Grant B, Davie A, Taggart J, Picchi N, Bradley C, Prodohl PA, Leclercq E & Migaud H (2016) Seasonal changes in broodstock spawning performance and egg quality in ballan wrasse (Labrus bergylta), *Aquaculture*, 464, pp. 505-514.

DOI: 10.1016/j.aquaculture.2016.07.027

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

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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 paired mating 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.

Keywords: Cleaner fish; ballan wrasse; broodstock management; parental contribution, spawning patterns, fatty acid

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 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-secured supply of farmed cleaner fish. Ballan wrasse is a monandric 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 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

69 evidence, with gonad maturation starting in November extending over a 2 month period,
70 typically from April to July, depending on geographic location (Muncaster et al. 2010).
71 Commercial hatcheries currently rely on the natural spawning of captive wild harems
72 maintained under controlled photo-thermal conditions. Ballan wrasse spawn adhesive,
73 spherical, benthic eggs of approximately 1 mm in diameter (D'Arcy et al. 2012). Hatcheries
74 use artificial turf laid within broodstock tanks as a spawning substrate for the collection and
75 incubation of eggs with hatching reported at 72 degree days (DD) post-fertilization (Ottesen et
76 al. 2012). A description of the spawning periodicity of captive ballan wrasse along with
77 potential fluctuations in egg quality over a full spawning season has not been reported but
78 represents an important first step to rationalise and optimise hatchery operations as with any
79 intensively cultured finfish species (Migaud et al. 2013).
80 Currently, there are no standard protocols to determine egg quality for ballan wrasse;
81 commonly used quality indicators across marine finfish species include, but are not limited to,
82 egg size, fertilization and hatching rates, and the biochemical composition of eggs including
83 lipids and fatty acids (FA) composition in particular (Bobe and Labbe, 2010; Migaud et al.
84 2013). Egg diameter in many multiple batch spawning species has been reported to reduce in
85 size as the spawning season progresses (Bagenal, 1971; McEvoy & McEvoy, 1992) which may
86 indicate an exhaustion of an individual females' physiological and nutritional condition
87 (Trippel, 1998). Fatty acids, predominantly docosahexaenoic acid (22:6n-3; DHA),
88 eicosapentaenoic acid (20:5n-3; EPA) and arachidonic acid (20:4n-6; ARA), usually correlate
89 well with egg viability, egg development, hatching and larval survival (Rainuzzo et al. 1997;
90 Sargent et al. 1999; Tveiten et al. 2004). However, no single parameter can define egg quality,
91 so therefore it is vital to benchmark and assess several quality indicators to help improve
92 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 to (3) apply microsatellite markers to investigate parental contribution in naturally 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.

2. Materials and methods

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 (SNP) 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 commercial spawning tanks: three tanks as Machrihanish (Tanks M1, M2 and M3) and one Dorset (Tank D1) origin (Table 1). Fish were anaesthetised (Tricaine Methane Sulphonate; MS-222; 40 ppm; 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). As was standard production practice presumed sex ratios were manipulated based on morphological data where possible 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 at 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 for waste removal daily. 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.

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 presence of 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 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 pictured 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.

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.

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 harems 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,

193 Lanarkshire, UK) and stocked into a 500 L flow-through incubator supplied with aerated
194 natural seawater (5 L / min; UV treated, filtered to 100 µm) and fitted with a 100 µm mesh
195 banjo filter at the outflow. Mean daily water temperature was 12.0 ± 0.4 ºC and DO = 96.0 ±
196 0.0 % over the incubation period. Eggs received two static bath treatments of bronopol (25
197 ppm, 1 h; Pyceze®; Novartis Animal Health^{Ltd}; Frimley, UK) at 2 and 4 DPF. Hatching was
198 induced by physical shock (gently scraping the eggs from the spawning substrate using a metal
199 spatula) when deemed optimal as per commercial hatchery practice at 6 to 7 DPF. Once all
200 mats were scraped, larvae were observed rising at the surface within 10 min and left untouched
201 for 1 hour to allow maximum hatching rate. The incubator was then drained into a condenser
202 fitted with a 50 µm mesh and larvae transferred to a container with a final volume of 30 L.
203 Larvae were gently mixed by light aeration and stirring, and replicated samples (n = 5 to 10)
204 of 100 ml separated. The total number of larvae per sample was counted and averaged across
205 replicate volumetric samples before translating the mean value to the batch total volume to
206 calculate the total number of hatched larvae in the batch.
207
208 **2.4 DNA extraction**
209 Fin clip biopsies were taken from each of the 39 broodstock fish within tanks M2 and M3 and
210 a sample of one hundred newly hatched larvae each originating from a single day spawning (n
211 = 6 spawning events from M2 and M3 which were the same batches used for batch fecundity
212 estimation). Samples were stored in 95 % ethanol at 4 °C until processed. Genomic DNA from
213 fin samples was isolated using a salt extraction method; approximately 0.5 cm^2 tissue was
214 added to 300 µl SSTNE buffer (0.30 M NaCl; 0.04 M Tris; 200 µM EDTA; 0.199 mM EGTA
215 (E3889, Sigma Aldrich); 4.89 mM spermidine (SO266, Sigma Aldrich); 1.4 mM spermine
216 (S1141, Sigma Aldrich)) a further 20 µl of SDS (10 %; L3771, Sigma Aldrich) and 5 µl
217 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 re-suspended in 100 µl 5 mM tris. A scaled down version of this protocol was used for larval extractions in the 96 well PCR plate format. 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.

