Yoshizaki, N. and Shibata, Y. 1997. Changes in the chorion and sperm entry into the micropyle during fertilization in the teleostean fish, *Oryzias latipes*. *Dev. Growth Differ,* 39, 33-41.

Jensen, N. R., Ireland, S. C., Siple, J. T., Williams, S. R. and Cain, K. D. 2008. Evaluation of egg incubation methods and larval feeding regimes for North American Burbot. *N. Am. J. Aquac,* 70, 162-170.

Jezierska, B., Lugowska, K., Witeska, M. and Sarnowski, P. 2000. Malformations of newly hatched common carp larvae. *Electron. J. Pol. Agric. Univ,* 3.

Johansson, N and Milbrink, G. 1976. Some effects of acidified water on the early development of roach (*Rutilus rutilus* L.) and perch (*Perca fluviatilis* L.)1. *JAWRA Journal of the American Water Resources Association*, 12, 39-48.

Kelly, N. I., Alzaid, A., Nash, G. W. and Gamperl, A. K. 2014. Metabolic depression in cunner (*Tautogolabrus adspersus*) is influenced by ontogeny, and enhances thermal tolerance. *PLoS One,* 9, e114765.

Khan, M. A. R., T.C. Moreira, F., Riu, J. and F. Sales, M. G. 2016. Plastic antibody for the electrochemical detection of bacterial surface proteins. *Sens. Actuators B Chem,* 233, 697-704.

Kim, K. H., Moon, H. N., Noh, Y. H. and Yeo, I. K. 2020. Influence of Osmolality and Acidity on Fertilized Eggs and Larvae of Olive Flounder (*Paralichthys olivaceus*). *Development & reproduction*, 24, 19-30.

Krise, W. F., Bulkowski-Cummings, L., Shellman, A. D., Kraus, K. A. and Gould, R. W. 1986. Increased walleye egg hatch and larval survival after protease treatment of eggs. *Progress Fish Cult,* 48, 95-100.

Kvenseth, P. G., Bjelland, R. M. and Kvenseth, A. M. 1996. Berggylte som lusespiser hos stor laks. *Norsk Fiskeoppdrett, 22,* 38-42

Kvenseth, P. G., Kvenseth, P. G. and Andreassen, J. 2003. Berggylt - en sterk medisin. *Norsk fiskeoppdrett 12A,* 17-20

Laurel, B. J., Hurst, T. P., Copeman, L. A. and Davis, M. W. 2008. The role of temperature on the growth and survival of early and late hatching Pacific cod larvae (*Gadus macrocephalus*). *J. Plankton Res,* 30, 1051-1060.

Legendre, M., Linhart, O. and Billard, R. 1996. Spawning and management of gametes, fertilized eggs and embryos in Siluroidei. *Aquat. Living Resour,* 9, 59-80.

Leitritz, E. and Lewis, R. C. 1976. *Trout and Salmon Culture (Hatchery Methods).* UC San Diego: Library – Scripps Digital Collection. 61-64

Linhart, O., Gela, D., Duda, P., Rodina, M. & Novák, V. 2000. Alcalase enzyme treatment for elimination of egg stickiness in tench, *Tinca tinca* L. *Aquac,* 191, 303-308.

Linhart, O., Rodina, M., Gela, D., Flajšhans, M. and Kocour, M. 2003a. Enzyme treatment for elimination of egg stickiness in tench (*Tinca Tinca* L.), European catfish (*Silurus Glanis* L.) and common carp (*Cyprinus Carpio* L.). *Fish Physiol. Biochem,* 28, 507-508.

Linhart, O., Rodina, M., Gela, D., Kocour, M. and Rodriguez, M. 2003b. Improvement of common carp artificial reproduction using enzyme for elimination of egg stickiness. *Aquat. Living Resour,* 16, 450-456.

Linhart, O., Štěch, L., Švarc, J., Rodina, M., Audebert, J., Grecu, J. and Billard, R. 2002. The culture of the European catfish, *Silurus glanis*, in the Czech Republic and in France. *Aquat. Living Resour,* 15, 139-144.

