Pilot Project To Acquire And Secure New Technical Capability To Produce All-Female Juvenile Halibut In Scotland

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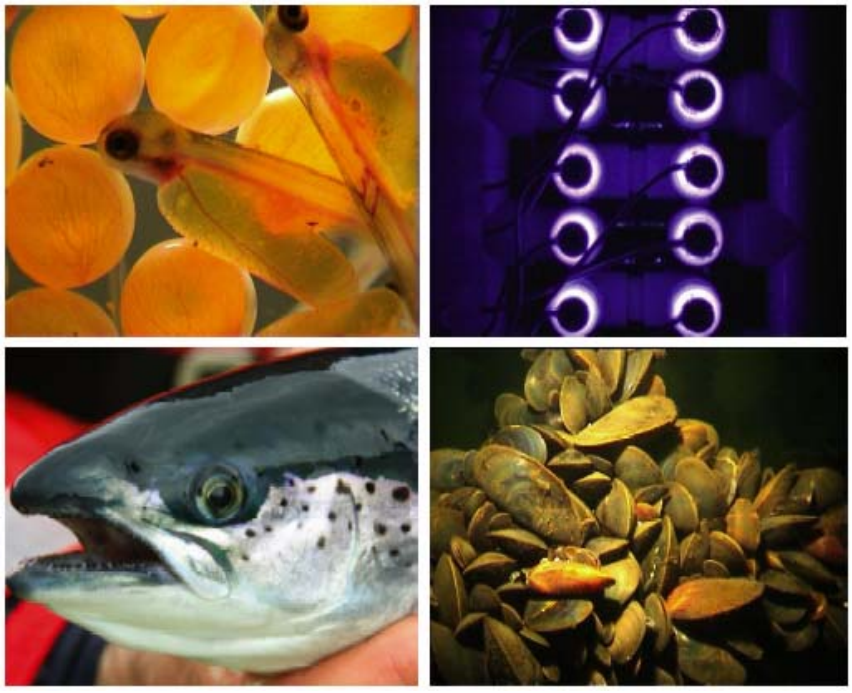

A REPORT COMMISSIONED BY SARF
AND PREPARED BY

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Published by the: Scottish Aquaculture Research Forum (SARF)

This report is available at: http://www.sarf.org.uk

Suggested Citation

Title: PILOT PROJECT TO ACQUIRE AND SECURE NEW TECHNICAL CAPABILITY TO PRODUCE ALL-FEMALE JUVENILE HALIBUT IN SCOTLAND

ISBN: 978-1-907266-41-6

First published: January 2012

SARF FINAL REPORT (027A)

"PILOT PROJECT TO ACQUIRE AND SECURE NEW TECHNICAL CAPABILITY TO PRODUCE ALL-FEMALE JUVENILE HALIBUT IN SCOTLAND"

This report was compiled by Dr. H. Migaud with the contribution of M. Cowan (sampling and histology), Dr. A. Davie (sampling and data analyses), Dr. A. Pino (validation of genetic markers) and Dr. D. Penman (support with the design of the study) from the Reproduction and Genetics Group, Institute of Aquaculture, University of Stirling, Stirling, UK.

1. INTRODUCTION

Monosex production is an effective management technique used to address the problem of early maturation in a number of commercially important aquaculture species which exhibit sexual dimorphism in growth and age at first maturation (Pandian & Kirankumar, 2003; Piferrer, 2001). In Atlantic halibut, *Hippoglossus hippoglossus*, it is the females which grow faster and larger than males and reach market size before maturation (Bjornsson, 1995; Imsland & Jonassen, 2005). Bjornsson (1995) demonstrated that female halibut reared in tanks under natural photoperiod matured at a mean weight of 12.7 kg whereas males matured at just 3.2 kg. Furthermore females showed a higher growth rate during the period of male maturation, 3.2 versus 1.4 kg/year for females and males respectively. Maturation is a major problem during ongrowing as energy is shifted into sexual development resulting in a loss in somatic growth and flesh quality and increased susceptibility to disease. Thus monosex production of female Atlantic halibut clearly holds great commercial and economic benefit for the industry. However, to date, little research has been done into the implementation of a monosex strategy in the European halibut industry as opposed to Canada where research led to the publication of a protocol for farmers to produce monosex commercially (Hendry et al., 2003).

The current most common way to produce all female fish populations is by indirect sex reversal and the use of 'neomales' (masculinised females) (Devlin & Nagahama, 2002). This technique requires that females are the homogametic sex. This is true in the case of Atlantic halibut where the gamete of the heterogametic male determines the sex of offspring at fertilisation although no specific sex chromosomes have been found yet (Hendry et al., 2002; Tvedt et al., 2009). Indirect sex reversal for the production of all-female populations is a two step process. The first consists of hormone treating juveniles with testosterone during the labile period which is the window following genetic sex determination but before phenotypic

differentiation, when gonads are still in an undifferentiated state. Exposure of individuals to exogenous steroids is aimed to over-ride natural endocrine signalling. Hormone treatment results in a predominantly male phenotypic sex population containing both normal males and masculinised females called 'neomales' which are genetically female fish but with testes and sperm, the sperm being carriers of only the female genotype. The second step of the process involves crossing these neomales (carrying the female genotype) with normal broodstock females to produce progeny consisting only of females. Indirect sex reversal has been successfully demonstrated in halibut in a study by Hendry et al. (2003), halibut lend themselves well to this as they start to differentiate after weaning at the time of first feeding, thus hormones can be administered in the feed (Hendry et al., 2003). Results from Hendry et al. (2002) indicated that halibut are sexually 'labile' from prior to hatch and the first signs of ovarian cavity formation appear at 38 mm fork length (L_F). In their study, male development was much later than females and there was still no differentiation of testis by 43.5 mm L_F, by 74 mm L_F spermatogonia started to appear along with interstitial tissue. Hendry et al. (2003) have demonstrated that treatment with in feed 17α-methylhydrotestosterone (MDHT) for six weeks at a mean size of 30 mm L_F successfully masculinised halibut. Monosex production by indirect sex reversal has already been successfully implemented in the commercial production of rainbow trout, *Oncorhynchus mykiss* (Kuzminski & Dobosz, 2010) and tilapia, *Oreochromis niloticus* (McAndrew, 1993).

