Development of broodstock management and husbandry tools for improved hatchery performance of ballan wrasse (*Labrus bergylta*).

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Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Abstract

Cleaner fish, including ballan wrasse (*Labrus bergylta*) have been proposed as a sustainable solution to sea lice infestations affecting farmed Atlantic salmon (*Salmo salar*) globally. However, in order to become sustainable, ballan wrasse need to be farmed. This thesis investigated the establishment of captive broodstock and protocols to optimise hatchery performance and productivity of ballan wrasse. High throughput sequencing was used to develop a panel of novel single nucleotide polymorphic markers (SNPs). These SNPs were used to investigate the phylogeographic structuring of ballan wrasse populations within northern geographic ranges including the UK and Norway. Results indicated fine scale population structuring within the UK suggesting that founder broodstock should be sourced locally to minimise the risk of genetic introgression with wild ballan wrasse. Secondly, captive breeding was benchmarked from harems to determine total egg production over the spawning season. Data quantified the spawning periodicity and seasonal changes in egg quality parameters. In addition, microsatellite markers identified the parental contribution to each spawning event of captive broodstock. Results confirmed, for the first time, the repeat-batch spawning behaviour and suggested that spawning events were single-paired matings. Furthermore, bottlenecks in commercial production were investigated including the benthic adhesive eggs and complex spawning behaviours of ballan wrasse within broodstock tanks. Experiments were conducted to optimise the spawning dynamics and egg productivity using fragmented spawning zones and coloured substrates. Finally, an effective bath treatment for removal of the adhesive gum layer of eggs using the proteolytic enzyme alcalase® was found to assist in egg disinfection and incubation. Overall, this research provides important baseline data on the management of broodstock and the optimisation of hatchery protocols to improve the commercial productivity and performance of ballan wrasse for use as a biological control of sea lice of farmed Atlantic salmon.

Keywords: *Labrus bergylta*, cleaner fish, phylogeographic structure, broodstock management, benthic spawning, egg quality, behaviour, degumming

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List of Abbreviations

ARA Arachidonic acid

BW Body weight

C1 Control tank 1

C2 Control tank 2

D1 Dorset 1

DD Degree days

DHA Docosahexaenoic acid

DO Dissolved oxygen

DPF Days post-fertilization

D Tajima's D

EPA Eicosapentaenoic acid

FA Fatty acid

FAME Fatty acid methyl esters

F_{IS} Inbreeding coefficient

F_S Fu's Fs

F_{ST} Fixation index

GLT Gum layer thickness

H haplotype diversity

H_e Expected heterozygosity

H_o Observed heterozygosity

HWE Hardy-Weinberg equilibrium

ISI Interspawning interval

K clusters

KASP Kompetitive allele specific PCR

M1 Machrihanish 1

M2 Machrihanish 2

M3 Machrihanish 3

mtDNA Mitochondrial DNA

MTSS Mean total spawning score

N Number

NGS Next generation sequencing

N_H Number of haplotypes

OD Oocyte diameter

PCR Polymerase chain reaction

qPCR Quantitative PCR

RADseq Restriction site associated DNA sequencing

SD Standard deviation

SE Standard error

SNP Single nucleotide polymorphism

SNPP Simulated natural photoperiod

SP Spawning period

SW Spawning window

T1 Treatment tank 1

T2 Treatment tank 2

TL Total length

TSP Targeted SNP panel

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known parental genotypes within each spawning tank and assume that all female/male parent combinations were equally likely to occur. Numbers in brackets represent the potential different families possible, given the number of males and females present in each tank.

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1 Chapter 1: General Introduction

Chapter 1: General Introduction

1.1 The sea lice challenge

Commercial production of Atlantic salmon (*Salmo salar L.*) has increased substantially over the last two decades with annual global production exceeding 2 million tonnes in 2013 (FAO, 2016). The Scottish salmon industry accounted for over 163,000 tonnes of the 2013 global production and in 2014 increased by 9.7 % to its highest ever recorded production level of 179,022 tonnes (The Scottish Government, 2015; www.gov.scot). One of the main obstacles to the expansion of sea-cage based salmon production is salmon lice (*Lepeophtheirus salmonis K.* and *Caligus spp*), which are ectoparasites reported as the most damaging health threat to the salmon farming industry in all of Europe and the Americas since the 1970's (Rae, 2002; Costello, 2006). Sea lice infestations are a major threat to fish welfare and the overall profitability of salmon production (Gharbi et al., 2015). At a global level the salmon farming industry spends over US$ 480 million annually on sea lice mitigation, and in Scotland alone, estimates for the economic impact of sea lice range from 7-10 % of total production (Roth, 2000; Rae, 2002; Costello, 2009). However, these figures do not take into account the value of indirect production losses due to stress, reduced growth (Gharbi et al., 2015), or the potential implications lice may pose as vectors for transmission of other important fish pathogens such as infectious salmon anaemia virus (ISA) (Oelckers et al., 2014).

The life cycle of *L. salmonis*, the louse species of main concern in Europe, consists of eight successive developmental stages, two of which are the infectious, pelagic free swimming nauplii stages (Pike & Wadsworth, 1999; Heuch et al., 1995; Costello et al., 2006). Sea lice pathogenicity is caused by feeding on the mucus, tissue, and blood of salmonid hosts causing skin lesions, osmotic imbalance, reduced growth, secondary infections, and in severe cases, mortality (Wootten et al., 1982; Grimnes & Jackobsen, 1996; Johnson et al., 2004; Frazer et al., 2012). Although treatment thresholds differ slightly between countries, the Scottish Code of Good Practice for Scottish Finfish Aquaculture (CoGP, 2016) states that sea lice treatment must commence when levels at an individual cage site reach an average of 0.5 adult female *L. salmonis* per fish during the period of 1st February – 30th June and an average of 1.0 adult female *L. salmonis* per fish during the period of 1st July – 31st January. These criteria are measured through

weekly on site lice counts with a target to reduce the overall number of gravid (or egg bearing) female lice.

To remain below allowed lice levels, the effective mitigation, management, and control of sea lice infections require good husbandry practices in the form of integrated pest management practices (IPM) (Grant, 2002; Mordue & Pike, 2002) which include a range of treatment options and/or operational practices such as fallowing, year class separation and use of chemotherapeutants. Two main types of chemical treatments for lice are in use, separated by their administration route either via bath treatment or in-feed additives. Bath treatments include the use of chemicals such as pyrethroids, hydrogen peroxide, and organophosphates, while avermectins are administered as in-feed additives (Burridge et al., 2010; Aaen et al., 2015). These treatments, although effective, are ultimately released into the wider aquatic environment and may negatively affect native marine organisms in the area surrounding salmon cages (Johnston et al., 2004). Furthermore, misuse and overuse of chemical treatments has led to parasite resistance (Roth, 2000; Rae, 2002; Burridge et al., 2010; Igboeli et al., 2012) whereby many of the commonly used compounds are not 100% effective on lice due to reduced sensitivity of the parasite (Aaen et al., 2015).

Widespread resistance to many of the commercially available chemical treatments has led to the investigation and development of a range of alternative non-chemical methods of lice control. Alternative treatment solutions for sea lice include the development of vaccines, which have the potential to be a cost-effective method for lice control; however research into lice vaccines is still ongoing (Raynard et al., 2002). Also, selective breeding for lice resistant salmon has been identified as a potential alternative to chemical treatments; however, while preliminary studies are promising, this work is currently in the developmental stages (Gharbi et al., 2015). Other management techniques utilise specific behavioural characteristics of sea lice, such as its occurrence predominantly in the upper part of the water column (Heuche et al., 1995), to develop technologies that prevent or limit the physical contact of farmed salmon and lice. For example, snorkel cages are one new approach, where salmon are restricted from accessing the surface for swim bladder inflation except through a vertical chamber that is impermeable to sea lice larvae (Stien et al., 2016). Also in development are closed sea cages where ‘lice-free’ water is pumped in from the deep (Strand et al., 2013), along with the use of tarpaulin

"skirts" to wrap around the top part of cages to divert surface water (Frank et al., 2015), and sub-surface feeding and lights to entice salmon to deeper waters (Frenzl et al., 2014). Mechanisms for physically removing lice from the salmon include 'Optical delousing' using lasers which can detect and remove individual lice as the salmon swim past submerged laser units. Although this technology is still in development and not widely implemented, the goal is to use it as a continuous delousing method to keep baseline lice numbers down (Stingray Marine Solutions, http://en.stingray.no/page/6018/Optical_Delousing). While many alternatives for pest management are being developed, the use of cleanerfish is considered as the most effective and sustainable option in the short to medium term.

The use of cleaner fish for the biological control of sea lice has been gaining importance in recent years as it represents an environmentally friendly alternative to chemical treatments (Roth, 2000; Mordue & Pike, 2002; Rae, 2002). Various cleaner fish species such as wrasse (*Labridae*) or lumpfish (*Cyclopterus lumpus L.*) (Imsland et al., 2014) are introduced into salmon cages and swim among the salmon picking off and eating sea lice (Darwall et al., 1992). This delousing behaviour is believed to be less stressful to salmon than cumbersome bath treatments and potentially more effective than in-feed treatments as heavily infected fish are often non-feeding and may not obtain accurate chemical dosage (Deady et al., 1995; Costello, 2006). Cleaner fish are already implemented on many cage sites throughout the UK, Ireland and Norway; however the main limitation has been the use of wild caught fish. Thus, in order to increase the sustainability of the technology and to provide a continuous supply, recent industrial effort has been focused on commercial production of cleaner fish, with an initial focus on ballan wrasse (*Labrus bergylta*) along with more recent interest in lumpfish.

1.2 Cleanerfish in salmon farming

The use of wrasse as a biological control of sea lice was first demonstrated in Norway in 1987, where three species of wild caught wrasse, goldsinny (*Ctenolabrus repestris L.*), rock cook (*Centrolabrus exoletus L.*), and female cuckoo wrasse (*Labrus mixtus L.*), were shown to successfully remove sea lice from farmed salmon in a trial setting (Bjordal, 1990). The apparent efficacy of this method meant that it was rapidly implemented such that by 1994 there were over 130 salmon farms in Norway using a

total of 1.5 million wild caught mixed species wrasse while in Scotland approximately 30 farms were using 150,000 wild caught wrasse (Costello, 1996).

Following the initial interest and implementation of cleaner fish for delousing in the mid to late 90's, this solution was replaced with the development and release of new highly effective antiparasitic drugs (Skiftesvik et al., 2014). However, following the recent increase in parasite resistance to many of the chemical treatments (Lees et al., 2009), cleaner fish are widely considered to be the alternative treatment strategy which is closest to industry wide deployment. As such, there has been a resurgence in cleanerfish with numbers of wild caught wrasse (various species) being deployed surpassing 10 million on Norwegian farms alone in 2010 (Norwegian Directorate of Fisheries statistics, 2013). Such sustained fisheries pressure on the wild stocks is considered unsustainable in the long term, but also such a supply of animals could be considered a biosecurity risk for salmon farms (Skiftesvik et al., 2014). Thus, the salmon farming industry has recognised the need for a sustainable, disease free, supply of farmed cleaner fish as a vital production goal. As such, hatcheries have been established in the UK and Norway with the initial interest being mainly focused on ballan wrasse (D'Arcy et al., 2012; Skiftesvik et al., 2013); however, commercial interest has recently been gaining for lumpfish was well (Imsland et al., 2014).

The five main wrasse species commonly found in northern European waters include goldsinny, rock cook, cuckoo, corkwing (*Crenilabrus melops L.*), and ballan (Fig. 1.1). Goldsinny wrasse are small, typically ranging from 100 – 200 mm in length as adults (Sayer et al., 1995) and are orange- red in colour with a very distinctive black patch on the dorsal edge of the caudal peduncle (Sayer & Treasurer, 1996). Rock cook are a similar size to goldsinny, ranging between 100 – 140 mm in length and are typically red/brown on the dorsal side and lighter in colour on the ventral side (Quignard & Pras, 1986). Corkwing are typically larger than goldsinny and rock cook, with average lengths between 235 and 280 mm (Quignard & Pras, 1986; Darwall et al., 1992). Furthermore, corkwing wrasse are sexually dimorphic with females generally displaying a dull green or brown colour and males often red or brown with distinctive blue and red stripes on the head (Quignard & Pras, 1986). Cuckoo wrasse can reach a maximum length of approximately 350 mm (Darwall et al., 1992) and, similarly, show marked sexual dimorphism with mature adult females usually displaying a red-brown or orange colour

with dark and light patches along the dorsal edge and mature males typically orange – yellow in colour with bright blue stripes (Sayer & Treasurer, 1996). Finally, the ballan wrasse is the largest of the five common wrasse species local to European waters, which can reach a maximum length of 600 mm, although lengths of 300 – 500 mm are common (Quignard & Pras, 1986; Darwall et al., 1992).

Figure 1.1. Five common wrasse species found in northern European waters; top left, goldsinny; top right, ballan; middle left, rock cook; middle right, female cuckoo; bottom left, corkwing; bottom right, male cuckoo.

Of the five common wrasse species native to European waters, ballan wrasse are the fastest growing and the most robust (Sayer & Treasurer, 1996) and therefore the favoured cleaner fish species for the salmon farming industry. Ballan wrasse would be the least abundant of the common wrasse species, although data on wild populations are scarce to date, and local fisheries are clearly insufficient to meet the increasing demand for the aquaculture industry (Skiftesvik et al., 2014). Therefore, in order to reduce the fishing pressure on wild stocks and to provide a consistent supply to the salmon farming industry, substantial effort has been put towards the intensive culture of ballan wrasse

over recent times (D'Arcy et al., 2012; Skiftesvik et al., 2013). Currently, the demand for cleaner fish by salmon farming companies is at an all-time high due to increases in production and resistance to chemotherapeutants. In 2014, Scotland alone stocked approximately 48.1 million smolts to sea and, based on the recommended 5 % wrasse to salmon stocking ratio (Skiftesvik et al., 2013), this number of smolts would require approximately 2.5 million farmed ballan wrasse over a salmon on growing cycle at sea (12-20 months). However, hatchery protocols have not yet been optimised and thus sustainable commercial upscaling of production to this level is only possible if some of the bottlenecks associated with broodstock management, egg incubation, and larval and juvenile rearing are overcome.

1.3 Farming ballan wrasse – domestication of a 'new' species

The successful establishment and domestication of a new species to aquaculture requires understanding of the reproductive biology of the species, the development of tools to monitor performance and a set of protocols to optimise productivity and quality (Migaud et al., 2013). The process of domestication is very similar between marine fish species, although species specific reproductive strategies means that different problems must be addressed to fine-tune solutions to commercial imperatives.

When starting the process of domestication, the first step is to source broodstock that will then be conditioned to captive spawning. Thought needs to be given as to where wild broodstock should be sourced from; taking into account what the natural range of the species is and if there are any underlying aspects of the population genetics that may influence the choice of populations for establishing captive breeding populations. This requires some understanding of wild population structuring to make informed decisions.

The next important factor is to establish captive spawning; to do so, the appropriate conditioning of the captive broodstock populations, including many environmental, social, and nutritional factors must be considered to promote captive spawning and ensure optimal egg productivity and quality. Without successful captive spawning, production is reliant on collection of eggs, larvae, or fry (Bromage, 1995). In order to establish successful captive spawning, the reproductive strategy and traits must be characterised in relation to seasonality (Dipper & Pullin, 1979; Karlsen & Holm, 1994) and to sustain year round production of eggs (Migaud et al 2013). Wherever possible, it

is best to maintain broodstock under controlled conditions that closely or best represents that of the species' wild habitat and optimisation of these conditions can lead to reduced stress and better overall performance (Mousa & Mousa, 2006). Parameters such as photoperiod, temperature, water quality, stocking density and broodstock nutrition can be optimised but differ widely between species. Therefore, the establishment of so-called "best practices" for a given species requires extensive research and development.

Once broodstock have been sourced and conditioned leading to captive spawning, the next important step is to be able to quantify egg production and assess quality. Quantifying production, with respect to spawning pattern and seasonality and fecundity, is important to ultimately determine the scale of the broodstock required to define the commercially viable productivity level of the hatchery. Furthermore, getting a handle on aspects of the quality of spawning, particularly fertilization and hatching rates, and the biochemical composition of eggs gives a baseline level against which to compare future egg production.

With respect to the domestication of ballan wrasse, although a level of production has been achieved thus far, the industry is still in its infancy and therefore requires investigation into the baseline information for the establishment of broodstock management and husbandry protocols to improve the overall hatchery performance.

1.4 Natural distribution and genetics of ballan wrasse

The wrasse family is highly diverse and represents over 600 species in more than 60 genera (Parenti & Randall, 2000). Ballan wrasse is the largest of the wrasse species in northern Europe inhabiting coastal regions from Morocco to mid-Norway (Quignard & Pras, 1986; Sayer & Treasurer 1996) (Fig. 1.2) and are typically found at a depth of 10 - 50 m around offshore reefs and rocky shores (Artüz, 2005).

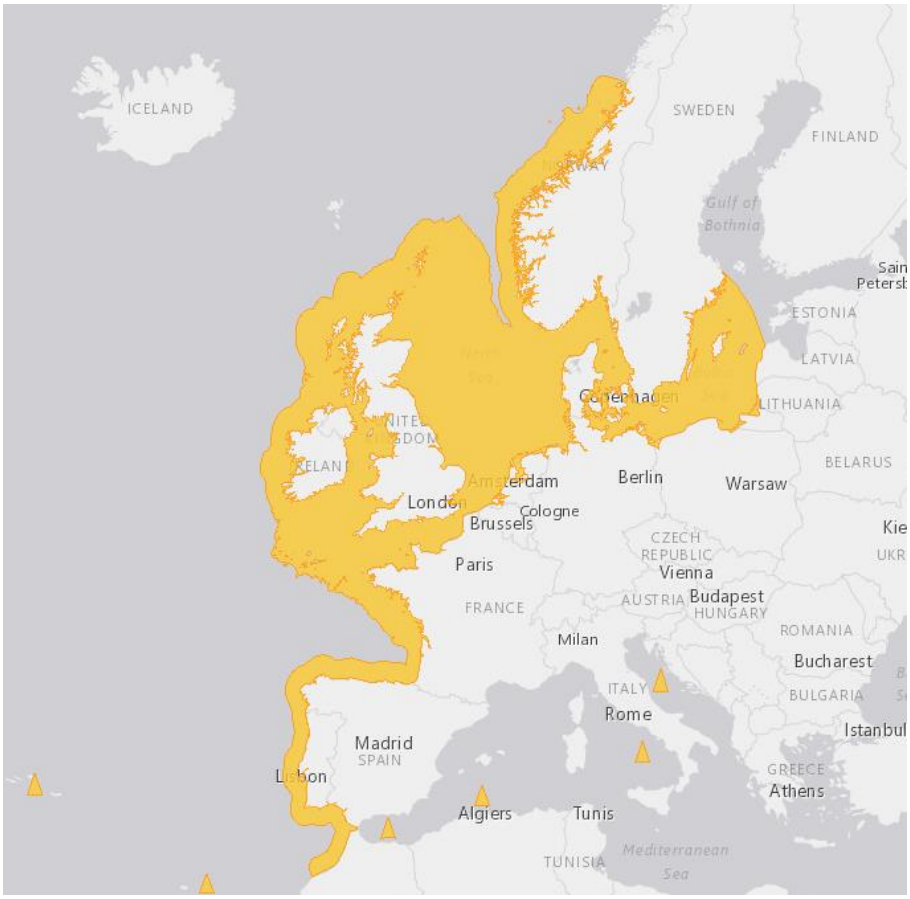

Figure 1.2. Distribution of ballan wrasse, *Labrus bergylta*. Yellow triangles represent unverified records; (Pollard, 2010).

Reaching up to 60 cm in length and 2 kg in weight, feeding on various molluscs and crustaceans (Dipper, 1987; Darwall et al., 1992), this species typically matures between 6-9 years of age and has an average lifespan of about 17 years; however, a maximum age of 25 years has been reported (Dipper et al., 1977; Sayer & Treasurer 1996). Furthermore, ballan wrasse have been found to have many different external colours (green, orange, red, blue) and pattern (plain and spotted) variations, with large prominent body scales (Sayer & Treasurer 1996) and no apparent external sexual dimorphism (Fig. 1.3). Previous research has suggested that plain and spotted individuals may be differentiated and coexist in sympatry based on growth trajectories, size at age, and otolith length to body length, and even goes as far to recommend, as a precautionary measure, that they are considered as two separate management units from a fishery perspective (Villegas-Ríos et al., 2013b). Furthermore, a recent study has found evidence of highly significant genetic differences between plain and spotted individuals from

Northwest Spain through the use of microsatellite markers (Quintela et al., 2016). However, further investigation is required to conclude as evidence shows that there is enough genetic overlap between the two morphotypes to suggest that plain and spotted forms may not be subspecies.

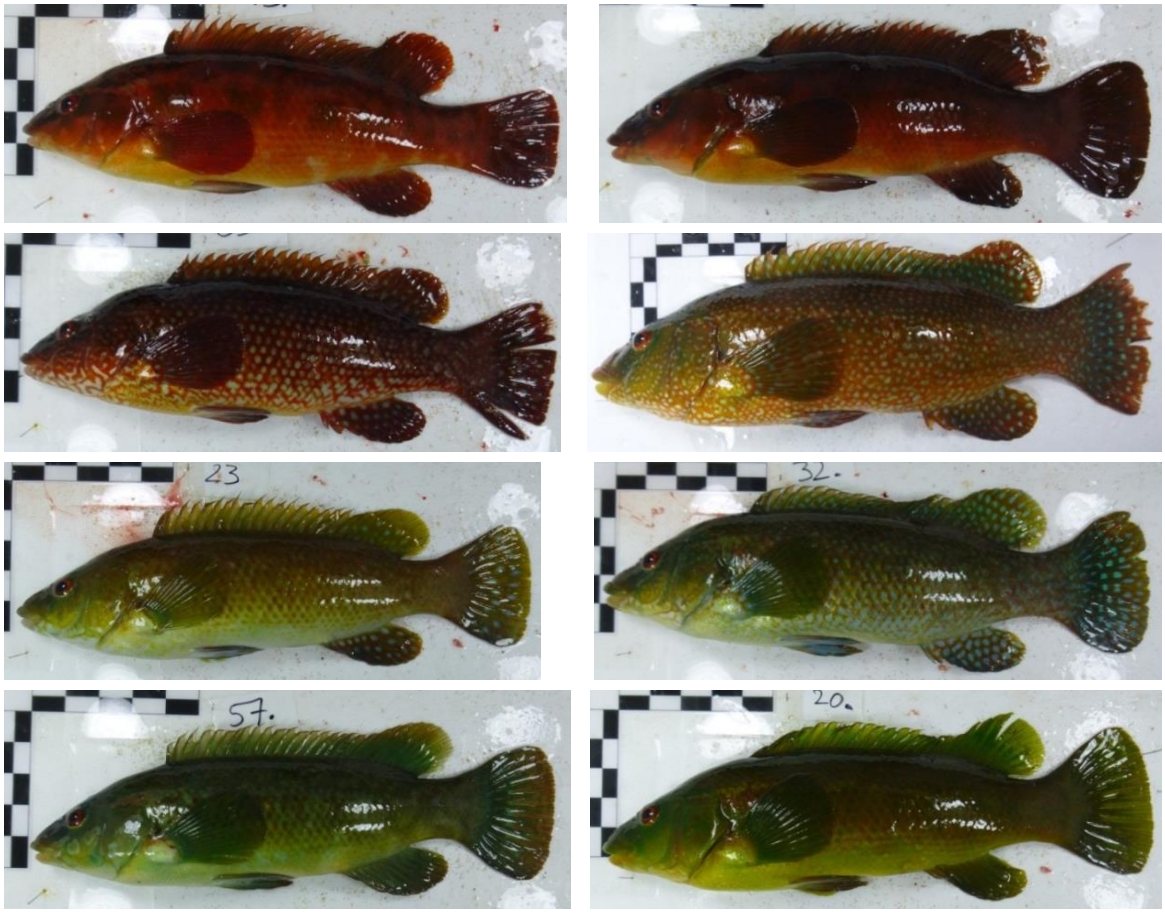

Figure 1.3. Colour and pattern variation of wild caught ballan wrasse from various locations across the west coast of Scotland, Shetland, and Norway; top to bottom: left hand images are females, right hand images are males; as part of sampling from Leclercq et al., (2014b).

The application of wrasse as cleaner fish in the salmon farming industry has led to the movement of various wrasse species out with their local geographical area of origin. The main commercial and ecological concerns relate to the implication of this work on the genetic management of both wild and farmed stocks with respect to escape events and the possibility of introgression into local populations. Within such a wide geographical range, reportedly from Morocco to Norway (Quinard & Pras, 1986), relatively little is

understood about what geographic structuring is present in wild ballan wrasse populations and what the implications may be of such structuring for broodstock choice and fish deployment. Given what is known about the reproductive physiology of ballan wrasse, discussed later in this chapter, territorial harem spawning (Darwall et al., 1992), skewed sex ratio (Dipper et al., 1977), benthic adhesive eggs (D'Arcy et al., 2012; Ottessen et al., 2012) and high site fidelity (Villegas-Ríos et al., 2013c; Morel et al., 2013), this would suggest limited dispersal and potentially strong differentiation between local populations.

D'Arcy et al., (2013) were the first to report on the phylogeographic structuring of ballan wrasse across its northern range in Norway, the UK and Ireland, using mitochondrial DNA (control region) sequence data. The authors presented evidence of population differentiation which segregated an Atlantic (Scotland & Ireland) and a Scandinavian (Norwegian) population, with local differentiation within these clades being apparently weak. Similar phylogeographic structuring was reported in the corkwing wrasse, a species with similar biological characteristics (Sayer & Treasurer 1996), in the northern Atlantic, showing a clear differentiation between Atlantic and Scandinavian populations through use of mtDNA (Robalo et al., 2012). The authors reported a marked difference between the genetic differentiation: higher in the southern populations and lower in northern populations (Robalo et al., 2012). On the other hand, a study looking into the genetic variation in goldsinny wrasse, showing similar biological characteristics to ballan and corkwing wrasse, although with planktonic eggs (Darwall et al., 1992) i.e. higher dispersal potential, found that significant differentiation exists among goldsinny wrasse from various regions in Norway. These authors went further to suggest that based on their results; local genetic differentiation exists between separate fjord systems within the same regions and that there is reason to expect that repeated transplantations of mature goldsinny wrasse from different geographic areas may cause unwanted gene flow to local populations (Sundt & Jørstad, 1998). However, the methodology used in this case predates the structuring analysis used today and, therefore, may not be entirely comparable.

From the limited evidence it would appear that there is a discernible level of population structuring across the natural range of ballan wrasse (D'Arcy et al., 2013); however, these results are not clearly definitive and have somewhat limited coverage within the

area of production interest (UK and Norway). D'Arcy et al., (2013), also concluded by highlighting the need for further investigation as well as the development of additional nuclear markers including microsatellites and single nucleotide polymorphisms (SNPs) directly from ballan wrasse populations to both aid in the selection of individuals for breeding programmes and to help understand and minimise the potential impacts of the interactions between both translocated wild and farmed individuals and local wild populations.

1.5 Reproductive physiology, spawning, and egg development

The single greatest milestone in the establishment of any new species is spawning in captivity, leading, ultimately, to the closure of the lifecycle (Migaud et al., 2013). This spawning can be achieved through stripping of gametes from mature broodstock followed by artificial fertilisation, however the attainment of natural spawning in captivity is preferential (Bromage, 1995). As with any new species to aquaculture there are certain bottlenecks associated with successful and sustainable husbandry including establishment of breeding populations, gender identification and control, understanding the periodicity of spawning and measuring the seasonal variations in quality and quantity of egg production. To date, there is a general lack of published information pertaining to these areas and the overall commercial production of ballan wrasse.

Ballan wrasse are protogynous hermaphrodites, meaning they develop first as functional females and later develop into males (Dipper & Pullin, 1979; Muncaster et al., 2013). This is a common reproductive strategy among Labrid species (Robertson, 1972; Robertson & Warner, 1978; Warner & Robertson, 1978; Dipper & Pullin, 1979; Jones, 1980) with the sex change process being driven by, as yet, uncertain endogenous and/or exogenous drivers. Dipper & Pullin (1979) have proposed that social cues, in particular the absence of a dominant male may be a key driver in sex change in ballan wrasse though this remains to be functionally demonstrated. Protogynous hermaphroditism is also common in many grouper species including the dusky grouper (*Epinephelus marginatus L.*) (Marino et al., 2001) and the brown spotted grouper (*Epinephelus tauvina F.*) (Abu-Hakima, 1987). This reproductive strategy is thought to have developed within mating systems where larger older males have more reproductive success than smaller younger males (Warner, 1975; Warner, 1982). Villegas-Ríos et al., (2013a) reported that the age at 50 % sexual change for ballan wrasse is between 7.4 – 11.8 years. However, it

should be recognised that this study focused only on individuals at the southern limit of the species range and thus may not necessarily reflect the age/growth structuring observed within the colder northern territories.

Ballan wrasse are believed to exhibit a haremic mating system where 6-8 females live in a loose community within the reproductive territory of a dominant male although data are very scarce (Sjolander et al., 1972). This type of reproductive strategy is associated with strong site fidelity (Morel et al., 2013) and a skewed sex ratio ranging between 6 – 10 % males (Dipper et al., 1977; Sjolander et al., 1972). In captivity, social interactions, with respect to stocking densities and sex ratios, can have major consequences on spontaneous spawning in captivity for both male and female fish in general (Mylonas et al., 2010). Current practice within commercial wrasse hatcheries is to establish small breeding populations prior to the spawning season with approximately 10 % males to reflect wild spawning harems. However, the identification of gender, particularly out with the spawning season, in this monomorphic species has been an area of concern with regards to setting up spawning populations. Methods such as ultrasound and biopsy have proven effective for gender determination in ballan wrasse during, as well as just prior to spawning (Lein & Helland, 2014), but require specialist equipment and technical experience. Leclercq et al., (2014b) validated an alternative methodology exploiting geometric morphometric analysis based on truss network analysis of landmark data points for identification of gender with 91 % accuracy among wild-caught individuals. Through a combination of these methods, along with routine tagging of broodfish, it should be possible to validate gender and, thereafter, maintain accurate stock records which will allow subsequent manipulation of stock structure, however there remains a lack of understanding of the optimum harem structure. What is clear however, is that due to their naturally low abundance (~ 10 % in wild populations; Quignard & Pras 1986; Darwall et al., 1992) males will likely be the limiting factor in the establishment of breeding populations. The issue of maintaining a sex-balanced broodstock has been overcome in other commercially produced species such as the dusky grouper, where through the use of hormonal therapies females can be induced to become functional males (Conceição et al., 2008). However, until a similar management strategy is validated to alleviate this bottleneck, clear guidance on the performance of males and, thus, the optimal usage of limited stocks will be essential to optimise productivity in the hatchery environment.

Early research had originally classed ballan wrasse as a group-synchronous single spawning species (Dipper & Pullen, 1979) although more recently, Muncaster et al., (2010) suggested that they should be classed as group-synchronous multiple batch spawners, based on histological evidence, with gonad maturation starting in November and peaking during the spawning season which occurs during the late spring and early summer. These authors went further to examine the reproductive cycle of ballan wrasse and classified the stages of ovarian development into six stages based on histological condition and leading oocyte diameter: I Immature or recovering, II Early maturing, III Early yolk, IV Late yolk, V Mature and VI Resorbing. In this study, it was shown that ovarian development in this Norwegian population began in late autumn to early winter with the growth of previtellogenic oocytes and the formation of cortical alveoli. Vitellogenic oocytes then were observed from January to June and ovaries containing post-ovulatory follicles were present between May and June (Muncaster et al., 2010). However, the potential multiple batch spawning nature of ballan wrasse has not yet been confirmed in the literature through the demonstration of an individual spawning more than once over the course of a single spawning season. Multiple batch spawning is common in many temperate marine species (Mananós et al., 2002) such as turbot (*Scophthalmus maximus L.*) (Jones, 1974), Atlantic cod (*Gadus morhua L.*) (Kjesbu et al., 1990) and Atlantic halibut (*Hippoglossus hippoglossus L.*) (Haug & Gullisken, 1988). McEvoy and McEvoy (1992), describe several advantages for multiple batch spawning fish including increased fecundity (Burt et al., 1988), spreading the risk of egg and larval predation over a longer time period, the impact of larvae on prey items and alleviating the risk of larval hatching during unfavourable environmental conditions.

While the reproductive axis of ballan wrasse appears fully functional in captivity with commercial hatcheries reporting spontaneous natural spawning (*Personal communication*, Mr. Paul Featherstone), there is a clear lack of understanding of the periodicity of spawning within a given spawning season. Furthermore, ambient captive spawning has been reported to occur between April and May (Ottesen et al., 2013), depending on latitude, however out of season production of eggs has also been achieved by housing broodstock under advanced and delayed photo-thermal regimes (*Personal communication*, Mr. Paul Featherstone). However, limited published studies clearly identify the spawning periodicity, i.e. the timing of spawning, and potential fluctuations in egg quantity of captive ballan wrasse across a given season in order to establish

commercial productivity. Therefore, the establishment of baseline data on this marine haremic spawning species is necessary to improve the understanding of the reproductive biology.