Lönning, S., Kjörsvik, E. and Davenport, J. 1984. The hardening process of the egg chorion of the cod, *Gadus morhua* L., and lumpsucker, *Cyclopterus lumpus* L. *J. Fish Biol,* 24, 505-522.

Macalood, J., Vicente, H., Boniao, R., Gorospe, J. & Roa, E. 2013. Chemical analysis of *Carica papapya* L. crude latex. *American Journal of Plant Sciences,* 04**,** 1941-1948

Mansour, N., Lahnsteiner, F. and Patzner, R. 2008. Physiological and biochemical investigations on egg stickiness in common carp. *Anim. Reprod. Sci,* 114, 256-268.

Mansour, N., Lahnsteiner, F. and Patzner, R. A. 2009. Ovarian fluid plays an essential role in attachment of Eurasian perch, *Perca fluviatilis* eggs. *Theriogenology,* 71, 586-593.

Marion, G. M., Millero, F. J., Camões, M. F., Spitzer, P., Feistel, R. and Chen, C. T. A. 2011. pH of seawater. *Mar. Chem,* 126, 89-96.

Mohammadi, Z., Shalavi, S. and Jafarzadeh, H. 2013. Ethylenediaminetetraacetic acid in endodontics. *Eur J Dent,* 7, s135-s142.

Mordue, A. J. and Birkett, M. A. 2009. A review of host finding behaviour in the parasitic sea louse, *Lepeophtheirus salmonis* (Caligidae: Copepoda). *J. Fish Dis,* 32, 3-13.

Muncaster, S., Norberg, B. and Andersson, E. 2013. Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *J Fish Biol,* 82, 1858-1870.

Myksvoll, M. S., Sandvik, A. D., Albretsen, J., Asplin, L., Johnsen, I. A., Karlsen, Ø., Kristensen, N. M., Melsom, A., Skardhamar, J. and Ådlandsvik, B. 2018. Evaluation of a national operational salmon lice monitoring system - from physics to fish. *PLoS One,* 13, e0201338.

Norwegian Directorate of Fisheries. 2020. *Matfiskproduksjon av laks, regnbueørret og ørret (Food fish production of salmon, rainbow trout and trout)* [Online]. Available: https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Akvakulturstatistikk-tidsserier/Laks-regnbueoerret-og-oerret/Matfiskproduksjon [Accessed 10 September 2021].

Neitali, B., Amiri, B., Kalbassi, M. and Nouri, A. 2014. A Comparative Study on the effect of trypsin treatment and clay method on jelly coat removal of the persian sturgeon (*Acipenser persicus*) ova. *Journal of Fisheries, Iranian Journal of Natural Resources,* 67, 109-122.

Olaussen, J. O. 2018. Environmental problems and regulation in the aquaculture industry. Insights from Norway. *Mar. Policy,* 98, 158-163.

Ottesen, O. H. and Bolla, S. 1998. Combined effects of temperature and salinity on development and survival of Atlantic halibut larvae. *Aquac Int,* 6, 103-120.

Ottesen, O. H., Dunaevskaya, E. and D'arcy, J. 2012. Development of *Labrus bergylta* (Ascanius 1767) larvae from hatching to metamorphosis. *J. Aquac. Res. Dev,* 3.

Ottesen, O. H., Treasurer, J., Fitzgerald, R., D'arcy, J., Maguire, J., O'neill, D., Rebours, C. and Al, E. 2011. Ecofish: rearing ballan wrasse for sea lice control. *Fish Farmer Magazine.*

Pähler, A., Banerjee, A., Dattagupta, J. K., Fujiwara, T., Lindner, K., Pal, G. P., Suck, D., Weber, G. and Saenger, W. 1984. Three-dimensional structure of fungal proteinase K reveals similarity to bacterial subtilisin. *EMBO Rep,* 3, 1311-1314.

Paterson, E. and Nwachi, O. 2014. Use of pineapple juice in the elimination of egg stickiness in mudfish (*Heterobranchus bidorsalis*). *Journal of Global Bio sciences and Biotechnology,* 3, 161-163.