2.5 DNA microsatellites and PCR amplifications

Seven polymorphic DNA microsatellites (Table 2) were chosen from the limited number of loci reported for ballan wrasse (Quintela et al. 2014). 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.

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 ^{TM} 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 (He and Ho), the inbreeding coefficient (F_{IS}) and the probability of identity (PI) for each locus were calculated using GenAlEx 6.502 (Peakall and Smouse 2006, 2012).

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. Results

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 populations 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 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. 1).

The total number of SW for isolated spawning populations ranged from 4-6, with individual SW's 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 D1 spawning population was different with SW starting on average 5.6 ± 0.8 days (range = 3-8 days) prior to tanks M1-M3 (Fig. 1).

In each SW throughout the season and for all spawning populations, 85 % of all matts collected were scored 1 (n = 611/723 mats) and 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. 2 a-d; Table 3b).

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. 2 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 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.

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 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. Similarly, GLT was 0.12 ± 0.002 mm with no 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).

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 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, however 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 difference between population. However the ARA to EPA ratio ranged from 0.28 to 0.31 with the ratio being signficiatly 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 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.

3.4 Genotyping and parental contribution

Mean predictive assignment rates among families ranged from 81 to 83 % between tanks (Table 7). Of the 600 larvae from tanks M2 and M3 that were screened, complete genetic profiles were obtained for 587 individuals. Of these genotyped offspring, 88 % assigned to at least one family without error tolerance. When the genotyping model allowed for a single allele mismatch all individuals were successfully assigned to families, with 83 % unambiguously assigned to a single family which correlates to the predicted assignment rates (Table 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 larvae were assigned to a single mating pair (Table 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.

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

368 population. This coincides with evidence of peak egg production occurring in May as
369 previously reported in Norwegian captive ballan wrasse broodstock (Muncaster et al. 2010).
370 Ballan wrasse have previously been proposed to be group synchronous multiple batch spawners
371 based on histological examination of ovaries (Muncaster et al. 2010), however empirical
372 evidence of spawning pattern and rhythmicity during a full spawning season was lacking. The
373 spawning rhythmicity of captive ballan wrasse in this study was characterised by a succession
374 of spawning windows of 1-6 days followed by longer interspawning intervals of 8-15 days with
375 a total of 4 to 6 spawning windows over the spawning season. Such regular spawning rhythms
376 are suggestive of a "multiple or repetitive spawning" reproductive strategy as previously
377 proposed in the species. This is further supported by the fact that the total number of spawning
378 dates for all spawning populations exceeded the total number of presumed females in each
379 tank; therefore it must be assumed that at least some of the females would have spawned on
380 more than one occasion within the spawning season. This is ultimately supported by
381 genotyping analysis which clearly identified a single female being the predominant contributor
382 during two separate spawning events, in two separate spawning windows. Repeat or multiple
383 batch spawning is a common spawning strategy for cultured temperate marine teleosts
384 including Atlantic halibut (*Hippoglossus hippoglossus*), which produce several batches of eggs
385 at regular intervals of 3-4 days over a 2-4 month period (Nordberg et al. 1991; Bromage et al.
386 2000; Brown et al. 2006) and Atlantic cod (*Gadus morhua*), which spawn egg batches every
387 few days for up to a 2 month period (Kjesbu, 1989).
388 Despite the differences between the four spawning populations in the number and duration of
389 SW and ISI, there was no difference in the overall duration of spawning periods. The average
390 SP across all tanks lasted on average 14 days, which is, by definition, equivalent to a semi-
391 lunar spawning cycle. Semi-lunar spawning cycles have been observed in two other Labrid
392 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 have been in captivity for 2-3 years under enclosed conditions and 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 females 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 authors 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*) (1.01 ± 0.03 mm) (Kožul et al. 2011), both species 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*) (2-7 % seasonal decrease, Wiborg, 1960), turbot (*Scophthalmus maximus*) (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