Ballan wrasse are benthic substrate spawners which lay eggs, approximately 1 mm in diameter, surrounded by an adhesive layer used for attachment to benthic substrate (Sjolander et al., 1972; D'Arcy et al., 2012). In the wild, female ballan wrasse have been observed cleaning rocky spawning sites (0.2 – 0.6 m^2) of algae while males patrolled the territory (Sjolander et al., 1972). Substrate spawning fish are known to be selective in their choice of spawning habitat and a variety of environmental factors have been shown to influence choice for spawning site (Snickars et al., 2010). For example, the distribution of spawning habitat for the Eurasian perch (*Perca fluviatilus* L.) was strongly dependent on specific habitat characteristics e.g. type of vegetation (Snickars et al., 2010). In addition, a clear relationship between sole (*Solea solea* L.) egg abundance and sediment type was found, with significantly higher egg densities found over sediment with a < 30 % gravel content (Eastwood et al., 2001). However, relatively little is known about the specific spawning behaviour of ballan wrasse with respect to the selection of spawning zone and substrate type as, in captivity, females actively seek out a substrate upon which to deposit adhesive eggs. A previous report indicated that various materials were examined as potential spawning substrates which included rugs, carpet tiles, AstroTurf matting, terracotta tiles and Perspex sheets; however, no preference was determined (Ottesen et al., 2013). Currently, some hatcheries use artificial spawning substrates, such as green low pile carpet mats, chosen based on availability, cost and practicality (e.g. cleaning and incubation). These artificial substrates are placed at the bottom of the spawning tanks for the collection of eggs and then removed from the tanks and placed into incubators until hatching occurs. The use of artificial spawning substrates is somewhat unique for a commercially cultured species and, as such, there is a general lack of an established methodology. However, given that field observations report females actively seeking and cleaning spawning sites (Sjolander et al., 1972; Artüz, 2005), it is fair to assume that the manner in which spawning substrates are presented and managed in the tank environment could well influence female activity and thus spawning productivity. Therefore, investigation into spawning substrates and methodology for their use represents an important step towards optimisation of broodstock management and hatchery practices.

The incubation of eggs, while adhered to spawning substrates, may pose a number of significant issues with respect to the disinfection and incubation of eggs, and not least prevents the assessment of simple management parameters like egg quality and quantity. A clear demonstration of the significance of this is the lack of quantification of egg productivity (total or batch fecundity) for ballan wrasse because adhered eggs negate standard volumetric production assessment. One potential way to overcome this challenge is to remove the adhesiveness prior to incubation. This is common practice in a number of cultured species (mainly freshwater) such as common carp (*Cyprinus carpio L.*) (Linhart et al., 2003a,c) and European catfish (*Silurus glanis L.*) (Linhart et al., 2003a), where various chemicals, including the enzyme alcalase ®, sodium sulfite, tannic acid, or L-cysteine can be applied to either remove egg adhesiveness or to prevent the eggs becoming adhesive in the first place (Kowtal et al., 1986; Rottmann et al., 1991; Ringle et al., 1992; Linhart et al., 2000; El-Gamal & El-Greisy, 2008). Therefore, it is important to establish if similar techniques can be adopted by ballan wrasse farmers which would greatly aid in the collection, disinfection and incubation of eggs.

Ballan wrasse eggs typically hatch at roughly 70 degree days (Celsius) post fertilization (Ottesen et al., 2012). Embryonic development has been described by D'Arcy et al (2012) and is similar to that of other demersal marine finfish species (Fuiman, 2002), including the brown wrasse (*Labrus merula L.*) (Dulcic et al., 1999). However, currently there are no standard measures of egg quality for either captive or wild ballan wrasse. This represents a further limitation to the successful commercial production of this species with a lack of baseline data measuring egg quality and quantity against which to compare future success. Many of the commonly used quality indicators across marine finfish species including egg size, fertilization and hatching rates, and the biochemical composition of eggs including lipids and fatty acids (FA) composition in particular (Migaud et al., 2013; Bobe & Labbe, 2010) have yet to be assessed for ballan wrasse. With such data, it would then be possible to monitor and assess seasonal changes in egg quality, determine individual batch fitness and would allow subsequent steps to optimise broodstock diets.

The establishment and optimisation of broodstock production and, thus, hatchery productivity is the first step in realising a sustainable farmed supply of ballan wrasse; however, it is by no means to only challenge faced in the species. As with all cold water

marine species the larval rearing and juvenile grow out is a long protracted business. The minimum size at which a farmed wrasse can be deployed on farm is 40 g, which currently takes 16-18 months from hatch. Live feeds, weaning, formulated feeds, environmental conditions and disease challenges are poorly understood and all need optimisation. However, before this can be meaningfully addressed a reliable supply of larval ballan is required; thus, research and development of broodstock management and optimisation of broodstock performance are the first vital steps in the establishment of the farming of ballan wrasse.

1.6 Research aims

The detrimental effects of sea lice to the salmon farming industry along with the growing need for alternatives to chemical treatments due to resistance has led to the increased use of cleaner fish, such as ballan wrasse, for the biological control of lice. In order to meet the high demand for ballan wrasse and to alleviate pressure on wild fisheries, the long term sustainable option is commercial production of this species. However, commercial production of ballan wrasse is in its infancy with the need for establishment of methods to implement and optimise broodstock management strategies and secure productivity in the hatchery. Only when these are addressed can the farming of ballan wrasse become a commercial reality. As such, the overarching aim of this thesis is to undertake research to address gaps in knowledge in key aspects of the species biology and develop tools and protocols to help implement broodstock management practices. Results will subsequently be used in the commercial hatcheries to enhance and sustain productivity of farmed ballan wrasse. The specific aims of the thesis are as follows:

1. To describe the population structuring of wild ballan wrasse across a geographical range pertinent to the UK salmon farming industry, and subsequently inform on selection of wild broodstock for establishment of commercial breeding programmes that minimises risk associated with any potential escapes on introgression with local wild populations.

2. To describe the spawning dynamics of captive broodstock populations, with respect to periodicity, productivity and parental contribution along with identifying potential variations in egg quality. New knowledge will help confirming if the species is multiple batch spawning, and, importantly, will

inform on the scale of a broodstock population required to achieve commercial reliable productivity as well as best broodstock management strategies.

3. To determine if ballan wrasse have preferences for substrate colour or areas when spawning spontaneously in the tank, and if optimisation of such preferences including fragmentation of the tank spawning area could potentially lead to increased productivity within the hatchery.

4. To determine an effective method for removal of adhesiveness of ballan wrasse eggs, which would ultimately aid in collection, disinfection, and incubation protocols.

Altogether, this research will give important baseline information for the establishment of broodstock management and husbandry protocols to improve the overall hatchery performance of ballan wrasse.

2 Chapter 2: Genetic population structure of wild ballan wrasse (*Labrus bergylta*) in the UK and Norway: implications for broodstock management.

Keywords: cleaner fish; SNP discovery; phylogeographic structure; population genetics.

Abstract

The natural range of ballan wrasse reportedly extends along the Eastern Atlantic seaboard from Morocco to Norway. Within such a wide geographic range it is not fully apparent what genetic structuring is present and what the implications may be of such structuring for broodstock choice and fish deployment. Given what is understood about the natural reproductive physiology of ballan wrasse, (benthic harem spawners with female skewed sex ratio), it is expected that clear differentiation between populations may be evident, perhaps more so than previously reported through the use of mitochondrial DNA sequence data. The aim of this study was to identify a panel of single nucleotide polymorphisms (SNPs) for assessment of genetic structuring of ballan wrasse in wild populations throughout the UK and Norway pertinent to the cleanerfish industry. Through the use of restriction site associated DNA (RAD) sequencing, over 15,000 potentially informative SNPs were identified and resolved down to a panel of 215 SNPs that were successful in differentiating the four sampled populations: three UK and one Norwegian. A closer examination showed that a panel of 11 highly informative SNPs proved sufficient to robustly differentiate stocks sampled from the mainland UK, Shetland, and Norway; however, this was not able to differentiate between populations within the mainland UK. SNP analysis revealed a more detailed level of structuring than the mtDNA analysis, completed on a subset of individuals from each location. This possibly alludes to local adaptation within the two previously reported clades and moreover, the increased precision in detecting low levels of genetic differentiation through use of large numbers of SNPs. With respect to the genetic management of farmed stocks, based on the SNP analysis, a precautionary approach is suggested, i.e. to source founder populations for commercial hatcheries as locally as possible to areas where farmed wrasse will be deployed. However, the potential risks that escapees may pose to local wild populations are thought to be relatively low.

2.1 Introduction

The ballan wrasse, *Labrus bergylta,* has been targeted for use as a cleaner fish for the biological control of sea lice (*Lepeophtheirus salmonis* and *Caligus* spp.) on farmed Atlantic salmon (*Salmo salar*) throughout the UK, Ireland, and Norway. The proven effectiveness of ballan wrasse for lice control (Skiftesvik et al., 2013; Leclercq et al., 2014a) is well established and has led to a substantial increase in the demand for cleaner fish since their implementation in the 1970's (Bjoral, 1991; Skiftesvik et al., 2014). This increased demand has been met in recent years, in part, by the establishment of dedicated wrasse hatcheries in the UK and Norway. When establishing a new species hatchery, the genetic basis of the founder population is an important concern both in terms of best practice of broodstock management but also in terms of considering the potential impact of hatchery progeny on native wild stocks in the area they are to be deployed (Sundt & Jørstad, 1998; Johnson, 2000; Doupe & Lymbery, 2000). Relatively little is understood about population structuring for ballan wrasse across its native range and what the implications may be for broodstock choice and subsequent fish deployment.

Ballan wrasse is a protogynous hermaphroditic species (Dipper et al., 1977; Darwall et al., 1992) with a reportedly wide natural geographic range extending along the Eastern Atlantic seaboard from Morocco to Norway (Quignard & Pras, 1986). Ballan wrasse typically inhabit rocky shores, reefs and kelp beds (Figueiredo et al., 2005) at depths of 10 - 50 m (Artüz, 2005). In the wild, this species exhibits a haremic style mating system with a skewed sex ratio (in favour of females) (Sjölander et al., 1972; Dipper et al., 1977) and spawns benthic adhesive eggs (D'Arcy et al., 2012). Furthermore, ballan wrasse display sedentary behaviour and high site fidelity (Morel et al., 2013) with relatively small estimated home range sizes of 0.133 ± 0.072 km^2 (Villegas-Ríos et al., 2013c). Biological characteristics such as these could potentially limit the mixing within wild populations and, as such, it is not unreasonable to presume that strong population genetic structuring could be evident between different geographic locations across the species natural range.

There is limited work, to date, looking at the phylogeographic structuring of wild ballan wrasse populations. D'Arcy et al., (2013) compared mitochondrial DNA (mtDNA) control region sequence variance to examine genetic diversity and population structuring in ballan wrasse from multiple locations in Ireland, Scotland, and Southern Norway.

These authors reported the presence of two highly divergent clades, however both clades had representation from individuals from all sampling locations; which, they suggested, may be a result of these two main lineages already existing before wrasse colonized the sampled areas. In addition, it was found that the highest proportion of variation (83.81 %) was found to be between regions (Irish, Scottish, and Norwegian) and a relatively low differentiation between populations within regions existed. This was further supported by the highly significant segregation between the Norwegian sites from all other sites. Additionally, through use of mtDNA, it was found that the low levels of population structuring around Irish and Scottish locations was comparable to that found in the sixbar wrasse (*Thallasoma hardwicki B.*) in the South China Sea (Chen et al., 2004), the bluehead wrasse (*Thalassoma bifasciatum B.*) in the Caribbean (Haney et al., 2007) and the fourline wrasse (*Larabicus quadrilineatus R.*) in the Red Sea (Froukh & Kochzius, 2007). Comparable studies conducted on corkwing wrasse (*Symphodus melops*), a species with similar reproductive characteristics (Potts, 1984), revealed comparable grouping of Atlantic and Scandinavian populations. Moreover, there was significant variation (7.24 %) found between the Atlantic and Scandinavian groups and again, based on pairwise differences, all Scandinavian populations were found to be significantly different from non-Scandinavian populations (Robalo et al., 2011). Both of these studies remarked that the Scandinavian wrasse populations showed much higher genetic diversity than southern populations, suggesting that a historic demographic event may have caused a population bottleneck within southern populations followed by more recent expansion to the northern population limits (Robalo et al., 2011, D'Arcy et al., 2013). On the other hand, Sundt and Jørstad (1998) were able to find local genetic differentiation in goldsinny wrasse (*Ctenolabrus rupestris*) populations between geographically separated fjord systems within the same regions of Norway. However, the reproductive characteristics of goldsinny wrasse (planktonic eggs) might suggest a potentially higher juvenile dispersal rate than ballan wrasse (Darwall et al., 1992). Furthermore, the methodology used in this case was based on the analysis of polymorphic tissue enzymes, and while there were significant differences between geographically distinct allele frequencies, the methods used in this case predate the modern structuring analysis used today.

The recent study by D'Arcy et al., (2013) on population genetics in ballan wrasse was relatively limited by the power of the methods used and even goes on to suggest the need

for further investigation of population structure using additional markers. Other markers including, but not limited to allozymes, microsatellites (Shaw et al., 1999) or single nucleotide polymorphisms (SNPs) (Helyar et al., 2011) can be used in studies of this nature looking at population structure in marine teleost species. Although each type of genetic marker has important applications, each also has limitations which need to be taken into consideration along with the practicality of methodology and availability of resources, when making the choice of marker for a study. For example, Shaw et al., (1999) investigated population structuring in Atlantic herring (*Clupea harengus* L.) populations from Norwegian waters and the Barents Sea along with differentiation between Atlantic and Pacific herring (*Clupea pallasi* L.) using three different types of markers; microsatellites, allozyme, and mtDNA. This study aimed to make direct comparisons of the resulting datasets to identify the utility of the three types of markers for detecting different levels of structuring within the species. Results from the first portion of the study assessing population structuring of Atlantic herring in Norwegian waters and the Barents Sea showed that the three marker types showed broadly the same level off structuring based on pairwise comparisons of F_{ST} values, although mtDNA was found the be generally less informative. However, using the same markers to assess the level of genetic differentiation between Atlantic and Pacific herring stocks showed conflicting results between the three marker types with allozyme and mtDNA datasets showing significant levels of differentiation where microsatellites indicated an overlap between the stocks. Overall, these authors suggested that microsatellites may detect structuring at a fine scale, but are less informative at the larger scale (Shaw et al., 1999).

SNPs are gaining popularity in population ecology as they are highly informative markers and the most widespread type of sequence variation in genomes (Lao et al., 2006; Helyar et al., 2011). While SNPs can generate similar statistical power to both microsatellites and mtDNA, they can often provide broader genome coverage and higher quality data (Morin et al., 2004). Advances in modern techniques such as next generation sequencing (NGS) (Davey et al., 2011), along with the associated bioinformatics tools for analysing these datasets, has created a renaissance in population genetics of non-model species (Helyar et al., 2011). Development of this technology has allowed for the direct analyses of sequence variations such as SNPs as opposed to markers such as microsatellites (Helyar et al., 2011). Furthermore, the fast and efficient detection of SNPs in non-model species is becoming more cost effective and widely available due to new

methodologies such as restriction site associated DNA (RAD-tag) sequencing (Miller et al., 2007; Baird et al., 2008). Large volume SNP analysis has been used to reveal both large and fine scale population structure in many commercially important and non-model marine species. For example, the European hake (*Merluccius merluccius L.*), based on SNP analysis, was confirmed to have a genetic break between Atlantic and Mediterranean basins and weak differentiation within the basins. Further analysis of a panel of outlier loci revealed significant divergence between Atlantic and Mediterranean populations along with fine-scale population structuring (Milano et al., 2014). Another example of the use of large volume SNP analysis is that of the American lobster (*Homarus americanus M.*), where RAD sequencing methods were used to develop a panel of 10,156 SNPs to look at the genetic structure and population assignment for over 500 specimens across the species' natural range. Results from this showed a total of 11 genetically distinct populations and weak, fine-scaled population structuring within each region (Benestan et al., 2015). This study, and others like it, demonstrates that the use of large numbers of SNPs can improve the definition of fine-scale population structuring along with the population assignment success where there may be weak genetic structure.

The aim of this current study was, therefore, to investigate the potential fine-scale structuring of wild ballan wrasse populations in the northern extent of its natural range (UK and Norway), where commercial exploitation is at its greatest, through the application of high throughput SNP sequencing methods. The study had an iterative approach whereby novel SNP markers, potentially informative of geographical location, were identified through a genotyping by sequencing approach using restriction site associated DNA (RAD) sequencing. Thereafter, a reduced panel of informative markers were screened across a wide population sample to improve our understanding of population structuring within the species in comparison to the previously reported mitochondrial haplotype sequencing methodology. Combined, such a dataset should give greater insight into the population structuring of the species which will be discussed in context of management of wild stocks as well as selection of broodstock for hatchery production.

2.2 Materials and Methods

2.2.1 Location and collection of samples

Wild ballan wrasse (*n* = 50 per location) were collected over a 6 week period from July to August, 2012 from one location in Rong, Norway (Location A: 60° 33’ N; 04° 53’ E); three coastal locations in Scotland, UK - Shetland (Location B: 60° 08’ N; 01° 17’ W), Ardtoe (Location C: 56° 46’N; 05° 53’ W), Machrihanish (Location D: 55° 25’ N; 05° 44’ W); and from Dorset, England (Location E: 50° 44’N; 02° 22’W, *n* = 50) (Figure 2.1). Fish were landed live using modified creel pots and gillnets; upon landing fish were maintained locally in flow-through systems under ambient photo-thermal conditions for a few days until sampling commenced. Each individual was killed with a lethal dose of anaesthesia (MS-222, 100 ppm) and a 5 mm fin clip was taken and stored in 100 % ethanol for genetic analysis

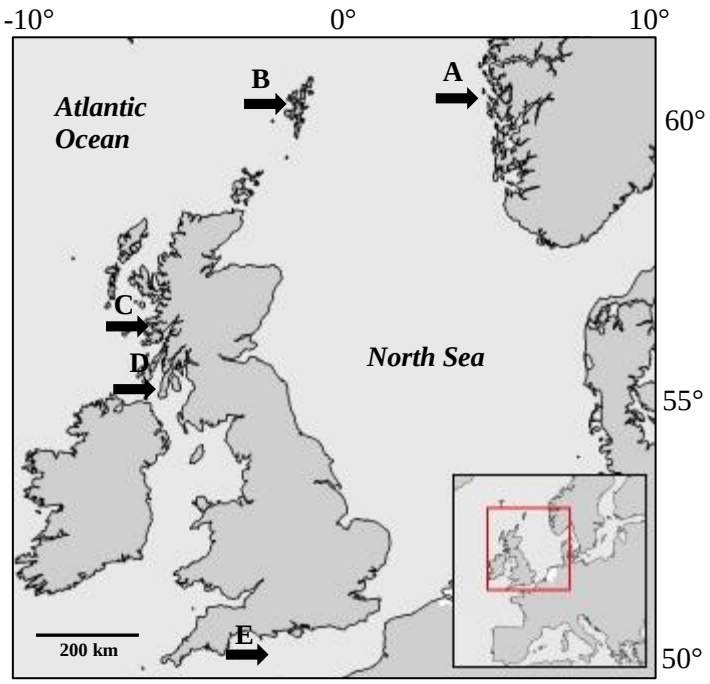

Figure 2.1. Sampling locations in Norway (A, Rong), Scotland (B; Shetland, C; Ardtoe, D; Machrihanish) and England (E; Dorset).

2.2.2 DNA extraction

Genomic DNA from fin clips was isolated using a modified salt extraction method (Aljanabi & Martinez, 1997). Approximately 0.5 cm^2 tissue was added to 300 µl SSTNE buffer (0.30 M NaCl; 0.04 M Tris; 200 µM EDTA; 0.199 mM EGTA (E3889, Sigma Aldrich), 4.89 mM spermidine (SO266, Sigma Aldrich), 1.4 mM spermine (S1141, Sigma Aldrich)), 20 µl of SDS (10 %; L3771, Sigma Aldrich) and 5 µl proteinase K (10 mg/ml; P2308, Sigma Aldrich). This was mixed well. Following a 4 hour digestion at 55 °C, samples were incubated at 70 °C to inactivate proteinase K. Once samples were cooled to room temperature, 20 µl of RNase A (2 mg/ml; R6148, Sigma Aldrich) was added to each sample. Following an additional 1 hour (37 °C) incubation, 200 µl of 5 mM NaCl was added for protein precipitation. 400 µl of supernatant was retained, transferred to fresh tubes, and an equal volume of isopropanol added and mixed well. Samples were then centrifuged for 10 minutes at 4 °C, and 10,000 X G to form a pellet. The DNA pellet was then washed overnight with 72 % ethanol, dried, and resuspended in 100 µl 5 mM/L Tris, pH 8.5. Each sample concentration was quantified using a

Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA), and molecular weight was assessed by agarose gel electrophoresis (Figure 2.2). In the case of RAD analysis (see section 2.3) double stranded DNA concentrations were then more accurately measured using a Qubit dsDNA Broad Range Assay kit and Quibit Fluorometer (Invitrogen, Paisley UK). Fin clip samples yielded an average of 150 ng/µl and were diluted to a concentration of 50 ng/µL before storage at 4 °C for up to 6 months before analysis.

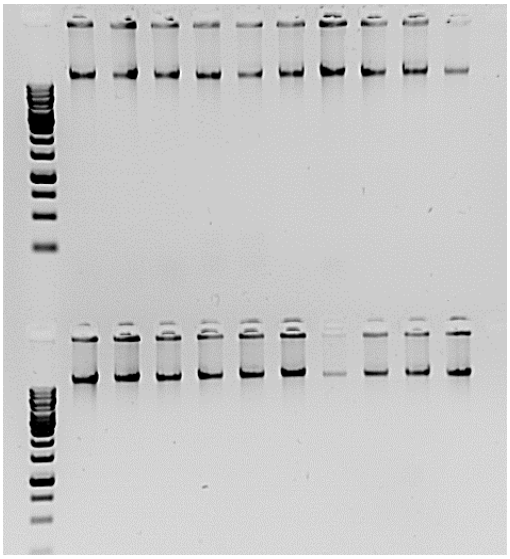

Figure 2.2. Example of a 0.8 % agarose gel electrophoresis, each lane contains 1 µl (50 ng) genomic DNA and run against a 1 kb ladder (top and bottom left lanes).

2.2.3 RAD library formation and sequencing

20 individuals each from locations A-D (Fig. 2.1; *n* = 80 total) were selected, with a balanced sex ratio where possible (1:1 male:female sex ratio apart from Ardtoe – all female), for construction of RAD library. RAD library construction protocol followed the methodology described by Baird et al., (2008) with modifications summarised by Palaiokostas et al., (2013). In summary, each sample (0.2 µg DNA) was digested at 37°C for 30 minutes with *Sbf* I (recognising the CCTGCA|GG motif) high fidelity restriction enzyme (New England Biolabs; NEB). The reactions were then heat inactivated at 65°C for 20 minutes. Individual specific P1 adapters, each with a unique 5-7 bp barcode (Table 2.1), were ligated to the *Sbf* I digested DNA at room temperature for 60 minutes by adding 0.5 µL 100 nmol/L P1 adapter, 0.12 µL 100 mmol/L rATP (Promega), 0.2 µL 10×

Reaction Buffer 2 (NEB), 0.1 μL T4 ligase (NEB, 2 M U/mL) and reaction volumes made up to 2 μL with nuclease-free water for each sample. Following heat inactivation at 65°C for 20 minutes, the ligation reactions were slowly cooled to room temperature (over 1 hour) then 5 pools of 16 individual samples were combined prior to shearing. Shearing (Covaris S2 sonication) and initial size selection (200-500 bp) by agarose gel separation was followed by gel purification, end repair, dA overhang addition, P2 paired-end adapter ligation (Table 2.1), library amplification, exactly as in the original RAD protocol (Baird et al., 2008; Etter et al., 2011). A total of 200 μL of each amplified library (14 PCR cycles) was size selected (c. 300-550 bp) by gel electrophoresis. Following a final gel elution step into 20 μL EB buffer (MinElute Gel Purification Kit, Qiagen), the libraries were transferred to The GenePool Genomics Facility at the University of Edinburgh, UK, for quality control and high-throughput sequencing on the Illumina Highseq platform using the 100bp paired end read HiSeq V3.0 chemistry (Illumina, Little Chesterford, UK).

Table 2.1. Sample origins and RAD barcodes; details each sample used: sample ID, RAD barcode used, number of raw reads and number of RAD-tags.

Sample ID	Location	Barcode 1	Barcode 2	Raw Reads	RAD-tags
Rong_1	A	TCGAG	TAGCA	3779600	62836
Rong_2	A	ATCGA	GAGATC	2221854	60337
Rong_3	A	TGCAACA	GAGATC	3351304	62493
Rong_4	A	GCATT	CGATA	2504068	61311
Rong_5	A	CGTATCA	AGTCA	2580206	59852
Rong_6	A	TCTCTCA	AGTCA	3351774	62566
Rong_7	A	GTCAC	GAGATC	2408784	61495
Rong_8	A	TGACC	AGCTGA	2063752	57105
Rong_9	A	ACTGCAC	GAGATC	2263114	60806
Rong_10	A	TCTCTCA	AGCTGA	2253862	58586
Rong_11	A	CACAGAC	CGATA	2525860	61284
Rong_12	A	TGACC	CGATA	4177312	62864
Rong_13	A	ATCGA	CGATA	2034394	58929
Rong_14	A	GTACACA	AGTCA	3169152	62051
Rong_15	A	GTACACA	AGCTGA	3308380	62733
Rong_16	A	TCTCTCA	GAGATC	3171336	62227
Rong_17	A	TCGAG	GAGATC	2768882	61020
Rong_18	A	ACTGCAC	CGATA	2193184	58819
Rong_19	A	CGTATCA	CGATA	3053878	62202
Rong_20	A	CATGA	GAGATC	2763392	61940
Shetland_1	B	TCGAG	AGCTGA	2465812	59659

Shetland_2	B	ACTGCAC	AGTCA	2127302	62082
Shetland_3	B	GTCAC	CGATA	2657150	62766
Shetland_4	B	TGCAACA	CGATA	2383092	59980
Shetland_5	B	GCATT	GAGATC	2510820	62185
Shetland_6	B	GTACACA	CGATA	3048666	63372
Shetland_7	B	CTCTTCA	TAGCA	2580780	59894
Shetland_8	B	CACAGAC	AGCTGA	2301770	59244
Shetland_9	B	CACAGAC	AGTCA	3241636	63391
Shetland_10	B	GATCG	AGCTGA	1698386	55167
Shetland_11	B	CACAGAC	GAGATC	2021926	61272
Shetland_12	B	GTACACA	GAGATC	2498630	59773
Shetland_13	B	CACAGAC	TAGCA	3002716	61366
Shetland_14	B	ACTGCAC	TAGCA	2430612	59684
Shetland_15	B	TCGAG	AGTCA	3226382	61536
Shetland_16	B	TCTCTCA	CGATA	2021536	58665
Shetland_17	B	CTAGGAC	TAGCA	2496028	59991
Shetland_18	B	CTAGGAC	AGCTGA	1700640	55927
Shetland_19	B	CGTATCA	TAGCA	2772824	60922
Shetland_20	B	GCATT	AGTCA	2944140	61709
Ardtoe_1	C	TCAGA	TAGCA	2002922	57839
Ardtoe_2	C	TCTCTCA	TAGCA	1430234	54589
Ardtoe_3	C	CATGA	CGATA	2670512	62574
Ardtoe_4	C	CGTATCA	GAGATC	2599974	62150
Ardtoe_5	C	GATCG	TAGCA	2458068	58771
Ardtoe_6	C	CTAGGAC	AGTCA	3123870	63110
Ardtoe_7	C	TGCAACA	AGCTGA	3296680	62908
Ardtoe_8	C	ATCGA	AGCTGA	3647934	62972
Ardtoe_9	C	TGACC	AGTCA	3288632	63515
Ardtoe_10	C	GCATT	AGCTGA	4116690	64876
Ardtoe_11	C	CATGA	TAGCA	4001486	63607
Ardtoe_12	C	GATCG	GAGATC	2187102	61377
Ardtoe_13	C	GTACACA	TAGCA	2959740	61363
Ardtoe_14	C	CTCTTCA	AGTCA	2907164	62937
Ardtoe_15	C	ATCGA	TAGCA	3737984	63865
Ardtoe_16	C	ACTGCAC	AGCTGA	3235266	63527
Ardtoe_17	C	ATCGA	AGTCA	1976752	57429
Ardtoe_18	C	GATCG	CGATA	3433078	63807
Ardtoe_19	C	TCAGA	CGATA	2793680	62601
Ardtoe_20	C	TCGAG	CGATA	2806756	63074
Mach_1	D	GCATT	TAGCA	3400100	62054
Mach_2	D	TGACC	TAGCA	2208842	57039
Mach_3	D	CTAGGAC	GAGATC	3275162	63376
Mach_4	D	CTAGGAC	CGATA	2785590	62843
Mach_5	D	GTCAC	TAGCA	1211812	46286
Mach_6	D	TCAGA	GAGATC	2887114	62604
Mach_7	D	CTCTTCA	GAGATC	2626382	62667

Mach_8	D	CTCTTCA	CGATA	2048390	60411
Mach_9	D	CATGA	AGCTGA	1707548	54309
Mach_10	D	GATCG	AGTCA	1612630	58340
Mach_11	D	TGCAACA	TAGCA	2791220	60104
Mach_12	D	TGCAACA	AGTCA	3434134	63492
Mach_13	D	TGACC	GAGATC	3431612	63267
Mach_14	D	TCAGA	AGTCA	2059830	58482
Mach_15	D	CTCTTCA	AGCTGA	2914598	62830
Mach_16	D	GTCAC	AGTCA	1678368	59103
Mach_17	D	TCAGA	AGCTGA	1793686	56208
Mach_18	D	CGTATCA	AGCTGA	1374976	48899
Mach_19	D	GTCAC	AGCTGA	1712016	55797
Mach_20	D	CATGA	AGTCA	1386048	48898

2.2.4 SNP verification assays

Based on the analysis of the RAD sequencing experiment a panel of 11 SNPs were identified as being highly informative for population structuring across the study range. These SNPs were translated into an allele-specific PCR genotyping assay (KASP^{TM} v4.0, LGC Genomics, UK). SNP assays were designed using the KASP on demand genotyping system (LGC Genomics Ltd) for 11 SNPs (Table 2.2). Genotyping assays were carried in a 5 µl final PCR reaction volume using 2.5 µl KASP 2x reaction mix, 0.07 µl assay, and 2.5 µl DNA (~25 ng DNA). Thermocycling conditions were as follows: 94 °C for 15 min, 94 °C for 20 sec, touchdown over 65 – 57 °C for 60 sec (10 cycles dropping 0.8 °C per cycle), and 26 cycles of 94 °C for 20 sec, 57 °C for 60 sec. All assays were run within a 96 well plate where individual genotype assignment was determined through reading the fluorescence emission of the allele specific fluorophores for each sample and compared to non-template control reactions, using the Quantica qPCR thermal cycler (Bibby Scientific, UK) and endpoint genotyping software. All 11 SNPs were measured in a total 50 individuals from all 5 locations, samples from Machrihanish, Ardtoe, Shetland and Rong consisted of the 20 individuals previously included in the RADseq experiment in addition to 30 new individuals from these locations.

Table 2.2. KASP assay primer sequences: Allele-specific primers and common primer designed for the allele-specific PCR genotyping assay of the 11 KASP markers.