Quignard, J.-P. and A. Pras, 1986. Labridae. In P.J.P. Whitehead, M.-L. Bauchot, J.-C. Hureau, J. Nielsen and E. Tortonese (eds.) Fishes of the north-eastern Atlantic and the Mediterranean. UNESCO, Paris. Vol. 2. 919-942

Ringle, J. P., Nickum, J. G., & Moore, A. 1992. Chemical separation of channel catfish egg masses. *The Progressive Fish-Culturist,* 54, 73-80.

Sarker, P. K., Talukdar, S., Deb, P., Sayem, S. M. A. and Mohsina, K. 2013. Optimization and partial characterization of culture conditions for the production of alkaline protease from *Bacillus licheniformis* P003. *SpringerPlus,* 2, 506.

Shchepak, L. 2011. Effects of different temperatures and salinities on development of fertilized eggs of Ballan wrasse. Master of Science thesis. Faculty of Biosciences and Aquaculture. University of Nordland

Shibata, Y., Iwamatsu, T., Oba, Y., Kobayashi, D., Tanaka, M., Nagahama, Y., Suzuki, N. and Yoshikuni, M. 2000. Identification and cDNA cloning of alveolin, an extracellular metalloproteinase, which induces chorion hardening of medaka (*Oryzias latipes*) eggs upon fertilization. *J. Biol. Chem,* 275, 8349-8354.

Southern Regional Aquaculture Center 2012. *Development Of Baitfish, Goldfish, And Ornamental Fish Hatchery Methods.* Stoneville.
Available:https://srac.msstate.edu/pdfs/Research%20Pubs/Hatchery%20Methods.pdf

Stavrakaki, M. G. 2013. Ballan wrasse (*Labrus bergylta*) larvae and live-feed quality; effects on growth and expression of genes related to mitochondrial functions. Master of Science. Department of Biology. Norwegian University of Science and Technology.

Stenberg, J. A., Sundh, I., Becher, P. G., Björkman, C., Dubey, M., Egan, P. A., Friberg, H., Gil, J. F., Jensen, D. F., Jonsson, M., Karlsson, M., Khalil, S., Ninkovic, V., Rehermann, G., Vetukuri, R. R. and Viketoft, M. 2021. When is it biological control? A framework of definitions, mechanisms, and classifications. *J Pest Sci,* 94, 665-676.

Stoorvogel, W., Geuze, H. J., Griffith, J. M., Schwartz, A. L. and Strous, G. J. 1989. Relations between the intracellular pathways of the receptors for transferrin, asialoglycoprotein, and mannose 6-phosphate in human hepatoma cells. *Int. J. Cell Biol,* 108, 2137-2148.

Thai, B. and Ngo, T. 2004. Use of pineapple juice for elimination of egg stickiness of common carp (*Cyprinus carpio* L.). *Asian Fish. Sci,* 17, 159-162.

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O., Nilsen, F., Horsberg, T. and Jackson, D. 2013. Salmon lice - impact on wild salmonids and salmon aquaculture. *J. Fish Dis,* 36.

Treasurer, J. 2012. Diseases of north European wrasse (Labridae) and possible interactions with cohabited farmed salmon, *Salmo salar* L. *J. Fish Dis,* 35, 555-562.

Treasurer, J. 2013. Use of wrasse in sea lice control. Report to Scottish Aquaculture Research Forum (SARF). Sarf068

Treasurer, J. W. 2018. *Cleaner fish biology and aquaculture applications.* Available at: https://www.perlego.com/book/2014531/cleaner-fish-biology-and-aquaculture-applications-pdf

Treves-Brown, K. M. 2000. *Applied Fish Pharmacology*, Springer, Dordrecht.

Verma, S., Dixit, R. and Pandey, K. C. 2016. Cysteine proteases: modes of activation and future prospects as pharmacological targets. *Front. Pharmacol,* 7.

Vollset, K. W., Krontveit, R. I., Jansen, P. A., Finstad, B., Barlaup, B. T., Skilbrei, O. T., Krkošek, M., Romunstad, P., Aunsmo, A., Jensen, A. J. and Dohoo, I. 2016. Impacts of parasites on marine survival of Atlantic salmon: a meta-analysis. *Fish and Fisheries,* 17, 714-730.