443 place a large physiological demand on spawning fish therefore depleting energy sources over
444 the course of the spawning season (Izquierdo et al. 2001).
445 While egg diameter remained consistent, a declining trend in mean gum layer thickness was
446 observed over the spawning season for the four spawning populations studied, however, only
447 significantly for one population (M1) which represented a 32 % decline from the first SW to
448 the last. To date, there is a lack of literature on seasonal changes in egg adhesiveness for marine
449 teleosts. There was no clear reduction observed in the 'stickiness' of egg batches over the
450 season as a whole; however, casual observation suggested that eggs appeared to become 'less
451 sticky' during the later stages of incubation, just prior to hatch. Similarly, in the green wrasse,
452 the adhesive gum layer has been shown to lose its stickiness and separate from the eggs a few
453 hours prior to hatching (Kožul et al. 2011). Further studies should be performed to determine
454 the role of the adhesive gum layer in ballan wrasse eggs and look at potential removal methods
455 for incubation purposes as is common commercial practice with many freshwater species that
456 spawn adhesive eggs (Linhart et al. 2003).
457 Another indicator of egg quality in fish is lipid and FA contents derived directly from
458 broodstock diet (Sargent et al. 1999; Migaud et al. 2013). They are required for the formation
459 of cell membranes and are a major source of metabolic energy (Sargent et al. 2002). In addition,
460 they play important roles in spawning, egg quality, in terms of successful embryo and larval
461 growth and development, hatching, and overall survival (Rainuzzo et al. 1997; Sargent et al.
462 2002; Tocher, 2003). Ballan wrasse egg FA composition in this study remained generally
463 consistent throughout the spawning season and across spawning windows, although subtle
464 variances were observed. Such variability in FA between spawning populations and spawning
465 windows could potentially be due to genetic or nutritional variability between individual
466 spawning fish. However, of the 63 egg batches collected, inclusive of all spawning populations,
467 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*) (Bruce et al. 1999) and common snook (*Centropomus undecimalis*) (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 (allele no., observed size range, He, Ho, F_{IS} and PI) 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

493 (Pompanon et al. 2005). The predictive FAP, which looks at the resolving power of parental
494 genotypic data sets (Taggart, 2007), indicated that the 7 loci panel used would not be
495 unambiguously discriminating, and the low level of multiple matches found, 99 out of 587
496 larvae (17 %) was similar to that predicted by FAP. The parental assignment results from the
497 exclusion based FAP analysis indicated that, overall, within the six spawning dates analysed
498 for the two spawning populations, only 19.5 % of females and 50 % of males within tanks
499 actually contributed to the progeny. Bearing in mind that larval samples were taken
500 immediately post hatch, this should be a reliable and robust estimate of parental contribution,
501 as larvae were not subjected to any active (hatchery practice) or natural (selective mortality)
502 grading. On all three spawning dates in M3 there was only a single male contributing to all
503 assigned larvae and within the three spawning dates for M2 there was one male contributing to
504 64 % of assigned larvae and a further two males showing a lower level of contribution. The
505 highly skewed male contribution is suggestive that male dominance is occurring within these
506 spawning populations which is supported by observations of territorial male behaviour.
507 Furthermore, these results support the harem mating behaviour reported from studies of wild
508 fish with territorial males courting and mating with several females (Sjölander et al. 1972;
509 Hilldén, 1984). Overall, the parentage assignment results highlight the need for further research
510 to be conducted on a larger scale and with improved assignment methods so that it can be
511 integrated as a management tool within hatcheries to test the social, environmental, or
512 hormonal manipulations on breeding activity.
513 As a whole this research provides the first detailed study on the spawning performance of
514 captive ballan wrasse. Results showed clear spawning rhythms and confirmed that ballan
515 wrasse is a multiple batch spawning species. In addition, parental contribution confirms the
516 social hierarchical structuring in captive ballan wrasse, which should be taken into
517 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.

Acknowledgments

This research was co-funded by Innovate UK (Project ref. 81199), Marine Harvest (Scotland)^{Ltd} and Scottish Sea Farms. The authors would like to thank Nicolas Picci for his invaluable help with sample collection, all staff at the Machrihanish Marine Farm, as well as the supporting staff at the Machrihanish Marine Environmental Research Laboratory (University of Stirling).

528 **References**

529 Bagenal, T.B., (1971). The inter-relation of the size of fish eggs, the date of spawning and the
530 production cycle. *Journal of Fish Biology* 3, 207-219.

531 Bjordal, Å., (1990). Wrasse as cleaner-fish for farmed salmon. *Progressive Underwater*
532 *Science* 16, 17-28.

533 Bobe, J., & Labbe, C., (2010). Egg and sperm quality in fish. *General and Comparative*
534 *Endocrinology* 165, 535-548.

535 Bromage, N., Mazorra, C., Bruce, M., & Brown, N., (2000). Halibut culture. In: Stickney, R.R.
536 (Ed.), *Encyclopaedia of Aquaculture*. Wiley and Sons, New York, 425-432.

537 Brown, N., Shields, R.J., & Bromage, N.R., (2006). The influence of water temperature on
538 spawning patterns and egg quality in the Atlantic halibut (*Hippoglossus hippoglossus L.*).
539 *Aquaculture* 261, 993-1002.