ID	Primer: AlleleFAM	Primer: AlleleHEX	Primer: Common	Allele FAM	Allele HEX
Lbergylta_657	GCATCAAATATATTGTGTTCTAAGTACATCT	GCATCAAATATATTGTGTTCTAAGTACATCA	TCTTGGAATTTGAAACAAGTAGAGCACTTT	T	A
Lbergylta_10502	AAAATGGTGATAAACCTCTTTAAAGTCCA	AATGGTGATAAACCTCTTTAAAGTCCC	CGCGCTGCTCAGATAATTAGCACAT	A	C
Lbergylta_15162	CACCGGGACCCCGCGG	GCACCGGGACCCCGCGA	TTAAACTCTTCCGGTTCGGGCCAAA	C	T
Lbergylta_20096	GTCTACCACGCCACAGTCTAC	GGTCTACCACGCCACAGTCTAT	GTAGGTGCGTGCCGCAGAGTT	C	T
Lbergylta_31601	TTGATCCCATAAGACACTGCTTCA	TGATCCCATAAGACACTGCTTCG	GCTCCTCTGATCTGTAGTGTCACTT	T	C
Lbergylta_32985	GAGGAGAAACGTAATAAAAGCAGGG	CGAGGAGAAACGTAATAAAAGCAGGA	CTGTCGGTTTTAAAAGTCACCCGGTT	C	T
Lbergylta_37284	TGGTGAACATCATGAGTGCGTGA	GGTGAACATCATGAGTGCGTGG	ACAGTGGAGCTGTTGACCAACACTA	A	G
Lbergylta_41588	CGCGGCGTTCAGCACGAAG	CGCGGCGTTCAGCACGAAC	TGCGCCTCACCTGGAGCGAA	C	G
Lbergylta_46143	TAATCCTTCACAGGAAAATTCAGTG	GCTTAATCCTTCACAGGAAAATTCAGTT	AGAAATTCTGGTGATTTTCATCTCATGCTA	G	T
Lbergylta_4156	GCAGTCAAAACCAGGTCCG	CCTGCAGTCAAAACCAGGTCCA	AAATCTGCACCATGTGAAGAAACCAGTT	C	T
Lbergylta_42488	AAAGTGGATTTTTTTTATGTGAACTTCCC	GAAAGTGGATTTTTTTTATGTGAACTTCCT	GAACAGTGAGCCGGTCTTTGCTTT	C	T

2.2.5 Summary statistics

For both the global RAD analysis (215 loci; 80 individuals; 4 locations) and targeted SNP panel (TSP) datasets (11 loci; 249 individuals; 5 locations) population genetic statistics including pairwise genetic differentiation (F_{ST}) and inbreeding coefficient (F_{IS}) (Weir & Cockerham, 1984), tests for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium, population differentiation using the exact G test using a Markov Chain Monte Carlo approach (1000 de-memorisations, 100 batches, 1000 iterations), and estimates of expected (H_e) and observed (H_o) heterozygosities were performed in GENEPOP 4.3 (Raymond & Rousset, 1995; Rousset, 2008). Type I error rates for HWE tests were corrected for multiple comparisons using the sequential Bonferroni procedure (Rice, 1989). ARLEQUIN 3.5.2 was used to identify putative loci under selection (outlier loci) in the global dataset using the hierarchical island model with 20,000 simulations and 100 demes per group (Excoffier & Lischer, 2010) in addition to F_{IS} estimates and corrected average pairwise differentiation.

2.2.6 Population structure analysis

To determine the level of genetic structure within populations both the global and TSP datasets were tested for the presence of distinct genetic clusters (K) using the Bayesian clustering method with MCMC inference using the STRUCTURE 2.3.4 software (Prichard et al., 2000). Structure analyses were performed on the complete global and TSP datasets comprising of all 4 and 5 populations, respectively, and then in a hierarchical manner where each population was analysed separately to determine if fine structuring existed within populations which may have been obscured by the major axes of structure on the overall datasets (Evanno et al., 2005). The Bayesian clustering model estimates the most likely K by minimising overall deviation from HWE and linkage equilibrium within each K. For this analysis, an admixture model with correlated allele frequencies was used to replicate the most plausible type of population connectivity and gene flow between sampling locations. Furthermore, the LOCPRIOR option was utilised to allow the model to include prior information about the sampling locations. A range of K values, K = 1 - 4 and K = 1 - 5 for the full and TSP datasets, respectively, were tested with a burn-in of 50,000 followed by 100,000 MCMC repetitions with 10 replicates for each K value. In order to identify the most likely K value, two methods were used along with a comprehensive biological interpretation; first, the most likely K was determined

by the ΔK statistic (Evanno et al., 2005) using STRUCTURE HARVESTER v0.6.94 (Earl et al., 2012); and second, the posterior probability values were calculated for each K within STRUCTURE and displayed in CLUMPAK 1.1 (Jakobsson & Rosenberg, 2007). The optimal K for each analysis was then run using the same model with a burn-in of 50,000 and then 500,000 MCMC repetitions with 10 replicates for each chosen most likely K value. Results of the model runs were assimilated, averaged and displayed using CLUMPAC 1.1.

2.2.7 Mitochondrial DNA extraction and processing

Twenty individuals from each of the five sampling locations were selected for further investigation of population structuring through sequencing of mitochondrial DNA (mtDNA) control region data. Following the protocol of D'Arcy et al., (2013), primer A 5'-TTCCACCTCTAACTCCCAAAGCTAG-3' (Lee et al., 1995) and TDKD 5'-CCTGAAGTAGGAACCAGATG-3' (Kocher et al., 1993) were used to amplify a portion of the mtDNA control region of wild ballan wrasse. Each amplification was carried out in a volume of 7.5 µl, containing ~ 40 ng of genomic DNA, 3.75 µl 2 X Q5 HiFi hotstart taq polymerase (New England BioLabs; M0494S), 0.37 µl each of both forward and reverse primers and 2.25 µl H_20. Thermal cycling conditions included an initial denaturation step of 30 seconds at 95 °C, followed by 40 cycles of 95 °C for 10 s, 59 °C for 20 s, 72 °C for 20 s, followed by a final extension step for 2 minutes at 72 °C. Each PCR was quality checked by gel electrophoresis using 1µl of the reaction volume (Fig. 2.3). The remaining volume of each PCR was cleaned up prior to sequencing with the Rapid PCR Cleanup Enzyme Set (New England BioLabs; E2622), by adding 1µl each of the cleanup enzymes, Exonuclease 1 and rASP. Thermal cycling conditions for cleanup included an incubation time of 5 minutes at 37 °C followed by a 10 minute heat inactivation at 80 °C. Each amplified DNA fragment was sequenced using both the forward and reverse primer independently by a commercial sequencing service (lightrun GATC, Germany). Resulting homologous sequences from all individuals were aligned using MEGA 6.06 (Tamura et al., 2013). A number of individuals (20 % of the total sample size) were resequenced to ensure the consistency of the sequencing process. Phylogenetic relationships between haplotypes were examined using a haplotype network, constructed with the median-joining network algorithm in POPART 1.7 (available at http://popart.otago.ac.nz). The program Arlequin 3.5.1.3 (Excoffier and Lischer, 2010) was utilised to calculate the number of polymorphic sites, haplotype diversity, nucleotide

diversity and number of haplotypes in addition to neutrality tests; Fu's Fs (Fu, 1997) and Tajima's D (Tajima, 1989). Analysis of Molecular variance (AMOVA), using F-statistics based on haplotype frequencies, was performed in Arlequin with all British sites nested within one group and the Norwegian site as a second group, to assess any potential substructure. Significance tests were carried out after 10,100 permutations. Finally, pairwise Fst's were calculated between sample sites based on haplotype frequencies, with significance calculated after 10,100 permutations in Arlequin. Overall nucleotide and haplotype diversity were calculated in DnaSP v5 (Librado and Rozas, 2009)

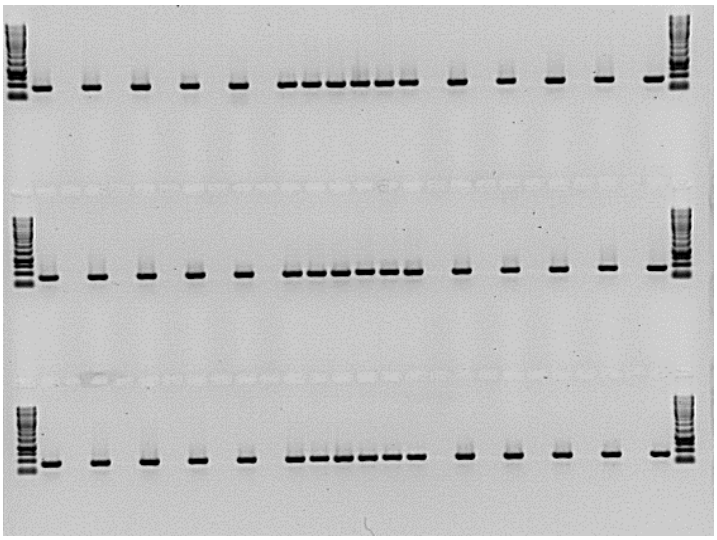

Figure 2.3. Example of an 0.8 % agarose gel electrophoresis of containing 1µl of 250 - 300 bp mtDNA control region amplified by PCR; Outer lanes on each row contain a 100 bp ladder, used to estimate the size of the PCR products.

2.3 Results

2.3.1 "Genotyping by sequencing" experiment

The DNA from 80 individuals (*n* = 20 per location; locations A-D) was analysed, barcoded, pooled, and sequenced in a single lane of an Illumina HiSeq 2000 Sequencer (Table 2.1). Sequencing effort generated a total of 297,493,266 raw reads and following quality filtering, which included removing low quality sequences, ambiguous barcodes and orphaned paired-end reads, 211,089,518 reads were used by the Stacks package

(Catchen et al., 2013) for the creation of individual paired-end markers. In total, this resolved down to a panel of 15,630 single nucleotide polymorphisms (SNPs) (Fig. 2.4).

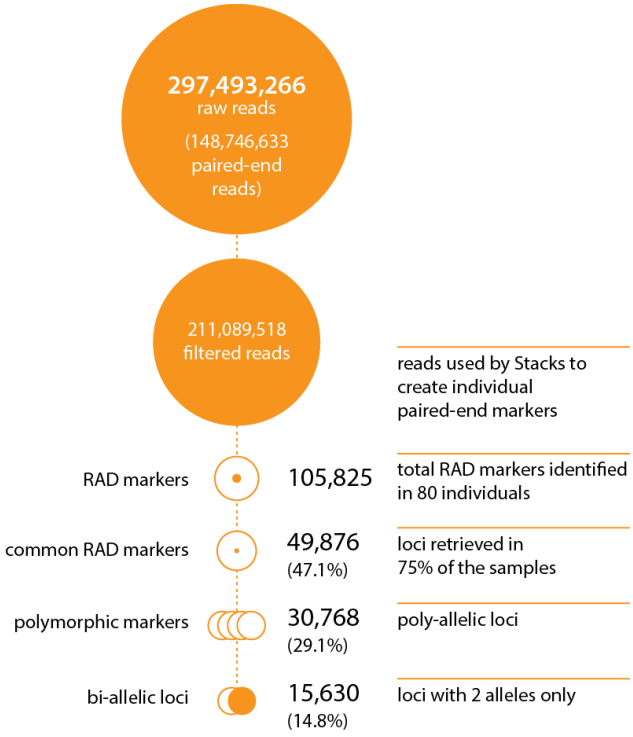

Figure 2.4. Sequencing and RAD tag summary: Details of the number of reads before and after filters (orange disks) followed by the reconstructed number of RAD markers, common RAD markers, polymorphic markers, and bi-allelic loci.

2.3.2 SNP panel investigation and population structuring

2.3.2.1 Global SNP panel

The average F_{ST} was 0.0533 across all 15,630 bi-allelic SNPs with a range of 0.00 – 0.99 (Table 2.3). A large proportion of SNPs were relatively uninformative and after

removing loci with a F_{ST} value of < 0.27 to form a more manageable dataset, the resulting set of 215 SNPs (hereafter referred to as the 'global dataset') had an average F_{ST} value of 0.3875.

Table 2.3. Locus F_{ST} (Weir & Cockerham, 1984) frequency distribution for 15,630 SNPs across 80 individuals, 20 each from locations A-D.

Fst	N	%
0.00 - 0.09	14178	90.710
0.10 - 0.19	1018	6.513
0.20 - 0.29	261	1.670
0.30 - 0.39	99	0.633
0.40 - 0.49	49	0.313
0.50 - 0.59	16	0.102
0.60 - 0.69	5	0.032
0.70 - 0.79	2	0.013
0.80 - 0.89	0	0.000
0.90 - 0.99	2	0.013
1	0	0.000
Total	15630	

A total of 601 tests for deviation from HWE were performed across all samples in the global dataset. Prior to and following sequential Bonferroni correction, 66 (10.9 %) and 4 (0.6 %) tests were significant, respectively. Furthermore, outlier detection revealed two potential loci under selection, SNP 214 (30549, F_{ST} 0.9232) and SNP 215 (11118, F_{ST} 0.9492) based on F_{ST} values, which were subsequently removed to create a 'neutral' dataset containing 213 SNPs with an overall F_{ST} value of 0.3808. All analyses were performed on the neutral dataset with no differences in overall population or genetic structure; therefore all results are reported for the global dataset containing all 215 SNPs.

To assess the genetic relatedness of ballan wrasse populations average F_{ST} for pairwise comparisons of all sampled populations were calculated based on the 215 SNPs within the global dataset; all pairwise F_{ST} values were found to be statistically significantly different from 0 at the alpha = 0.05 level (Table 2.4). Furthermore, there were no fixed alleles detected across the four sampled populations (i.e. markers specific to a given geographic location.

The Rong and Shetland populations were clearly differentiated from each other (F_{ST} 0.5082) and from both of the mainland UK based populations (Ardtoe and Machrihanish, F_{ST} 0.0884 – 0.5595). The UK based populations were only weakly differentiated from one another (F_{ST} 0.0199) and, although statistically significant, this may not be biologically significant as the value is particularly close to zero.

Table 2.4. Pairwise F_{ST} values (below diagonal) and corrected average pairwise differences (above diagonal) among ballan wrasse locations based on 80 individuals, 20 each from each location across 215 SNPs; * = Highly significant (p<0.001).

	Rong	Shetland	Ardtoe	Machrihanish
Rong	X	48.882*	46.987*	44.818*
Shetland	0.5082*	x	2.9946*	1.7864*
Ardtoe	0.4958*	0.0884*	X	0.6245*
Machrihanish	0.4878*	0.5595*	0.0199*	x

This level of structuring, based on the global dataset, was loosely supported by the initial structure analysis which suggested that the most likely number of genetic clusters across all four populations was three (K = 3), which corresponded to the Rong, Shetland, and UK (Ardtoe and Machrihanish) sampling locations. However, when a hierarchical approach was used to define clusters there was clear evidence of differentiation between the two 'UK' populations, resulting in K = 4 as the most likely number of clusters within the dataset which corresponded to the four main sampling locations (Fig. 2.5A). Based on a typical output for proportion of membership in each cluster, 97.7 % of Rong individuals were assigned to a single cluster with the remaining 2.3 % distributed between the other three defined clusters which very clearly defining a Rong grouping (Table 2.5). Similarly, 94.8 % of Shetland individuals were assigned to a single cluster with the remainder defined in the remaining clusters. However, the lower level of differentiation between the UK populations is further shown by 75.9 % and 49.6 % of Ardtoe and Machrihanish individuals, respectively, assigned to the same cluster. While the majority of individuals in both the UK populations were assigned to the same cluster, almost 40 % of Machrihanish individuals resolved to a final cluster suggesting a low level of differentiation between the two populations.

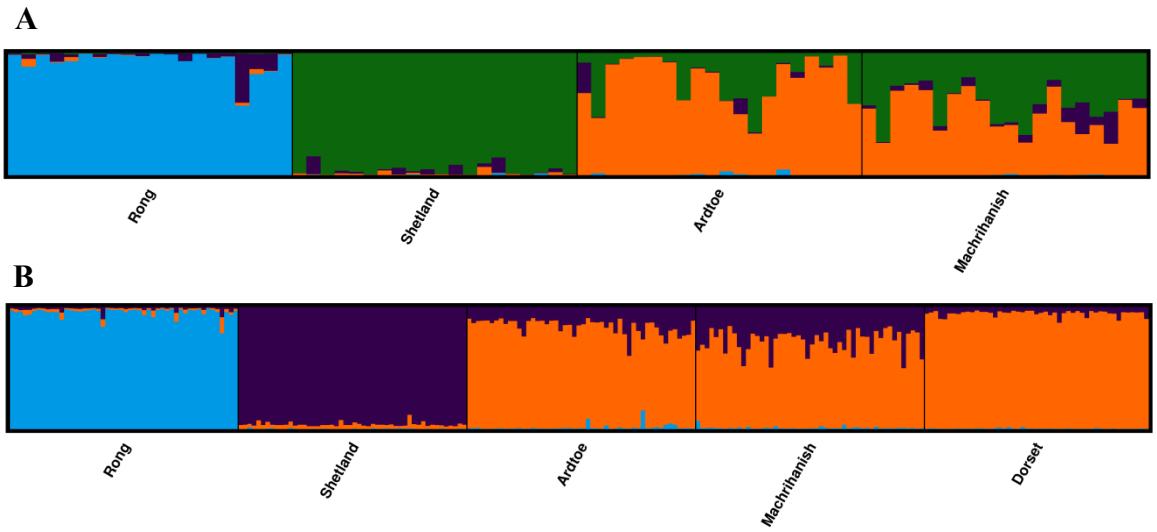

Figure 2.5. Proportional membership of each individual wrasse (represented by one vertical line) in the four clusters (K = 4) as identified by STRUCTURE in the global dataset (A, 215 SNPs, 80 individuals; 4 locations) and the TSP dataset (K = 3) (B, 11 SNPs, 250 individuals; 5 locations). Black lines separate population locations labelled below each figure.

Table 2.5. Typical example output from a single (one of ten runs) of proportion of membership of individuals within each inferred cluster (K = 4) as defined by STRUCTURE within the global dataset (215 SNPs).

	Inferred clusters				
	1	2	3	4	Number of Individuals
Rong	0.977	0.009	0.002	0.012	20
Shetland	0.003	0.044	0.948	0.005	20
Ardtoe	0.009	0.034	0.198	0.759	20
Machrihanish	0.002	0.102	0.399	0.496	20

2.3.2.2 Targeted SNP panel

From the global dataset, 11 highly informative SNPs were selected to form the TSP and translated into individual genotyping assays that were then applied to a further 30 individuals per location (Locations A-D) and 50 individuals from a previously untested location (Location E). The TSP had an average F_{ST} value of 0.3068 and ranged between 0.1206 – 0.5193. A total of 38 tests for deviation for HWE were performed across samples in the TSP dataset with 3 (7.8 %) and 1 (2.6 %) significant tests before and after sequential bonferroni correction, respectively. Significant tests following correction for multiple testing included one marker within one population in the TSP and only one significant test involved a SNP likely to be affected by selection; therefore we do not expect departure from HWE within the TSP dataset to affect downstream analysis. Levels of H_O and H_E were comparable across the five sampled locations and ranged between 0.21 – 0.35; however, observed heterozygosity was higher than the expected heterozygosity in two out of the five populations (Table 2.6). Both Ardtoe and Machrihanish had a highly positive inbreeding coefficient (F_{IS} = 0.12 and 0.11 for Ardtoe and Machrihanish, respectively; Table 2.6), indicating that these populations had an excess of homozygotes. The deficit of heterozygotes in these two populations may be explained by their close geographic proximity which may have allowed a level of mixing between populations or localised population structuring.

Table 2.6. Summary statistics for 5 populations of ballan wrasse in the UK and Norway calculated over 11 microsatellite loci. H_o = observed heterozygosity, H_e = expected heterozygosity; F_{IS} = inbreeding coefficient; SD = Standard deviation. * Denotes significant $p < 0.05$.

Population	*n*	H_o± SD	H_e± SD	F_{IS} ± SD
Rong	50	0.35 ± 0.19	0.34 ± 0.18	-0.01 ± 0.13
Shetland	50	0.30 ± 0.14	0.29 ± 0.14	-0.04 ± 0.12
Ardtoe	50	0.21 ± 0.12	0.25 ± 0.15	0.12 ± 0.18*
Machrihanish	50	0.25 ± 0.16	0.28 ± 0.17	0.11 ± 0.20*
Dorset	49	0.27 ± 0.17	0.28 ± 0.15	-0.05 ± 0.35

Analysis of population differentiation and structure within the TSP dataset indicated that two (Rong and Shetland) out of the five sampling locations were genetically differentiated based on pairwise F_{ST} values. All pairwise comparisons of the five sampling locations ranged from 0.0023 (Ardtoe vs Dorset) to 0.5413 (Rong vs Shetland) (Table 2.7). Overall 7 out of 10 of the pairwise comparisons of genetic differentiation between sampling locations were significant which resolved to three genetically distinguishable populations among the five sampling sites. Two out of three of the putative populations corresponded to unique sampling locations (Rong and Shetland) and the last grouped together neighbouring sampling locations (Ardtoe, Machrihanish, and Dorset) hereafter referred to as the 'UK' population. This structuring was supported by Bayesian clustering analysis which revealed three clear clusters, consistent with the Rong and Shetland and UK sampling sites (Fig. 2.5B). A typical example of assignment to clusters defined for Rong and Shetland populations contained 97.2 and 97.0% of individuals from each location, respectively (Table 2.8). The third and final 'UK' cluster defined for the TSP dataset contained 87.4, 72.3, and 96.6 % of individuals from Ardtoe, Machrihanish and Dorset respectively.

Table 2.7. Pairwise F_{ST} values (below diagonal) and corrected average pairwise differences (above diagonal) among ballan wrasse locations based on 249 individuals, 50 each from locations A-D and 49 individuals from location E across 11 SNPs; * = Highly significant (p<0.001).

	Rong	Shetland	Ardtoe	Machrihanish	Dorset
Rong	X	3.6454*	3.0252*	2.6969*	2.7469*
Shetland	0.5413*	X	0.2776*	0.2085*	0.3755*
Ardtoe	0.4881*	0.0955*	x	0.0345	0.0063
Machrihanish	0.4465*	0.0693*	0.0118	X	0.0147
Dorset	0.4615*	0.1239*	0.0023	0.0050	x

Table 2.8. Typical example output from a single (one of ten runs) proportion of membership of individuals within each inferred cluster as defined by STRUCTURE within the TSP dataset (11 SNPs).

	Inferred Clusters			
	1	2	3	Number of Individuals
Rong	0.025	0.003	0.972	50
Shetland	0.97	0.026	0.004	50
Ardtoe	0.122	0.874	0.004	50
Machrihanish	0.267	0.723	0.01	50
Dorset	0.031	0.966	0.003	49

2.3.3 mtDNA sequencing for population structuring

After removing PCR primer sequence data at the 3- and 5- ends, 381 bp of mtDNA control region sequences were obtained for 100 ballan wrasse from 5 locations. In total, 49 haplotypes were identified among the sampled areas (Table 2.9), 19 of which were previously reported by D'Arcy et al., (2013) and 30 of which were novel within the sampled areas. The most common haplotype was present in all four of the UK populations but was absent in the Norwegian population, with the highest representation in Shetland samples, followed by equal representation in both Dorset and Machrihanish populations and rare in Ardtoe populations. Moreover, Rong samples were represented by 9 haplotypes that were completely absent in all UK sampling sites. All sampled areas were characterised by a large proportion of unique haplotypes, which accounted for a total of 33 out of 49 (67.3 %; Table 2.9). The overall haplotype diversity was 0.9590 ± 0.01 and the overall nucleotide diversity was 0.0303 ± 0.0015. Haplotype diversity within the UK sampling areas ranged between 0.8895 and 0.9842 and was lower in the Norwegian site (0.6526). Nucleotide diversity followed a similar pattern with higher diversity within UK sampling sites (0.0206 – 0.2986) than the Norwegian sampling site (0.0107) (Table 2.10).

Table 2.9. Frequencies of the 49 haplotypes in the 5 sampled areas. Top half of the table represents previously established haplotypes from D'Arcy et al., (2013), bottom half of table represents novel haplotypes within sampled individuals.

Haplotype	Total Frequency	Dorset	Machrihanish	Ardtoe	Shetland	Rong	GenBank Accession No.
Haplo01	12					12	KC477846
Haplo02	14	3	3	2	6		KC477847
Haplo04	4		1		3		KC477849
Haplo05	1					1	KC477850
Haplo06	3	1	1	1			KC477851
Haplo07	2			1	1		KC477852
Haplo08	6	2	2	1	1		KC477853
Haplo10	2		1	1			KC477855
Haplo11	1			1			KC477856
Haplo13	1		1				KC477858
Haplo14	1					1	KC477859
Haplo15	1					1	KC477860
Haplo19	2	1	1				KC477864
Haplo20	2		1		1		KC477865
Haplo22	2	1	1				KC477867
Haplo27	1	1					KC477872
Haplo58	3	2		1			KC477903
Haplo69	1	1					KC477914
Haplo75	1					1	KC477920
Haplo93	4	2	1		1		
Haplo94	3	1		1	1		
Haplo95	2		2				
Haplo96	2			2			
Haplo97	2			2			
Haplo98	1	1					
Haplo99	1	1					
Haplo100	1	1					
Haplo101	1	1					
Haplo102	1	1					
Haplo103	1		1				
Haplo104	1		1				
Haplo105	1		1				
Haplo106	1			1			
Haplo107	1			1			
Haplo108	1			1			
Haplo109	1			1			
Haplo110	1			1			
Haplo111	1			1			

Haplo112	1			1		
Haplo113	1				1	
Haplo114	1				1	
Haplo115	1				1	
Haplo116	1				1	
Haplo117	1				1	
Haplo118	1				1	
Haplo119	1					1
Haplo120	1					1
Haplo121	1					1
Haplo122	1					1
Total	98	20	18	20	20	20

Tests for selective neutrality, including both Fu's Fs and Tajima's D revealed departure from selective neutrality (based on both negative and significant values) in two of the UK sites (Fu's Fs) and the Norwegian site (Tajima's D), whereas no deviation from selective neutrality was seen in the remaining two UK sites (Table 2.10).

Table 2.10. Descriptive statistics and diversity indices of 381 bp of the mitochondrial control region of ballan wrasse from five locations. *N*, number of individuals; N_H, number of haplotypes; Blue, clade I; Red, clade II; *H*, haplotype diversity, ∏, nucleotide diversity; *D*, Tajima's D; F_s, Fu's F_s; *$p < 0.05$, ** $p < 0.01$

	Rong	Shetland	Ardtoe	Machrihanish	Dorset
N	20	20	20	20	20
N_H	9	12	17	16	15
Clade I vs II frequencies					
Polymorphic sites	28	32	39	36	41
H	0.6526 ± 0.1225	0.8895 ± 0.0548	0.9842 ± 0.0205	0.9737 ± 0.025	0.9684 ± 0.254
∏	0.0107 ± 0.0062	0.0206 ± 0.0112	0.0279 ± 0.01486	0.0250 ± 0.0134	0.2987 ± 0.0158
D	-2.0644**	-0.4041	0.0129	-0.2082	0.0850
F_s	-0.7148	-0.9370	-5.0178*	-4.1942*	-2.2341

A haplotype network was created to understand the phylogenetic relationships among mtDNA control region sequence data from individuals within the 5 sampling locations (Fig. 2.6). The haplotype network showed a star-like formation with many individual haplotypes linked by a single or a few mutational steps and, furthermore, was dominated by two main clusters of haplotypes (clades) which were separated by 12 mutational steps. Each of the two main clades included individuals from all 5 of the sampling locations. Frequencies of Clade I versus Clade II haplotypes within each of the sampling locations is represented in Table 2.10. Clade I was made up of many low frequency haplotypes and one more common haplotype that was present within all UK sampling sites and absent from Norwegian sampling sites. Similarly, Clade II was characterised by many low frequency haplotypes, however, the most common haplotype was only present within the Norwegian sampling site (Fig. 2.6).

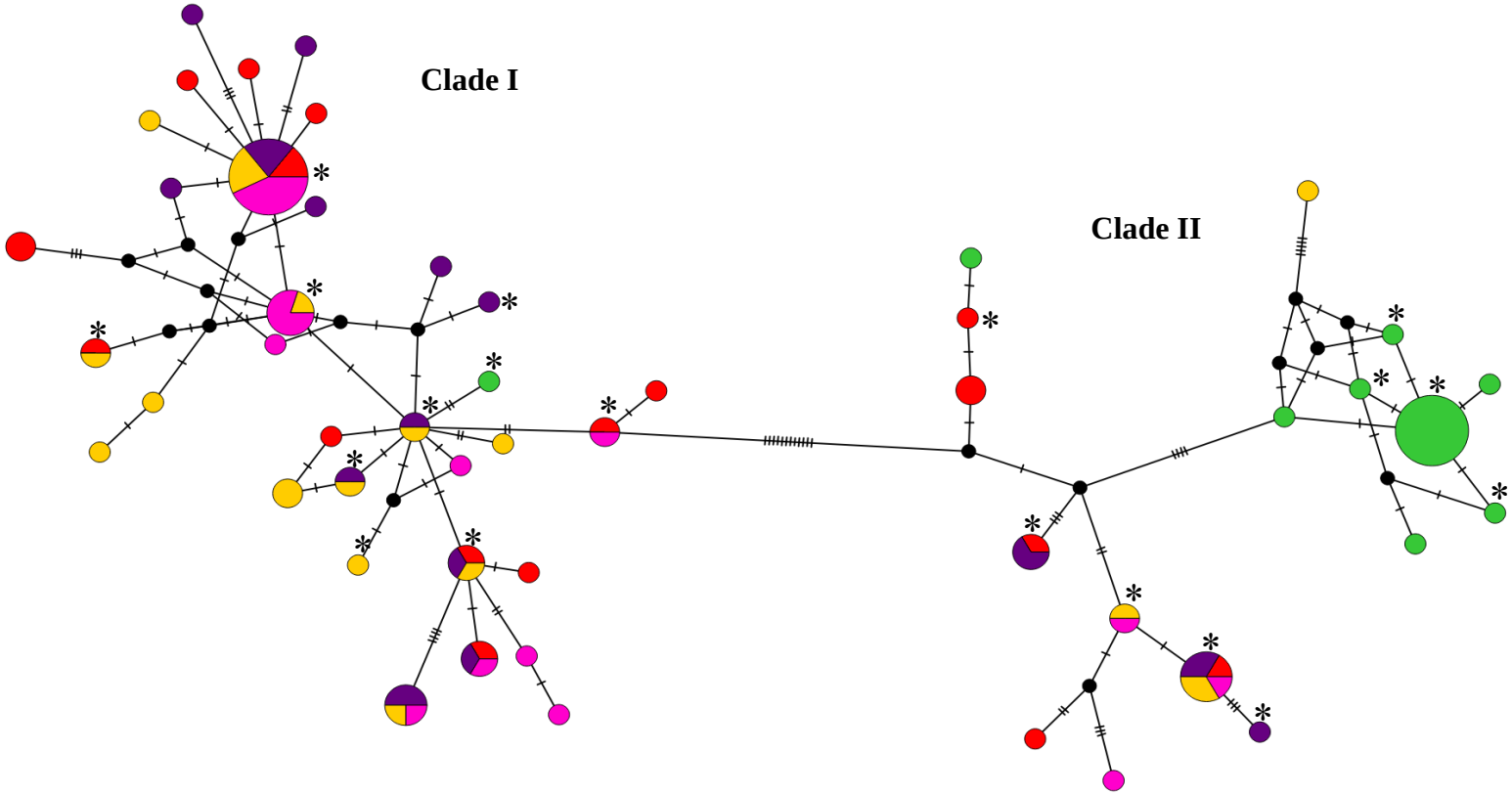

Figure 2.6. Network of haplotypes. Node size reflects the frequency of its occurrence (green, Rong; pink, Shetland; red, Ardtoe; yellow, Machrihanish; purple, Dorset). Dashes on lines indicate the number of mutational steps between nodes; small black circles represent missing or un-sampled haplotypes; * represents previously reported haplotypes from D'Arcy et al., (2013).

A hierarchical AMOVA was performed among sampling locations nested within UK (inclusive of Shetland, Ardtoe, Machrihanish, and Dorset) and Norwegian regions. The greatest variance observed was found to be within populations (82.3 %; F_{ST} = 0.177; p < 0.001; Table 2.11). However, no significant variation was found among regions or populations within regions. Correspondingly, the pairwise F_{ST} values, based on haplotype frequencies, revealed strong differentiation between regions however not within regions, with only the Norwegian sampling site showing significant differentiation from all UK sites (Table 2.12).

Table 2.11. Results from AMOVA. Regions = Norway and UK.

Source of Variation	d.f.	Sum of squares	Observed Partition			
			Variance components	% variation	F statistic	*p*-value
Among regions	1	3.487	0.0932	17.17	Fct = 0.1717	0.1929 ± 0.0043
Among populations within regions	3	1.513	0.0029	0.53	Fsc = 0.0064	0.3531 ± 0.0044
Within populations	95	42.45	0.4468	82.3	Fst=0.1769	0.0000 ± 0.0000
Total	99	47.45	0.5429			

Table 2.12. Population pairwise F_{ST}. Top diagonal = F_{ST} values of 5 independent populations when run for 10,000 permutations at 0.05 significance level; Lower diagonal = *p* values based on 10,100 permutations; * = Highly significant ($p<0.001$).

	Ardtoe	Machrihanish	Shetland	Dorset	Rong
Ardtoe	X	-0.0041	0.0267	-0.0065	0.1816*
Machrihanish	0.7205 ±0.0043	X	0.0037	-0.0168	0.1868*
Shetland	0.0649 ± 0.0025	0.3737 ± 0.0047	X	0.0144	0.2290*
Dorset	0.7844 ± 0.0036	0.9677 ± 0.0017	0.1988 ± 0.0039	X	0.1895*
Rong	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0000 ± 0.0000	X

2.4 Discussion

Understanding and measuring genetic diversity and fine scale structuring in marine fish populations is an important prerequisite for the effective management, conservation, and monitoring of wild stocks. The past idea that marine fish with a pelagic larval phase are typically panmictic over their geographical range due to a demographically open ocean territory (Ward et al., 1994; Jennings et al., 2001) has changed over the years due to the analyses of population dynamics, concluding the existence of genetically separated units and suggesting significant population structuring in some marine fishes (Hauser & Carvalho, 2008). The main goal of this study therefore was to identify a panel of SNPs to help better delineate fine scale genetic structure in wild populations of ballan wrasse throughout the UK and Norway. Moreover, this study endeavoured to provide the tools and insight into 1) genetic management of farmed stocks with respect to selection of broodstock for commercial hatcheries and 2) assess the possible risks that escapees may pose to local wild populations. Through the use of RAD sequencing, a large number of novel SNPs were identified from 20 individuals from 4 geographic locations, of which 215 were found to be informative for geographic location in ballan wrasse, showing significant differentiation between Norwegian, Shetland, and both mainland UK origin populations. This represents a level of segregation which is more detailed than previously reported for this species (D'Arcy et al., 2013). A closer examination using a panel of 11 SNPs, within a larger population sampling which included a fifth UK sampling location, revealed fine scale structuring across the study range with clear differentiation of Norwegian and Shetland individuals from the mainland UK populations, but did not support clear sub-structuring between mainland UK populations. As such, this work provides improved phylogeographic definitions that have clear implications for the selection of founder populations for hatchery production of the species.