Yamagami, K., Hamazaki, T. S., Yasumasut, S., Masuda, K. and Luchi, I. 1992. Molecular and Cellular Basis of Formation, Hardening, and Breakdown of the Egg Envelope in Fish. In: Jeon, K. W. and Friedlander, M. (eds.) *International Review of Cytology*.

Yamamoto, T. S. 1956. Digestion of egg envelopes and their chemical properties of the Lamprey's egg, *Lampetra japonica*.

Appendices

Appendix 1. Solution preparation

Table 1. Amount of enzyme added to 300ml seawater to achieve the desired concentration for each replicate for the main experiment.

Treatments	Specific activity	Stock solution	Volume of enzyme for 300ml solution	Achieved Concentration
Alcalase® Enzyme, *Bacillus licheniformis*	≥0.75 Anson units/mL	-	6ml	2%
Trypsin, Bovine pancreas	≥7,500 BAEE units/mg solid	400mg in 100 ml Seawater	20ml	2000 U/ml
			2ml	200 U/ml
			0.2ml	20 U/ml
Papain, Papaya latex	2.9 U/mg	27g in 157ml Seawater	30ml	50 U/ml
			6ml	10 U/ml
			3ml	5 U/ml
Pineapple juice (Bromelain)	-	100%	48ml	16%
			24ml	8%
			12ml	4%
Proteinase K, *Tritirachium album*	30.0 mAnsonU/mg	80mg in 100ml Seawater	11.25ml	900 µAu/ml
			8.75ml	700 µAu/ml
			5ml	400 µAu/ml
Acidic seawater	-	-	using 2M-NaoH	pH 3.38

Appendix 2. Egg counted on mats before and after exposure to enzymatic treatments.

Table.2: Number of eggs on the mat piece before and after the enzymatic exposure of 30 min with the percentage of total eggs detached by the treatments in the main experiment.

Enzymes	Replicates	No. of eggs on mat before treatment	No. of eggs on mat after treatment	No. of eggs detached after 30 min of exposure	% Of eggs detached from the mats upon exposure (Mean±SD)
Alcalase	**H1**	335	77	255	68 ± 8.2
	H2	146	53	88	
	H3	358	134	221	
	H4	166	43	123	
T 2000U	**H5**	180	97	14	12 ± 7.9
	H6	90	27	21	
	H7	159	90	17	
	H8	324	216	18	

T 200U	**H9**	242	195	5	2 ± 1.3
	H10	136	82	2	
	H11	251	182	0	
	H12	167	109	5	
T 20U	**H13**	375	264	1	1 ± 0.7
	H14	243	171	1	
	H15	109	45	0	
	H16	125	63	2	
Pap 50U	**I1**	98	7	88	88 ± 5.7
	I2	57	12	45	
	I3	173	14	156	
	I4	170	15	155	
Pap 10U	**I5**	69	14	52	78 ± 9.9
	I6	146	22	124	
	I7	118	15	101	
	I8	110	34	71	
Pap 5U	**I9**	71	8	63	84 ± 6.6
	I10	91	13	75	
	I11	183	40	139	
	I12	137	12	124	
Pro 900U	**I13**	158	98	6	14 ± 10
	I14	113	52	8	
	I15	85	30	15	
	I16	100	28	26	
Pro 700 U	**J1**	241	122	17	5 ± 2.3
	J2	113	44	8	
	J3	214	97	7	
	J4	230	130	7	
Pro 400U	**J5**	200	115	5	2 ± 0.7
	J6	122	80	2	
	J7	211	118	6	
	J8	317	194	4	
Pine 16%	**J9**	226	119	11	5 ± 1.6
	J10	192	79	10	
	J11	116	37	4	
	J12	271	160	20	
Pine 8%	**J13**	183	76	16	16 ± 7.3
	J14	230	116	42	
	J15	120	60	30	
	J16	88	35	10	
Pine 4%	**K1**	186	57	40	10 ± 7.8
	K2	245	117	22	
	K3	157	87	13	

	K4	282	126	9	
Acidic 3.38	**K5**	351	207	3	1 ± 0.3
	K6	109	30	1	
	K7	196	103	2	
	K8	319	200	5	
Control	**K9**	357	206	6	1 ± 0.6
	K10	174	98	2	
	K11	287	175	1	
	K12	170	76	3	

Appendix 3. Opaque solution of papain from papaya latex.