540 Bruce, M., Oyen, F., Bell, G., Asturiano, J.F., Farndale, B., Carrillo, M., Zanuy, S., Ramos, J.,
541 & Bromage, N., (1999). Development of broodstock diets for the European sea bass
542 (*Dicentrarchus labrax*) with special emphasis on the importance of n – 3 and n – 6 highly
543 unsaturated fatty acid to reproductive performance. *Aquaculture* 177, 85-97.

544 Chistiakov, D.A., Hellemans, B., & Volckaert, F.A.M., (2005). Microsatellites and their
545 genomic distribution, evolution, function and applications: a review with special reference
546 to fish genetics. *Aquaculture* 225, 1-29.

547 Christie, W.W., (2003). *Lipid Analysis: Isolation, separation, identification and structural*
548 *analysis of lipids*, Third edition. Oil Press, Bridgewater, England.

549 Costello, M., (2006). Ecology of sea lice parasitic on farmed and wild fish. *Trends in*
550 *Parasitology* 22, 475-483.

551 Costello, M., (2009). The global economic cost of sea lice to the salmonid farming industry.
552 *Journal of Fish Diseases* 32, 115-118.

553 D'Arcy, J., Dunaevskaya, E., Treasurer, J.W., Ottesen, O., Maguire, J., Zhuravleva, N.,
554 Karlsen, A., Rebours, C., & Fitzgerald, R.D., (2012). Embryonic development in ballan
555 wrasse *Labrus bergylta*. *Journal of Fish Biology* 81, 1101-1110.
556 Dipper, F.A., Bridges, C.R., & Menz, A., (1977). Age, growth and feeding in the ballan wrasse
557 *Labrus bergylta* Ascanius 1767. *Journal of Fish Biology* 11, 105-120.
558 Dipper, F.A., & Pullin, R.S.V., (1979). Gonochorism and sex-inversion in British labridae
559 (Pisces). *Journal of Zoology* 187, 97-112.
560 Dipper, F., (1987). *British Sea Fishes*. London: Underwater World Publication Ltd.
561 Dulčić, J., Kožul, V., Kraljević, B., Skaramuca, B., Glamuzina, B., & Ré, P., (1999).
562 Embryonic and larval development of the brown wrasse *Labrus merula* (Pisces: Labridae).
563 *Journal of Marine Biology Association of the United Kingdom* 79, 327-332.
564 Evans, D.H., & Claiborne, J.B., (2006). *The Physiology of Fishes*: Third Edition. Boca Raton,
565 Florida: CRC Press.
566 Folch, J.M., Lees, M., & Sloane Standley, G.H., (1957). A simple method for the isolation and
567 purification of total lipids from animal tissues. *Journal of Biological Chemistry* 226, 497-
568 509.
569 Fraser, A.J., Gamble, J.C., & Sargent, J.R., (1988). Changes in lipid content, lipid class
570 composition and fatty acid composition of developing eggs and unfed larvae of cod (*Gadus*
571 *morhua*). *Marine Biology* 99, 307-313.
572 Hamre, K., Nordgreen, A., Grøtan, E., & Breck, O., (2013). A holistic approach to development
573 of diets for ballan wrasse (*Labrus bergylta*) – a new species in aquaculture. *PeerJ*, DOI
574 10.7717/peerj.99.
575 Hilldén, N.O., (1984). Behavioural ecology of the labrid fishes (*Teleostei: labridae*) at
576 Tjärnöon the Swedish west coast. PhD. University of Stockholm.

577 Izquierdo, M.S., Fernández-Palacios, H., & Tacon, A.G.J., (2001). Effect of broodstock
578 nutrition on reproductive performance of fish. *Aquaculture* 197, 25-42.
579 Kjesbu, O.S., (1989). The spawning activity of cod (*Gadus morhua* L.). *Journal of Fish Biology*
580 34, 195-206.
581 Kožul, V., Glavić, N., Tutman, P., Bolotin, J., & Onofri, V., (2011). The spawning, embryonic
582 and early larval development of the green wrasse *Labrus viridis* (Linnaeus, 1758) (Labridae)
583 in controlled conditions. *Animal Reproduction Science* 125, 196-203.
584 Kvenseth, P.G., Sjømatsenter, N., Andreassen, J., & Solgaard, J., (2003). Ballan wrasse –
585 strong medicine. In *Cleanerfish* (Hjelme, A. N., ed.) 18-22. Berger: Norsk Fiskeoppdrett
586 A/S.
587 Leclercq, E., Davie, A., & Migaud, H., (2014a). Delousing efficiency of farmed ballan wrasse
588 (*Labrus bergylta*) against *Lepeophtheirus salmonis* infecting Atlantic salmon (*Salmo salar*)
589 post-smolts. *Pest Management Science* 70, 1274-1282.
590 Leclercq, E., Grant, B., Davie, A., & Migaud, H., (2014b). Gender distribution, sexual size
591 dimorphism and morphometric sexing in ballan wrasse *Labrus bergylta*. *Journal of Fish*
592 *Biology* 84, 1842-1862.
593 Linhart, O., Rodina, M., Gela, D., Faljšhans, M., & Kocour, M., (2003). Enzyme treatment for
594 elimination of egg stickiness in tench (*Tinca Tinca* L.) European catfish (*Silurus Galanis*
595 L.) and common carp (*Cyprinus Carpio* L.). *Fish Physiology and Biochemistry* 28, 507-508.
596 McEvoy, L.A., & McEvoy, J., (1991). Size fluctuation in the eggs and newly hatched larvae of
597 captive turbot (*Scophthalmus maximus*). *Journal of the Marine Biological Association of*
598 *the United Kingdom* 71, 679-690.
599 McEvoy, L.A., & McEvoy, J., (1992). Multiple spawning in several commercial fish species
600 and its consequences for fisheries management, cultivation and experimentation. *Journal of*
601 *Fish Biology* 41, 125-136.