The level of population structuring shown through analysis of mtDNA was comparable to results determined by D'Arcy et al., (2013), whereby two main clades were identified, each with an uneven representation of individuals from UK and Norwegian populations. The Norwegian population was clearly differentiated from UK populations; however, population structuring was not evident between the UK mainland and Shetland populations using this analysis method. Furthermore, both haplotype and nucleotide diversity were lower in the Norwegian origin population than in UK populations which was also demonstrated by D'Arcy et al., (2013), and similarly by Robalo et al., (2011) for

Scandinavian vs. Atlantic populations of corkwing wrasse. Although population divergence times and time to most recent ancestor was not estimated within this study, both D'Arcy et al., (2013) and Robalo et al., (2012) suggested that this sharp contrast in haplotype and nucleotide diversity between Norwegian/Scandinavian and UK populations may be a result of population expansion occurring more recently in Northern regions following the last glacial maxima which ended approximately 18-25 thousand years ago (Bernatchez & Wilson, 1998; Taberlet et al., 1998). Furthermore, the same authors suggested that Norwegian populations, which happen to be at the northern most geographical limit of the species range, may have been colonised on a more recent timescale and by fewer individuals than UK populations. One interesting difference between the current study and D'Arcy et al., (2013) is the presence of mixed origin haplotypes. Within this study, all haplotypes were either specific to Norwegian or UK derived individuals whereas D'Arcy et al., (2013) reported 2 haplotypes occurring which had a combination of both UK and Norwegian origin individuals (6% and 8% inclusion of the minor location within haplotypes). As will be discussed further below, the SNP based analysis suggests clear population differentiation of the Norwegian vs. all other sampling locations and thus it would be fair to anticipate clearly segregated mitochondrial DNA haplotypes as was observed in the current study. The low abundance of "mixed" individuals may have negated their detection in the current study, however there is a possibility that the occurrence of mixed location haplotypes in D'arcy et al., (2013) may in fact be due to technical error. The authors sequenced using a standard "non-proofreading" Taq polymerase enzyme (Promega GoTaq) which has a reasonably high error rate (*circa* 1:3-4000 bases), whereas in the current study, a high performance proofreading enzyme was used (New England Biolabs Q5) with a greatly reduced error rate (*circa* = 1:1,000,000 bases). Thus, there is a *circa* 250-300 fold lower chance of sequencing error using the current methodology which is a technical consideration that should not be overlooked when comparing such studies. Overall, mtDNA analysis, both in this study and in D'Arcy et al., (2013) are in agreement and show clear differentiation between Norwegian and UK populations, however they both fail to detect any finer scale structuring within UK populations.

Mitochondrial DNA has been widely accepted as an effective tool in many population genetic and phylogenetic studies (Zhang & Hewitt, 2003; Hurst & Jiggins, 2005). Advantages to the use of mtDNA include its high stability over time, due to its high copy

number and circular genome, which makes it easy to obtain DNA from small or highly degraded samples. Furthermore, the relatively low cost for analysing large numbers of individuals makes it a widely applied methodology (Veeramah & Hammer, 2014). However, the fact that mtDNA is maternally inherited giving only a single genealogy, although useful for reconstruction of individual gene trees, only provides information for historical processes in females rather than the underlying population as a whole (Hurst & Jiggins, 2005). Moreover, genetic diversity can often be underestimated by mtDNA markers as the effective population size for mtDNA is smaller than that of nuclear DNA which leads to a faster lineage sorting rate and higher allele extinction rate (Zhang & Hewitt, 2003). On the other hand, nuclear markers such as SNPs are rapidly becoming the markers of choice for population genetic studies due to their large abundance throughout the genome, occurring as frequently as one SNP every few hundred base pairs (Morin et al., 2004). Furthermore, SNPs are associated with low-scoring error rates and a high level of information content for population structure analysis (Brumfield et al., 2003; Helyar et al., 2011). However, the use of SNPs in nonmodel species was, until recently, limited by the technical aspects of SNP discovery, genotyping and analysis of the associated large datasets (Garvin et al., 2010). Recent improvements in the cost, accuracy and speed of high throughput sequencing, along with enhancements in the associated bioinformatics software pipelines for analysing large datasets, has revolutionised the discovery of SNPs and their application in population structuring studies in species that are data poor (Helyar et al., 2011; Davey et al, 2011; McCormack et al, 2013).

While the power of SNPs for population differentiation is widely recognised, at the beginning of this study there were no registered SNPs for ballan wrasse. The use of RADseq was employed in this study as an effective method that can identify and score thousands of genetic markers distributed across an entire targeted genome (Baird et al., 2008; Davey et al., 2011; McCormack et al., 2013). Furthermore, RADseq can be applied in population genetic studies in species with limited or non-existent sequence data, i.e. no reference genome (Davey & Blaxter, 2011). Through the use of this sequencing technology 15,630 bi-allelic SNPs were discovered that had never before been reported in this species. When this dataset was interrogated to look for SNPs that would be informative for geographic location within ballan wrasse populations it resulted in a panel of 215 potentially informative SNPs. This high volume SNP discovery is

comparable to that reported for the American lobster (*Homarus americanus*) through the use of RADseq, which resulted in a panel of 10,156 SNPs that were informative on population structuring across the study range (Benestan et al., 2015). Similarly, high volume SNP discovery through the use of RADseq technology has been successful in studies on Oregon threespine stickleback (*Gasterosteus aculeatus L.*) (Hohenlohe et al., 2010; Catchen et al., 2013) and rainbow trout (*Oncorhynchus mykiss W.*) (Sánchez et al., 2009) among other species.

Although there are many examples in the literature on marine fish species showing genetic homogeneity over large geographic scales of up to thousands of kilometres (Palumbi, 1992; Benzie, 1999; Walters et al., 2000; Chen et al., 2004), typically explained by the connectivity created by sea surface currents, there are also studies documenting some species with high genetic divergence among populations with no distinct barriers to dispersal (Bell et al., 1982; McMilliam & Palumbi, 1995; Shulman & Berminghan, 1995; Walters et al., 2000; Chen et al., 2004). Similarly, within the current study, based on the global SNP panel, results revealed clear evidence of genetic structuring in ballan wrasse sampled across the species' northern range. The existence of a hierarchical genetic structure was evident, first separating populations in Norway and Shetland from the mainland UK and then separating the two populations within the mainland UK. Therefore, four distinct populations were resolved from the four initial sampling locations throughout the UK and Norway, revealing finer-spatial scale genetic structuring than previously reported for this species. All pairwise F_{ST} values were highly statistically significant; however, the highest differentiation was between the Norwegian population and all other populations (F_{ST} range = 0.4878 – 0.5082) which mirrored the findings of the mitochondrial DNA haplotypes study.

To improve the resolution and thus power of the comparison, further analysis of more individuals from each of the original populations (n raised from 20 to 50 per location), as well as a previously untested fifth population was performed using a TSP. The 11 SNPs chosen for the TSP were selected based primarily on their population differentiation strength (average F_{ST} = 0.27) as well as technical limitations due to the allele specific PCR assay design in association with the available sequence information flanking the identified SNP. While the TSP was able to detect significant differences between the Norwegian and Shetland populations from the mainland UK populations (inclusive of

Ardtoe, Machrihanish and Dorset) it was not able to differentiate between the three mainland populations. Results based on the STRUCTURE output alluded to a possible finer scale differentiation between the English and Scottish mainland populations; however this was not supported by analysis of pairwise F_{ST}. Furthermore, both of the Scottish mainland populations had highly significant inbreeding coefficients (F_{IS} = 0.11 and 0.12 for Ardtoe and Machrihanish, respectively). Positive F_{IS} values such as this indicate that individuals within a population are more closely related than expected under the assumption of random mating (Keller & Waller, 2002; Holsinger & Wier, 2009). However, at this stage there is no way to determine if the highly significant F_{IS} levels are due to discrete inbreeding within each of the two populations or if the relatedness of individuals may be due to the initial selection of individuals from a relatively close proximity to each other.

It should be recognised that the TSP represents only a single formulation of the possible combinations of the 215 SNPs that were identified as being informative for geographic location. It is possible that if alternative SNPs were analysed, the panel performance and thus structuring definitions may have differed. Future work could possibly explore this further though a re-analysis of the global data output focusing on Machrihanish and Ardtoe datasets to identify SNPs more specifically associated with this local differentiation, and subsequently apply them in the larger dataset. Notwithstanding these technical limitations, the current analysis alludes to a level of population structuring never before reported in ballan wrasse which provides a greater insight in to the ecology of the species as well as having practical implications for the exploitation of the species.

Many factors can be responsible for the genetic structure of marine teleost species over a range of levels from biological factors, such as the dispersal potential of eggs, larvae, and adult fish (Froukh & Kochzius, 2007), to environmental factors such as recent and past climatic changes (Perry et al., 2005), and even including anthropological implications such as those imposed by fishing pressures and both direct and indirect damages to marine ecosystems (Halpern et al., 2008). At a geographical scale, results from this study indicate that there is a major population break in the North Sea, which concurs with suggestions by D'Arcy et al., (2013), but also there is a clear break between the Shetland population from Norway and mainland UK populations, as well followed by low levels of differentiation between mainland UK populations. This may indicate that deep waters

between the mainland UK and Shetland (50-100 m) as well as between Shetland and Norway (100-200 + m) may be one of the main barriers to gene flow in ballan wrasse within the studied areas. This is not an unexpected result, when taking the biological aspects of this species into consideration; benthic substrate spawners (D'Arcy et al., 2012), territorial harem spawning (Sjolander et al., 1972; Dipper et al., 1977), high site fidelity (Morel et al., 2013) and relatively small estimated home range sizes of 0.133 ± 0.072 km^2 (Villegas-Ríos et al., 2013c). Furthermore, ballan wrasse are typically found in areas no deeper than 50 m (Artrüz, 2005). Therefore, it is safe to assume that the main form of larval dispersal is through local oceanic currents. Moreover, the low levels of differentiation between the mainland UK populations (namely Ardtoe and Machrihanish) may suggest the potential for marked genetic connectivity among sites and putative populations which could potentially be a result of varying inshore water currents and relatively close geographic distance between sampling sites (approximately 240-280 km between Ardtoe and Machrihanish).

Ultimately, the main driver for this study was to inform the collection of founder populations for commercial wrasse hatcheries to secure a consistent supply of farmed cleaner fish for the salmon industry. The sampling design within this study focused on obtaining samples belonging to different spatial units currently associated with the harvest as well as deployment of both wild and farmed ballan wrasse in the context of the UK salmon farming industry. When the current results are considered as a whole, the most pragmatic interpretation would be to advise against large geographical translocations of ballan wrasse and likewise, it would be prudent to source broodstock for commercial hatcheries as locally as possible to where farmed generations may be deployed. If broodstock sourcing and subsequent farmed wrasse deployment adhered to the "Mainland UK", "Shetland" and "Norwegian" groupings it would minimise the risk of possible introgression impact. Furthermore, every precaution possible should be taken to limit the escape of farmed individuals from cage sites. While escape events caused by technical errors or adverse weather conditions are regrettably unavoidable, good farming practice can go a long way to prevent escapes from occurring. Nevertheless, there is always a potential risk of farmed ballan wrasse mixing with wild populations as has been documented for farmed Atlantic salmon escaping and mixing with wild populations in Ireland (Clifford et al., 1998; Crozier, 2000), North America (Bourret et al., 2011) and Norway (Glover et al., 2012; Glover et al., 2013). Glover et al., (2013) describes the

large-scale invasion in wild Atlantic salmon populations by farmed escapees as one of the most significant examples of human-mediated increased straying rates for any organism. The potential impacts of farmed escapes on wild populations, in the case of Atlantic salmon, have been described on many levels from the biological risks such as the direct competition with wild fish for mating, territory, and prey, to the risks associated with pathogen transmission from farmed to wild salmon (Naylor et al., 2005). Furthermore, the impacts of genetic interactions of farmed escaped salmon with their wild counterparts can be both direct and indirect which result in an overall loss of fitness or recruitment in wild populations (Hindar et al., 2006; Ferguson et al., 2007). Interbreeding between wild and farmed fish inevitably results in the mixing of gene pools and if the subsequent offspring go on to reproduce this can eventually lead to a wild population composed entirely of individuals that have descended from farmed escapees. Repeated occurrences of such interbreeding would ultimately result in the loss of genetic diversity of wild salmon and their ability to survive and adapt to environmental change (Naylor et al., 2005).

On the other hand, unlike Atlantic salmon escapees, ballan wrasse would not need to migrate to freshwater to spawn and therefore would have the potential to establish breeding populations within the direct vicinity of the cage sites. However, with the recommended stocking ratio of ballan wrasse to farmed salmon sitting at approximately 1 - 5 % (Skiftesvik et al., 2013; Leclercq et al., 2014a) this represents a much lower level of risk. Moreover, of the 15,630 markers identified there was no evidence of fixed alleles or markers specific to a given population within the ballan wrasse populations tested. Therefore, introgression would have to be evidenced through homogeneity of allele frequencies, which requires significant admixture to show a measurable shift (Glover et al., 2013). With respect to the animals themselves, farmed stocks are generally 2 years of age (40 g) when stocked to sea cages (*personal communication*, Mr. Paul Featherstone) and first sexual maturity (as females) is not reached until 6+ years (Leclercq et al., 2014b). Therefore when considered as a whole, the likelihood of farmed ballan wrasse escaping, surviving > 4 years to reach first maturation, establishing within local breeding populations, and contributing to local recruitment should ultimately be considered highly unlikely.

Altogether, this study has made a significant technical contribution to the development of analytical methods to support the conservation as well as on-farm management of the species. The identification of over 15K novel genetic markers specific to the species is the first step towards developing a genomics toolkit that is a corner stone of modern broodstock management (Migaud et al., 2013). Thereafter, the application of a subset of these SNPs has provided new finer scale definitions of population structuring among wild ballan wrasse populations throughout the UK and Norway than has previously been reported for this species (D'Arcy et al., 2013). As such, it provides clear steerage as to the most pragmatic approach to sourcing of founder broodstocks to minimise the perceived risk of subsequent possible interaction of farmed and wild stocks. Moreover, this work provides a step forward in the development of sustainable cleaner fish production and practices which are rapidly becoming highly sought after by the growing salmon farming industry.

3 Chapter 3: Seasonal changes in broodstock spawning performance and egg quality in ballan wrasse (*Labrus bergylta*).

RESEARCH ARTICLE

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Keywords: Cleaner fish; ballan wrasse; broodstock management; parental contribution.

Abstract

Sea lice continue to be one of the largest issues for the salmon farming industry and the use of ballan wrasse (*Labrus bergylta*) as a biological control is considered to be one of the most sustainable solutions in development. Broodstock management has proved challenging, in the initial phases, due to the significant lack of understanding of basic reproductive physiology and behaviour in the species. The aim of the study was to monitor captive breeding populations throughout a spawning season to examine timing and duration of spawning, quantify egg production, and look at seasonal changes in egg quality parameters as well as investigate the parental contribution to spawning events. A clear spawning rhythm was shown with 3-5 spawning periods inclusive of spawning windows lasting 1-9 days followed by interspawning intervals of 8-12 days. Fertilization rate remained consistently high (> 87.5 %) over the spawning season and did not differ significantly between spawning populations. Hatch rate was variable (0-97.5 %), but peaked in the middle of the spawning season. Mean oocyte diameter and gum layer thickness decreased slightly over the spawning season with no significant differences between spawning populations. Fatty acid (FA) profile of eggs remained consistent throughout the season and with the exception of high levels of ARA (3.8 ± 0.5 % of total FA) the FA profile was similar to that observed in other marine fish species. Parental contribution analysis showed 3 out of 6 spawning events to be single pair matings while the remaining 3 had contributions from multiple parents. Furthermore, the proposed multiple batch spawning nature of this species was confirmed with proof of a single female contributing to two separate spawning events. Overall this work represents the first comprehensive dataset of spawning activity of captive ballan wrasse, and as such and will be helpful in formulating sustainable broodstock management plans for the species.

3.1 Introduction

Sea lice (*Lepeophtheirus salmonis* and *Caligus* spp.) have been reported as the most harmful ectoparasites to the Atlantic salmon (*Salmo salar*) farming industry (Costello, 2006) with an estimated total economic cost ranging from 4 to 10 % of production value globally (Rae, 2002) which translates to approximately € 33 million in Scotland alone (Costello, 2009). Parasitic sea lice feed on the mucus, tissue and blood of their hosts leading to stress, reduced growth performance, and a risk of secondary infections and mortalities. The use of wild wrasse as a biological control of sea lice was first implemented in Norway in 1989 (Bjordal, 1990) followed by Scotland in 1990 (Sayer et al., 1993; Rae, 2002). The method has gained new incentive in recent years across the European salmon industry in an effort to establish effective integrated pest management practices (IPM) with minimal reliance on chemotherapeutants (Leclercq et al., 2014a).

Ballan wrasse (*Labrus bergylta*) is the fastest growing of five wrasse species commonly found in northern European coastal waters (Treasurer, 2002) and further is regarded as the most robust and active in winter (Sayer et al., 1996; Kvenseth et al., 2003). It has, therefore, been selected by the salmon industry as the prime labrid species for the development of a sustainable, steady and bio-secure supply of farmed cleaner fish. Ballan wrasse is a protogynous hermaphrodite with no apparent external sexual dimorphism (Dipper, 1987; Evans & Claiborne, 2006; Muncaster et al., 2013; Leclercq et al., 2014b). The species exhibits a haremic mating system (Sjölander et al., 1972) and a skewed sex-ratio of approximately 10 % males in wild populations (Dipper, 1987). Protogynous sex change, thought to be driven predominantly by social cues (Dipper & Pullin, 1979; Hilldén, 1984; Muncaster et al., 2013), is reported to occur from 5 - 6 years of age with an age and size at 50 % sex-change of 10.8 years, 636 g and 342 mm (total length) in northern Europe (Dipper et al., 1977; Leclercq et al., 2014b). Ballan wrasse have been classified as a group-synchronous multiple-batch spawning species, based on histological evidence, with gonad maturation starting in November and spawning extending over a 2 month period, typically from April to July, depending on geographic location (Muncaster et al., 2010).

Commercial hatcheries currently rely on the natural spawning of captive wild harems maintained under controlled photo-thermal conditions. Ballan wrasse spawn adhesive, spherical, benthic eggs of approximately 1 mm in diameter (D'Arcy et al., 2012).

Hatcheries use artificial turf laid within broodstock tanks as a spawning substrate for the collection and incubation of eggs with hatching reported at 72 degree days (DD) post-fertilization (Ottesen et al., 2012). A description of the spawning periodicity of captive ballan wrasse along with potential fluctuations in egg quality over a full spawning season has not been reported but represents an important first step to rationalise and optimise hatchery operations as with any intensively cultured finfish species (Migaud et al., 2013).

Currently, there are no standard protocols to determine egg quality for ballan wrasse. Commonly used quality indicators across marine finfish species include, but are not limited to, egg size, fertilization and hatching rates, and the biochemical composition of eggs including lipids and fatty acids (FA) composition in particular (Bobe & Labbe, 2010; Migaud et al., 2013). Egg diameter in many multiple batch spawning species has been reported to reduce in size as the spawning season progresses (Bagenal, 1971; McEvoy & McEvoy, 1992) which may indicate an exhaustion of an individual female's physiological and nutritional condition (Trippel, 1998). Fatty acids, predominantly docosahexaenoic acid (22:6n-3; DHA), eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; ARA), usually correlate well with egg viability, egg development, hatching and larval survival (Rainuzzo et al., 1997; Sargent et al., 1999; Tveiten et al., 2004). However, no single parameter can define egg quality, so therefore it is vital to benchmark and assess several quality indicators to help improve husbandry techniques and overall hatchery productivity (Migaud et al., 2013).

Assessing the parental contribution to daily spawning events in naturally spawning harems is also an important milestone to assist hatcheries in establishing the optimal spawning populations. Furthermore, assessment of parental contribution could give further evidence to support the multiple-batch spawning nature of this species as proposed by Muncaster et al., (2010). Polymorphic microsatellite DNA markers have been used as a tool for parental assignment in many marine aquaculture species (Chistiakov et al., 2005) and a panel of DNA microsatellite markers have previously been developed for ballan wrasse (Quintela et al., 2014) but as yet, have not been applied in a broodstock management context.

The aims of this study were to: (1) describe for the first time the spawning dynamics of captive ballan wrasse; (2) identify potential variations in egg quality parameters over a full spawning season with the view to get accurate estimates of hatchery production; and

(3) apply microsatellite markers to investigate parental contribution in captive spawning ballan wrasse harems, all within a commercial production context. Together, this research serves to further optimise and develop standardised protocols for the establishment of broodstock populations and egg quality parameters to aid in the overall improvement of ballan wrasse hatchery productivity.

3.2 Materials and Methods

3.2.1 Experimental fish and system

Wild broodstock were captured using modified lobster creels off shore from Machrihanish in 2011 (55º 17'N, 5º 20'W; Scotland UK) and Dorset in 2012 (50º 44'N, 2º 20'W, England UK) and transferred to Machrihanish Marine Farm (Machrihanish, Scotland) where the study was performed. Prior to the start of the study, Dorset broodstock were overwintered in a common conditioning tank, kept on a simulated natural photoperiod (SNPP) at ambient temperature (6-10 °C) and fed daily to satiation on an industry standard extruded pellet (Symbio Wrasse Diet, 6.5 mm diameter; Biomar^{Ltd}, Grangemouth, Scotland UK). Machrihanish broodstock were overwintered in a common conditioning tank, kept under SNP and at a constant 12 °C. Fish were fed daily to satiation with a mixture of langoustine (*Nephrops norvegicus*) tails and mussels (*Mytilus edulis*).

In January 2013, spawning harems were established in four experimental spawning tanks: three tanks of Machrihanish fish (Tanks M1, M2 and M3) and one of Dorset (Tank D1) origin (Table 3.1).

Ta**ble 3.1.** Description of ballan wrasse broodstock used in the study including origin, sex ratio and size parameters.

	M1	M2	M3	D1
Spawning harems				
Fish (n)	10	19	20	28
Presumed males (n)	3	5	3	8
Presumed female (n)	7	14	17	20
Male body-weight (g)	1373.3 ± 126.5	945.0 ± 54.5	1258.3 ± 8.2	1215.0 ± 64.7
Female body-weight (g)	957.1 ± 59.4	665.0 ± 31.8	673.5 ± 55.4	767.3 ± 33.9

Fish were anaesthetised (Tricaine Methane Sulphonate; MS-222; 40 ppm; Pharmaq^{Ltd}, Hampshire, UK), measured for total body weight (BW ± 1 g) and total length (TL ± 1 mm) and assigned to a presumptive gender based on body size and morphological parameters (Leclercq et al., 2014b). As was standard production practice, presumed sex ratios were manipulated where possible (based on morphological data) to reach approximately 25 % males (range = 15-35 %) in each tank. Circular spawning tanks of 7 m^3 were adjacent and connected onto a single indoor recirculating system (TMC System 10,000; Tropical Marine Centre, Chorleywood, UK) equipped with protein skimmer, mechanical filters (100 μm), biofilters, UV disinfection and photo-thermal control. The system received a ~ 20 % pumped ashore natural seawater exchange daily and the water inflow into each tank was set at 66 L/min (50 % renewal / h). Fish were kept on SNP with a targeted constant water temperature of 12 °C. Water quality parameters were checked daily and averaged over the spawning season: temperature of 12.2 ± 0.07 ºC; salinity of 33.3 ± 0.1 ppt; dissolved oxygen (DO) of 94.1 ± 0.99 % saturation; and pH of 8.0 ± 0.03. Fish were fed a mixture of fresh langoustine tails and mussels and tanks were siphoned daily for waste removal. Artificial spawning substrates (Miami Gel carpet, 70x40 cm; MDC, Glasgow, Scotland UK) were placed within each tank (*n* = 16-20 / tank) in addition to artificial kelp and PVC pipes as hides.

3.2.2 Sampling schedule and parameters

From 1st of April to 25th of June 2013, spawning substrates within each tank were removed and visually inspected daily for presence of spawned eggs at 9 am. Mats without eggs were immediately returned to the tank while mats with adhered eggs were replaced by new ones and transferred into a holding bath freshly filled with seawater from the rearing system. Each mat was visually inspected and given a subjective score of egg quantity as follows: 1: Low density of eggs and variable coverage, i.e. few eggs scattered over the mat; 2: High density of eggs but low coverage, i.e. many eggs clustered together on a portion of the mat; 3: High density of eggs and high coverage, i.e. many eggs covering the whole mat. A daily 'spawning score' for relative egg quantity per day per tank was given as the sum of the individual subjective mat scores.

For each daily spawn, a representative sample of eggs was removed by gentle scraping from across all spawned mats was randomly collected and pooled within a petri-dish

previously filled with 10 ml rearing water for assessment of fertilisation and hatching rates, egg diameter (ED) and gum layer thickness (GLT), and lipid content and fatty acid profile as follows.

A sub-sample of 40 eggs was randomly taken for assessment of fertilisation and hatching rates according to Thorsen et al., (2003). Eggs were individually placed into wells of a sterile 96-well microplate (Sarstedt 96U, Newton, NC, USA) pre-filled with 200 µl of rearing water freshly filtered to 0.2 µm and kept at 12 °C. Eggs were inspected upon collection (GX Stereo microscope; XTL3T, GT Vision, Suffolk, UK) for presence of cell cleavage indicating fertilization. Well plates were then numbered, covered, sealed to prevent evaporation and incubated (LMS Cooled Incubator, LMS Ltd, Kent, UK) at 12 °C in darkness. Eggs were individually examined at 108 DD post-fertilization (PF) to allow sufficient time for hatching, previously reported to initiate at 72 DD PF in ballan wrasse (Ottesen et al., 2012). The number of hatched larvae was counted and expressed as the proportion of sampled eggs (n = 40 eggs) to define the hatching rate of each daily spawn.

A sub-sample of eggs was placed into a plastic petri dish with 5 ml of filtered seawater and immediately photographed (Fig. 3.1) using a digital microscope camera (1x magnification, GXCam3, GT Vision, Suffolk, UK) fitted onto a stereo microscope and connected to a computer. Pictures were subsequently uploaded onto an image analysis software (ImageJ® 1.47v, National Institutes of Health, USA) and a total of 30 eggs was examined to determine developmental stage according to D'Arcy et al., (2012) and measured as follows. Egg diameter was determined as the average diameter of the chorion measured from two perpendicular lines passing through the egg centre while gum layer thickness (GLT) was determined by measuring the total egg diameter then dividing the difference between total and chorion diameter in two.

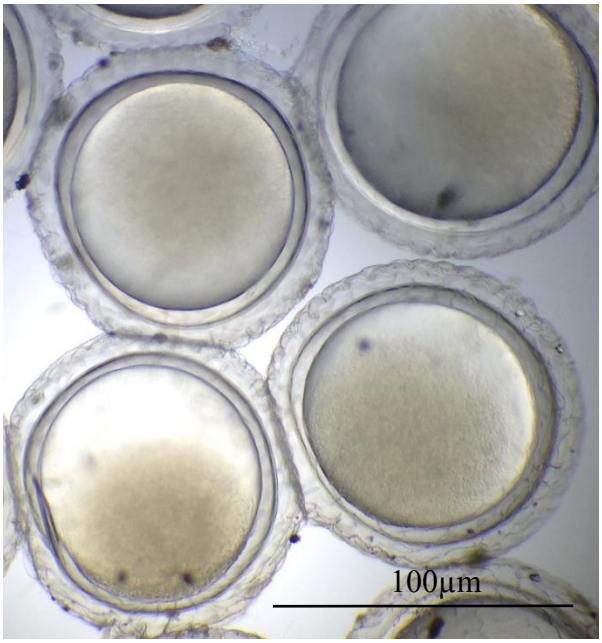

Figure 3.1. Ballan wrasse eggs photographed for measurement of egg diameter and gum layer thickness.

A last sub-sample of approximately 100 eggs was stored in a glass vial pre-filled with 20 ml chloroform methanol (2:1 v/v) and stored at -20 ºC for analysis of lipid content and fatty acid composition. Lipid extraction was carried out using the Folch et al., (1957) protocol. The fatty acid composition was determined by subjecting the lipid fraction to acid-catalysed transesterification (Christie, 2003) resulting in fatty acid methyl esters (FAME) which were purified by thin-layer chromatography on silica-coated glass plates using the developing solvent iso-hexane:diethyl ether (90:1 v/v) with 0.01 % BHT as antioxidant. The FAME were then analysed by capillary gas chromatography.

3.2.3 Batch fecundity

The total number of eggs collected from a single day and tank was numerically estimated on six separate dates by back calculation of the volumetric count of larval density hatched in isolation corrected by the batch hatching rate (based on well plate hatch rate) in order to estimate a harem's daily fecundity and assess the relative performance of the subjective egg quantity scoring system. For each of the six spawning dates, all egg mats were subjected to a static formalin bath treatment (100 ppm, 1 h; 36.6 % formaldehyde solution, Fisher Scientific, Lanarkshire, UK) and stocked into a 500 L flow-through incubator supplied with aerated natural seawater (5 L / min; UV treated, filtered to 100

µm) and fitted with a 100 µm mesh banjo filter at the outflow. Mean daily water temperature was 12.0 ± 0.4 ºC and DO = 96.0 ± 0.0 % over the incubation period. Eggs received two static bath treatments of bronopol (25 ppm, 1 h; Pyceze®; Novartis Animal Health^{Ltd}; Frimley, UK) at 2 and 4 DPF. Hatching was induced by physical shock (gently scraping the eggs from the spawning substrate using a metal spatula) when deemed optimal as per commercial hatchery practice at 6 to 7 DPF. Once all mats were scraped, larvae were observed rising at the surface within 10 min and left untouched for 1 hour to allow maximum hatching rate. The incubator was then drained into a condenser fitted with a 50 µm mesh and larvae transferred to a container with a final volume of 30 L. Larvae were gently mixed by light aeration and stirring, and replicated samples (n = 5 to 10) of 100 ml separated. The total number of larvae per sample was counted and averaged across replicate volumetric samples before translating the mean value to the batch total volume to calculate the total number of hatched larvae in the batch.

3.2.4 DNA extraction

Fin clip biopsies were taken from each of the 39 broodstock fish within tanks M2 and M3 and a sample of one hundred newly hatched larvae each originating from a single day spawning (n = 6 spawning events from M2 and M3 which were the same batches used for batch fecundity estimation). Samples were stored in 95 % ethanol at 4 °C until processed. Genomic DNA from fin samples was isolated using a salt extraction method; approximately 0.25 cm^2 tissue was added to 300 µl SSTNE buffer (0.30 M NaCl; 0.04 M Tris; 200 µM EDTA; 0.199 mM EGTA (E3889, Sigma Aldrich); 4.89 mM spermidine (SO266, Sigma Aldrich); 1.4 mM spermine (S1141, Sigma Aldrich)) a further 20 µl of SDS (10 %; L3771, Sigma Aldrich) and 5 µl proteinase K (10 mg/ml; P2308, Sigma Aldrich) was added and mixed well. Following a 4 hour digestion at 55 °C, samples were incubated at 70 °C to inactivate proteinase K. 20 µl of RNase A (2 mg/ml; R6148, Sigma Aldrich) was added to each sample. Following an additional 1 hour (37 °C) incubation, 200 µl of 5 mM NaCl was added for protein precipitation. 400 µl of supernatant was retained, transferred to fresh tubes, and an equal volume of isopropanol added and mixed well. Samples were then centrifuged for 10 minutes, 4 °C, at 10,000 g to form a pellet. The pellet was then washed overnight with 72 % ethanol, dried, and resuspended in 100 µl of 5 mM Tris. A scaled down version of this protocol was used for larval extractions in the 96 well PCR plate format and resuspended in 10 µl of 5 mM Tris. DNA was quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA).

Broodstock fin clip and whole body larval samples yielded an average of 150 ng/µl and 10 ng/µl of DNA, respectively. Genomic DNA was stored at 4 °C for up to 6 months before PCR amplification.