Figure 1: Papain solution from papaya latex with high amount of insoluble material making it opaque.

Appendix 4. Degummed eggs from the exposure of papain from papaya latex.

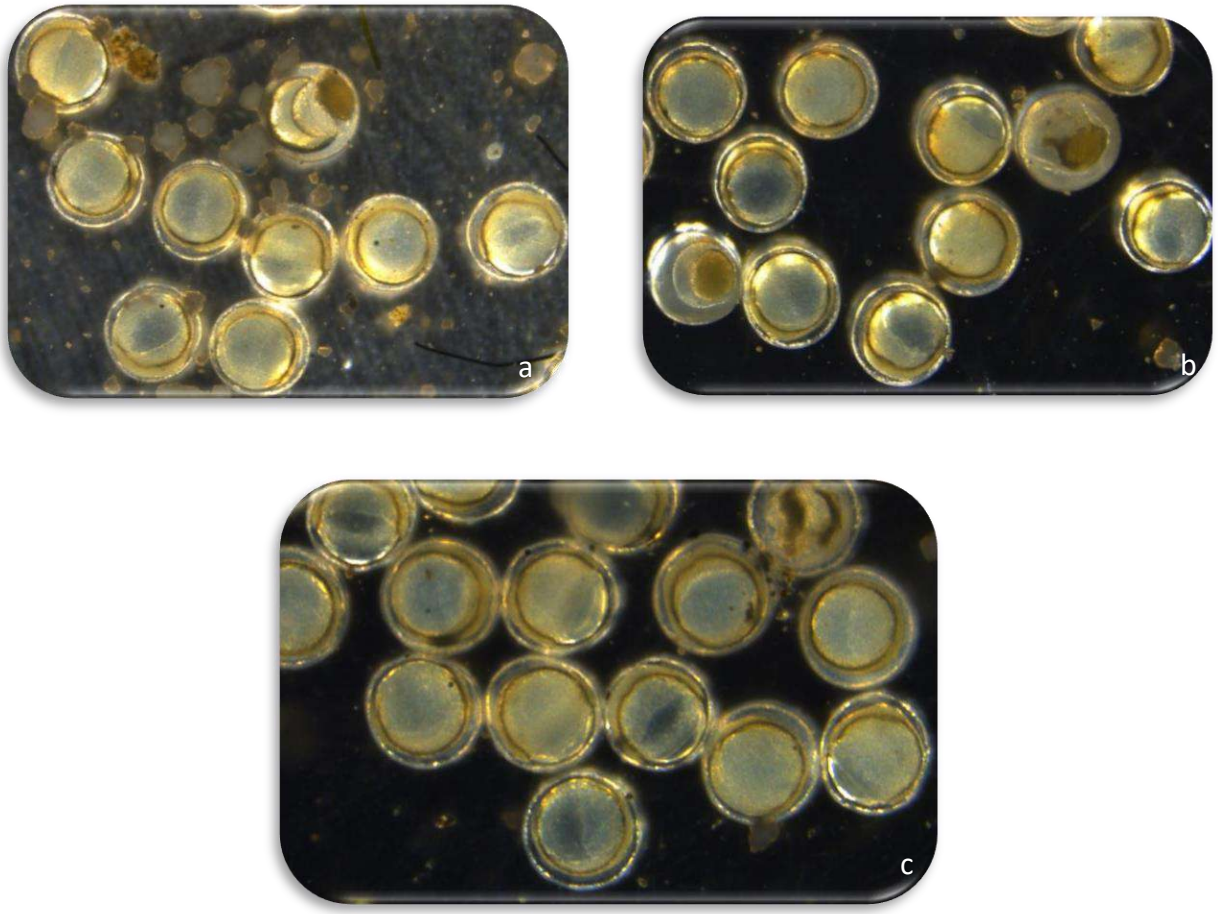


Figure 2: Degummed eggs from the exposure of papain from papaya latex for 30 minutes. a) Papain 50 U/ml, b) Papain 10 U/ml, c) Papain 5 U/ml.

NTNU

Norwegian University of
Science and Technology