602 Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., &
603 Carrillo, M., (2013). Gamete quality and broodstock management in temperate fish. *Reviews*
604 *in Aquaculture* 5, 194-223.
605 Muncaster, S., Andersson, E., Kjebsu, O.S., Taraner, G.L., Skiftesvik, A.B., & Norberg, B.,
606 (2010). The reproductive cycle of female ballan wrasse (*Labrus bergylta*) in high latitude,
607 temperate waters. *Journal of Fish Biology* 77, 494-511.
608 Muncaster, S., Norberg, B., & Andersson, E., (2013). Natural sex change in the temperate
609 protogynous ballan wrasse *Labrus bergylta*. *Journal of Fish Biology* 82, 1858-1870.
610 Nordberg, B., Valkner, V., Huse, J., Karlsen, I., & Grung, G.R., (1991). Ovulatory rhythms
611 and egg viability in the Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture* 97, 365-
612 371.
613 Ottesen, O.H., Dunaevskaya, E., & D'Arcy, J., (2012). Development of *Labrus bergylta*
614 (Ascanius 1767) larvae from hatching to metamorphosis. *Aquaculture Research and*
615 *Development* 3, 1-4.
616 Peakall, R. and Smouse P.E. (2006) GENALEX 6: genetic analysis in Excel. Population
617 genetic software for teaching and research. *Molecular Ecology Notes*. 6, 288-295.
618 Peakall, R. and Smouse P.E. (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic
619 software for teaching and research-an update. *Bioinformatics* 28, 2537-2539.
620 Pickova, J., Dutta, P.C., Larsson, P.O., & Kiessling, A., (1997). Early embryonic cleavage
621 pattern, hatching success and egg lipid fatty acid composition: comparison between two cod
622 stocks. *Canadian Journal of Fisheries and Aquatic Science* 54, 2410-2416.
623 Pompanon, F., Bonin, A., Bellemain, E., & Taberlet, P., (2005). Genotyping errors: causes,
624 consequences and solutions. *Nature Reviews Genetics* 6, 847-859.

625 Quintela, M., Danielsen, E.A., Svåsand, T., Knutsen, H., Skiftesvik, A. B., & Glover, K.,
626 (2014). Isolation and characterisation of twenty microsatellite loci for the ballan wrasse,
627 *Labrus bertylta*. *Conservation Genetic Resources* 6, 425-428.
628 Rae, G., (2002). Sea louse control in Scotland, past and present. *Pest Management Science* 58,
629 515-520.
630 Rainuzzo, J.R., Reitan, K.I., & Olsen, Y., (1997). The significance of lipids at early stages of
631 marine fish: a review. *Aquaculture* 155, 103-115.
632 Robertson, R.R., Petersen, C.W., & Brawn, J.D., (1990). Lunar reproductive cycles of benthic-
633 brooding reef fishes: Reflections of larval biology or adult biology? *Ecological Monographs*
634 60, 311-329.
635 Ross, R.M., (1983). Annual, semilunar and diel reproductive rhythms in the Hawaiian labrid
636 *Thalassoma duperrey. Marine Biology* 72, 311-318.
637 Sargent, J.R., Tocher, D.R., & Bell, J.D., (2002). The lipids, In: Halver, J.R., Hardy R.W., (Ed),
638 *Fish Nutrition*, 3nd edition, 181-257. Academic Press, San Diego, CA.
639 Sargent, J.R., McEvoy, L., Estevez, A., Bell, J.G., Bell, M.V., Henderson, J.R., & Tocher, D.R.,
640 (1999). Lipid nutrition of marine fish during early development: current status and future
641 directions. *Aquaculture* 179, 217-229.
642 Sayer, M.D.J., Gibson, R.D., & Atkinson, R.J.A., (1993). Distribution and density of
643 populations of goldsinny wrasse (*Ctenolabrus rupestris*) on the west coast of Scotland.
644 *Journal of Fish Biology* 43, 157-167.
645 Sayer, M.D.J., Treasurer, J.W., & Costello, M.J., (1996). *Wrasse: biology and use in*
646 *aquaculture.* Blackwell Science Ltd. Oxford, 3-13.
647 Sjölander, S., Larson, H., Engstrom, J., (1972). On the reproductive behaviour of two labrid
648 fishes, the ballan wrasse (*Labrus bergylta*) and Jago's goldsinny (*Ctenolabrus rupestris*).
649 *Revue Comporte Animale* 6, 43-51.