The objective of this study was to establish the first UK population of broodstock neomales for the production of monosex (all-female) halibut populations by indirect sex reversal based on the published protocol by Hendry et al. (2003). To do so, 1) halibut juveniles were sexed reversed (MDHT in-feed treatment in spring 2007), 2) fish were on grown and sexed using histology to determine the efficiency of the treatment (spring 2008), then 3) a selected population with clear phenotype shift was monitored up to their first maturation in comparison to a normal mixed sex population (spring 2010), 4) selected

maturing (MDHT treated) individuals were selected and crossed with normal females (spring 2010) to identify neomales through progeny testing (spring 2011) and finally 5) a set of genetic markers was validated for parentage discrimination in future communal rearing during commercial scale progeny testing. Tasks 1 to 3 were funded by a SARF project (SARF 027, PhD project of Mairi Cowan) and Crown Estate (facilities at Machrihanish) with the support of BMFA. Task 4 and 5 were funded by an extension to the SARF 027 project, designated SARF 027a.

2. MATERIALS AND METHODS

2.1. Fish stock and initial rearing conditions

Weaned mixed sex halibut larvae (mean total length ± SE of 40.1 ± 0.2 mm, mean wet weight ± SE of 0.5 ± 0.01 g) were obtained from a commercial halibut hatchery and transferred to MERL (55:44^{0}N, 5:44^{0}W) for hormonal treatment. Six tanks were prepared, each with 230 halibut juveniles. Tanks were part of a seawater flow-through system with water running at a flow rate of approximately 50 L min^{-1} at ambient temperatures and filtered to 60 μm.

2.2. Experimental conditions

In-feed hormone treatments started on the 16th August 2007 (one day following transfer) and continued for a maximum of 6 weeks, until the 28th September 2007. Three treatments were tested in duplicate: 1) 6 weeks steroid free diet (control conditions), 2) 6 weeks MDHT in-feed (5 ppm) and 3) 3 weeks MDHT in-Feed (10 ppm) followed by 3 weeks steroid-free diet. Food was provided by automated feeders which were programmed to shake pellets into the tanks every 12 minutes throughout the 24 hour cycle to ensure that fish could feed to satiation.

To incorporate steroids into the manufactured feed, two stock solutions of MDHT (Sigma–Aldritch Co Ltd, Poole, UK) (dissolved in 100% ethanol) were made up at 5ppm (based on published protocol) and 10ppm (experimental protocol) (Hendry et al., 2003). Trays containing manufactured feed (Low Energy Marine Larval diet, EWOS, West Lothian, UK) were prepared and covered in the appropriate MDHT solution (2.5 ml solution / g feed). These were then left in a fume extraction cupboard overnight to facilitate evaporation of the ethanol. Dividers were placed between the trays to prevent any potential steroid contamination. The same process was conducted with the control diets but with steroid free

ethanol. Diets were individually bagged and stored at 4°C, all diets were used within 7 weeks of their production.

2.3. Sampling regime

Five sampling time-points were conducted throughout the 6 week hormonal treatment period, these included: baseline sample (pre-treatment, day 0), 12, 22, 33 and 43 day post treatment onset. At each sampling point, for each tank, 10 fish were culled by lethal anaesthesia and weight/length taken. Length was then taken for another 50 halibut/tank which were anaesthetised (1:10,000 concentration of 2-phenoxyethanol, Sigma-Aldrich Co Ltd, Poole, UK) and then returned back to respective tanks. Water, feed and fish samples were also taken for analysis of MDHT content by high performance liquid chromatography (HPLC) however the analytical technique employed did not have a high enough sensitivity to allow detection of MDHT in any of the samples. Halibut were transported to Otterferry Seafish Ltd on the 17th of October (3 weeks later) for ongrowing prior to the screening for sexual phenotype.

2.4 Ongrowing of MDHT treated fish and sex determination

Halibut have been maintained in commercial ongrowing facilities (Otterferry Seafish Ltd., Argyll, UK) and monitored for growth performance. Fish were initially maintained in separate tanks according to their respective treatment replicates however once they had reached a mean weight of 28.4 ± 0.4 g (mean ± SE) they were marked with panjet dye and replicates were pooled according to treatment (3 tanks in total, approximately 300 fish/tank, 6th December, 2007) due to space limitations at the farm facilities. The panjet mark was re-applied approximately every 1.5 months to retain the identity of fish treatments. Once halibut had reached a weight of 79 ± 2.95 g (mean ± SE) (14th February 2008), 20 individuals from each treatment were sampled for histological determination of sex to ensure that gonadal differentiation had occurred. The posterior gut cavity (containing the region of gonadal

development) was dissected from individuals, fixed in 10% neutral buffered formalin, processed by histology and stained using haemotoxylin and eosin. Following confirmation that sex could be determined at this stage, a further 60 individuals / treatment were sacrificed and sexed (21st March 2008). Thus a mean total of 80 individuals per treatment were sampled for sex determination however, due to a loss during processing or difficulty in sex identification owing to the small size of gonads, 3-4 samples per treatment could not be assessed.

On the 15th of May 2008, at a mean size of 180.8 ± 3.1 g (mean ± SE), 60 control fish (30 / replicate) and 150 5ppm fish (75 / replicate) were tagged with a passive integrated transponder tag (Fish Eagle Co., Lechlade, UK) and only these individuals were monitored for weight/length thereafter. All the remaining fish from these treatments and those from the 10 ppm treatment were culled. At the subsequent sampling points, tagged individuals were selected at random, anaesthetised and their weight and length taken, a minimum of 20 individuals/treatment were sampled. At the final sampling point on 8th April 2010 all tagged fish were measured and owing to the presence of maturing males, individuals were classified into maturing or immature cohorts (Table 1).

Table 1: Number of tagged control and 5 ppm fish sampled at each time point. Fish have been classed into a maturing or immature group according to their maturation status in April 2010.

Date		**Immature**		**Mature**	
Year	**Date**	**Control**	**5 ppm**	**Control**	**5 ppm**
2008	15th May	56	47	4	12
	9th October	18	34	2	8
2009	24th April	21	40	0	13
	12th November	18	31	2	10
2010	8th April	53	105	4	33

The specific growth rate (SGR) of immature individuals has been calculated retrospectively for tagged fish in the months leading up to 8th April 2010. SGR was calculated according to the equation: $SGR = (e^{g} - 1) \times 100$, where $g = [LN(weight_{end}) - LN(weight_{start})] \times$ number of days.

2.5. Progeny testing

In March 2010, crosses were performed between 7 males from the hormone treated population and normal female broodstocks. Microscopic analysis of eggs from crosses indicated successful fertilisation by these males. Eggs were then maintained in separate incubators for each of the 7 males. Progeny from 4 of the male crosses survived through yolk sac absorption, live feeding and weaning. Ongrowing took place at OFS with progeny from the 4 males being reared in 4 separate tanks. Phenotypic sex ratio was assessed in February-March 2011 once fish reached a suitable size (>50g) for histological sexing of the gonads. Sampling was performed in first week of March 2011 and consisted in sacrificing 30 (family

A & B) or 70 (family C & D) individuals/family for histology and blood sampling for genotyping (total of 200 offsprings).