3.2.5 DNA microsatellites and PCR amplifications

Seven polymorphic DNA microsatellites (Table 3.2) were chosen from the limited number of loci reported for ballan wrasse (Quintela et al., 2014) and amplified as two separate panels. The microsatellite panels were selected in a manner to maximise the number of microsatellites and associated alleles. Forward primers were fluorescently labelled for automated detection of PCR products. The loci were amplified as 2 separate multiplex PCR reactions: Multiplex 1 used markers WR-A111, WR-A107, WR-A113, and WR-A103; multiplex 2 used markers WR-A228, WR-A224, and WR-A203. The 3.5 µl reaction contained 5-10 ng of DNA template, 1.75 µl 2x concentrated Plain Combi PPP Master Mix (C211, TOP-BIO), 0.67 µl PCR H_2O (TOP-BIO, 18 Mohm.cm, ultrafiltered) and for multiplex 1: 0.03µM of each primer for WR-A111, WR-A113, and WR-A103, and 0.015 µM of each primer for WR-A107; Multiplex 2: 0.04 µM of each primer for WR-A228, WR-A224, and WR-A203. The PCR amplification program was: initial denaturation at 95 °C for 15 min, 25 cycles at 94 °C for 30 s, 56 °C for 90 s, 72 °C for 1 min, and final extension step at 60 °C for 30 min. PCR reaction products were stored at 4 °C until genotyped.

Table 3.2. Details of the seven polymorphic microsatellite markers used in the present study inclusive of M2 and M3 spawning populations (*n* = 39 fish), "reported" allele observations are from Quintela et al., (2014).

Locus	Primer sequence (5'-3')	Fluorescent Label	Repeats	No. of alleles observed	No. of alleles reported	Allele size range observed (bp)	Allele size range reported (bp)	H_e	H_o	F_{IS}	PI	Multiplex
Wr-A103	F: TGGTTGCTACCAAATCATG	6FAM	$(GTT)_9$	7	6	186-197	191-200	0.824	0.872	-0.068	0.055	1
	R: GGGACAGAATGAAATATCTCTG											
Wr-A107	F: GAAAGAGACGGACAGAGACA	NED	$(AAC)_9$	3	3	185-194	188-197	0.319	0.282	0.121	0.501	1
	R: CGTCCCTATTTCATTGTCAC											
Wr-A111	F: ATCCAACAAATGGACTTAGTCA	VIC	$(TCTA)_{18}$	8	10	199-240	198-238	0.740	0.641	0.106	0.097	1
	R: AAACGGAGACCAGTGGAG											
Wr-A113	F: TTGGAATCAAACAACCTCTC	PET	$(GTT)_{17}$	8	7	195-223	198-288	0.751	0.795	-0.059	0.090	1
	R: GAGCCTACAAATTATCATTGGT											
Wr-A203	F: GATAGCGGGATAAAAGAAGATC	6FAM	$(GTT)_{14}$	11	12	155-208	168-219	0.760	0.795	-0.051	0.078	2
	R: TTCTATTTGGCAACCTTTACAC											
Wr-A224	F: GGACTGGGAACAGTTAAGATG	NED	$(ATC)_9$	5	4	171-193	174-195	0.563	0.590	-0.048	0.280	2
	R: CATGCGAGAGTTTTTCAAAG											
Wr-A228	F:AGGAAAACAGAGCCTACAAATT	VIC	$(AAC)_{12}$	8	7	163-190	167-196	0.751	0.795	-0.059	0.090	2
	R: CTTGCTCCAGAACATTTCAG											

3.2.6 Genotyping and parentage analyses

Parental samples were PCR amplified and genotyped on two separate occasions to obtain high quality scores. Larval samples were screened only once, and samples were excluded where PCR amplification had clearly failed. Following PCR, the amplified DNA fragments were diluted one-seventh with double-distilled H_2O and 1 µl of this dilution was added to 9 µl of HiDi formamide (Life Technologies; www.lifetechnologies.com) mixed with Gene Scan 600-LIZ size standard (Life Technologies), as per standard ABI 3730xl genotyping protocol. Allele peaks were detected using ABI Genescan ™ software, and genotyping data were interpreted using an exclusion-based program called the Family Analysis Programme (FAP) described by Taggart (2007). The number of observed alleles per locus, the expected and observed heterozygosity (H_e and H_o), the inbreeding coefficient (F_{IS}) and the probability of identity (PI) for each locus were calculated using GenAlEx 6.502 (Peakall & Smouse 2006; 2012).

3.2.7 Statistical analysis

Where applicable, all figures were presented as mean ± standard error (SE). Minitab 16 (Minitab, Coventry, UK) and Instat were used for statistical analysis. All data sets were checked for normality using the Anderson-Darling and the Kolmogorov-Smirnov test and arcsine-transformed when normality was not confirmed. The data for days per spawning window, inter-spawning interval (ISI), spawning score, fertilization rate, hatch rate, ED, GLT, and % FA of total FA were analysed using a one way ANOVA and a Tukey test for significant differences between tanks, spawning windows and spawning periods. Linear regression analysis was performed for ED and GLT data. All percentage data were arcsine-transformed. A probability level of $P < 0.05$ was considered significant in all tests.

3.3 Results

3.3.1 Spawning patterns and estimated egg quantity

The spawning season started on the 9th of April and lasted until the 17th of June 2013 inclusive across the experimental population and averaged 58.5 ± 4.8 days with a total of 14, 11, 12 and 26 days of spawning in M1, M2, M3 and D1 respectively (Table 3.3a.). The spawning pattern of all four spawning populations was characterised by a series of spawning periods (SP); each SP consisted of a series of days where spawning occurred,

referred to as a 'spawning window' (SW) followed by a series of days without spawning, referred to as the 'inter-spawning interval' (ISI) (Fig. 3.2).

Table 3.3. Ballan wrasse broodstock spawning performance in the four spawning populations studied: (a) Spawning dynamic; Spawning windows (SW), Inter-spawning intervals (ISI) and spawning period; (b) Relative egg production given as mean number of mats collected per day, mean daily spawning score and the estimated seasonal egg production based on the mean number of eggs per unit of subjective spawning score (Table 3.4); and (c) Egg quality; fertilization rate (%), hatch rate (%). *Note:* Superscripts represent significant differences between spawning populations for each given parameter (all p values < 0.05).

	M1	M2	M3	D1
a. Spawning dynamic				
Spawning season (n days)	64	56	46	68
Total number of spawning days	14	11	12	26
Number of SW (n)	5	5	4	6
Length of SW (n days)	3.6 ± 0.7^{ab}	2.4 ± 0.7^{b}	4.5 ± 1.2^{ab}	6.0 ± 1.0^{a}
Spawning days within SW (n days)	2.8 ± 0.4^{ab}	2.2 ± 0.5^{b}	3.0 ± 0.4^{ab}	4.3 ± 0.6^{a}
Duration of ISI (n days)	12.5 ± 1.0^{a}	12.0 ± 0.4^{a}	11.0 ± 1.5^{ab}	8.0 ± 1.0^{b}
Spawning period (n days)	15.3 ± 1.2	14.5 ± 0.6	14.3 ± 1.4	12.8 ± 0.6
b. Egg Production				
Number of mats per spawning day	12.5 ± 1.4	11.5 ± 3.5	9.6 ± 2.8	11.8 ± 1.1
Daily spawning score	13.4 ± 1.6	11.9 ± 1.5	10.7 ± 1.3	15.0 ± 1.6
Total score (whole season)	187	136	128	389
Estimated seasonal egg production*	1,061,524	772,018	726,605	2,208,197
c. Egg quality				
Fertilization rate (%)	98.8 ± 0.01	96.9 ± 0.01	99.6 ± 0.00	99.3 ± 0.00
Hatching rate (%)	61.2 ± 0.06^{ab}	46.8 ± 0.11^{b}	75.8 ± 0.07^{a}	67.0 ± 003^{ab}

* Estimation based on results presented in Table 3.4

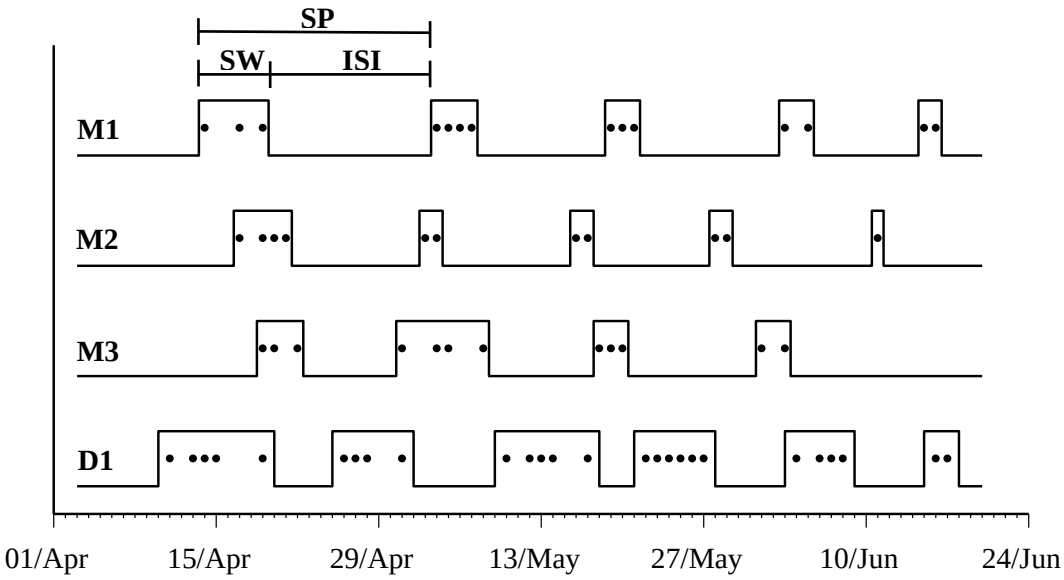

Figure 3.2. Spawning dynamics for M1, M2, M3 and D1 including spawning period (SP), spawning window (SW), and inter spawning interval (ISI). Each point on the graph represents a single spawning date.

The total number of SW for isolated spawning populations ranged from 4-6, with individual SWs varying in length from 1 to 9 days. Mean SW duration of population M2 was significantly shorter than that of D1 and, inversely, the mean ISI duration was significantly shorter for D1 than for M1 and M2. However, average SP (SW + ISI) lasted 14.2 ± 0.5 days (*n* = 16 SP) with no significant differences between spawning populations.

The M1, M2 and M3 spawning populations followed a similar spawning pattern with an average of 5.2 ± 0.7 days (range of 4-7 days) between the SW start dates for tanks M1, M2, and M3. The SW in the D1 spawning population started on average 5.6 ± 0.8 days (range = 3-8 days) prior to tanks M1-M3 (Fig. 3.2).

In each SW throughout the season and for all spawning populations, 85 % of all mats collected were scored 1 (*n* = 611/723 mats), 14 % (*n* = 103/723 mats) were classed as score 2 and a final 1 % (*n* = 8/723 mats) were score 3. Individual values for the number of mats and corresponding scores varied between SW and between spawning populations (Fig. 3.3 a-d; Table 3.3b).

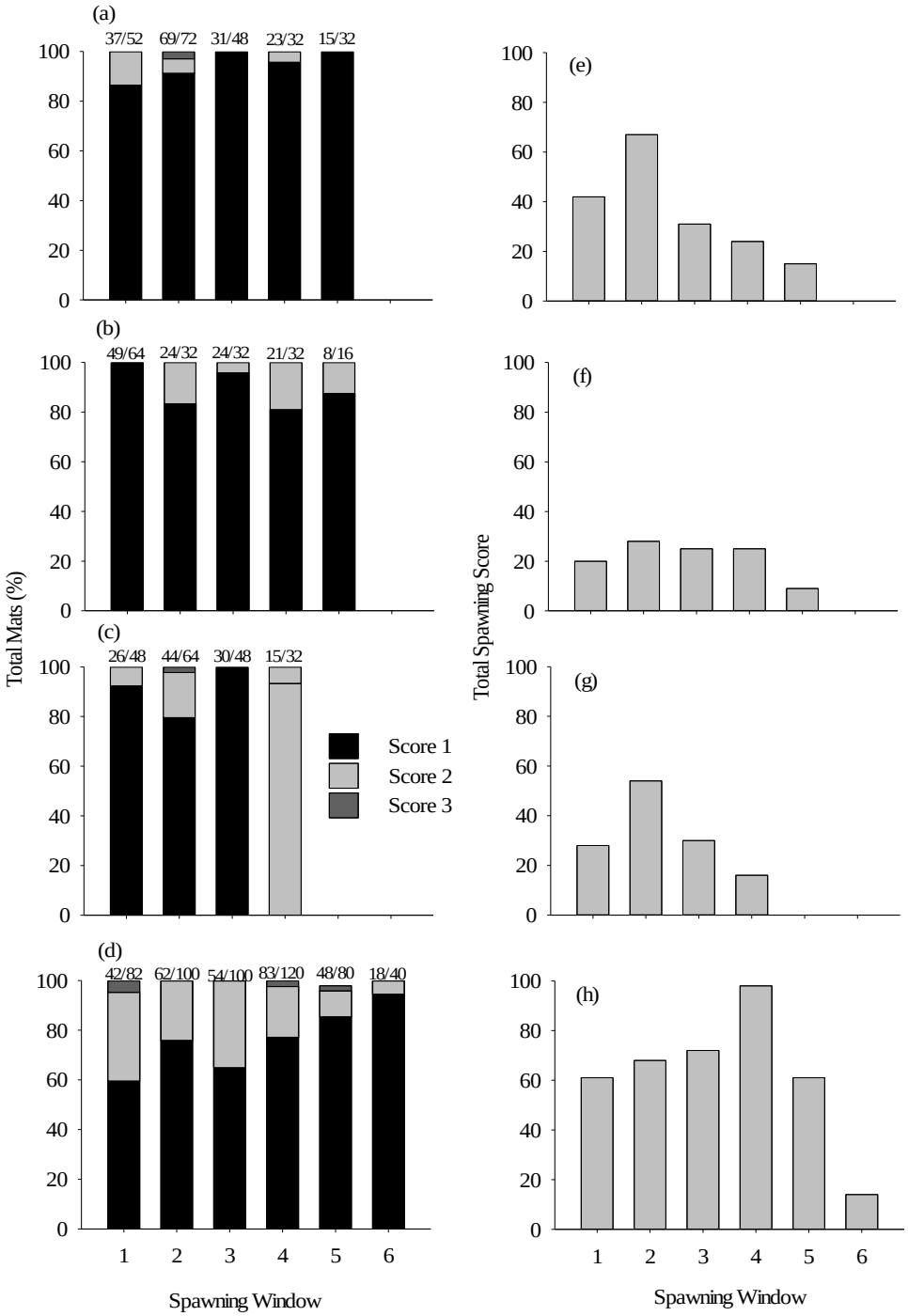

Figure 3.3. Proportion of the total number of mats collected per spawning window (SW) defined as score 1, 2, or 3: (a) M1; (b) M2; (c) M3; (d) D1. *Note*: numbers above each bar represent the total number of mats collected / total number of mats offered in each SW; and total spawning score per (SW) for each population: (e) M1; (f) M2; (g) M3; (h) D1.

The total subjective score of daily egg quantity per SW was highest during the 2nd SW for tanks M1, M2 and M3 and during the 4th SW for D1 (Fig. 3.3 e-h). For all spawning populations the least productive SW was the last to occur with total spawning score reduced by an average of 75.9 ± 4.2 % compared to their respective most productive SW.

The total number of eggs estimated from volumetric counts varied from 25,063 to 74,080 and from 4,177 to 7,347 eggs per unit of subjective egg quantity score (mean = 5677 ± 558; n = 6) across daily egg batches incubated for numerical estimation (Table 3.4). Based on this estimated egg quantity per unit of subjective score, the presumed seasonal egg production per population was as follows: M1 = 1,061,524 eggs; M2 = 772,018 eggs; M3 = 726,605 eggs; and D1 = 2,208,197 eggs.

Table 3.4. Hatch rate (%, Mean ± SEM, *n* = 5 larval counts performed), volumetric counts, estimated larval number, estimated egg number using back calculation of larval number and well plate hatch rate; spawning score and estimated egg number per unit of spawning score from 6 individual egg batches, three each from M2 and M3.

	M2			M3		
Spawning Date	16/05/2013	17/05/2013	29/05/2013	08/05/2013	18/05/2013	19/05/2013
Hatch rate (%)	92.5	85.0	45.0	75.0	80.0	90.0
Volumetric larval count (per 100ml)	161 ± 23	205 ± 17	82 ± 2	278 ± 51	67 ± 3	176 ± 37
Estimated larvae number	48,375	61,613	24,750	55,560	20,050	52,900
Estimated egg number	52,297	72,485	55,000	74,080	25,063	58,777
Spawning score	8	17	11	11	6	8
Estimated egg number per unit of spawning score	6,537	4,264	5,000	6,735	4,177	7,347
Mean egg number per unit of subjective spawning score (*n* = 6 batches)	5676.6 ± 558.4					

3.3.2 Egg quality

Fertilization rates remained consistently high in all four spawning populations throughout the season (overall mean batch fertilisation rate 98.6 ± 0.7 %; min to max range: 87.5 to 100.0 %) with there being no significant differences between tanks (Table 3.3c). Hatching rates were highly variable between daily egg batches and spawning populations (range = 0-97.5 %) with population mean hatch rates being significantly lower for M2 compared to M3.

Mean egg diameter was 0.95 ± 0.004 mm and decreased slightly, although not significantly, throughout the spawning season with no significant differences found between populations. GLT was 0.12 ± 0.002 mm with no significant differences between populations and showed an overall decreasing trend over the spawning season in all four spawning populations. However, linear regression between mean GLT over time showed that only M1 was characterised by a significant negative slope ($r^2 = 0.68$, $n = 14$, $p < 0.001$) (Fig. 3.4).

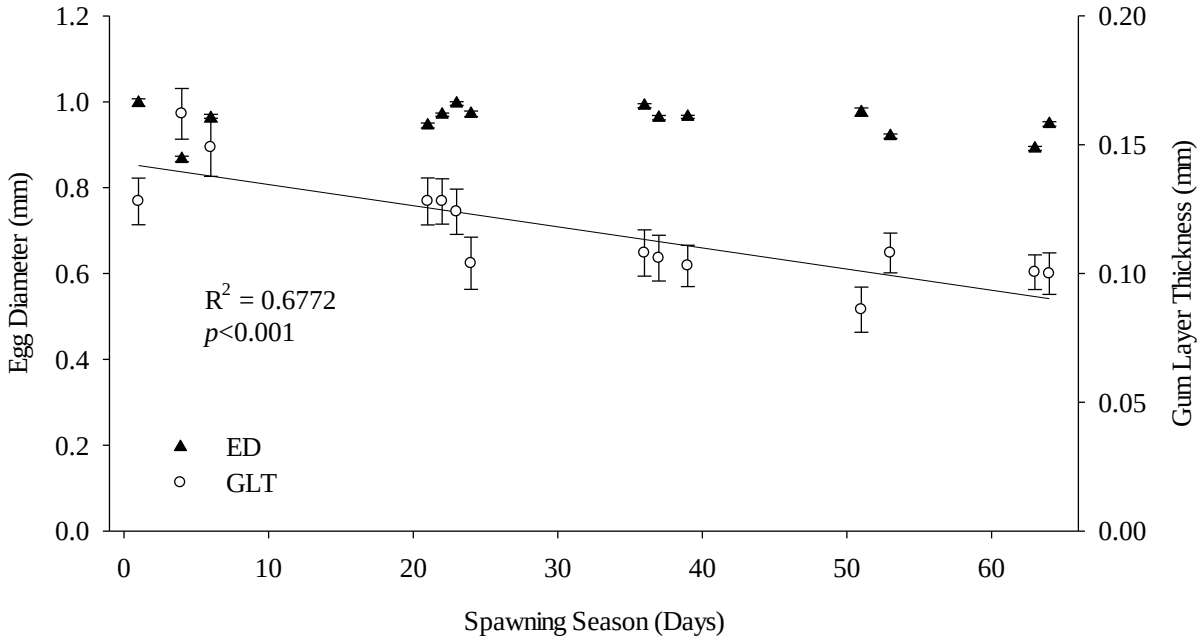

Figure 3.4. Mean egg diameter (ED) ± SE and mean gum layer thickness (GLT) ± SE over the spawning season for M1.

3.3.3 Fatty acid profile

The most abundant saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) observed in ballan wrasse eggs were palmitic acid (16:0), oleic acid (18:1*n*-9) and docosahexaenoic acid (DHA) (22:6*n*-3), respectively (Table 3.5). SFA accounted for an average of 32.5 ± 4.0 % of the total fatty acids in ballan wrasse eggs. MUFA ranged from 18.4 to 29.4 % of the total fatty acids, and significant differences were seen between spawning populations. Within the PUFA, the *n*-3 were more abundant than the *n*-6 and significant differences were observed between spawning populations for total *n*-6 PUFA. The mean EPA to DHA ratio was 1.72 ± 0.02 with there being no significant differences between populations. However, the ARA to EPA ratio ranged from 0.28 to 0.31 with the ratio being significantly higher in D1 compared to M3.

There was little variation in the main FA classes over the course of the spawning season. However, significant differences were seen in ARA between SW for M2, EPA for D1 and for DHA:EPA for M3 and D1 (Table 3.6). Furthermore, there was an overall decreasing trend, although not significant, from the first to the last SW in all four tanks for ARA, EPA, and DHA, with the exception of DHA in the M1 and M3 spawning populations.

Table 3.5. Captive ballan wrasse egg fatty acid composition for each of the four broodstock populations, values averaged over the season, per tank. *Note:* Superscripts represent significant differences between spawning populations for each parameter (all *p* values < 0.05).

% Fatty Acid of total fatty acid				
Fatty Acid	**M 1**	**M2**	**M3**	**D 1**
14:0	1.50 ± 0.07	1.39 ± 0.06	1.30 ± 0.06	1.39 ± 0.04
15:0	0.38 ± 0.01^{a}	0.39 ± 0.01^{a}	0.36 ± 0.01^{ab}	0.33 ± 0.01^{b}
16:0	25.96 ± 0.27	25.46 ± 0.26	25.41 ± 0.27	25.46 ± 0.20
18:0	5.13 ± 0.20	4.94 ± 0.15	5.15 ± 0.20	5.07 ± 0.11
20:0	0.02 ± 0.01^{ab}	0.04 ± 0.02^{a}	0.01 ± 0.01^{ab}	0.00 ± 0.00^{b}
22:0	0.15 ± 0.04	0.17 ± 0.03	0.11 ± 0.02	0.12 ± 0.02
Σ Saturated	33.15 ± 0.36	32.39 ± 0.30	32.34 ± 0.26	32.38 ± 0.20
16:1n-9	1.29 ± 0.06^{ab}	1.51 ± 0.12^{ab}	1.19 ± 0.04^{b}	1.60 ± 0.11^{a}
16:1n-7	3.73 ± 0.19	4.47 ± 0.29	3.47 ± 0.28	4.24 ± 0.24
18:1n-9	11.91 ± 0.34^{ab}	12.04 ± 0.30^{ab}	11.37 ± 0.19^{b}	12.63 ± 0.27^{a}
18:1n-7	3.86 ± 0.14	4.30 ± 0.15	3.97 ± 0.16	4.27 ± 0.08
20:1n-11	0.17 ± 0.11	0.08 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
20:1n-9	1.01 ± 0.10	0.95 ± 0.06	1.12 ± 0.05	1.10 ± 0.03
20:1n-7	0.19 ± 0.01	0.20 ± 0.02	0.19 ± 0.01	0.17 ± 0.01
Σ Monounsaturated	22.25 ± 0.57^{ab}	23.55 ± 0.79^{ab}	21.32 ± 0.62^{b}	24.06 ± 0.54^{a}
18:2n-6	1.07 ± 0.10^{c}	1.35 ± 0.07^{ab}	1.59 ± 0.10^{a}	1.19 ± 0.04^{bc}
18:3n-6	0.03 ± 0.03^{b}	0.07 ± 0.01^{a}	0.03 ± 0.01^{ab}	0.02 ± 0.01^{b}
20:2n-6	0.24 ± 0.02^{b}	0.27 ± 0.02^{b}	0.33 ± 0.02^{a}	0.24 ± 0.01^{b}
20:3n-6	0.11 0.02^{b}	0.14 ± 0.01^{ab}	0.17 ± 0.01^{a}	0.14 ± 0.01^{ab}
20:4n-6 ARA	3.49 ± 0.06^{b}	3.74 ± 0.14^{ab}	3.94 ± 0.12^{a}	3.82 ± 0.09^{ab}
22:4n-6	0.22 ± 0.01	0.25 ± 0.01	0.22 ± 0.01	0.25 ± 0.01
22:5n-6	0.30 ± 0.01	0.32 ± 0.01	0.30 ± 0.01	0.30 ± 0.02
Σ n-6 PUFA	5.45 ± 0.16^{c}	6.14 ± 0.18^{ab}	6.57 ± 0.20^{a}	5.97 ± 0.09^{b}
18:3n-3	0.21 ± 0.02	0.21 ± 0.01	0.23 ± 0.01	0.18 ± 0.01
18:4n-3	0.11 ± 0.01	0.15 ± 0.01	0.17 ± 0.01	0.13 ± 0.01
20:4n-3	0.24 ± 0.01^{b}	0.28 ± 0.01^{ab}	0.31 ± 0.01^{a}	0.26 ± 0.01^{b}
20:5n-3 EPA	12.67 ± 0.19^{ab}	12.69 ± 0.42^{ab}	13.59 ± 0.20^{a}	12.30 ± 0.19^{b}
22:5n-3	2.36 ± 0.09	2.09 ± 0.07	2.08 ± 0.35	2.25 ± 0.10
22:6n-3 DHA	22.40 ± 0.33	21.3 ± 0.54	22.24 ± 0.50	21.32 ± 0.32
Σ n-3 PUFA	38.00 ± 0.50	36.72 ± 0.85	38.61 ± 0.54	36.44 ± 0.49
16:2	0.14 ± 0.01	0.15 ± 0.00	0.14 ± 0.00	0.15 ± 0.01
16:3	0.35 ± 0.03	0.39 ± 0.02	0.35 ± 0.02	0.33 ± 0.02
16:4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
Σ	0.48 ± 0.03	0.53 ± 0.03	0.49 ± 0.02	0.49 ± 0.02
16:0 DMA	0.14 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
18:0DMA	0.34 ± 0.01	0.36 ± 0.01	0.34 ± 0.01	0.36 ± 0.01
18:1DMA	0.19 ± 0.01	0.20 ± 0.01	0.20 ± 0.01	0.19 ± 0.01
Σ	0.67 ± 0.02	0.67 ± 0.01	0.67 ± 0.01	0.67 ± 0.02
Σ PUFA	43.93 ± 0.39^{ab}	43.39 ± 0.93^{ab}	45.67 ± 0.51^{a}	42.90 ± 0.53^{b}
Σ FA	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00
EPA/DHA	1.77 ± 0.03	1.69 ± 0.05	1.64 ± 0.04	1.74 ± 0.02
ARA/EPA	0.28 ± 0.01^{b}	0.30 ± 0.01^{ab}	0.29 ± 0.01^{b}	0.31± 0.00^{a}

Table 3.6. Mean egg fatty acid composition per spawning window (SW) for each spawning population. *Note:* Superscripts represent significant differences between SW (all p values < 0.05).

Tank	SW (days)	ARA	EPA	DHA	DHA:EPA
M1	1 (3)	3.49 ± 0.15	12.97 ± 0.72	22.17 ± 0.97	1.71 ± 0.07
	2 (4)	3.59 ± 0.13	12.87 ± 0.20	23.11 ± 0.62	1.79 ± 0.06
	3 (3)	3.39 ± 0.16	12.82 ± 0.37	22.14 ± 0.53	1.72 ± 0.01
	4 (2)	3.47 ± 0.02	11.91 ± 0.19	21.38 ± 0.92	1.79 ± 0.04
	5 (2)	3.41 ± 0.13	12.32 ± 0.16	22.74 ± 0.76	1.84 ± 0.08
M2	1 (4)	4.27 ± 0.13^{a}	13.64 ± 0.49	21.28 ± 0.89	1.57 ± 0.10
	2 (2)	3.77 ± 0.32^{ab}	13.38 ± 1.73	22.36 ± 0.82	1.69 ± 0.15
	3 (2)	3.46 ± 0.28^{ab}	12.58 ± 1.05	21.56 ± 1.53	1.71 ± 0.03
	4 (2)	3.12 ± 0.07^{ab}	11.63 ± 0.36	21.71 ± 1.20	1.86 ± 0.04
	5 (1)	3.20 ± 0.04^{b}	10.84 ± 0.42	19.45 ± 2.14	1.78 ± 0.12
M3	1 (3)	4.14 ± 0.16	13.82 ± 0.16	20.70 ± 0.92	1.05 ± 0.08^{b}
	2 (4)	3.98 ± 0.30	13.82 ± 0.40	22.55 ± 0.52	1.63 ± 0.04^{ab}
	3 (3)	3.84 ± 0.26	13.41 ± 0.63	23.11 ± 1.33	1.72 ± 0.02^{ab}
	4 (2)	3.59 ± 0.004	12.92 ± 0.22	23.37 ± 0.20	1.80 ± 0.04^{a}
D1	1 (5)	4.05 ± 0.15	12.78 ± 0.19^{ab}	20.48 ± 0.50	1.60 ± 0.04^{b}
	2 (4)	4.11 ± 0.14	13.26 ± 0.33^{a}	22.31 ± 0.66	1.68 ± 0.05^{ab}
	3 (5)	3.83 ± 0.11	12.39 ± 0.22^{ab}	22.21 ± 0.54	1.79 ± 0.03^{a}
	4 (6)	3.44 ± 0.14	11.48 ± 0.39^{b}	20.91 ± 0.72	1.82 ± 0.05^{a}
	5 (4)	3.64 ± 0.38	11.28 ± 0.74^{b}	20.61 ± 1.29	1.82 ± 0.03^{a}
	6 (2)	3.24 ± 0.16	10.98 ± .26^{b}	18.75 ± 0.35	1.71 ± 0.01^{ab}

3.3.4 Genotyping and parental contribution

Mean predictive assignment rates among families ranged from 81 to 83 % between tanks (Table 3.7). Of the 600 larvae from tanks M2 and M3 that were screened, genetic profiles were obtained for 587 individuals. Of these genotyped offspring, 88 % were assigned to at least one family without error tolerance. When the genotyping model allowed for a single allele mismatch (Occasional single allele mismatches were observed across the panel of loci and not associated with a single locus) all individuals were successfully assigned to families, with 83 % unambiguously assigned to a single family which correlates to the predicted assignment rates (Table 3.7). A further 17 % of individuals were assigned to multiple families, however, in all multiple-match cases, at least one of the candidate families was a previously confirmed spawning pair.

Analysis of parental contribution was performed on 27 % (n = 3/11) and 25 % (n = 3/12) of spawning events from M2 and M3, respectively (based on the 488 offspring assigned to single match families, allowing up to one allele mismatch). Results indicated that in three out of the six total spawning events analysed, all single mismatch and unambiguously assigned larvae were assigned to a single mating pair (Table 3.8). Two spawning events showed evidence of two mating pairs where two separate females had spawned with a common male. Finally, in the last of the six spawning events, 97 % (n = 93/96) of offspring were assigned to a single mating pair, with the remaining 3 % of offspring assigned to three different mating pairs. Parental contribution during these spawning events was not even across the populations with only 22 % of females (n = 3/14) and 60 % (n = 3/5) of males present in tank M2 and 24 % of females (n = 4/17) and 33 % (n = 1/3) of males present in tank M3 actually contributing to the offspring analysed. Furthermore, one out of the 7 spawning females (female 13, tank M2) was shown to have spawned twice; once in each of the SW assessed and with a different male on each occasion. All larvae in tank M2 were assigned to a single male (individual 05) during the first two spawning dates which were grouped within a single SW, but the third spawning date, which was in a separate SW, had a different male (individual 12) as the main contributor. There was only one male assigned to all larvae from tank M3, both within and between the two SW.

Table 3.7. Computation of the resolving power of microsatellite panels within two (M2 and M3) of the broodstock tanks. The proportion of offspring per family that should be unambiguously assignable to a single family are given. Seven loci are considered for all individuals. The calculations, performed using FAP (Taggart, 2007), were based on the known parental genotypes within each spawning tank and assume that all female/male parent combinations were equally likely to occur. Numbers in brackets represent the potential different families possible, given the number of males and females present in each tank (sex was verified by gonadal biopsy sampling).

		Tank M2 (70)	Tank M3 (36)
All 7 loci	Mean	0.83	0.81
	SD	0.17	0.16
	Min	0.43	0.47
	Max	1.00	1.00

Table 3.8. Parental contribution to the ballan wrasse larval samples taken from six separate spawning dates, as determined by exclusion based parentage based on the genotyping of 7 DNA microsatellites. *Note:* Format '**35**/5' where the first number (in bold) refers to both the total number of offspring assigned unambiguously and those assigned allowing up to one allelic mismatch and the second number (not bold) refers to offspring assigned to multiple families, with one of the potential families being that of the previously identified single-match family. Shaded area implies that the spawning dates occurred within the same spawning window.

Spawning pair (Female x Male)	No. of larvae assigned		
M2	16/05/2013	17/05/2013	14/06/2013
04x05	**35**/5		**1**/2
10x05	**42**/18		**1**/0
13x05		**85**/4	
13x11			**1**/0
13x12			**93**/1
Total no. larvae genotyped	**77**/100	**85**/89	**96**/99
M3	08/05/2013	18/05/2013	19/05/2013
26x24	**13**/2		
27x24			**73**/27
30x24		**67**/33	
36x24	**77**/7		
Total no. larvae genotyped	**90**/99	**67**/100	**73**/100

3.4 Discussion

The present study describes for the first time the spawning periodicity of captive ballan wrasse harems throughout an entire spawning season, along with the seasonal variation in reproductive performances including fecundity, egg quality, and parental contributions. This type of dataset is important when trying to close the captive lifecycle for any new species in order to develop hatchery protocols and increase spawning productivity.