650 Taggart, J.B., (2007). FAP: an exclusion-based parental assignment program with enhanced
651 predictive functions. *Molecular Ecology Notes* 7, 412-415.
652 Taylor, M.H., (1984). Lunar synchronization of fish reproduction. *Transactions of the*
653 *American Fisheries Society* 113, 484-493.
654 Thorsen, A., Trippel, E.A., & Lambert, Y., (2003). Experimental methods to monitor the
655 production and quality of eggs of captive marine fish. *Journal of Northwest Atlantic*
656 *Fisheries Science* 33, 55-70.
657 Tocher, D.R., (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Reviews*
658 *in Fisheries Science* 11, 107-184.
659 Tocher, D.R., Fraser, A.J., Sargent, J.R., & Gamble, J.C., (1985). Fatty acid composition of
660 phospholipids and neutral lipids during embryonic and early larval development in Atlantic
661 herring (*Clupea harengus* L.). *Lipids* 20, 69-74.
662 Treasurer, J.W., (2002). A review of potential pathogens of sea lice and the application of
663 cleaner fish in biological control. *Pest Management Science* 58, 546-558.
664 Trippel, E.A., (1998). Egg size and viability and seasonal offspring production of young
665 Atlantic cod. *Transactions of the American Fisheries Society* 127, 339-359.
666 Tveiten, H., Jobling, M., & Andreassen, I., (2004). Influence of egg lipids and fatty acids on
667 egg viability, and their utilization during embryonic development of spotted wolf-fish,
668 *Anarhichas minor* Olafsen. *Aquaculture Research* 35, 152-161.
669 Warner, R.R., (1982). Mating systems, sex change and sexual demography in the rainbow
670 wrasse, *Thalassoma lucasanum. Copeia* 1982, 653-661.
671 Wiborg, K.F., (1960). Investigations on eggs and larvae of commercial fishes in Norwegian
672 costal and offshore waters in 1957-58. *Fiskeridirektorates Skrifter Serie Havundersokelser*
673 21, 1-27.

674 Yanes-Roca, C., Rhody, N., Mystrom, M., & Main, K.L., (2009). Effects of fatty acid
675 composition and spawning season patterns on egg quality and larval survival in common
676 snook (*Centropomus undecimalis*). *Aquaculture* 287, 335-340.
677

678 **Table 1.** Description of ballan wrasse broodstock used in the study including origin, sex ratio
679 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

680

Table 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	Allele size range observed (bp)	H_e	H_o	F_{IS}	PI	Multiplex
Wr-A103	F: TGGTTGCTACCAAATCATG	6FAM	$(GTT)_9$	7	186-197	0.824	0.872	-0.068	0.055	1
	R: GGGACAGAATGAAATATCTCTG									
Wr-A107	F: GAAAGAGACGGACAGAGACA	NED	$(AAC)_9$	3	185-194	0.319	0.282	0.121	0.501	1
	R: CGTCCCTATTTCATTGTCAC									
Wr-A111	F: ATCCAACAAATGGACTTAGTCA	VIC	$(TCTA)_{18}$	8	199-240	0.740	0.641	0.106	0.097	1
	R: AAACGGAGACCAGTGGAG									
Wr-A113	F: TTGGAATCAAACAACCTCTC	PET	$(GTT)_{17}$	8	195-223	0.751	0.795	-0.059	0.090	1
	R: GAGCCTACAAATTATCATTGGT									
Wr-A203	F: GATAGCGGGATAAAAGAAGATC	6FAM	$(GTT)_{14}$	11	155-208	0.760	0.795	-0.051	0.078	2
	R: TTCTATTTGGCAACCTTTACAC									
Wr-A224	F: GGACTGGGAACAGTTAAGATG	NED	$(ATC)_9$	5	171-193	0.563	0.590	-0.048	0.280	2
	R: CATGCGAGAGTTTTTCAAAG									
Wr-A228	F:AGGAAAACAGAGCCTACAAATT	VIC	$(AAC)_{12}$	8	163-190	0.751	0.795	-0.059	0.090	2
	R: CTTGCTCCAGAACATTTCAG									

Table 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 sore (Table 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 4

693 **Table 4**. Hatch rate (%, Mean ± SEM, *n* = 5 larval counts performed), volumetric counts, estimated larval number, estimated egg number using
694 back calculation of larval number and well plate hatch rate; spawning score and estimated egg number per unit of spawning score from 6 individual
695 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					

696

Table 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}

703 **Table 6**. Mean egg fatty acid composition per spawning window (SW) for each spawning
704 population. *Note:* Superscripts represent significant differences between SW (all p values <
705 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}

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Table 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.