2.6 Validation of genotyping assays

The work focused on the validation of the genotyping tools to be used for the parentage assignment of the communal rearing for the progeny testing. This step is essential to guarantee that we can discriminate individuals from different crosses when the progeny testing will be done at a commercial scale (2011-2012). Ten microsatellites were analysed. Using the linkage groups found by Reid et al. (2007), linked markers were not selected (Table 2). Blood samples were withdrawn from 8 males (300 µL of blood, 700 µL of ethanol) which included the 7 males incorporated in the progeny testing. DNA extraction was performed using phenol: chloroform protocol (Taggart et al. 1992). The final volume in each aliquot was 40 µL with a final concentration of 50 ng/µL. PCR conditions were as follows: DNA 9 µL, Buffer 13.5 µL, dNTP 13.5 µL, $MgCl_2$ 3.78 µL, Primer tailed* 2 µL (minimum amount in a PCR mix), Primer labeled 4.05 µL, Primer F/R 4.05 µL, TAQ 1.35 µL, Water 83.77 µL. Primer annealing temperatures were 56ºC for Hhi C17, I29, D34, J42, A44, 53 and 57 and 57ºC for Hhi 3, 52 and 60 (Table 3). The results were analyzed using Cervus 3.0 (Kalinowski *et al.*, 2007) to check the suitability of these markers in parentage assessment.

Table 2: List of microsatellites analysed.

Locus	Author	Link group (Reid et al., 2007)
HhiC17	McGowan and Reith, 1999	AH-3
HhiI29	McGowan and Reith, 1999	AH-10
HhiD34	McGowan and Reith, 1999	AH-20
HhiJ42	McGowan and Reith, 1999	AH-1
HhiA44	McGowan and Reith, 1999	AH-8
Hhi-3	Coughlan et al., 2000	AH-12
Hhi-52	Coughlan et al., 2000	AH-7
Hhi-53	Coughlan et al., 2000	AH-22
Hhi-57	Coughlan et al., 2000	AH-13
Hhi-60	Coughlan et al., 2000	AH-18

Table 3: Technical details of the microsatellite loci described in the literature.

Locus	**Primer sequence (5´-3´)**	**Repeat motif**	T[a]
HhiC17	F: 5´-TTAGGTCTGATCACCGCTATG R: 3´-GTTTACAAAGGTTTCTGATGGC		55ºC
HhiI29	F: 5´-GCTTCGGTTACACCTTTGC R: 3´-AGGACAGTGAGGATGTCCG		55ºC
HhiD34	F: 5´-GCCTGGTCTCATTGTGTTCC R: 3´-AGGTTAAATGATTTCCTGAAGCTG		55ºC
HhiJ42	F: 5´-CACAAACTCAAGATGTTGCG R: 3´-AAGCTCACTGGAAAATAATACCC		55ºC
HhiA44	F: 5´- CAACTGTGGGTATGTGCCTG R: 3´- GTGTCAGCACTGTGCTTAAACC		55ºC
Hhi-3	F: 5'- TCAGACAGGAAGGAAGTTTGG R: 5'- CCTCTCGGAATCACACACAG	$(CA)_{32}$	57ºC
Hhi-52	F: 5'- ATTGAGAAAGCAAATGTACGACC R: 5'- GTTCTTTTTATGTGAGCGACTGTG	$(CTGTAACATACAACAA)_3$ $(CTGTAACATACAA)_2$	57ºC
Hhi-53	F: 5'- ACCAACAGTGACACATAGCTCCT R: 5'- ATGCTAATGGGCTCTAAAATC	$(CA)_{29}$	55ºC
Hhi-57	F: 5'- GATTGCTGCTGTTGCCTC R: 5'- TCCGCTGCTCCCTCTA	$(CA)_2CT(CA)_2CT(CA)_3CT(CA)_5CTCACG$ $(CA)_5GA(CA)_5GA(CA)_{11}GA\ (CA)_5GA(CA)_4$	55ºC
Hhi-60	F: 5'- CAGACAAAAACTCACACACGCTC R: 5'- GCAAGTTTCATATAGGGGGTCAGAC	$(CA)_{12}$	57ºC

2.7. Genotyping and parentage assignment

A total of two hundred offspring and thirteen breeders (four males and three females) have been analyzed in this study. (Note that even female F1, for which the blood extraction could only be performed after dying, has been successfully genotyped.) The progeny has been distributed in four groups (A, B, C and D). Groups are composed by 30, 30, 70 and 70 individuals, respectively. Each group has only one putative male and two to three putative females. The total number of males that contributed to the offspring is four (one for each tank) and the total number of putative females is five (two of these five females were not sampled). The DNA extraction has been performed from whole blood extracted from the caudal vein (30%) and pure ethanol (70%) and the DNA has been isolated using a modified Chelex® 100 resin procedure (Walsh et al., 1991).

A set of ten microsatellite loci previously described in the bibliography and validated above were analyzed to infer the paternity relationships (see section 2.6). PCR reactions were been performed in a MJ Research PTC-100 thermocycler using the following protocol: Initial denature step of 10 minutes at 96ºC, 40 cycles of 30 seconds at 94ºC, 40 seconds at primer annealing temperature, 60 seconds at 72ºC and a final extension step of 10 minutes at 72ºC.

The specific annealing temperature, $MgCl_2$ and Primer concentrations for each locus are given in Table 4. Alleles have been scored on an ABI PRISM 3730 automatic sequencer (Applied Biosystems) in two multiplex groups composed by five markers. Loci were labeled by four different marker dye labels according to their size. Alleles were categorized using the software GeneMapper v 4.0 (Applied Biosystems).

Table 4: Set of primers and amplification conditions of the 10 loci microsatellites used in this study.