Spawning occurred from early April to mid-June with a peak in egg production, based on the highest number of spawning days within a given spawning window, occurring in early May for the three Machrihanish origin populations, and in late May for the Dorset origin spawning population. This coincides with evidence of peak egg production occurring in May as previously reported in Norwegian captive ballan wrasse broodstock (Muncaster et al., 2010). Ballan wrasse have previously been characterised as group synchronous multiple batch spawner based on histological examination of ovaries (Muncaster et al., 2010), however empirical evidence of spawning pattern and rhythmicity during a full spawning season was lacking. The spawning rhythmicity of captive ballan wrasse in this study was characterised by a succession of spawning windows of 1-6 days followed by longer interspawning intervals of 8-15 days with a total of 4 to 6 spawning windows over the spawning season. Such regular spawning rhythms is suggestive of a "multiple or repetitive spawning" reproductive strategy as previously proposed in the species. This is further supported by the fact that the total number of spawning dates for all spawning populations exceeded the total number of presumed females in each tank; therefore, it must be assumed that at least some of the females would have spawned on more than one occasion within the spawning season. This is ultimately supported by genotyping analysis which clearly identified a single female being the predominant contributor during two separate spawning events, in two separate spawning windows. Repeat or multiple batch spawning is a common spawning strategy for cultured temperate marine teleosts including Atlantic halibut (*Hippoglossus hippoglossus*), which produce several batches of eggs at regular intervals of 3-4 days over a 2-4 month period (Nordberg et al., 1991; Bromage et al., 2000; Brown et al., 2006) and Atlantic cod (*Gadus morhua*), which spawn egg batches every few days for up to a 2 month period (Kjesbu, 1989).

Despite the differences between the four spawning populations in the number and duration of SW and ISI, there was no difference in the overall duration of spawning periods. The average SP across all tanks lasted on average 14 days, which is equivalent to a semi-lunar spawning cycle. Semi-lunar spawning cycles have been observed in two other Labrid species, *Thalassoma duperrey* and *Thalassoma lucasanum*, where peak spawning occurs on spring tides and on or around the quarter moon (Warner, 1982; Ross, 1983; Taylor, 1984). Lunar reproductive cycles are common among marine fish and, as suggested by Robertson et al., (1990) and Taylor (1984), moonlight or tidal regime may play a role in dispersal of eggs or newly hatched larvae when conditions are best for predator avoidance and/or parental care. However, the broodstock in this study had been in captivity for 2-3 years under enclosed conditions and were not directly exposed to lunar cycles therefore these rhythms are either endogenous or other unidentified zeitgebers are providing a synchronising cue.

Due to the adhesive properties of spawned ballan wrasse eggs, the direct quantification of individual egg batches has proven very difficult and could not be measured volumetrically as is common hatchery practice with other marine fish species releasing pelagic eggs. After numerous attempts at quantifying eggs while adhered to egg mats (using image analysis or scraping), it was concluded that a subjective 'spawning score' of relative egg quantity and coverage across the egg mat was a more suitable and reproducible method. Due to limited facilities and commercial constraints, it was not possible to incubate and hatch each egg batch separately for volumetric counts of larvae, thus larval counts were obtained from 6 random separate batches throughout the season.

The differences in spawning scores, i.e. egg dispersal over the spawning substrates, between batches and spawning populations cannot be explained at this stage, but it may be down to the number of females contributing to each egg batch or potential variation in individual female spawning behaviour. Furthermore, it is possible that not all eggs from an individual batch were adhered directly to the egg mats collected as the entire tank bottom was not covered with spawning substrate.

Using the total seasonal spawning score per tank, an estimation of population seasonal fecundity was found to range between 726,605 and 2,208,197 eggs per spawning population. However, this does not take into account the number of females per tank and without knowing how many females actually spawned on a given day or how many

batches each individual female spawned, it is not possible to estimate total or batch fecundity to an individual level. That said, this estimation is deemed vital to give baseline information for hatchery management to forecast overall broodstock productivity and be able to compare estimates of productivity from one season to the next.

Fertilization rate is a commonly used early indicator of egg batch quality in marine fish species (Thorsen et al., 2003). However, in this study, fertilization rates, when measured at collection (less than 24 hours post spawning) remained consistently high throughout the spawning season for all spawning populations. This did not correlate with individual batch hatch rates which were highly variable between spawning windows and spawning populations. Therefore, it must be concluded that in this study fertilisation rate, assessed within 24 hours of spawning, is not a valid early indicator of egg batch quality and, thus, the author would encourage future studies in ballan wrasse to perform such measurements at a later stage post spawning and then re-examine the predictive power of fertilisation rate as a quality indicator.

Mean total egg diameter in this study was marginally smaller (0.95 ± 0.004 mm) than previously reported for Norwegian origin ballan wrasse eggs (measured at comparable developmental stages) (1.05 ± 0.04 by Ottesen et al., 2012); however, it was similar to egg diameter reported for the brown wrasse (*Labrus merula*) (0.93 ± 0.05 mm) (Dulčić et al., 1999), and smaller to that seen in the green wrasse (*Labrus viridis L.*) (1.01 ± 0.03 mm) (Kožul et al., 2011), both of which also spawn adhesive benthic eggs. Egg size did not appear to vary along the spawning season, as opposed to findings in other batch spawning species such as Atlantic cod (~11 % seasonal decrease, Trippel, 1998), Arctic cod (*Arctogadus glacialis P.*) (2-7 % seasonal decrease, Wiborg, 1960), turbot (*Scophthalmus maximus L.*) (McEvoy & McEvoy, 1991) and halibut (Bagenal, 1971). Seasonal reduction in egg size has been supposedly linked to physiological effects from the maternal component (Trippel, 1998), as batch spawning may place a large physiological demand on spawning fish therefore depleting energy sources over the course of the spawning season (Izquierdo et al., 2001).

While egg diameter remained consistent, a declining trend in mean gum layer thickness was observed over the spawning season for the four spawning populations studied, however, only significantly for one population (M1) which represented a 32 % decline from the first SW to the last. To date, there is a lack of literature on seasonal changes in

egg adhesiveness for marine teleosts. There was no clear reduction observed in the 'stickiness' of egg batches over the season as a whole; however, casual observation suggested that eggs appeared to become 'less sticky' during the later stages of incubation, just prior to hatch. Similarly, in the green wrasse, the adhesive gum layer has been shown to lose its stickiness and separate from the eggs a few hours prior to hatching (Kožul et al., 2011). Further studies should be performed to determine the role of the adhesive gum layer in ballan wrasse eggs and look at potential removal methods for incubation purposes as is common commercial practice with many freshwater species that spawn adhesive eggs (Linhart et al., 2003a).

Another indicator of egg quality in fish is lipid and FA contents derived directly from broodstock diet (Sargent et al., 1999; Migaud et al., 2013). They are required for the formation of cell membranes and are a major source of metabolic energy (Sargent et al., 2002). In addition, they play important roles in spawning, egg quality, in terms of successful embryo and larval growth and development, hatching, and overall survival (Rainuzzo et al., 1997; Sargent et al., 2002; Tocher, 2003). Ballan wrasse egg FA composition in this study remained generally consistent throughout the spawning season and across spawning windows, although subtle variances were observed. Such variability in FA between spawning populations and spawning windows could potentially be due to genetic or nutritional variability between individual spawning fish. However, of the 63 egg batches collected, inclusive of all spawning populations, no direct correlation was found between any FA and fertilisation or hatch rates. Therefore the observed PUFA variance (DHA, EPA, ARA and DHA:EPA) was independent of these quality assessments. This was an unexpected result as DHA in particular and EPA have been linked to fertilization and hatching success in many other marine teleost species including cod (Pickova et al., 1997), sea bass (*Dicentrarchus labrax L.*) (Bruce et al., 1999) and common snook (*Centropomus undecimalis B.*) (Yanes-Roca et al., 2009).

Lipid content and FA composition of fish eggs are known to vary considerably between species (Sargent et al., 2002). With the exception of the high levels of ARA (~3.8 ± 0.5 % of total FA) compared to ~ 2.5 % total FA in other marine species, ballan wrasse egg FA profile observed in this study fits the general profile for marine fish (Tocher et al., 1985; Fraser et al., 1988; Sargent et al., 2002). The relative levels of EPA observed in captive ballan eggs, in this study, were similar to that reported for wild ballan wrasse (12

± 1 %) and the levels of ARA, DHA, and DHA:EPA ratio were lower than those previously reported for wild ballan wrasse (6 ± 2; 30 ± 4; and 2.5 ± 0.5 %, respectively) (Hamre et al., 2013). However, this comparison is not straightforward as in the previously published study samples were taken from female gonads just prior to spawning. Future research should aim to obtain more egg samples from wild ballan wrasse as well as benchmark egg quality more comprehensively.

Given the spontaneous spawning behaviour of ballan wrasse in captivity, it is difficult to determine parental contribution to egg batches. Therefore, a seven loci microsatellite panel was selected from an original pool of 20 previously published (Quintela et al., 2014). The panel performed well, and provided robust genotyping data for all of the parents assessed as well as the majority of larvae. Loci performance (number of alleles and observed size range) was generally comparable with Quintela et al., (2104), which demonstrates these markers can be used effectively, more widely across the species natural range. The exclusion-based FAP had a higher level of single-match assignment (83 %) when a single allelic mismatch was tolerated, which is the general level of acceptance for the expected low level of error (Pompanon et al., 2005). The predictive FAP, which looks at the resolving power of parental genotypic data sets (Taggart, 2007), indicated that the 7 loci panel used would not be unambiguously discriminating, and the low level of multiple matches found, 99 out of 587 larvae (17 %) was similar to that predicted by FAP. The parental assignment results from the exclusion-based FAP analysis indicated that, overall, within the six spawning dates analysed for the two spawning populations, only 19.5 % of females and 50 % of males within tanks actually contributed to the progeny. Bearing in mind that larval samples were taken immediately post-hatch, this should be a reliable and robust estimate of parental contribution, as larvae were not subjected to any active (hatchery practice) or natural (selective mortality) grading. On all three spawning dates in M3, there was only a single male contributing to all assigned larvae and within the three spawning dates for M2 there was one male contributing to 64 % of assigned larvae and a further two males showing a lower level of contribution. The highly skewed male contribution is suggestive that male dominance is occurring within these spawning populations which is supported by observations of territorial male behaviour. Furthermore, these results support the harem mating behaviour reported from studies of wild fish with territorial males courting and mating with several females (Sjölander et al., 1972; Hilldén, 1984). It should be noted that when the same 7

loci panel was tested in larger harem sizes (e.g. tank D1, n = 28 individuals) the performance of the panel weakened with predictive FAP dropping <80% (data not shown). It would therefore be prudent that further research be conducted to develop a stronger performing microsatellite panel with improved assignment confidence so that it can be integrated more widely as a management tool within hatcheries to test the social, environmental, or hormonal manipulations on breeding activity.

As a whole this research provides the first detailed study on the spawning performance of captive ballan wrasse. Results showed clear spawning rhythms and confirmed that ballan wrasse is a multiple batch spawning species. In addition, parental contribution confirms the social hierarchical structuring in captive ballan wrasse, which should be taken into consideration when establishing spawning populations. Finally, the analysis of egg batch quality provide the first data to serve as a comparison in future commercial batches. The knowledge gained on ballan wrasse reproductive performances and egg quality is critical for the development of broodstock management programs to secure a sustainable supply of farmed fish to combat sea lice.

4 Chapter 4: Effects of spawning area fragmentation and substrate colour on spawning activity of captive ballan wrasse (*Labrus bergylta*) broodstock.

Keywords: Benthic spawning, behaviour, substrate colour, area fragmentation.

Abstract

Ballan wrasse (Labrus *bergylta*) display clear spawning behaviour and seek out benthic substrates to deposit their adhesive eggs. Understanding the spawning behaviour and optimizing the captive conditions of commercial broodstock is vital for the successful establishment of breeding populations and to maximise egg productivity in captivity. This study aimed to determine if ballan wrasse have a preference for spawning substrates with respect to colour or position within light blue spawning tanks and, if so, whether this could be used to increase overall productivity of captive spawning populations. Four spawning populations were monitored for a six week period through the ambient spawning season at the Marine Harvest wrasse hatchery, Machrihanish. Treatment and control tanks were set up with four separate spawning zones, each containing four artificial spawning substrates. Spawning zones in treatment tanks each contained different coloured substrates (red, blue, grey and green) while control tanks spawning zones all contained hatchery standard green spawning substrates. After an initial three-week period, the location of the coloured mats within the experimental tanks was interchanged. While spawning was equally distributed across spawning zones in control tanks, in the experimental tanks, a preference for red spawning substrates was observed, irrespective of location within the tanks. This was shown through both the highest number of spawning events recorded within red spawning zones (37.5 – 38.6 %) and the highest proportion of the total spawning score per tank (40.9 – 49.3 %) recorded on red spawning substrates (followed by blue and green). Least preferred were grey spawning substrates with both lowest overall spawning events (9.3 – 10.5 %) and proportion of total spawning scores (1.4 – 8.2 %). Mean total spawning scores per spawning zone were comparable across zones and tanks, irrespective of location and colour. These data are suggestive of an active selection by ballan wrasse for spawning substrates with preference for colours with increased contrast from tank background colour. In commercial production, green spawning substrates are routinely used and while present results suggested an effect of substrate colour on spawning and egg productivity, further work is needed to confirm these effects before implementing new industry protocols.

4.1 Introduction

The first step in a successful broodstock management program is the identification of the optimal conditions required for a species to breed in captivity and produce good quality gametes (Mylonas et al., 2010). As with any emerging or new species to aquaculture, there is a period of trial and error with respect to spawning and hatchery conditions in an effort to find the best practices. Therefore, questions and bottlenecks surrounding ballan wrasse broodstock spawning behaviour and overall success in a commercial hatchery environment remain. Some of the more pressing areas of concern are the spawning behaviour and optimisation of overall spawning conditions to maximize egg production. Following a detailed study into captive broodstock spawning performance and egg quality (Chapter 3), one potential area highlighted for further investigation was spawning substrate selection. By determining if ballan wrasse have a colour or position preference for spawning substrates within tanks, optimisation may lead to increased overall spawning productivity and fertilisation, while also improving egg retrieval from spawning tanks.

The process of collecting naturally spawned eggs in a commercial setting is a basic husbandry requirement. For species such as Atlantic cod (*Gadus morhua*) (Kjesbu et al., 1991) or Atlantic halibut (*Hippoglossus hippoglossus*) (Brown et al., 2006), the collection of floating eggs using passive egg collectors fitted on the surface overflow is relatively straightforward (Zohar et al., 1995; Liu et al., 2000). However, the retrieval of eggs in species that spawn adhesive eggs is more of a challenge. For example, commercial pond culture of the common carp (*Cyprinus carpio*) includes setting up spawning ponds supplied with artificial substrates for spawning such as plants and vegetation, conifer branches, synthetic brushes, or plastic sheets that can be removed and incubated elsewhere (Rothbard & Yaron, 1995; Mananos et al., 2009). The issue of adhesive eggs in some commercially cultured species is overcome by manually stripping broodstock of gametes and applying chemicals for removing or preventing egg adhesiveness as discussed in Chapter 4. However, manual stripping of gametes, especially for a multiple batch spawning species with asynchronous ovarian development, can be time consuming and requires an accurate assessment of the stage of gametogenesis to prevent post-ovulatory aging and therefore the collection of suboptimal quality eggs (Mananos et al., 2009; Mylonas et al., 2010). Spontaneous spawning in captive ballan wrasse is clearly a positive reproductive trait. This is not always the case

in all cultured species, for example, in turbot (*Scophthalmus maximus* L.; Mugnier et al., 2000) or Atlantic halibut where interventions such as hormonal induction of spawning and/or manual stripping are required for obtaining eggs (Kjørsvik & Holmefjord, 1995; Mananos et al., 2009; Mylonas et al., 2010). Therefore, where possible, spontaneous spawning in captivity should be promoted as it allows fish to express their natural spawning behaviour and to release gametes in synchrony which may, ultimately, lead to high fertilization success (Mylonas et al., 2010).

L. bergylta display clear spawning behaviour and, in captivity, actively seek out a spawning substrate to deposit adhesive eggs. In the wild, ballan wrasse, along with other Labrid species such as the green wrasse (*Laburs viridis;* Kožul et al., 2011), have been reported to spawn eggs over nests or rocks covered with algae (Artüz, 2005). However, relatively little is known about the specific spawning behaviour of wild ballan wrasse with respect to the selection of spawning areas and substrates. The harem style mating strategy of this species, whereby males appear to defend territories with several females (Sjölander et al., 1972; Dipper, 1987), might suggest active investment into mate and/or territory choice. However, location and type of substrate could also play an important role on spawning success.

Substrate spawning fish are known to be selective in their choice of spawning habitat and a variety of environmental factors have been shown to influence choice for spawning site including, but not limited to, water temperature, salinity, depth, wave action, and indeed substrate and/or vegetation type (Snickars et al., 2010). For example, the distribution of spawning habitat for the Eurasian perch (*Perca fluviatilus* L.) was strongly dependent on specific habitat characteristics e.g. type of vegetation (Snickars et al., 2010). In addition, a clear relationship between sole (*Solea solea* L.) egg abundance and sediment type was found, with significantly higher egg densities found over sediment with a < 30 % gravel content (Eastwood et al., 2001).

Selection of spawning sites in some pelagic spawning wrasse species, such as the bluehead wrasse (*Thalassoma bifasciatum*), has also been linked to female choice of particular characteristics of the sites rather than the males occupying them (Warner et al., 1990). In the peacock wrasse (*Cirrhilabrus temminckii L.*) (Kohda et al., 2005) and the spotty wrasse (*Pseueolabrus celidotus B.*) (Jones, 1981) selection of spawning location has been linked to female choice based on territory depth.

Current practice within UK hatcheries successfully uses low pile, green carpet mats as spawning substrate. However, no real scientifically informed decision has been made with regards to the colour, type, or positioning of the mats in broodstock spawning tanks. The selection was based on availability, cost and practicality of cleaning and incubating. Early breeding trials looked at the use of different types of carpet tiles, terracotta tiles, and Perspex plastic sheets with no clear substrate preference established (Ottesen et al., 2013). Therefore, the aim of this study was to determine if ballan wrasse broodstock have a preference for the colour or position of spawning substrates within commercial spawning tanks and if so, determine if optimisation may lead to increased spawning activity and egg production. This research is working towards an overall applied goal of fine tuning and increasing ballan wrasse hatchery productivity.

4.2 Materials and Methods

4.2.1 Experimental fish and system

The spawning substrate colour preference trial took place during the ambient 2014 spawning season (March – June) at the Machrihanish Marine Farm (Machrihanish, Scotland). Following the 2013 ambient spawning season, during which the broodstock spawning performance and egg quality work took place (Chapter 3), Machrihanish wild caught broodstock were overwintered in common conditioning tanks, kept on a simulated natural photoperiod (SNP), at a constant temperature of 12 °C. Broodstock were fed daily to satiation on an industry standard extruded pelleted diet (Symbio Wrasse Diet, 6.5 mm diameter; Biomar^{Ltd}, Grangemouth, Scotland UK).

In March 2014, spawning harems were established in four experimental spawning tanks. Broodstock were anesthetised using Tricaine Methane Sulphonate (MS222) (Pharmaq Ltd., Hampshire UK), measured for total body-weight (BW ± 1 g) and total body-length (TL ± 1 mm) and assigned to a presumptive gender based on body-size and morphological parameters (Leclercq et al., 2014b) and, where possible, visual inspections of male or female gametes upon gentle manual stripping. Sex ratios were manipulated where possible to reach 10 % (range = 10 – 10.3 %) males in tanks to match reported sex ratios in the wild (Table 4.1).

Table 4.1. Description of ballan wrasse broodstock used in colour substrate preference experiment: sex ratio and size parameters; values represented as mean ± SD.

	Control		Colour treatment	
	Tank 1	**Tank 2**	**Tank 1**	**Tank 2**
Fish (n)	30	30	30	29
Males (n)	3	3	3	3
Females (n)	27	27	27	26
Male body-weight (g)	1307.3 ± 37.3	1337.7 ± 35.2	1297.0 ± 101.2	1166.3 ± 14.9
Male length (mm)	419.3 ± 16.0	416.7 ± 2.7	407.3 ± 5.5	396.7 ± 5.9
Female body-weight (g)	781.0 ± 47.0	812.1 ± 28.6	785.5 ± 35.0	786.3 ± 30.1
Female length (mm)	345.3 ± 6.8	351.7 ± 4.0	349.0 ± 5.0	348.3 ± 4.0

All spawning harems were established in 7 m³ round, light blue, fiberglass tanks fitted to a single indoor recirculation system (TMC System 10,000; Tropical Marine Centre, Chorleywood, UK) with protein skimmer, mechanical filtration to 100 µm, biofilters, UV disinfection and photo-thermal control. Flow rate to each tank was 66 L/min (50 % renewal / hour), approximately 20 % pumped ashore seawater exchange daily, and introduced in a clockwise direction. Broodstock were kept on ambient SNP with a water temperature of 12.20 °C ± 0.03 °C. Water quality parameters were checked daily and averaged over the spawning season: salinity 33.4 ± 0.12 ppt; oxygen 7.50 ± 0.04 mg/l, 85.60 ± 0.43 % saturation and pH 8.23 ± 0.05. Broodstock were fed to satiation on langoustine (*Nephrops norvegicus*) tails and tanks were siphoned daily for waste removal. Feed was introduced to tank from 'spawning zone D' (See description in section 4.2.2). Artificial spawning substrates (Miami Gel carpet, 70 x 40 cm; MDC, Glasgow, Scotland UK) were provided in each tank (n = 16 / tank) as well as artificial kelp and PVC pipes for shelter.

4.2.2 Experimental design and sampling

From the 4th of April, when spawning commenced, each of the four spawning tanks was set up with 10 green spawning substrates as per hatchery standard. Once, at least, a single day of spawning was observed in each of the four experimental tanks, each tank was then set up with four separated spawning zones (A-D) each supplied with four industry

standard spawning mats for deposit and collection of adhesive eggs. Spawning mats were arranged in such a way that each mat in a group of four overlapped to avoid gaps (Fig. 4.1A-C). Each tank was also fitted with PVC pipes and artificial seaweed made out of black tarpaulin for shelter, located in the centre of tanks.

In control tanks 1 and 2 (hereafter referred to as C1 and C2), all four spawning zones contained green spawning mats throughout the entire trial period (Fig. 4.2A).

Experimental Phase 1

During Phase 1 of the trial, (16^{th} April – 6^{th} May) each spawning zone (A-D) in colour treatment tanks 1 and 2 (hereafter referred to as T1 and T2) was allocated a different spawning substrate colour: Zone A = Green; Zone B = Grey; Zone C = Red; Zone D = Blue (Fig. 4.2B).

Experimental Phase 2

During Phase 2 (7^{th} – 28^{th} May), the location of coloured mats within T1 and T2 were interchanged to determine if preference of spawning was due to location within the tank or colour of the substrate: Zone A = Blue; Zone B = Red; Zone C = Grey; and Zone D = Green (Fig. 4.2C).

In all four tanks, spawning mats were individually assessed daily at 9 am for presence of eggs and scored from 1 to 3 based on relative abundance and distribution of eggs across the mat. The subjective scoring was done as follow, according to methodology developed in Chapter 3 (1: Low density of eggs and variable coverage, i.e. few eggs scattered over the mat; 2: High density of eggs but low coverage, i.e. many eggs clustered together on a portion of the mat; 3: High density of eggs and high coverage, i.e. many eggs covering the whole mat). A daily 'spawning score' per spawning zone (A-D) for relative egg quantity per day and per tank was determined as the sum of the individual subjective mat scores. Spawning mats without eggs were cleaned and returned to allocated spawning zones while spawning mats with eggs were removed to a seawater bath for later incubation and replaced with new mats of the same colour.

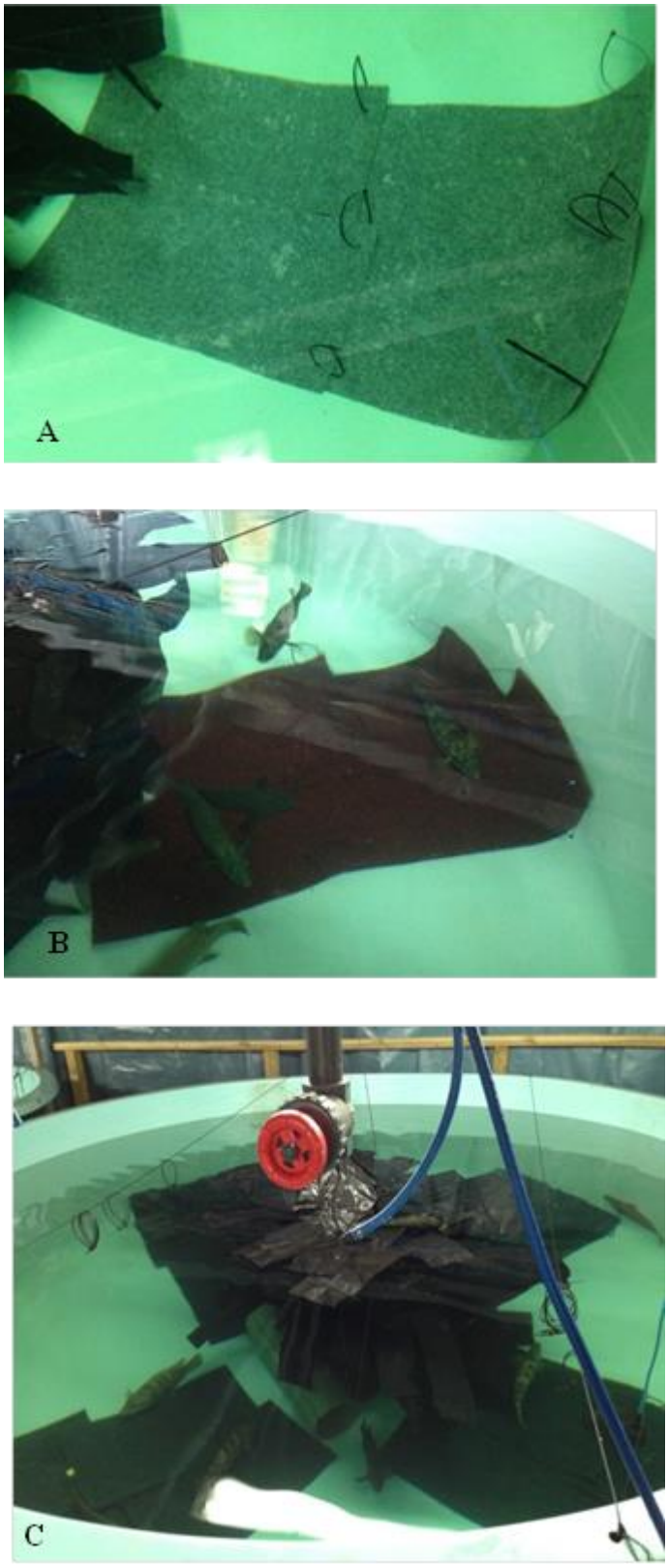

Figure 4.1. A. Grey spawning mats set up in overlapping fashion to avoid gaps; B. Red spawning mats with 6 ballan wrasse located within red spawning zone; C. Control spawning tank with 4 green spawning zones.

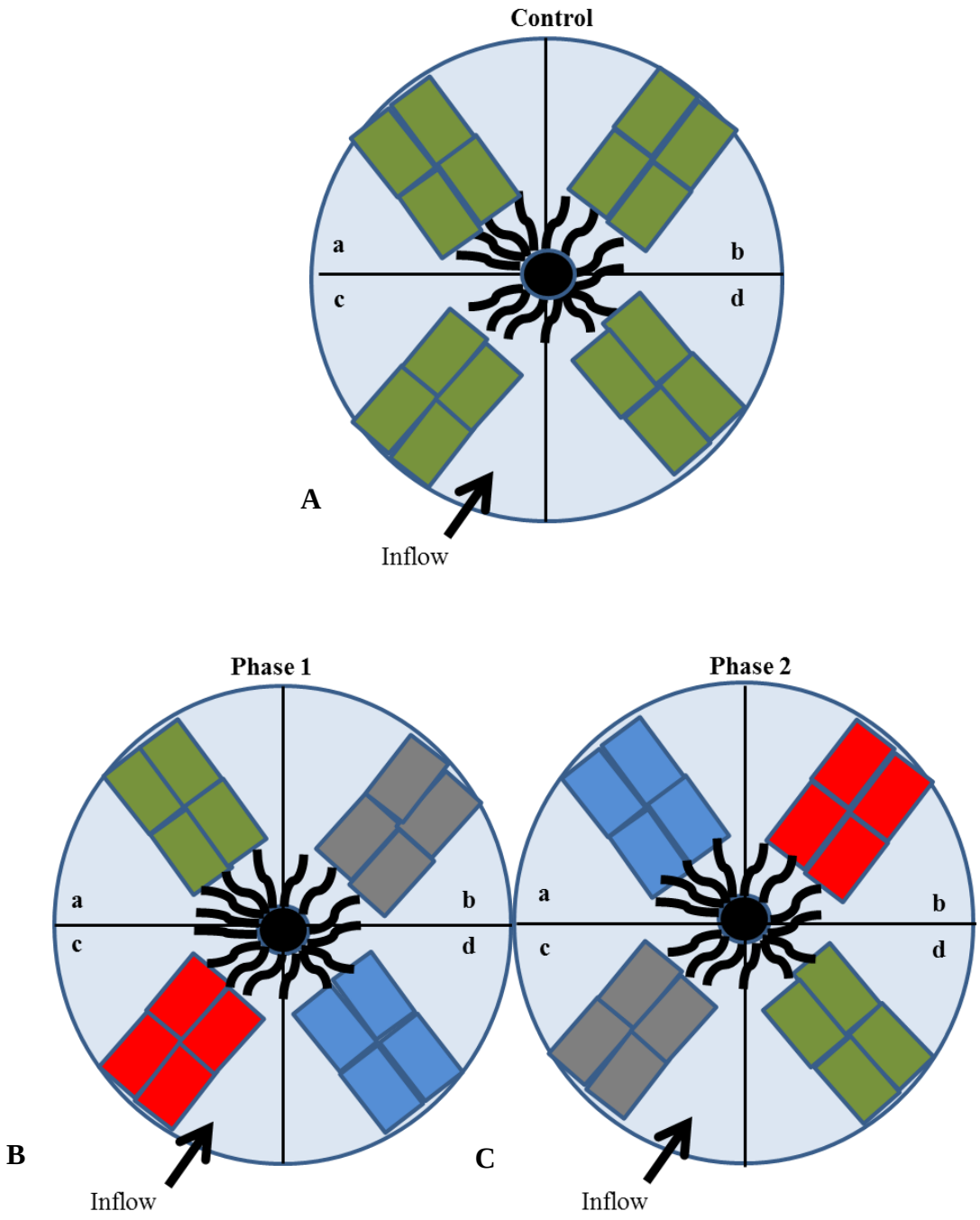

Figure 4.2. Diagrams representing the experimental setup of broodstock spawning tanks. A. Control tanks (C1 and C2) tanks equipped with 16 spawning mats set up with all four spawning zones (A-D) containing 4 green spawning mats each; B. Experimental phase 1 setup for T1 and T2; C. Phase 2 setup for T1 and T2. Control and trial tanks also contained artificial kelp and PCV hides.

4.2.3 Statistical Analysis

Statistical analysis was performed using Minitab version 16 statistical software (Minitab, Coventry, UK. http://www.minitab.com/en-us/products/minitab/). Normality of data was checked using the Anderson Darling test and a one way ANOVA with Tukey's family error comparison (95 % confidence interval) was used to test for significant differences between spawning scores from each tank. Data sets were log transformed when normality and/or homogeneity of variance was not confirmed. Replicate treatments were pooled where tests for equal variance between tanks revealed no variation between mean spawning scores. A Chi-Squared Goodness-of-Fit Test was used (assuming equal proportions of expected values) to test whether the observed frequencies of spawning events per colour differed from the expected frequencies. All statistical comparisons were performed using a significant level of 5 % ($p < 0.05$). Results are presented as mean ± standard deviation (SD).

4.3 Results

4.3.1 Spawning dynamic

From 16th April – 6th May, a total of 24, 17, 29, and 27 spawning days were observed in tanks C1, C2, T1 and T2, respectively (Fig. 4.3). Of the four spawning populations, C2 was the only to follow a similar spawning periodicity to that observed in the previous spawning season (Chapter 3). During the 6 week trial, C1 had two complete spawning periods (SP) of 12.0 ± 2.8 days; each SP consisted of an average spawning window (SW) of 6.0 ± 0.0 days where spawning occurred, followed by inter-spawning interval (ISI) of 6.0 ± 2.8 days without spawning. The remaining three spawning tanks (C1, T1, and T2) did not follow the same rhythmicity seen in the previous spawning season, making it difficult to assess the length of individual spawning periods. These three tanks spawned almost continuously throughout the trial period with no more than a 2 day gap between spawning days (mean 1.4 ± 0.5 days).