		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

716 **Table 8.** Parental contribution to the ballan wrasse larval samples taken from six separate
717 spawning dates, as determined by exclusion based parentage based on the genotyping of 7
718 DNA microsatellites. *Note:* Format '**35**/5' where first number (in bold) refers to both the total
719 number of offspring assigned unambiguously and those assigned allowing up to one allelic
720 mismatch and the second number (not bold) refers to offspring assigned to multiple families,
721 with one of the potential families being that of the previously identified single-match family.
722 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

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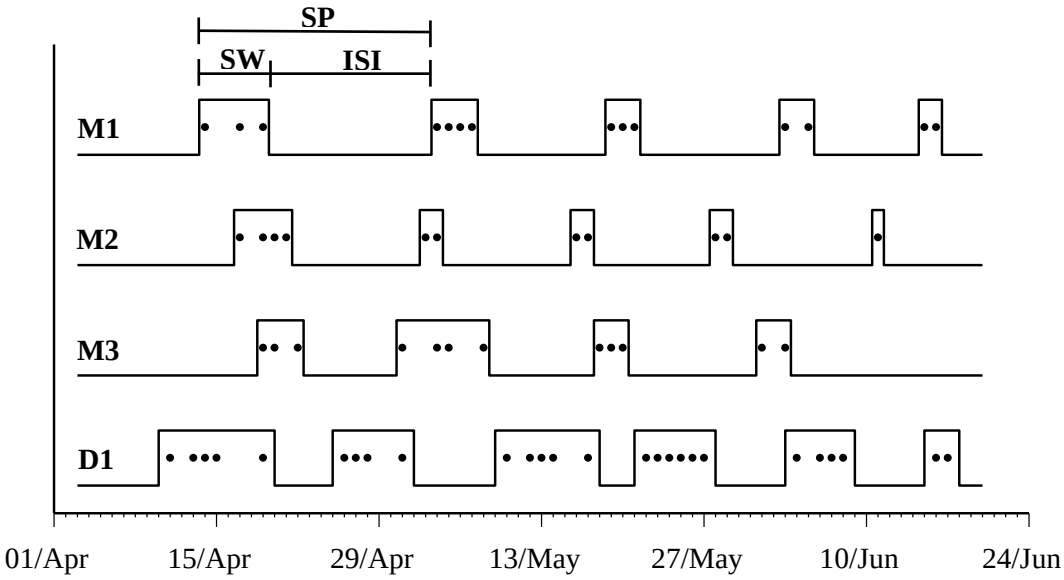
FIGURE LEGENDS

Figure 1. 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.

Figure 2. 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.

Figure 3. Mean egg diameter (ED) ± SE and mean gum layer thickness (GLT) ± SE over the spawning season for M1.