ABI groups	Loci	Primer sequence	Tº	Primer concentration	MgCl2 (mM)	Range
ABI group 1	HhiC17	F: 5´-TTAGGTCTGATCACCGCTATG R: 3´-GTTTACAAAGGTTTCTGATGGC	56ºC	2µM	1.4	124-138
	HhiI29	F: 5´-GCTTCGGTTACACCTTTGC R: 3´-AGGACAGTGAGGATGTCCG	56ºC	2µM	1.4	097-135
	HhiD34	F: 5´-GCCTGGTCTCATTGTGTTCC R: 3´-AGGTTAAATGATTTCCTGAAGCTG	56ºC	2µM	1.4	191-204
	HhiJ42	F: 5´-CACAAACTCAAGATGTTGCG R: 3´-AAGCTCACTGGAAAATAATACCC	56ºC	2µM	1.4	117-137
	HhiA44	F: 5´- CAACTGTGGGTATGTGCCTG R: 3´- GTGTCAGCACTGTGCTTAAACC	56ºC	2µM	1.4	143-216
ABI group 2	Hhi-3	F: 5'- TCAGACAGGAAGGAAGTTTGG R: 5'- CCTCTCGGAATCACACACAG	57ºC	2µM	1.4	187-211
	Hhi-52	F: 5'- ATTGAGAAAGCAAATGTACGACC R: 5'- GTTCTTTTTATGTGAGCGACTGTG	57ºC	2µM	1.4	136-167
	Hhi-53	F: 5'- ACCAACAGTGACACATAGCTCCT R: 5'- ATGCTAATGGGCTCTAAAATC	56ºC	2µM	1.4	224-255
	Hhi-57	F: 5'- GATTGCTGCTGTTGCCTC R: 5'- TCCGCTGCTCCCTCTA	56ºC	2µM	1.4	116-162
	Hhi-60	F: 5'- CAGACAAAAACTCACACACGCTC R: 5'- GCAAGTTTCATATAGGGGGTCAGAC	57ºC	2µM	1.4	118-136

*marker dye labels: Blue: FAM; Green: VIC; Yellow: NED; Red: PET.

Parentage assignment was performed using the exclusion-based approach implemented in the program CERVUS 3.0.3 (Kalinowski et al., 2007). We applied two of the available options in the package. The first option was the paternity assignment, using only the paternal information. The second option was the parent-pair assignment, using both female and male information. In both cases, the progeny were tested against the breeders from all the tanks—as opposed to performing single-tank analyses. This way, we simulated that all individuals were bred in the same tank. Genetic diversity estimators (heterozygosity, polymorphic information content (PIC), allele number) and the two cumulative non-exclusion probabilities (NE-1P, NE-2P) were calculated from the allele frequencies of the 13 breeders using CERVUS 3.0.3. The power of each locus and the combined probability over loci for not excluding a false candidate parent of a tested offspring without genotype information of any

true parent was termed as NE-1P probability and that one additionally knowing the genotype of one true parent was termed as NE-2P probability.

2.8. Statistical analysis and data presentation

For sex ratio determination, divergence from the expected 1:1 sex ratio was evaluated statistically using a Chi-square (χ^2) formula, with an χ^2 value of 3.84 ($p < 0.05$). All data were analysed using MINITAB ® version 15.0 (Minitab Ltd., Coventry, UK) statistical software. Length and weight (L/W) data were initially tested for normality using the Kolmogorov–Smirnov test and homogeneity of variances using Bartlett's test. L/W data throughout the hormone treatment period were compared by analysis of variance (ANOVA) manipulated using a General Linear Model (GLM) that included a comparison of treatment replicates (n=2) nested within the fixed treatment effect. L/W during ongrowing was compared between treatments (with replicates pooled as there were no significant differences between each) at each time point using a GLM.

3. RESULTS

3.1. Sex ratios of the MDHT treated fish

The control population exhibited the expected 1:1 phenotypic male to female sex ratio (52% male: 48 % female) whereas 97 % of the 5 ppm population and 70 % of the 10 ppm populations were confirmed as phenotypic males (Table 5). Chi square analysis confirmed that both in feed treatments significantly altered the natural sex ratio in favour of the male phenotype.

Table 5: Results of chi-squared analyses comparing control group sex ratios with MDHT (5 ppm and 10 ppm) treatments (Obs., observed sex ratio; Exp., expected sex ratio).

	Control		**5 ppm**		**10 ppm**	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
N	77	77	77	77	76	76
Male	40	38.5	75	38.5	53	38
Female	37	38.5	2	38.5	23	38
χ^2	0.12		69.2		11.8	
P	> 0.05		< 0.001		< 0.001	
df	1		1		1	
Sex Ratio	52 % male		97 % male		70 % male	

3.2. Growth performance and maturation of MDHT treated fish

Throughout the hormone treatment period, there were no significant differences in length and weight of halibut between steroid treatments over time (Fig. 1). Following the hormone

treatment period, the growth of halibut remained comparable up until November 2009 at which point individuals from the 5 ppm treatment showed a reduced length and weight in comparison to control individuals (Fig. 2) (*n.b.* 10ppm treatment fish were culled on April 2009 following sexing). By April 2010 the 5ppm fish were 84% of the mean weight of control fish (1968.3 ± 47.0 g and 2331.4 ± 81.0 g respectively, mean ± SE). Also at this timepoint, a number of maturing males were observed in the control (7%, 4 out of 60 individuals) and 5 ppm populations (21%, 32 out 150 individuals). Retrospective analysis of growth performance of the mature versus immature cohorts showed growth was comparable until April 2010, at which point their weight and length were significantly lower (Fig. 3). This was more pronounced in those under the control treatment which were just 66% of the mean weight of immature fish (1586 ± 277 g versus 2388 ± 80 g mature and immature mean weights ± SE respectively), in the 5 ppm treatment the mature individuals were equivalent to 71% of the immature mean weight (1508 ± 59g versus 2113 ± 51g). There were no significant differences in the SGR of tagged individuals that remained immature throughout the period measured, from May 2008 - April 2010 (Table 6).

Table 6: Specific growth rate of tagged halibut which have remained immature until April 2010. Data is presented as treatment mean ± SE (n = 6-31).

Date	Treatment	
	Control	**5 ppm**
May 08-Oct 08	0.69 ± 0.04	0.73 ± 0.02
Oct 08-Apr 09	0.38 ± 0.03	0.37 ± 0.02
Apr 09-Nov 09	0.25 ± 0.01	0.20 ± 0.02
Nov 09-Apr 10	0.16 ± 0.03	0.14 ± 0.02