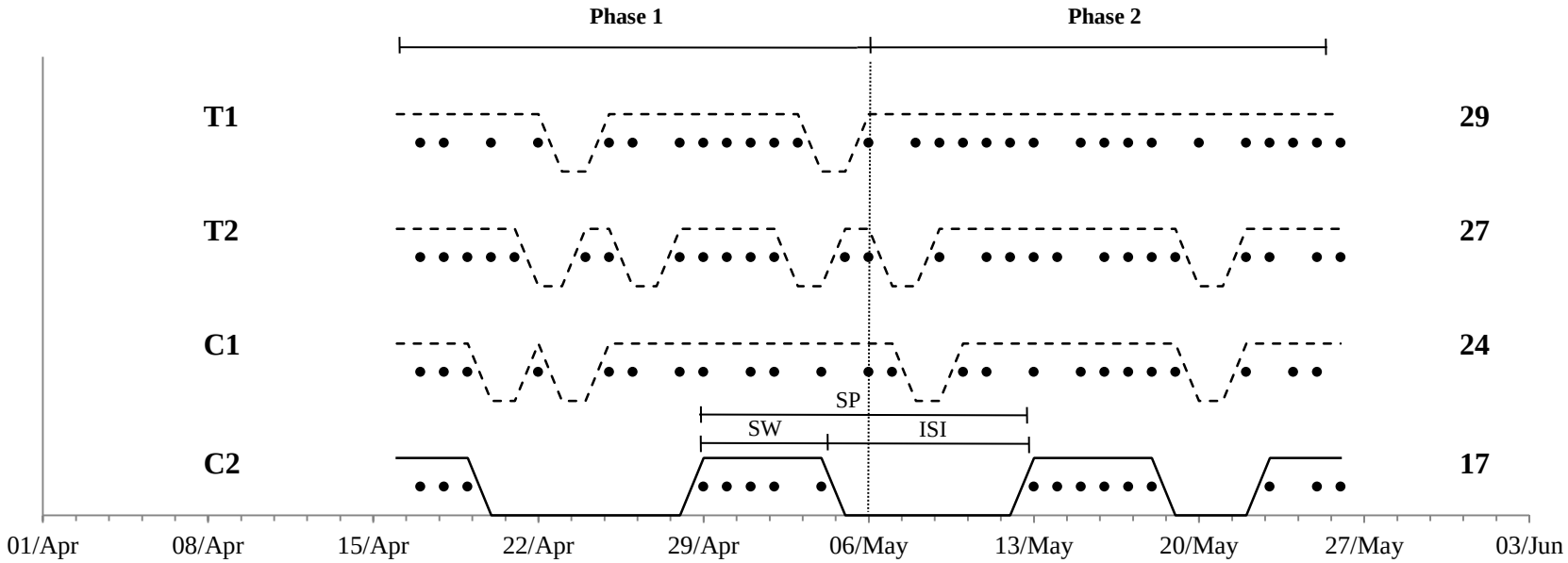

Figure 4.3. Spawning dynamics for C1, C2, T1 and T2 during experimental phases 1 and 2 including spawning period (SP), spawning window (SW), and inter spawning interval (ISI). Each point on the graph represents a single spawning date, total number of spawning days for each tank (inclusive of both phases) in bold on right hand side. Solid line represents clear spawning rhythm seen in C2 and broken lines represent lack of spawning synchrony seen in T1, T2, and C1.

4.3.2 Spawning colour and location preferences

The occurrence of spawning was evenly distributed across the four spawning locations in control tanks C1 and C2 and the chi-squared test goodness-of-fit showed that there was indeed an equal distribution for the four spawning locations (Table 4.2). Although not statistically significant, a slightly higher proportion of spawning events took place in location D (32 and 33 % for tanks C1 and C2, respectively) than locations A-B-C. Conversely, spawning in the colour treated tanks was not equally distributed in either T1 ((X^2 3, N = 64) = 11.5, $p < 0.05$) or T2 ((X^2 3, N = 57) = 11.84, $p < 0.05$), with the highest number of spawning events observed on red substrates followed by blue and green.

Table 4.2. Chi-squared goodness-of-fit results for four individual tests; tests in Tanks 1 and 2 sought to determine if preference was equal between the four spawning colours (inclusive of both trial phases) and tests for Control tanks 1 and 2 sought to determine if preference was equal between the four spawning locations. Observed spawning events (SE) per location or colour with expected values in brackets (assumed equal preference); degrees of freedom (DF) is equal to the number of levels (k) of the categorical variable minus 1: DF = k – 1. *Not significant (NS) at the 0.05 level.

Control 1			**Control 2**		**Treatment 1**			**Treatment 2**	
Location	Observed SE	Contribution to Chi	Observed SE	Contribution to Chi	Colour	Observed SE	Contribution to Chi	Observed SE	Contribution to Chi
A	10 (12.5)	0.5	9 (11.25)	0.45	Green	14 (16.0)	0.25	10 (14.25)	1.267
B	10 (12.5)	0.5	11(11.25)	0.005	Grey	6 (16.0)	6.25	6 (14.25)	4.776
C	14 (12.5)	0.18	10 (11.25)	0.13	Red	24 (16.0)	4	22 (14.25)	4.214
D	16 (12.5)	0.98	15 (11.25)	1.25	Blue	20 (16.0)	1	19 (14.25)	1.583
Total	50		45		**Total**	64		57	
DF		3		3	**DF**		3		3
Chi-Sq		2.61		1.84	**Chi-Sq**		11.5		11.84
P-Value		**0.540** *ns		**0.605** *ns	**P-Value**		**0.009**		**0.008**

4.3.3 Spawning score

The largest proportion of the total spawning score per tank, inclusive of all four spawning locations over both experimental phases, was from red spawning substrates in both T1 (40.9 %) and T2 (49.3 %) followed by blue spawning substrates (28.4 % and 30.5 %, for T1 and T2, respectively), green (22.6 % and 18.8 %, for T1 and T2, respectively) and finally grey (8.2 % and 1.4 %, for T1 and T2, respectively) (Fig. 4.3a, b).

During phase 1 of the trial, in both T1 and T2, spawning location 'B' (grey spawning substrate) received the lowest amount of spawning activity (6.8 % and 1.6 % of total spawning score, respectively) while location 'C' (red spawning substrate) received the highest (41.9 % and 48.4 %, respectively). During phase 2 of the trial, location 'B' (red spawning substrate) received again the highest amount of spawning activity (39.6 % and 50.6 % for T1 and T2, respectively) while location 'C' (grey spawning substrate) received the lowest spawning activity (9.9 % and 1.1 % for T1 and T2, respectively). In both control tanks, spawning activity appeared to be higher in location D with 37.6 % and 45.5 % of the total spawning scores per tank for C1 and C2, respectively (Fig. 4.3c, d). Furthermore, location A in both control tanks appeared to receive a lower amount of spawning activity with 14.1 % and 16.0 % of the total spawning score.

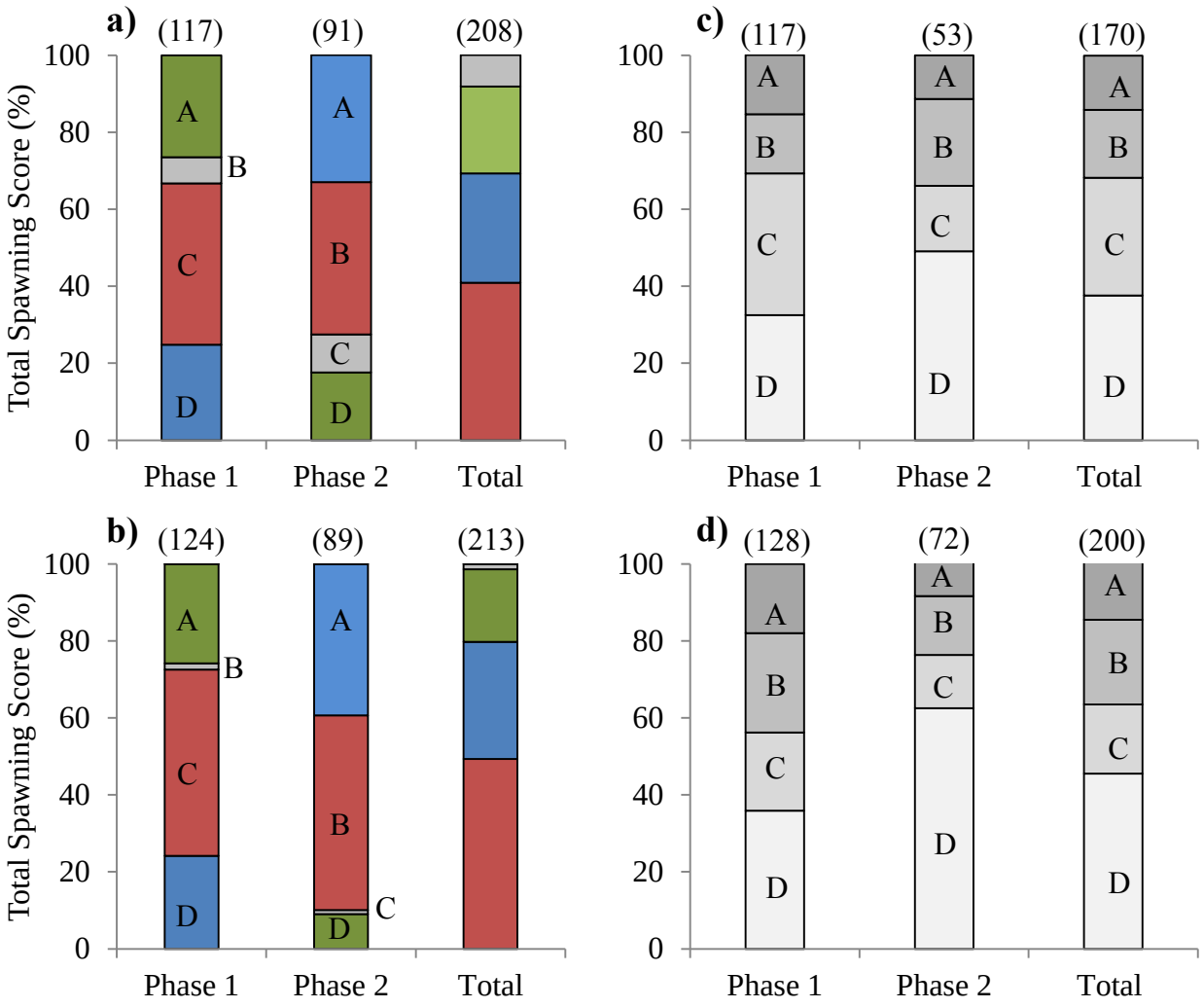

Figure 4.3. Percent of the total spawning score for each colour, location, and tank (**a**, Treatment 1; **b**, Treatment 2; **c**, Control 1; and **d**, Control 2) for both experimental phases. Total spawning score is shown on top of each bar and location within the tank (A, B, C, or D) listed within each bar section.

Mean total spawning score (MTSS) per spawning zone was calculated to compare the relative amount of eggs spawned within each colour or location per day. MTSS per spawning substrate colour (T1 and T2) and location (C1 and C2) were comparable across all four spawning tanks. However, while no significant differences in MTSS were seen between colours or locations, MTSS appeared to be slightly higher overall within red spawning zones in T1 and T2 and location 'D' in C1 and C2.

Table 4.3. Mean total spawning score (MTSS) per spawning substrate colour per day (T1 and T2) and per substrate location per day (C1 and C2) inclusive of both experimental phases. Values presented as mean ± SD, *N* = number of spawning days for each colour or location. *Note*: Superscripts represent significant differences between spawning scores.

Location	Control 1	*N*	Control 2	*N*	Colour	Treatment 1	*N*	Treatment 2	*N*
A	2.40 ± 1.17	10	4.11 ± 1.26	9	Red	3.54 ± 1.95	24	3.77 ± 1.54	30
B	3.00 ± 1.82	10	4.36 ± 2.42	11	Blue	2.95 ± 1.66	20	3.21 ± 1.54	19
C	3.71 ± 1.72	14	4.20 ± 2.04	10	Green	3.35 ± 1.94	14	3.10 ± 2.37	10
D	4.00 ± 1.82	16	4.86 ± 2.41	15	Grey	2.83 ± 1.16	6	1.66 ± 0.81	6

On a given spawning day, spawning often occurred within more than one spawning zone in each tank. In treatment tanks (inclusive of both T1 and T2) spawning most often occurred within two zones per day (35.7 %, 20/56 spawn days; Table 4.4) and least often within four spawning zones per day (8.9 %, 5/56 spawn days). On the other hand, in control tanks (inclusive of C1 and C2), spawning most often occurred within one spawning zone (36.6 %, 15/41 spawn days) followed closely by 26.8 % of spawning occurring within four spawning zones per day (11/41 spawn days).

The MTSS per day was significantly higher when 4 spawning zones were selected versus one spawning zone in treatment tanks (One-way ANOVA: DF = 3, F = 4.30, P = 0.015; Table 4.4). Similarly for control tanks, MTSS was higher when three and four spawning zones were selected than one and two spawning zones (One-way ANOVA: DF = 3, F = 27.48, P = 0.000). Although the MTSS per day increased based on the number of zones selected for spawning, the MTSS per zone was not significantly different irrespective of the number of zones selected in both treatment and control tanks.

Table 4.4. The mean total spawning scores (MTSS) per day or per zone based on the number of spawning zones (1-4) selected for spawning on a given day; percent (%) of the total spawning days where '*X*' spawning zones were selected for spawning in treatment and control populations (Pooled replicates: Treatment = T1 and T2, Control = C1 and C2). *n* = the number of days on which '*X*' spawning zones were selected for spawning. *Note:* Superscripts represent significant differences between MTSS based on the number of spawning zones selected (all *p* values < 0.05).

Controls					Colour treatments			
Number of spawning zones used per day	MTSS per day	MTSS per zone	(*n*) days	% total days	MTSS per day	MTSS per zone	(*n*) days	% total days
1	3.73 ± 1.62^{a}	3.73 ± 1.64^{a}	15	36.6	4.55 ± 2.24^{a}	4.55 ± 2.24^{a}	16	28.6
2	4.77 ± 2.22^{a}	2.38 ± 1.11^{a}	9	22	7.00 ± 3.35^{ab}	3.66 ± 3.35^{a}	20	35.7
3	13.83 ± 6.37^{b}	4.61 ± 2.12^{a}	6	14.6	8.83 ± 4.71^{ab}	3.50 ± 1.56^{a}	15	26.8
4	16.91 ± 5.72^{b}	4.22 ± 1.42^{a}	11	26.8	14.67 ± 3.79^{b}	2.94 ± 0.94^{a}	5	8.9

4.4 Discussion

This study tested the preference of captive ballan wrasse for spawning on different colour substrates and/or locations within broodstock tanks. This research is important for the successful establishment of wrasse breeding populations in captivity to maximise hatchery productivity and quality. In this study, the overall occurrence of spawning and proportion of the total spawning score per tank was significantly higher on red spawning substrates than grey substrates and blue/green to a lesser extent. In addition, spawning appeared to not be correlated to substrate position within the spawning tanks. This suggests ballan wrasse broodstock perceived the spawning zones differently depending on substrate colour and displayed a preference towards red spawning substrates which have an increased contrast against the tank background colour.

Only one out of the four spawning populations (C1) had a comparable spawning periodicity to that seen in the previous spawning season (Chapter 3) characterised by a series of spawning periods lasting approximately 12 days made of spawning windows lasting ~ 6 days followed by interspawning intervals of ~ 6 days where no spawning occurred. However, surprisingly, no such rhythm was observed in the other three spawning populations (T1, T2, and C1), where spawning occurred almost continuously throughout the trial period with no more than a two day gap between spawning days. The difference in spawning rhythmicity between the spawning populations observed in Chapter 3 and those within the current study may be partly explained by the number of females present in the spawning tanks. Overall, there were fewer females within each spawning population in Chapter 3 (Table 3.1) than in the current study and there was a strong correlation (R^2 = 75.4 %; P = 0.005; data not shown) between the number of females per tank and the overall number of spawning days per tank over the same given time period. However, it remains unclear at this stage whether the lack of spawning rhythmicity in three out of the four broodstock tanks within the current study was partly due to colour choice or fragmentation of the spawning area into four zones or individual spawning population variability. While it was not possible within this study to perform parentage assignment and assess overall spawning contribution, the overall number of spawning days was higher in colour treated tanks than controls, despite there being one less female present in the T2 spawning population. The fragmentation of spawning tanks in four different colour substrate zones has, therefore, potentially increased the

occurrence of spawning suggesting that more individuals spawned overall. Equally, increased spawning was observed in one of the control tanks probably due to the multiple spawning zones provided.

The preference for red spawning substrates was clear within both colour substrate tanks, supported not only by the highest overall occurrence of spawning, but also the largest proportion of the total spawning score. This was evident in both tanks during the first experimental phase. Similarly, when the location of the coloured spawning substrates was interchanged in the second experimental phase, again individuals within both tanks spawned preferentially on red mats followed by blue and green. The fact that individuals preferably spawned on red (and then blue/green) substrates over grey substrates suggests that they may have the ability to perceive the different colours or differences in contrast against the tank background. The ability to perceive colour, or colour vision, has been confirmed in many animals across most phyla, including many teleost species. Colour vision is used for specific behaviours such as object recognition or phototaxis (i.e. movement in response to light) and varies considerably between species (Kelber et al., 2003). However, in fish, this is generally studied in the context of predator prey interactions or for communication purposes such as attracting a mate or exhibiting dominance. For example, the Picasso triggerfish (*Rhinecanthus aculeatus L.*) was found to have colour vision over their spectral range and behavioural studies concluded that triggerfish were more likely to choose coloured over grey stimuli, irrespective of brightness. Moreover, it was found that triggerfish have a preferential foraging response to red stimuli followed by green over blue and yellow (Cheney et al., 2013). The preference for red stimuli has also been observed in the three-spined (*Gasterosteous aculeatus*) and nine-spined sticklebacks (*Pungitius pungitius L.*), where in a laboratory setting, males and females responded most strongly to red objects (Smith et al., 2004). It has also been shown that female three-spined sticklebacks preferably mate with males that have red pigmentation on their throat and jaw during the breading season (Bakker & Mundwiler, 1994), and that in a laboratory setting males can increase their reproductive success by decorating their nests with red objects (Östlund-Nilsson & Holmlund, 2003). Although the specific visual capabilities of ballan wrasse has not yet been described, Siebeck & Marshall (2000) reported on the transmission properties of the ocular media of 36 tropical wrasse species. Ocular media acts as a filter that limits light spectrum reaching photoreceptors and, therefore, represents an important first step to investigate

the visual system of fish (Thorpe et al., 1993). It was found that a large number of the wrasse species studied had yellow pigmentation in the cornea which has been shown to absorb high-intensity UV wavelengths and limits the sensitivity to short wavelengths (Siebeck & Marshall, 2000). However, with most wrasse species displaying diurnal behaviour, i.e. being most active during the day under high-intensity light conditions, this limitation for light below 500 nm is possibly not important (Thorpe et al., 1993; Randall et a. 1997). Furthermore, yellow pigmentation can enhance the contrast of prey items against a background which is particularly effective against a blue background (Douglas et al., 1998).

The relative amount of eggs per zone, based on the mean total spawning score and irrespective of colour or location, remained similar. This is not surprising as fecundity between females or individual batches of eggs is not expected to vary greatly and furthermore, all females were of comparable size and have the same origin. There was however, a slightly lower mean total spawning score on grey mats, potentially due to the fact that females were not actively selecting these mats to spawn but were possibly spawning on adjacent mats (green or blue mats) and some of the eggs were accidentally released or drifted to these locations. This can be corroborated by the fact that there was no more than a score one observed on any single grey mat collected from both of the experimental tanks.

Interestingly, results showed that on a given day, when spawning occurred within multiple zones, the relative amount of eggs, based on the overall spawning scores, was higher. Therefore, the most likely explanation is that multiple individuals were spawning when more than one spawning zone was selected which happened roughly 70 and 36 % of the time in colour substrate tanks and control tanks, respectively. This is evident through the mean total spawning scores remaining similar within each zone, irrespective of the number of zones selected on a given day and that the total spawning score per day increased based on the number of zones selected for spawning. At this stage, however, it cannot be determined if eggs within a given spawning zone belonged to a single female or if there were multiple females contributing in a single zone. This type of behaviour has been described in many species including the whitebelly damselfish (*Amblyglyphidodon leucogaster B.*), another demersal spawning species where territorial males display paternal care of eggs, and females preferentially spawned with males that already had 1-3

day old eggs within their guarded nesting site (Goulet, 1998). Additionally, female Mediterranean blenny (*Aidablennius sphynx V.*) prefer to mate with males that are already guarding eggs (Kraak & Groothus, 1994). However, the daily removal of spawning substrates and eggs from spawning tanks in the case of captive ballan wrasse, make it unlikely that the presence of eggs within a given spawning territory may influence the spawning site choice of other females. Moreover, in Chapter 3, it was shown through analysis of parental contribution that in 50 % of the daily spawning events assessed there was evidence of multiple females contributing to spawning, and the remaining showed evidence of only single female contribution. The main limitation in this result is that it was not possible to determine parental contributions to individual spawning events within this study. However, future research should focus on assessing parental contributions to spawning within fragmented spawning zones using the panel of 7 microsatellite markers selected and verified in Chapter 3 to verify if the daily number of female contributors increases with number and colour of spawning zones.

Whether the preference of colour substrate and/or location is influenced by male or female ballan wrasse is debateable at this stage. In fact, the relative roles of males and females in structuring mating systems are the subject of debate across many species (Warner, 1990). To the author's knowledge, Sjölander et al., (1972), has been the only source to report on the spawning behaviour of ballan wrasse in the wild, characterised by territorial males that court and mate with several females in pairwise spawning events, and provide parental care to eggs. While there were some anecdotal observations during this study of territorial behaviour between males, it would seem that in captivity, females may choose the location to spawn and the male will guard 'her' from other males within the tank and fertilize wherever she lays the eggs. This has also been suggested in the bluehead wrasse where territorial males concentrate their spawning effort in areas where females have chosen to mate (Warner, 1990). However, observations were made of both male and female aggression, with females chasing off other females from a particular spawning area and of males being aggressive towards other males, sometimes to the point of inflicting serious wounds which require treatment or removal of bullied males to other tanks. Male territorial behaviour with respect to spawning area is common among many wrasse species, including the spotty wrasse (Jones, 1981), concha wrasse (*Nelabrichthys ornatus C.*) (Andrew et al., 1996) and the rainbow wrasse (*Thalassoma lucasanum G.*), where males have been found to defend spawning territories of up to 10

m^2 from other males while spawning in single pair matings with multiple females within the territory (Warner, 1982). Territorial behaviour in spawning is often indicative of an environment where competitor densities are low enough so as to allow economic defendability and further, territory defence is presumed to have a high energetic cost (Warner, 1984). Although it is difficult to make direct comparisons to wild spawning behaviour as the captive environment clearly puts different pressures on fish, namely the forced interactions in smaller territories, the general lack of understanding of ballan wrasse spawning behaviour, with respect to the establishment of breeding populations, highlights the need for further research.

The results from this study indicate that captive ballan wrasse actively select substrates to spawn on and have a preference for spawning substrates with an increased colour contrast to the tank bottom. This result is directly applicable to commercial practices for broodstock management of ballan wrasse and although routine spawning already occurs on hatchery standard green substrate, the use of red spawning substrates may increase overall spawning activity. However, these results highlight the need for further research to assess the effects of fragmentation of spawning zones within tanks and the potential effects this may have on territorial behaviour and spawning which could be verified through analysis of parental contribution using the microsatellite panel developed in Chapter 3.

5 Chapter 5: Removal of the adhesive gum layer surrounding naturally fertilised ballan wrasse (*Labrus bergylta*) eggs.

RESEARCH ARTICLE

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Keywords: Cleaner fish, adhesive eggs, gum layer, hatching, enzyme alcalase®.

Abstract

Commercial production of ballan wrasse (*Labrus bergylta*) as a cleaner fish for the removal of sea lice (*Lepeophtheirus salmonis*) from farmed salmonids (*Salmo salar*) has increased due to its proven efficiency. One bottleneck in commercial hatchery production is working with the benthic adhesive eggs, which makes disinfection and incubation of eggs challenging; therefore, this study aimed to find a chemical or enzymatic treatment and process to remove the adhesive gum layer. Naturally spawned eggs were collected from artificial spawning substrates up to 24 hours post spawning from wild caught broodstock kept in captivity at the Marine Harvest, Machrihanish facility. Four treatments were tested: tannic acid (0.2, 0.1, and 0.05 %), sodium sulfite (2, 1, and 0.5 %), L-cysteine (2, and 1 %), and enzyme alcalase® (4.0, 3.0, 2.0, 1.0, and 0.5 %) *in vitro*. Eggs were exposed for 25 minutes while being continually agitated, and the proportion of "degummed" eggs was counted at the end of each time period. Enzyme alcalase® was the only treatment that proved successful in degumming eggs, with the time to complete degumming ($\geq$ 96 %) inversely related to enzyme concentration. Complete degumming occurred between 15 and 30 minutes for all enzyme alcalase® dose rates. Mean hatch rates for eggs treated with enzyme alcalase ® were not compromised by the treatment and in the highest dose tested were actually found to be significantly higher in treated eggs (73.7 ± 2.6 %) than controls (65.1 ± 4.4 %). The use of enzyme alcalase® proved effective in degumming ballan wrasse eggs without affecting hatch rates. However, translation of this method to *in situ* degumming and, thus, removal of eggs from spawning substrate on farm remains to be standardised.

5.1 Introduction

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

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

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

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

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

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

5.2 Materials and Methods

5.2.1 Broodstock management and egg collection

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

Each tank was furnished with artificial kelp and PVC pipes as shelters in addition to polypropylene carpets (*n* = 16, 70x40 cm; Miami Gel carpet, MDC, Glasgow, Scotland) as spawning substrate for collection of benthic eggs. Over the study duration (20th April to 3rd July 2013) which spanned the natural spawning season, spawning substrates were checked daily at 9 am for presence of eggs from natural spawning events. Random daily egg batches were selected from spawning pairs within the 10 spawning tanks; eggs used for each given trial (with replicates) were taken from single egg batches. Eggs were removed from spawning substrates using a metal spatula and placed into petri dishes prefilled with 20 ml of hatchery water (UV treated and filtered to 0.2 µm, hereafter referred to as hatchery water).

5.2.2 Treatment efficacy screening

Three different chemical compounds and one enzymatic treatment were tested at varying concentrations as preselected from the literature. Solutions of tannic acid (0.05, 0.1 and 0.2 % by weight; $C_{76}H_{52}O_{46}$; W304204; Sigma-Aldrich, UK), L-Cysteine (1.0 and 2.0 % by weight; $C_3H_7NO_2S$; W326205; Sigma-Aldrich, UK), sodium sulfite (0.5, 1.0 and 2.0

%; Na_2O_3S; S0505, Sigma-Aldrich, UK) and the proteolytic enzyme, Alcalase® *Bacillus Licheniformis* (4.0, 3.0, 2.0, 1.0 and 0.5 %; 126741-500; VWR, UK) were prepared using hatchery water. Sodium sulfite had a measurable effect on salinity (35 and 48 ppt at 0.5 and 2.0 %, respectively) such that each concentration was also prepared and tested in distilled freshwater (1, 3 and 21 ppt at 0.5, 1.0 and 2.0 %, respectively). The pH of each solution was adjusted to that of the hatchery water (pH 8.0) with 5 M HCl or 5 M NaOH using a calibrated pH-meter (Mettler Toledo, MP220/225), and salinity was measured using a hand-held refractometer.

5.2.3 Standardised experimental design

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

5.2.4 Optimisation of enzyme alcalase® treatment

Following initial screening, the proteolytic enzyme (Alcalase®) treatment was selected for further investigation to define the time required for complete degumming at each enzyme concentration. Each enzyme concentration previously tested (4.0, 3.0, 2.0, 1.0 and 0.5 %) was retested in triplicate and assessed on at least three intermediary time points against a non-treated control until maximum (100 %) degumming was reached. Time

points were initially selected based on observations of highest % degumming during the previous experiment; however, later time points were adjusted accordingly after degumming rate was assessed at the first time point, based on how fast degumming was occurring so as to not miss the point at which 100 % degumming was reached. A total of 12 petri-dishes per treatment were prepared according to the standardised methodology (Section 5.2.3); 9 of which were filled with enzyme solution at a single concentration and 3 with hatchery water prior to placing onto the horizontal orbital mixer at 240 rpm. At each time-point, 3 replicate and 1 control petri-dishes were measured. The same challenge protocol was repeated for each enzyme concentration tested.

5.2.5 Effect of enzyme alcalase® treatment on hatching rate

Based on results from the enzyme optimisation, hatching rate of eggs exposed to the three lowest enzyme dose-durations (0.5 %, 30 min; 1.0 %, 20 min; and 2.0 %, 10 min) were assessed following three discrete (independent egg batches) challenges against a control and in triplicate according to the standardised methodology (Section 5.2.3). For each challenge, a total of 6 petri-dishes ($n \approx 60$ eggs each) were exposed to a single enzyme dose-duration (Treatment, $n = 3$) or seawater (Control, $n = 3$). Upon completion of exposure time, all eggs were rinsed in hatchery water and placed into a 24-well microplate (5 eggs per well) pre-filled with 500 µl / well of hatchery water. Egg viability and stage of development were assessed under a stereomicroscope (GX Stereo microscope; XTL3T, GT Vision, Suffolk, UK). Microplates were covered, sealed, and incubated in darkness within a temperature controlled incubator (12 ± 1 °C; LMS Cooled Incubator, Model 305, Series 1, 60 L capacity, LMS Ltd, Kent, UK). The number of hatched larvae was counted (expressed as the proportion of stocked eggs per replicate to define the hatching rate) under a stereomicroscope at 9 days (108 °C days) post egg-collection to allow sufficient time for hatching to occur (Ottesen et al., 2012).

5.2.6 Progression of gum layer removal under enzyme alcalase® treatment

In order to look at the progression of gum layer removal over the course of enzyme exposure, replicate treatments of $n \approx 60$ eggs were exposed to 1 % enzyme alcalase and seawater controls ($n = 3$ replicates per time point; 0, 5, 10, 15, 20, and 25 minutes) following the previously described methodology (Section 5.2.3). At each designated time interval, the appropriate replicates for the treatment and control treatments were removed for the orbital shaker and then solutions were removed, eggs were rinsed in sea water,

and immediately photographed using a computer controlled digital microscope camera (1x magnification, GXCam3, GT Vision, Suffolk, UK) fitted onto a stereo microscope (GX Stereo microscope; XTL3T, GT Vision, Suffolk, UK). The breakdown of the adhesive gum layer was described using a 4 point subjective scale in n = 30 eggs per replicate explained further below to characterise the progression of the enzymatic treatment.

5.2.7 Statistical analysis

All figures were presented as mean ± standard deviation (SD). Statistical analysis was carried out with Minitab 16. All data sets were checked for normality using the Anderson Darling test and arc-sine transformation of percentage data was carried out before statistical analysis. Hatch rate and degumming data were analysed with a one way analysis of variance (ANOVA) and post-hoc analysis was carried out using Tukey's Multiple Comparison tests ($p \leq 0.05$).

5.3 Results

5.3.1 Treatment efficacy screening

Results of the screening tests indicated that enzyme alcalase® was the only treatment that liberated adhered eggs effectively (≥69%) (One-way ANOVA: DF = 5, F = 62.91, P = 0.000) (Table 5.1). The highest enzyme concentrations (3.0 - 4.0 %) resulted in 100 % liberation of eggs after 25 minute exposure time which will be referred to as "degumming rate" hereafter. In the same time period, even the lowest enzyme concentration (0.5 %) showed a 69.3 ± 5.5 % degumming rate. Visual counts at intermediary time points revealed that by 10 minutes the mean degumming rate in each of the enzyme alcalase® treatments was significantly higher than control treatments, which did not vary over the course of the exposure period (Fig. 5.1). Furthermore, eggs exposed to higher enzyme doses (3.0 and 4.0 %) were degummed faster than the lower doses (0.5, 1.0 and 2.0 %). Given the success of the enzyme treatment in removing the adhesive gum layer of ballan wrasse eggs, it was chosen for further investigation in subsequent experiments.

Table 5.1. Proportion of ballan wrasse eggs degummed following 25 minutes of exposure to candidate degumming solutions, each tested at varying concentrations (%). Control treatments for each concentration are pooled (*n* = X replicates). Values are expressed as mean ± SD (*n* = 3 replicates). Superscripts indicate significant differences between treatments and pooled controls.