735 **Figure 1**



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738 **Figure 2**

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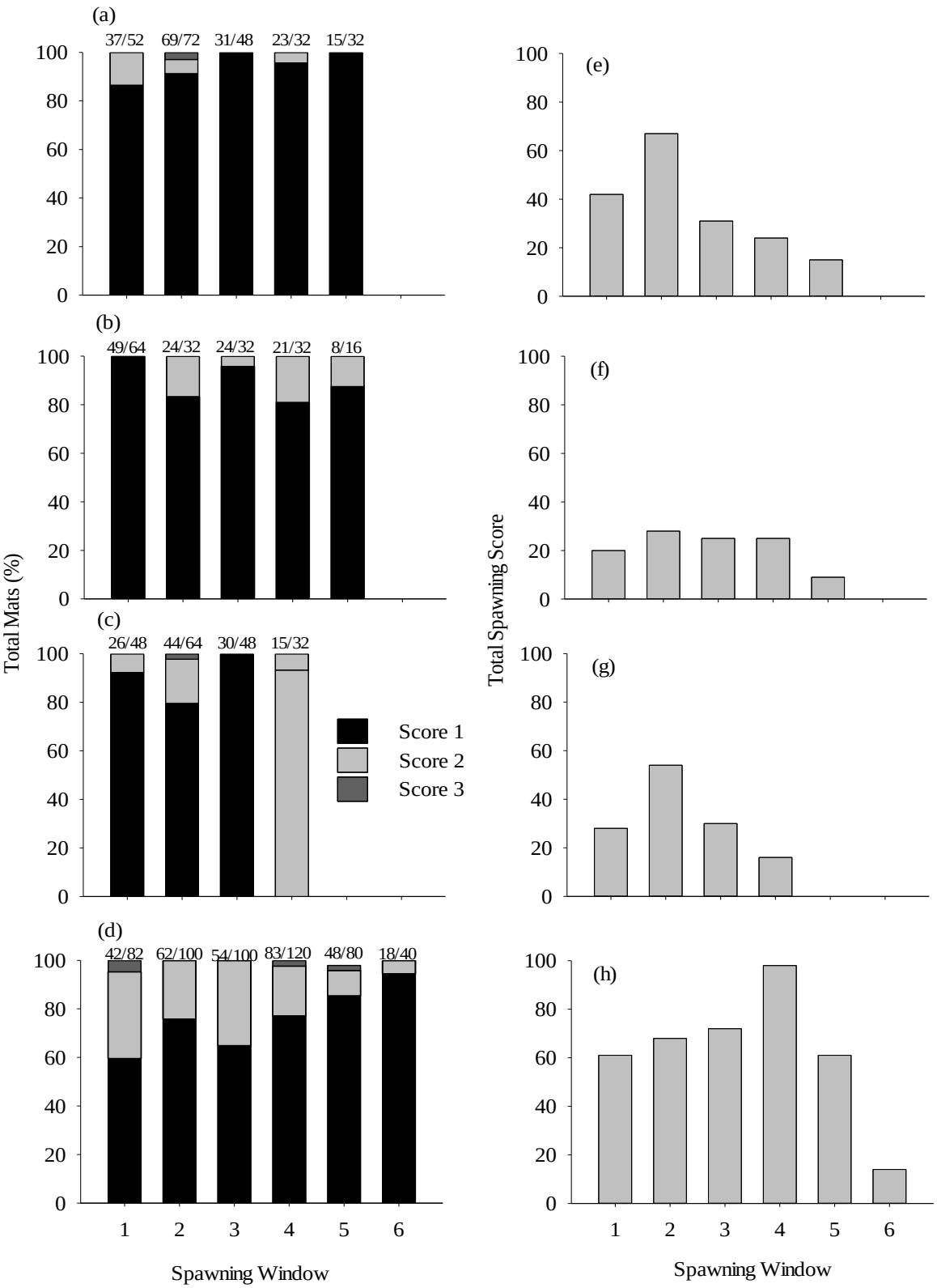
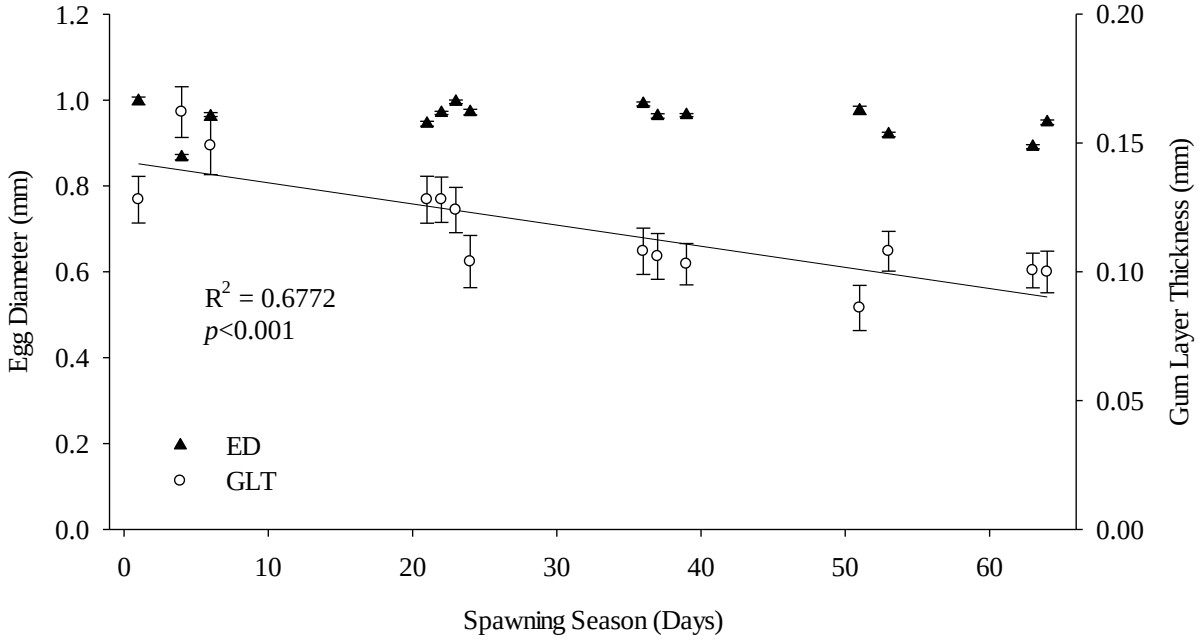


740

741 **Figure 3**



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