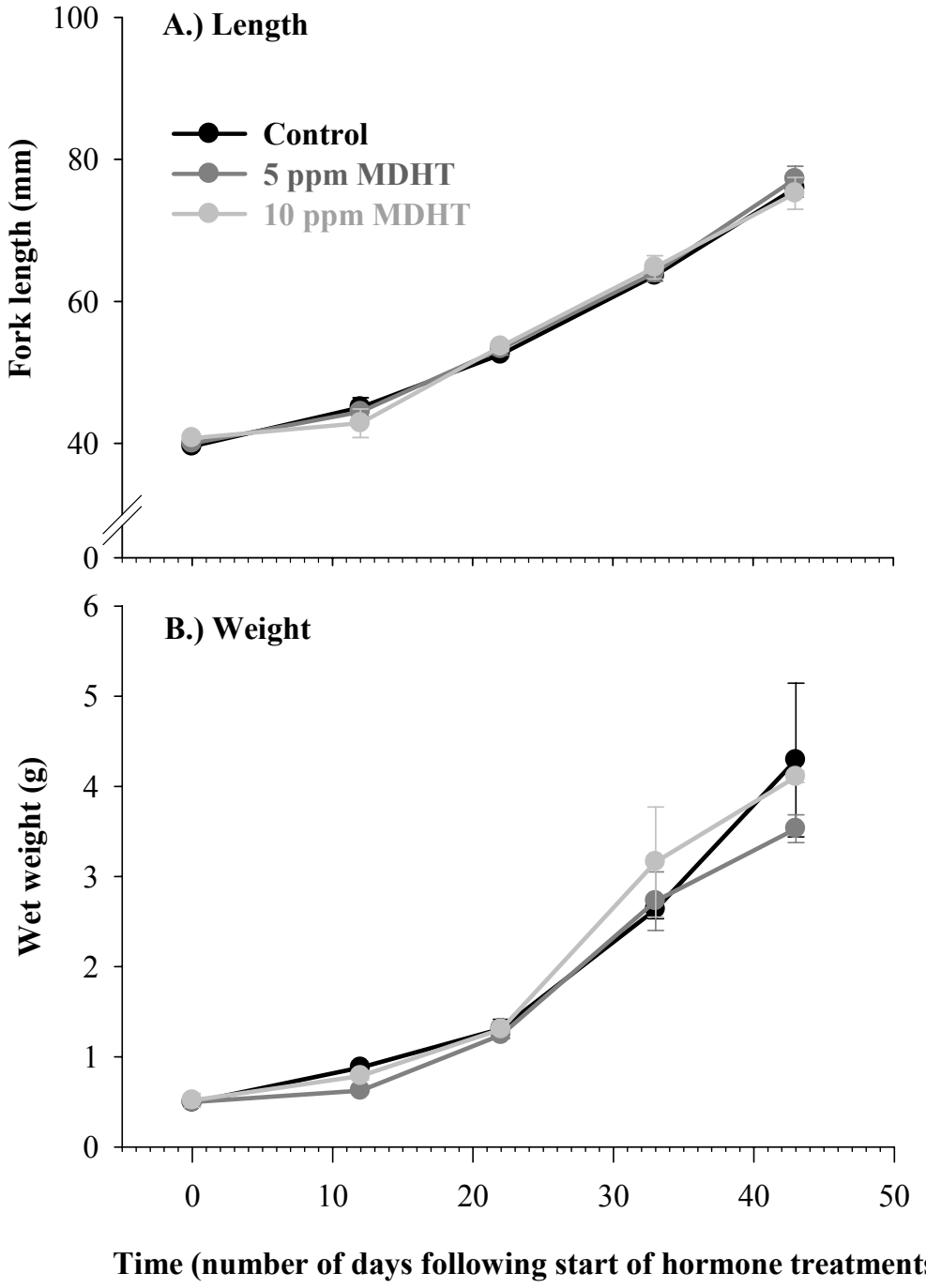

Figure 1. Length (a) and weight (b) of halibut treated (5 or 10 ppm) or not (control) with MDHT throughout the period of hormone treatment. Length and weight data presented as mean ± SD (n = 2, 60 or 10 individuals / replicate respectively). No significant differences were recorded between treatments.