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

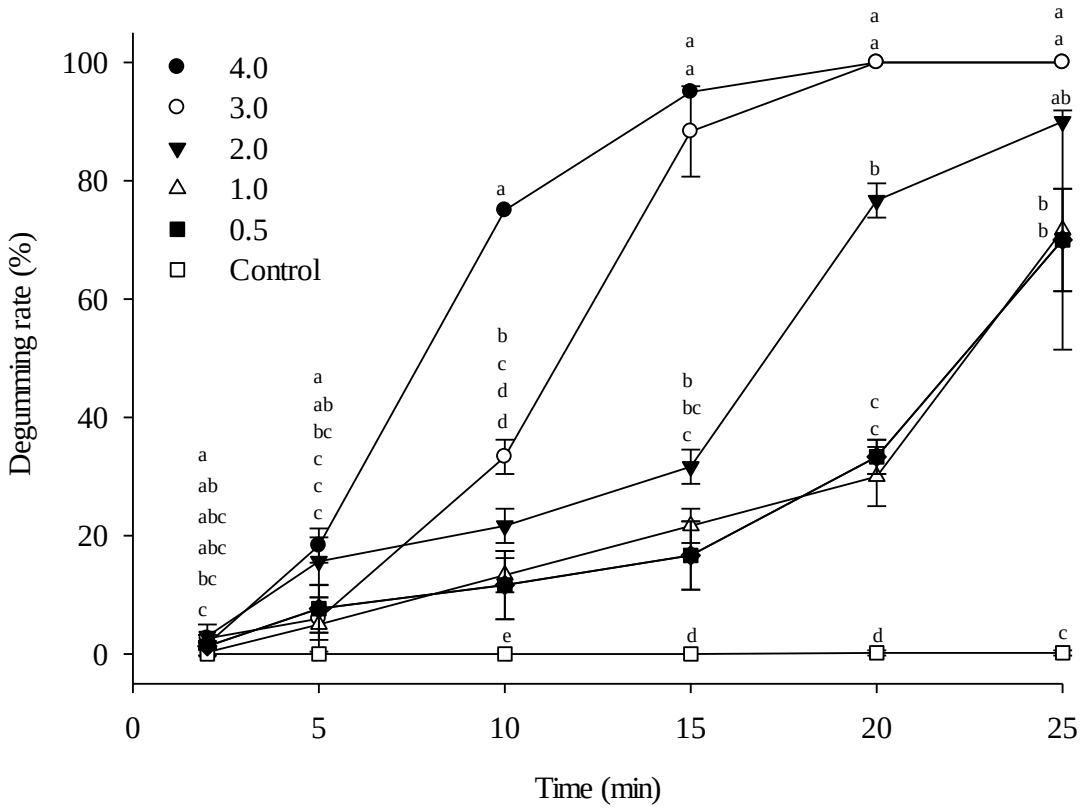

Figure 5.1. Proportion of ballan wrasse eggs degummed following exposure to enzyme alcalase (4.0, 3.0, 2.0, 1.0, and 0.5 %) compared to seawater control for 2, 5, 10, 15, 20 and 25 minutes. Superscripts indicate significant differences between treatments at each time point. Values are expressed as mean ± SD with *n* = 3 replicates for treatments and *n* = 5 replicates for control.

With respect to the other treatments, sodium sulfite was unsuccessful in freeing adhered eggs, with the highest degumming rate (11.1 ± 10.8 %) obtained with the 2 % sodium sulfite seawater solution (no freshwater based treatments showed higher than 0.3 ± 0.5 % degumming rate, data not shown). Similarly, tannic acid and L-cysteine solutions were both ineffective in degumming eggs during the 25 minute exposure time with the highest degumming rate of 0.7 ± 0.6 and 13.5 ± 7.2 % for tannic acid and L-Cysteine, respectively. Furthermore, the mean degumming rates for all concentrations of sodium sulfite, tannic acid, and L-Cysteine were not significantly higher than control treatments (One-way ANOVA: Sodium sulfite, DF = 6, F = 2.82, P = 0.052; tannic acid, DF = 3, F = 3.77, P = 0.059; L-Cysteine, DF = 2, F = 3.59, P = 0.108).

5.3.2 Optimisation of enzyme alcalase® treatment

The mean degumming rate was found to be inversely related to concentration of enzyme alcalase®, i.e. the higher the enzyme dose the shorter period of time for degumming. Complete degumming occurred between 25 - 30 minutes for the lowest enzyme dose rate (0.5 %), between 15 - 20 minutes for 1.0 - 2.0 %, between 15 – 17 minutes for 3.0 % and between 12 - 15 minutes for the highest enzyme dose (4.0 %) (Table 5.2).

Table 5.2. Degumming rate (Mean % ± SD (*n* = 3; control *n* = 1)) for each enzyme alcalase® concentration assessed at varying time points until complete degumming was achieved.

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

5.3.3 Impact of enzyme alcalase® treatment on hatching rate

Overall, mean hatch rates were statistically comparable in eggs treated with enzyme alcalase with respect to controls for 0.5 and 1.0 % treatments, while hatch rate was higher for the 2.0% treatment (One-way ANOVA: DF = 1, F = 8.79, P = 0.052) (Table 5.3).

Table 5.3. Hatching rate (%) of ballan wrasse eggs after exposure to varying dose/durations of enzyme alcalase treatment vs. control treatment. Values are expressed as mean ± SD (*n* = 3 replicates). Superscripts indicate significant differences between treatment and control.

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

5.3.4 Progression of gum layer removal under enzyme alcalase® treatment

Observation of the gum layer under enzyme alcalase® treatment revealed that the mode of action was not to erode the gum evenly from the outside, rather the gum layer was observed to swell and detach from the egg chorion before finally breaking open and then ultimately completely detaching from the egg. Therefore, a subjective four point scoring criteria was created as follows: 1.Gum layer fully intact; 2. Gum layer starting to swell and break down; 3. Gum layer swollen and partially removed from egg, although still attached; and 4. Gum later fully detached (clean egg). This was then used to characterise the progression of the gum layer removal (Fig. 5.2, 1-4).

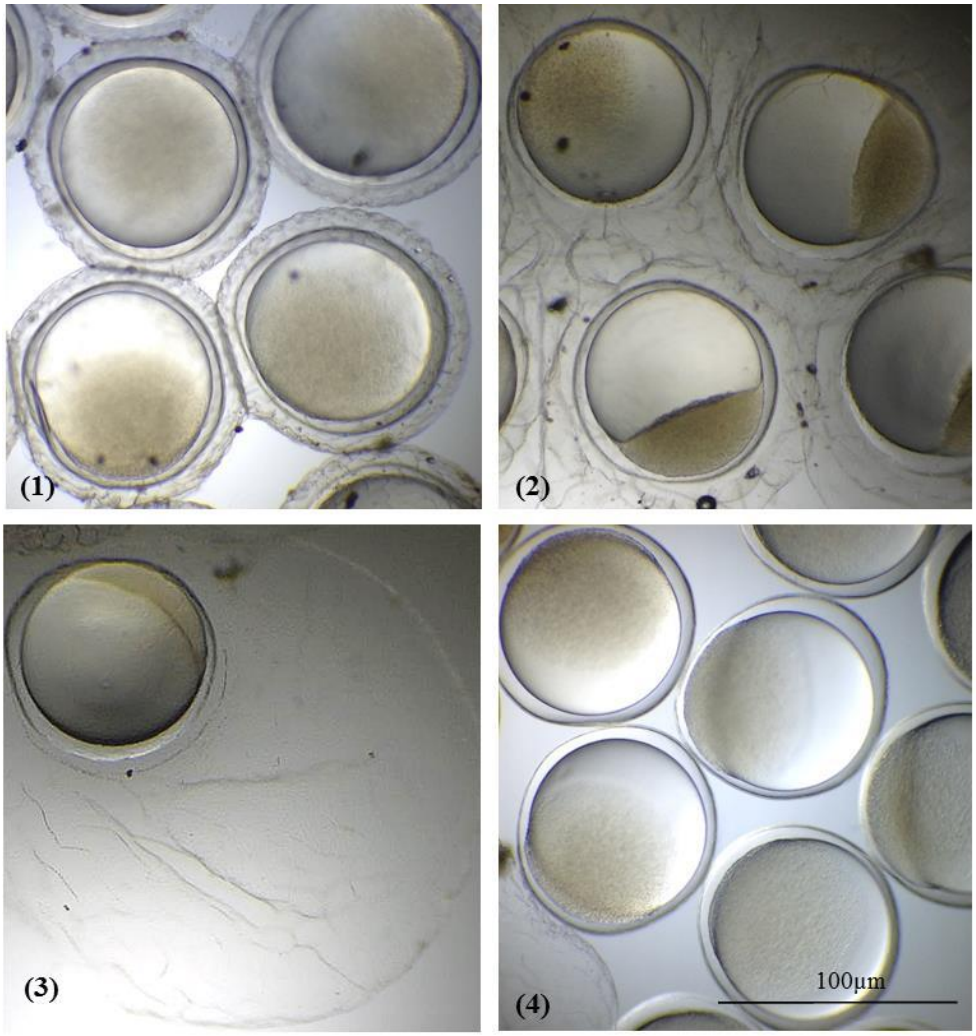

Figure 5.2. Ballan wrasse eggs at varying levels of gum layer removal during exposure to 1 % enzyme alcalase over a 25 minute time period: (1) Score 1; fully intact gum layer prior to enzyme exposure. (2) Score 2; gum layer starting to swell and break down after 15 minutes of enzyme exposure. (3) Score 3; gum layer swollen and partially removed from egg although still attached after 20 minutes of enzyme exposure. (4) Score 4; gum layer fully detached from egg (clean egg).

At the 0 and 5 minute time intervals for 1 % enzyme treatments and, at all time points, for control treatments 100 ± 0.0 % of eggs had a gum layer score of 1 (fully intact gum layer) (Fig. 5.3). Thereafter, the proportion of eggs with fully intact gum layers decreased as enzyme exposure time progressed. By the 25 minute time point, 93.3 ± 3.3 % of eggs exposed to the enzyme treatment had fully detached gum layers (Score 4). However, mean degumming rate started to increase from the 5 minute time point (5.1 ± 5.8 %) until complete degumming (100 ± 0.0 %) i.e. all eggs detatched from petri dish, at the 25 minute time point, suggesting that the gum layer does not have to be fully removed before an egg is detached from the substrate.

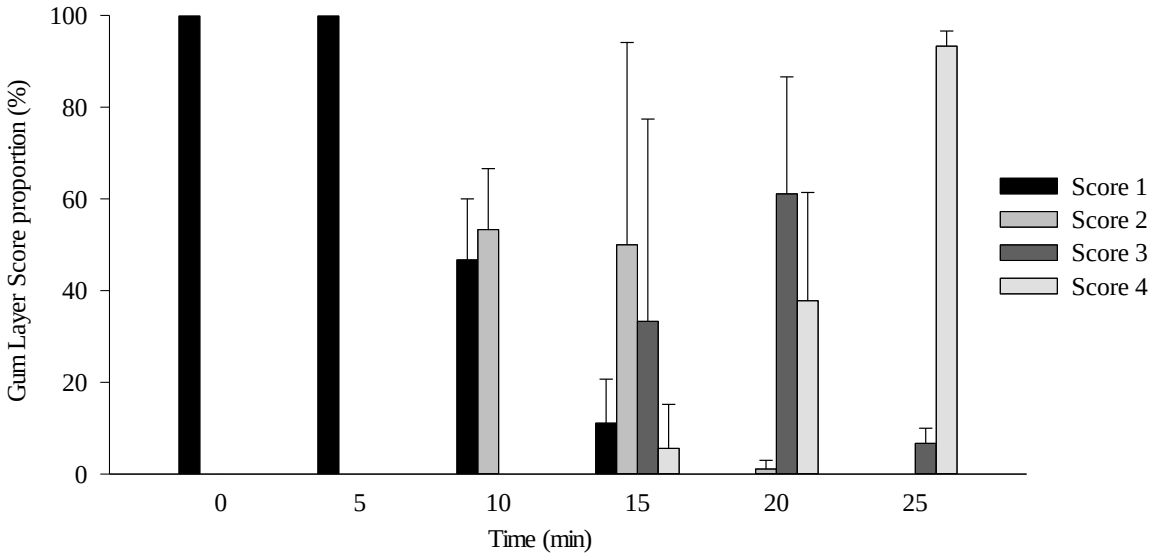

Figure 5.3. Proportion (Mean % ± SD (*n* = 3)) of ballan wrasse eggs classed with a gum layer score of 1-4 at 5 minute time intervals following exposure to 1% enzyme alcalase. Degumming rate (Mean % ± SD (*n* = 3)) at each time point was: 0 minutes = 0.0 ± 0.0; 5 minutes = 5.1 ± 5.8; 10 minutes = 46.2 ± 21.0; 15 minutes = 66.1 ± 14.0; 20 minutes = 89.7 ± 13.8; and 25 minutes = 100.0 ± 0.0.

5.4 Discussion

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

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

In this study, positive results were obtained using enzyme alcalase® for the removal of the adhesive gum layer around ballan wrasse eggs. During the initial screening, all dose rates tested were successful at degumming 69 - 100 % of eggs after the 25 minute exposure period. Subsequent trials demonstrated that the exposure time required for complete degumming was inversely related to the dose, with time to complete degumming ranging between 10 and 30 minutes. Subsequently, as all enzyme doses rates were effective, the lowest enzyme concentrations were then selected for further testing as using a lower enzyme concentration would be favourable to limit the costs of commercial upscaling.

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

Removal of the adhesive gum layer is not only important for egg handling purposes, but as these benthic eggs are laid on artificial substrates on the bottom of broodstock tanks they are in close proximity to waste feed and faecal matter that, in turn, causes excess bacterial and fungal loading in egg incubation tanks. Krise et al., (1986) pointed out that

the adhesive layer surrounding the chorion is particularly susceptible to viral, fungal, and bacterial pathogens and that removal of this can significantly reduce the development of such pathogens. Furthermore, routine disinfection and removal of dead eggs during egg incubation are important for improved biosecurity, both of which can be made easier and more effective by incubating eggs in suspension.

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

6 Chapter 6: General Discussion

6.1 Summary of Findings

In this section the main findings of each research chapter are summarised:

Chapter 2: Genetic population structure of wild ballan wrasse (*Labrus bergylta*) in the UK and Norway: implications for broodstock management.

- Over 15,000 novel SNPs were developed for ballan wrasse through the use of next generation RADseq technology.
- Identification of 30 novel haplotypes for ballan wrasse through mtDNA analysis from individuals within the sampled areas.
- A panel of 215 SNPs was successful in differentiating wild stocks of ballan wrasse from the mainland UK, Shetland and Norway, showing a higher level of population structuring than previously reported for this species.
- A reduced panel of 11 SNPs was able to differentiate Norwegian and Shetland populations from each other and mainland UK, however this panel was not able to detect local differentiation between mainland UK populations.

Chapter 3: Seasonal changes in broodstock spawning performance and egg quality in ballan wrasse (*Labrus bergylta*).

- First comprehensive dataset of spawning periodicity, reproductive characteristics and egg quality parameters of captive ballan wrasse, serving as a benchmark for the development of standardised hatchery practices to optimise broodstock performance.
- A clear spawning rhythm was observed with 3-5 spawning periods inclusive of spawning windows lasting 1-9 days followed by interspawning intervals of 8-12 days.

- Confirmation of multiple batch spawning nature of this species through genotyping analysis of parental contribution to spawning events.

Chapter 4: Effects of spawning area fragmentation and substrate colour on spawning activity of captive ballan wrasse (*Labrus bergylta*) broodstock.

- A preference for red spawning substrates was observed, with the highest number of spawning events and highest proportion of total spawning score on red spawning substrates, followed by blue and green; grey substrates were least preferred.

- Three out of the four spawning populations showed increased overall number of spawning days in comparison to the previous season's spawning rhythmicity, potentially due to fragmentation of spawning area.

Chapter 5: Removal of the adhesive gum layer surrounding naturally fertilised ballan wrasse (*Labrus bergylta*) eggs.

- Chemical screening emphasized success of enzyme alcalase® for degumming eggs.

- *In vitro* trials reveal that degumming rate was related to enzyme concentration.

- Enzyme treatment showed no negative impacts on hatch rates.

- *In situ* degumming and commercial upscaling still to be standardised.

6.2 Introduction

The ultimate objective of this thesis was to deliver important baseline information for the establishment of broodstock management and husbandry protocols to improve the overall hatchery production of ballan wrasse. The first aim of this research focused on providing insight into the genetic management of farmed stocks with respect to selection of broodstock for commercial breeding populations that would minimise the possible risks of escapees on wild populations (Chapter 2). The second aim was to assess and benchmark captive breeding performance and to establish effective broodstock management practices and protocols to aid in the successful commercial production of ballan wrasse (Chapter 3). This research explored aspects including spawning periodicity and the seasonal changes in egg quality parameters. In addition, the parental contribution to spawning events was determined to provide confirmation of the multiple batch spawning nature of this species. The third aim of this research was to investigate several highlighted bottlenecks in commercial hatchery production. The complex spawning behaviour of ballan wrasse with respect to preference for spawning substrate colour and position was explored to determine if optimisation of such factors may lead to increased productivity within the hatchery (Chapter 4). The final aim of this research was to determine an effective method for removal of adhesiveness of ballan wrasse eggs, which would ultimately aid in collection, disinfection and incubation protocols (Chapter 5). The following general discussion will evaluate the main findings and the wider implications of the research while elaborating on the limitations of the experimental work undertaken and identifying where future research opportunities exist.

6.3 Wild population structuring

Research was undertaken to develop a robust panel of markers through the use of high throughput RADseq technology to inform on the genetic structuring of wild ballan wrasse populations throughout the UK and Norway (Chapter 2). This work was fundamental both with respect to the selection of founder broodstock for captive breeding populations, but also to understand and minimise the potential implications that deployment of farmed ballan wrasse may have on local wild populations in the event of escapees from salmon farms. In addition, this work provides important ecologically based information on discrete population structuring of wild ballan wrasse from the northern geographic range of the species within the UK and Norway which could be used

for further dissemination and future conservation of the species. This work builds on research presented by D'Arcy et al., (2013) that alluded to a degree of population differentiation between Norwegian and UK ballan wrasse populations; however, D'Arcy's study was unable to differentiate between UK populations based on the methodology employed (mtDNA control region sequence data). However, given what is currently understood about the basic reproductive and life history traits of ballan wrasse, this result warranted further investigation through the development of genetic tools, in particular nuclear DNA markers, to identify differences between ballan wrasse populations from different geographic locations pertinent to the UK industry.

In Chapter 2, a subset of the >15,000 novel SNP markers identified through RAD sequencing were successful in differentiating wild populations of ballan wrasse from across the sampled range in the UK and Norway. However, when this panel was further reduced to only 11 potentially informative SNPs and applied across a wider sample range, no significant differentiation was found between the UK mainland populations while Shetland and Norwegian populations were still clearly differentiated. In other words, this suggests a higher degree of population structuring than previously reported for ballan wrasse; however, it is possible that if an alternative panel of SNPs were analysed, the structuring definitions for mainland UK populations may have differed. Therefore, future work in this respect should focus on re-analysis of SNPs from the vast number identified, with a more specific focus on mainland UK based datasets, to identify SNPs that may be more specifically associated with local differentiation and applied within the larger dataset. Furthermore, although this research gives new insight into the population structuring of ballan wrasse around the UK, it may be interesting to take this further to explore the ecological and environmental drivers behind the isolation of the Shetland population from the mainland UK populations. It is possible that depth, oceanic currents or geographic distance act as physical barriers to gene flow for isolated ballan wrasse populations. For example, it has been shown in the European flounder (*Platichthys flesus*) that oceanographic and bathymetric barriers are responsible for the marked genetic isolation of populations surrounding the Faroe Islands (Hemmer-Hansen et al., 2007). As such, a further investigation using samples from the northern mainland UK, Orkney, the Outer Hebrides, and Ireland may lead to a better understanding of the natural barriers of gene flow among UK ballan wrasse populations.

The practical implications for the level of genetic structuring revealed in this study imply that care needs to be taken when selecting founder individuals for the establishment of captive breeding populations. This research suggests that if the selection of source populations remains as local as possible to where farmed generations will be deployed this may reduce the potential risks associated with escapees and the consequences of potential introgression with local wild populations. Unlike the documented cases and observations of farmed Atlantic salmon escapees mixing with and having negative impacts on wild salmon populations (Naylor et al., 2005; Hindar et al., 2006; Ferguson et al., 2007); ballan wrasse escapees would, in theory, be capable of establishing and spawning in habitats directly surrounding the cage sites; i.e. they would not need to migrate or travel far to find suitable spawning locations. The fact that the 'natural' habitat for ballan wrasse would be in close proximity to cage sites means that escaped individuals may have an increased likelihood of local establishment. That being said, the risks of introgression of farmed with wild ballan wrasse populations are thought to be relatively low, based on deployment size and age at sexual maturity (Leclercq et al., 2014b). In addition, the absence of any fixed alleles within sampled populations means that it would require significant admixture of populations to show a measurable genetic shift (Glover et al., 2013). Moreover, with the extremely low suggested stock ratio for ballan wrasse (1 – 5 %; Skiftesvik et al., 2013; Leclercq et al., 2014a) this represents a low level of risk as it would take repeated mass escapee events to release any great number of farmed individuals to the surrounding environment. Nevertheless, should deployment of farmed ballan wrasse as cleaner fish for the salmon industry become regulated to a local status this research provides an important basis for establishing guidelines for such regulations.

6.4 Broodstock performance

The benchmarking of captive broodstock performance throughout an entire natural spawning season represented an important first step to rationalise spawning quantity and quality and to provide measures against which to compare future production (Chapter 3). Through the monitoring of captive breeding populations over the course of a spawning season, one of the most striking findings was the clear spawning rhythmicity observed which had not previously been reported for this species in captivity. The suggested semi-lunar spawning rhythm, although having been observed in other labrid species (Warner, 1982; Ross, 1983; Taylor, 1984), would require further investigation within captive

ballan wrasse to determine whether it is an endogenous rhythm or if there are other external environmental factors regulating it that would need to be optimised to insure effective spawning conditions (Migaud et al., 2013). However, the practical implication of such a defined spawning rhythm, from a hatchery perspective, is important as an effective management tool that allows for the prediction and/or planning of spawning. This, in turn, allows for the concentration and synchronisation of hatchery stocking which is vital for optimised larval density and live feed ratios. On the other hand, the clear spawning rhythmicity observed during the initial spawning season (Chapter 3) was not seen for all spawning populations during the second spawning season (Substrate preference; Chapter 4). While this discrepancy may have been due to the effects of the spawning substrate fragmentation and/or colour it is impossible to rule out at this point that the overall number of fish per tank, which was higher for the four spawning populations in the second season than the first, may have played an important role in increasing the overall number of spawning days as well.

Another of the main findings from this work was the confirmation of the multiple batch spawning nature of this species. This was originally proposed by Muncaster et al., (2010) through histological examinations of ovaries, however had, until now, not been proven through evidence of a single female spawning multiple times within a given season. Through the use of previously described microsatellite markers (Quintela et al., 2014) it was possible to determine the parental contribution to multiple spawning events which provided confirmation of multiple batch spawning within this species, while also providing evidence of a highly skewed male contribution that may be suggestive of male dominance within captive spawning populations. Male dominance and territorial behaviour in wild ballan wrasse is widely recognised (Sjölander et al., 1972; Dipper, 1987); however, in this respect the quantification of parental contribution gives new insight into the actual number of individuals contributing to spawning events which is critical information when trying to establish effective breeding populations with the optimum sex ratio. However, the conclusions drawn on parental contribution within this study were based on multiple spawning events from only two spawning populations. Therefore, in order to implement this as a practical management tool, future studies should focus on assessing parental contribution over the course of a spawning season as a whole and across a wider number of spawning populations to give a more robust

representation of individual contributions which is vital to demonstrate improved productivity of spawning populations.

An important factor in broodstock management is to be able to replace existing broodstock with equal or better quality fish to ensure continued and future production (Migaud et al., 2013). This ultimately can take two routes either through continued harvest of wild individuals for use as commercial broodstock or through the establishment of a dedicated breeding programme that would allow for selected farmed individuals to be kept on as future broodstock (Gjedrem et al., 2012). Both routes inevitably have positive and negative points; for example, the continued harvest of wild individuals may have negative impacts on recruitment in wild populations. Furthermore, there are potential risks associated with disease transfer when bringing wild fish into the farming environment. On the other hand, the continued use of wild broodstock for commercial spawning would mean that the resulting farmed individuals would be as genetically close as possible to their wild counterparts which could be considered positive in the event of escapees (Gjedrem et al., 2012). The establishment of dedicated breeding programmes allowing selective breeding to improve the quality of farmed strains (Migaud et al., 2013), would be an important step in the long term development of ballan wrasse aquaculture. This could be achieved using the previously described microsatellites (Quintela et al., 2014) or through the establishment of new markers based on the described RAD sequencing output (Chapter 2). However, selective breeding for traits such as, improved growth or disease resistance, which has been achieved in other species including Atlantic salmon (Gjedrem, 2005) and rainbow trout (Janssen et al., 2015), may not be applicable in the short term to ballan wrasse due slower growth and longer time to maturation. Therefore, at present it may be more beneficial for the ballan wrasse industry to maintain a diverse broodstock line while respecting geographic origin.

The initial survey of broodstock spawning performance highlighted the lack of understanding of the complex spawning behaviours of this benthic harem spawning species and how these factors are important when considering how to implement the optimal captive spawning environment for maximising ballan wrasse egg production. Substrate spawning fish are known to be selective in their choice of spawning habitat (Sinckars et al., 2010). This, along with the fact that captive ballan wrasse actively seek out a substrate upon which to deposit adhesive eggs prompted an investigation into the

spawning preferences of ballan wrasse with respect to the selection of colour and positioning of spawning substrates within broodstock tanks (Chapter 4). The results of this study indicate that ballan wrasse do in fact show preferences for specific coloured spawning substrates over others, namely red substrates over the hatchery standard green, blue or grey. Although the specific visual capabilities of ballan wrasse have not yet been reported in the literature, results from this study suggest that they can perceive the substrate, be it the specific colour or the contrast to the tank bottom, and actively seek to spawn in selected areas. Furthermore, the segmentation of spawning substrates into discrete zones within the tanks instead of a single spawning area may have led to increased overall productivity based on the total number of spawning days within the trial period. However, the increased number of spawning days, in comparison to the previous seasonal spawning rhythmicity, was only observed in three out of the four tank populations and therefore further investigation is required before reaching conclusions. However, these findings do indicate that, when given the choice, ballan wrasse have preferences for spawning substrate colour and potentially location within a captive spawning environment. Furthermore, the ability to provide a preferred spawning substrate may act as a way to concentrate spawning efforts to a particular location or within a tighter physical area within spawning tanks that would aid in the retrieval and incubation of eggs. Going forward, an important next step would be to combine this work with the use of parental contribution analysis to assess if the segmentation of spawning zones actually leads to an increased number of spawning pairs per day in a given tank which may ultimately increase commercial hatchery production.

The use of commercial hatchery and broodstock facilities for experimental work provided an in depth insight into ballan wrasse broodstock production. Furthermore, this allowed a greater understanding of how the hatchery and broodstock techniques identified by this research can be directly applied at a commercial level, therefore improving overall productivity. In addition, the exposure to commercial ballan wrasse production has helped to identify where future key research questions should be targeted. On the other hand, the use of commercial facilities, in particular broodfish, spawning tanks and eggs, often limited the capabilities of the experimental work as production pressures took priority over research. Great care had to be taken when designing and conducting experimental work within this commercial environment to reduce any negative impacts on spawning performance and egg production. Upon reflection, future

work may warrant the establishment of strictly experimental broodstock populations that would not impact on commercial production and thus experimental design.

6.5 Egg quality and handling

Reliable indicators of egg quality are important as these are the characteristics of the eggs that determine survival capacity (Migaud et al., 2013). Furthermore, in a hatchery setting, it is important to have an overall indication of individual egg batch fitness to determine whether it is worthwhile spending the time and resources on incubation (Bromage et al., 1994). The adhesiveness of ballan wrasse eggs poses an interesting challenge to the indication of egg quality as in many other marine species parameters such as sinking eggs can be used to identify non-viable eggs (Migaud et al., 2013), however for this species all eggs, viable or not, remain adhered to the spawning substrate. Some of the common egg quality indicators such as fertilization and hatch rates, egg size, and lipid and fatty acid composition (Bromage et al., 1994; Bobe & Labbe, 2010; Migaud et al., 2013) were quantified throughout the entire spawning season within four spawning populations of ballan wrasse (Chapter 3). The results from this study indicated that there was a general lack of correlation between egg quality, in terms of overall hatch rate, with the observed parameters (fertilization and hatch rates, egg size and biochemical composition of eggs); and moreover, these factors remained relatively consistent throughout the spawning season. This overall lack of correlation justifies further investigation to reliably define early indicators of egg quality for this species.

At the beginning of this PhD research project, the issue of adhesive eggs was considered to be an important bottleneck to commercial production and therefore posed an interesting research question. The incubation of eggs while adhered to spawning substrates was a challenge to commercial production and presented difficulties with respect to egg handing, disinfection and incubation of commercial ballan wrasse eggs. Furthermore, the adhesive nature of the eggs made an accurate quantification of egg numbers difficult, which is important for husbandry practices in order to determine production yields, but also for larval rearing purposes to have the correct stocking densities and live feed ratios. Many attempts were made at quantifying egg numbers directly on spawning substrates; however, these methods proved very labour intensive and therefore led to the cumbersome methodology of counting newly hatched larvae. This method again proved time consuming and potentially detrimental to the larvae

which are very delicate at this stage of development. These were some of the many drivers behind the work looking into the removal of adhesiveness in ballan wrasse eggs. The issue of egg adhesiveness has been overcome in other commercially important species (mainly freshwater) such as European catfish and common carp (Linhart *et al.*, 2003a, c) and similar methods were applied to ballan wrasse in a trial setting (Chapter 5). Through this, an effective method for removal of adhesiveness or 'degumming' ballan wrasse eggs was developed using enzyme alcalase®, without negatively impacting on hatch rates. The next stage of this research will be to commercially upscale the methodology identified; however, hatchery protocols and practices were developed to accommodate the incubation of whole egg mats and as yet the protocol developed for chemical degumming of eggs has not been commercially implemented within the UK.

6.6 Conclusions

This thesis presents important baseline information for the establishment of broodstock management and husbandry protocols to improve the overall hatchery production of farmed ballan wrasse. This research established a robust panel of novel SNP markers that were used to reveal a level of population structuring within the United Kingdom and that support the conclusion that founder broodstock should be sourced from geographic regions reflective of likely deployment. Also, this work benchmarked captive breeding performance over a natural spawning season to assess factors such as spawning periodicity, seasonal changes in egg quantity and quality, and the parental contribution to spawning events. Finally, this research advanced current knowledge of some of the technical aspects of ballan wrasse production with respect to optimizing the captive spawning conditions for breeding populations and methods for effectively degumming ballan wrasse eggs to aid in disinfection and incubation processes. Overall, this research provides important data on the management of broodstock and the optimisation of hatchery protocols to improve the commercial productivity and performance of ballan wrasse for use as a biological control of sea lice of farmed Atlantic salmon.

7 Publications and Conferences

7.1 Publications

Grant, B., Picci, N., Davie, A., Leclercq, E., & Migaud, H., (2016). Removal of adhesiveness in naturally fertilized ballan wrasse (*Labrus bergylta*) eggs. *Aquaculture* **456**, 44-49.

Grant, B., Picci, N., Davie, A., Taggart, J.B., Bradley, C., Prodohl, P., Leclercq, E., & Migaud, H., Seasonal changes in broodstock spawning performance and egg quality in ballan wrasse (*Labrus bergylta*). *Aquaculture* **464**, 505-514.

Leclercq, E., **Grant, B.,** Davie, A., & Migaud, H., (2014). Gender distribution, sexual size dimorphism and morphometric sexing in ballan wrasse *Labrus bergylta*. *Journal of Fish Biology* **84**, 1842-1862.

7.2 Conferences

Grant, B., Leclercq, E., Migaud H., & Davie A., (2012). Production and implementation of farmed ballan wrasse (*Labrus bergylta A.*) in the salmon farming industry' (Poster presentation) - University of Stirling Postgraduate Conference – October 2012, University of Stirling, Scotland.

Grant, B., Leclercq, E., Migaud H., & Davie A., (2012). Production and implementation of farmed ballan wrasse in the Scottish salmon industry (Oral presentation) - LARVITA Training School, CCMAR, University of Algarve (Faro, Portugal).

Grant, B., Leclercq, E., Migaud H., & Davie A., (2013). Ballan wrasse (*Labrus bergylta A.*) spawning dynamics, egg quality and egg degumming' (Oral Presentation) – UK and Norwegian Wrasse Workshop, October 2013, Machrihanish Marine Lab, Machrihanish, Scotland.

Grant, B., Leclerq E., Clark, W., Davie, A., & Migaud H., (2014). Tools and techniques to support Ballan Wrasse (*Labrus bergylta A.*) broodstock management. (Oral presentation) - 10th International Symposium of Reproductive Physiology of Fish, Olhão, Portugal, May 2014

Grant, B., Picci, N., Davie, A., & Migaud, H., (2014). Broodstock spawning performance of ballan wrasse (*Labrus bergylta A.*) (Poster presentation) - 10th International Symposium of Reproductive Physiology of Fish, Olhão, Portugal, May 2014

Grant, B., Picci, N., Davie, A., & Migaud, H., (2014). Tools and techniques to support Ballan Wrasse (*Labrus bergylta A.*) broodstock management and chemical removal of egg adhesiveness (Oral Presentation) – Centre for Environment, Fisheries and Aquaculture Science, CEFAS, Weymouth, UK, July 2014.

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