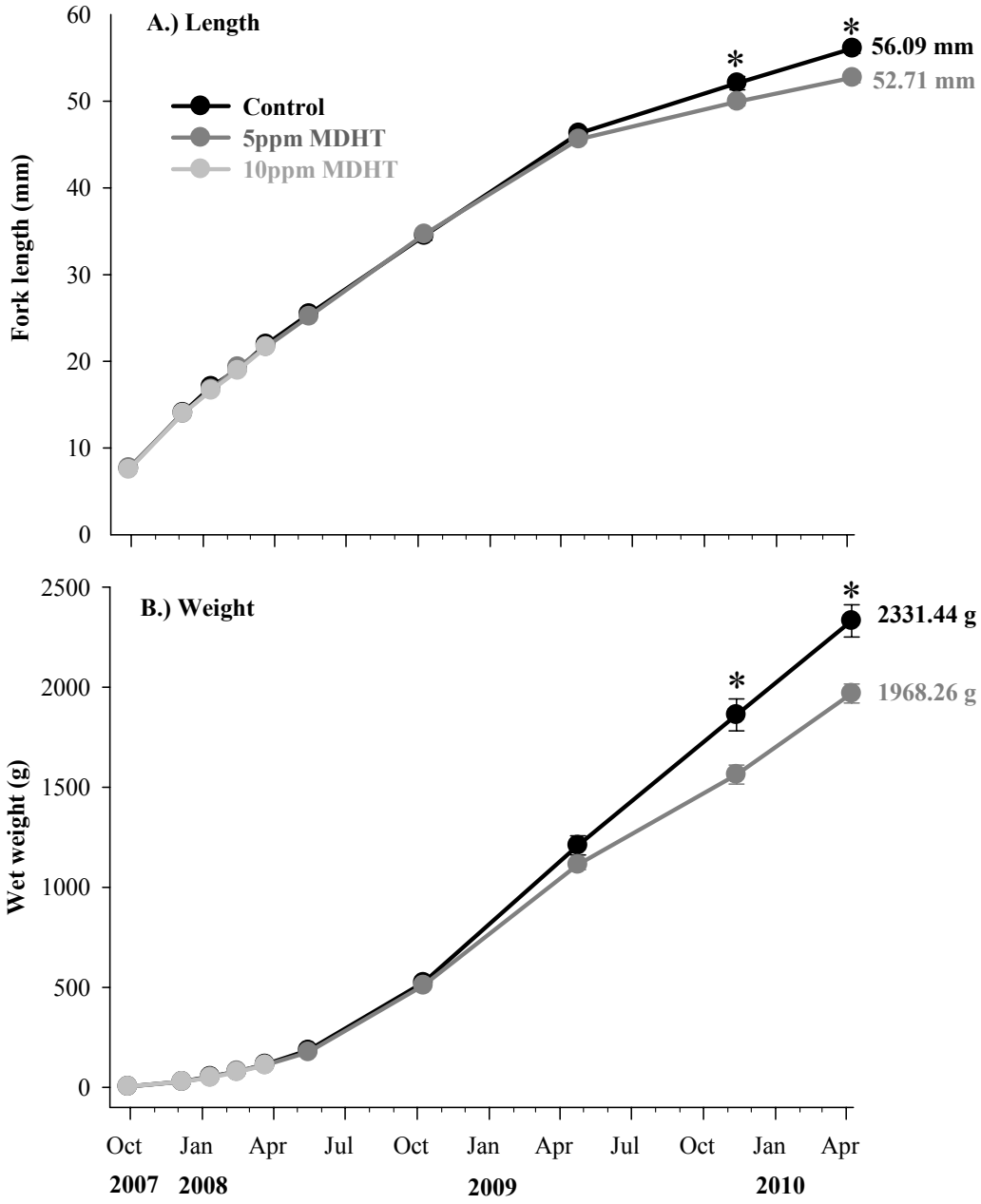

Figure 2. Length (a) and weight (b) of halibut treated (5 or 10 ppm) or not (control) with MDHT during ongrowing (data displayed from the last hormone treatment sampling on 28th Sep 2007 to the latest PIT-tag sample on 8th April 2010). Data for replicates is pooled and presented as treatment mean ± SE (10 ppm *n* = 20-100, 5 ppm *n* = 20-138, control ppm *n* = 20-83individuals / treatment). Significant differences between treatments at each timepoint are indicated by an asterix.

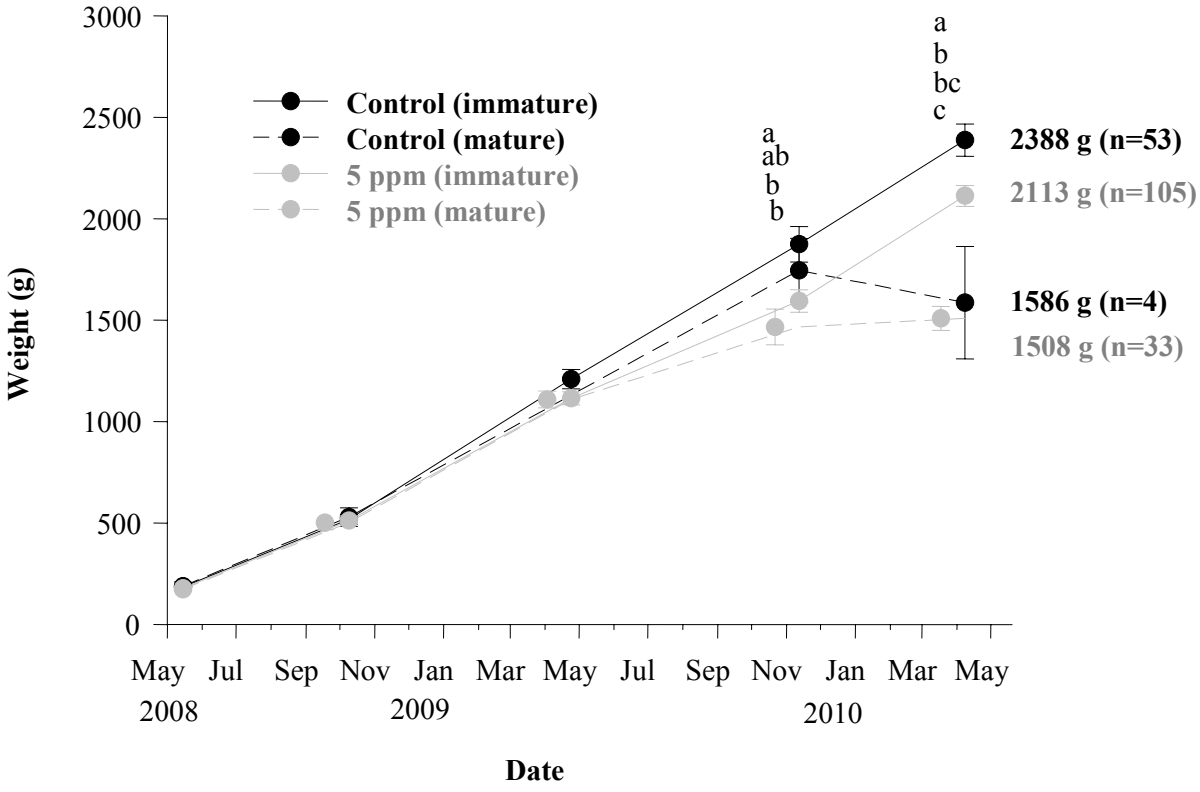

Figure 3. Weight of immature versus maturing halibut according to hormone treatment (control or 5 ppm). Data is presented as treatment mean ± SE (number of fish/timepoint is presented in table 2). Significant differences between treatments and maturation status are indicated by superscripts.

3.3 Progeny testing of 3 year old mature MDHT treated fish

Of the 7 tested males only four populations survived to allow sexing of the offspring at approximately 1 year of age. Among the four males crosses tested, two gave 100% female progeny (A and D, Table 6). The progeny of the crosses was determined through histological examinations of the gonad showing primary oocytes (oogonia) for females and spermatogonia for males (Figure 4). Mean length of females in batch A was significantly

shorter than batches B, C and D (Figure 7). As for weight, females of batches A and D showed lower weight than those from batches B and C.

Table 7: Results of chi-squared analyses comparing sex ratios between the progeny populations (A-D) from the four neomale crosses (Obs., observed sex ratio; Exp., expected sex ratio).

Population	**A**		**B**		**C**		**D**	
N	26		30		71		70	
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
Male	0	13	15	15	32	30.5	0	35
Female	26	13	15	15	29	30.5	70	35
χ^2	26		0		0.14		70	
P	< 0.001		> 0.05		> 0.05		< 0.001	
df	1		1		1		1	
Sex Ratio	100% female		50% female		48% female		100% female	

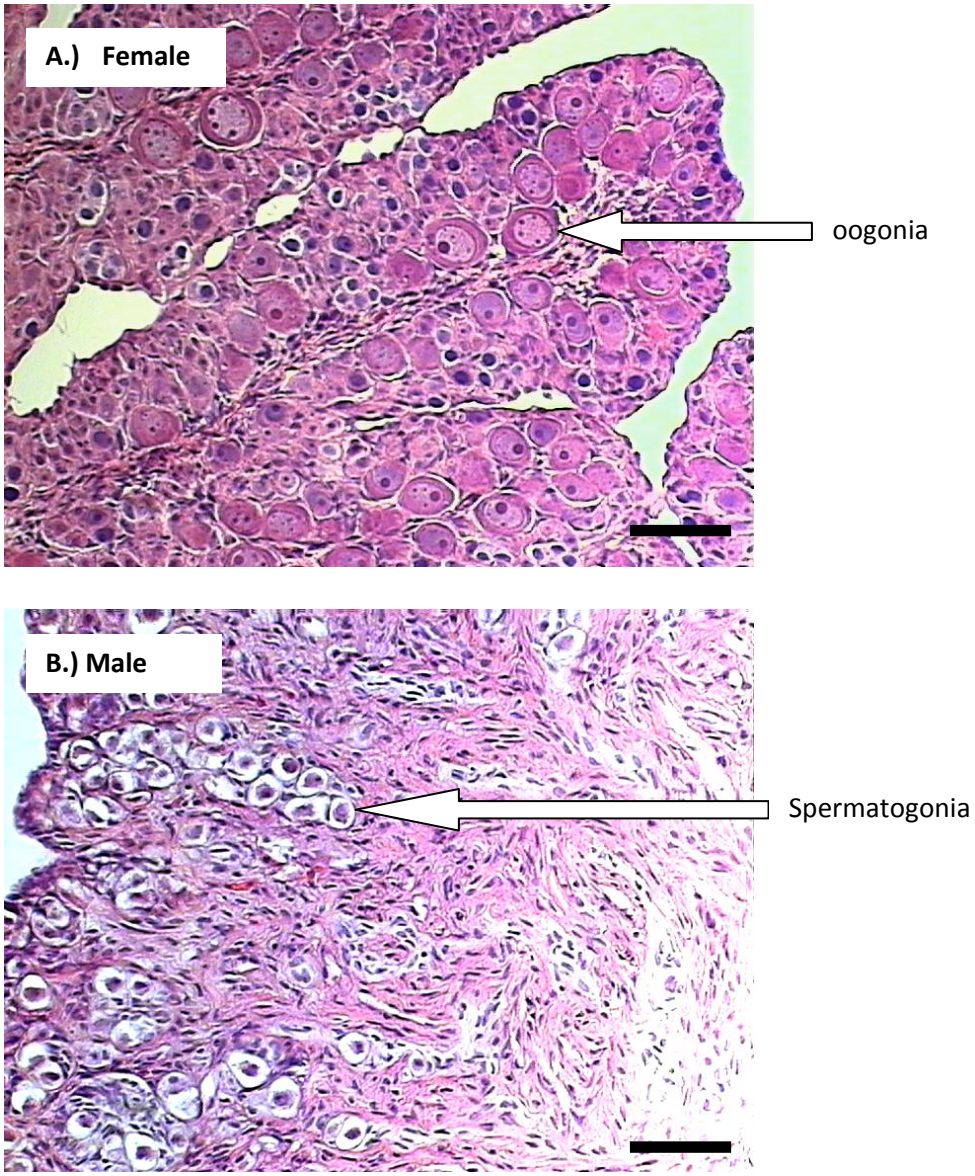

Figure 4. Histological sections of A.) female and B.) male gonads from halibut progeny. Scale bar represents 40μm.

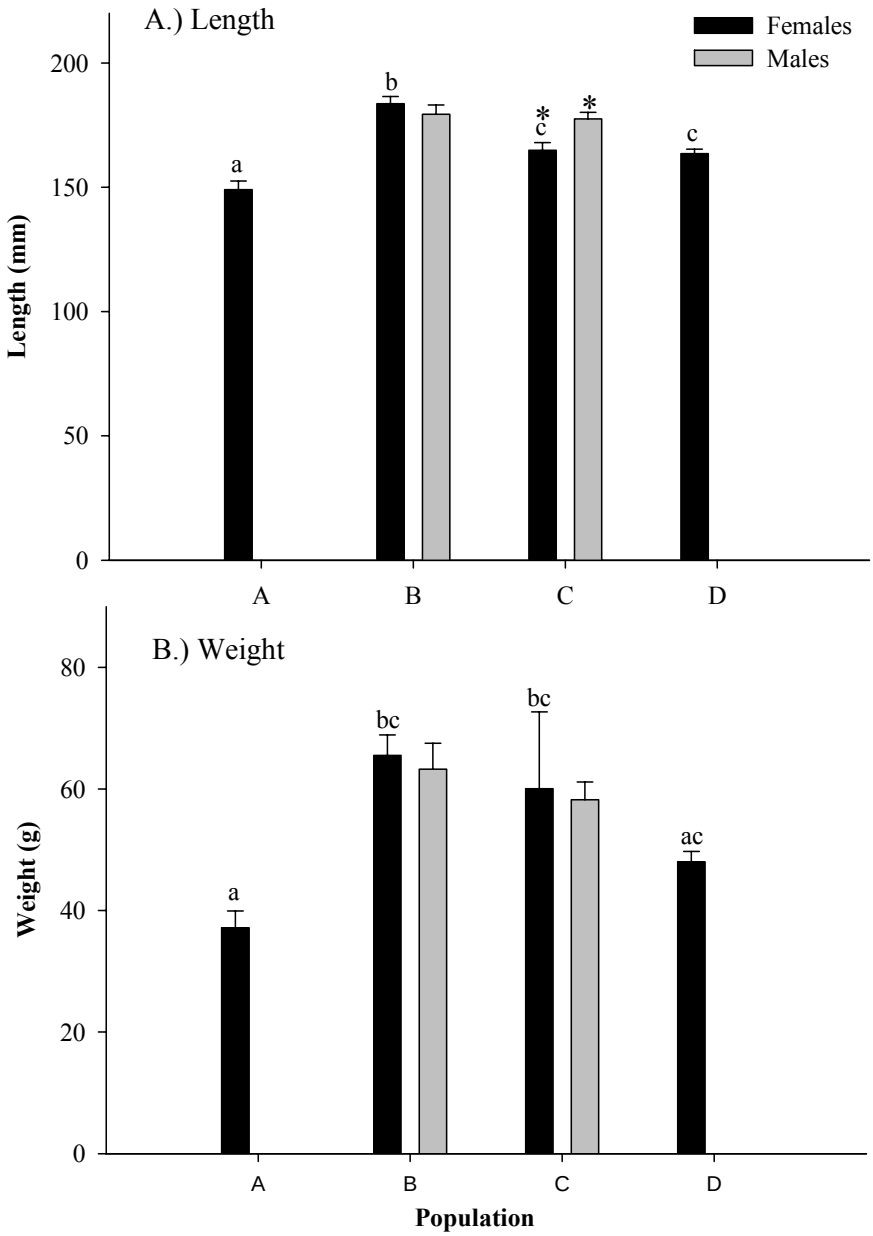

Figure 5. A.) Length and B.) weight of halibut progeny from the four male crosses. Data for females is presented for all four populations of progeny whereas data for males is only presented for populations B and C as there were no males present in populations A and D. Data is presented as mean ± SE (n = 13-70, see table 6 for exact numbers of males and females in each population). Significant differences in weight and length between female populations are indicated by lowercase lettering, significant differences between males and females within populations are indicated by an asterix.

3.4 Validation of genotyping assays

The number of alleles and He in each locus for the 10 microsatellites tested was very high in general. No significant differences between He and Ho were found, which suggests that no null alleles occurred (Table 8, 9). The genetic diversity of these 10 loci was shown to be suitable for parentage assessment of halibut progeny.

Table 8: Genetic diversity of the microsatellite described in the literature. N: number of individuals, Na: number of alleles, Ho: Observed heterozygosity, He: expected heterozygosity.

Locus	**N**	**Na**	**Ho**	**He**
HhiC17	110	22	0.89	0.95
HhiI29	110	14	0.85	0.86
HhiD34	110	9	0.8	0.73
HhiJ42	110	13	0.67	0.79
HhiA44	110	18	0.87	0.86
Hhi-3	20	16	0.95	0.88
Hhi-52	20	9	0.7	0.78
Hhi-53	20	14	0.9	0.94
Hhi-57	20	12	0.7	0.79
Hhi-60	20	8	0.8	0.82

The results were analyzed using Cervus 3.0 (Kalinowski *et al.*, 2007) to check the suitability of these markers in parentage assessment (Table 8).

Table 9: Results from Cervus 3.0 analyses (Kalinowski *et al.*, 2007). K: number of alleles, N: number of individuals, Hobs: observed heterozygosity, Hexp: expected heterozygosity, PIC: polymorphic information content, Ne-1p, Ne-2p and Ne-pp: non-exclusion probability for first, second or both parents respectively.

Locus	K	N	Hobs	Hexp	PIC	Ne-1p	Ne-2p	Ne-pp
Hhi 3	7	7	0.571	0.901	0.816	0.500	0.329	0.156
Hhi 52	5	8	0.875	0.725	0.641	0.726	0.546	0.352
Hhi 53	4	7	0.714	0.692	0.585	0.776	0.616	0.444
Hhi 60	7	7	0.571	0.802	0.716	0.637	0.454	0.254
Hhi 44	8	8	0.75	0.875	0.799	0.520	0.347	0.165
Hhi C17	2	8	0.125	0.458	0.337	0.908	0.831	0.742
Hhi D34	3	8	0.500	0.567	0.468	0.859	0.723	0.577
Hhi I29	5	8	0.875	0.817	0.727	0.637	0.459	0.278
Hhi J42	6	8	0.625	0.833	0.748	0.603	0.425	0.241

Combined exclusion probability using the parent pair (female and male) is 99.997% (Table 10). This probability is very high if we consider that the 8 males which were used in this test are related, therefore it appears that parentage assessment can be used as an effective tool for progeny testing halibut. All parents used in this project have been genotyped and samples from all future potential parents are being gathered in 2011 to ensure that this tool can be used in segregate parentage in future pooled rearing conditions.

Table 10: Exclusion probability using the set of 10 microsatellites for parentage assessment of communally reared halibut juveniles.

Number of individuals	**8**
Number of loci:	**9**
Mean number of alleles per locus	**5.22**
Mean proportion of individuals typed	**0.9583**
Mean expected heterozygosity	0.7412
Mean polymorphic information content (PIC)	0.6486
Combined non-exclusion probability (first parent)	0.02793296
Combined non-exclusion probability (second parent)	0.00203108
Combined non-exclusion probability (parent pair)	**0.00002956**

3.5 Genetic diversity and parentage assignment

-Genetic diversity:

The polymorphism observed in the 10 microsatellite loci analyzed in this study is not very high as compared to the results obtained in other halibut studies (McGowan and Reith, 1999 and Coughlan et al., 2000). In those studies, the number of alleles per locus ranged from 8 to 22 whereas in our study we obtained a maximum of 9 alleles in locus Hhi3 and a minimum of 3 alleles in locus HhiD34. This fact is due to different factors like the low number of samples analyzed (13 breeders) and the inbreeding relationships of the breeders.

In this study, we have obtained a combined probability over ten loci for not excluding a false candidate parent of a tested offspring without genotype information of any true parent of NE-1P=0.01293333 and an average non-exclusion probability for one candidate parent given the genotype of a known parent of the opposite sex of NE-2P=0.00054637. Therefore, using the whole set of 10 microsatellite loci we would expect a 98.7% probability to exclude a false parent when we do not know the other one, and a 99.99% probability to exclude a false parent given the genotype of a known parent of the opposite sex (Table 11). Thus it can be said that there is a very high probability of making categorical assignments of a progeny to a pair of parental individuals. However, it is known that several theoretical assumptions such as Hardy–Weinberg (HW) equilibrium, independent segregation among loci and Mendelian segregation are known to affect the theoretical predictions for the potential of parentage assignment of the microsatellites. In particular, inbreeding makes HW not to hold and our predictions to be biased upwards. Thus, we need to consider the probabilities we have obtained as upper limits.

-Parentage analysis:

Using paternity assignment we could determine the paternity of 77.61% of the individuals. However, for the remaining 22.39% we have obtained at least two putative males compatible

with the offspring genotype. Not surprisingly, these proportions differ significantly from our theoretical predictions (NE-1P=0.01293333, 98.7% percentage to exclude a false parent when we do not know the other one), which assume HW proportions for the parents.

Using parent-pair assignment, our results are closer to the theoretical predictions. When analyzing all individuals together we get to categorical assignments for 92.5% of the offspring. When using the additional information of paternity—coming from the experimental design (one only male per group), the percentage of categorical assignments raises to 98%. This value is very informative to us—it is the expected percentage of categorical assignments of a one-tank experiment in which we have sampled all putative parents (as opposed to lacking some of the females, as in our current analysis).

Table 11: Genetic diversity estimates (k: number of alleles, N: number of individuals, HObs: unbiased observed heterozygosity, HExp: unbiased expected heterozygosity, PIC: polymorphic information content), probabilities of non-exclusion (NE-1P: average non-exclusion probability for one candidate parent, NE-2P: average non-exclusion probability for one candidate parent given the genotype of a known parent fo the opposite sex, NE-PP: average non-exclusion probability for a candidate parent pair, NE-I: average non-exclusion probability for identity of two unrelated individuals, NE-SI: average non-exclusion probability for identity of two siblings) and frequency of null alleles (F Null) for the 10 microsatellite loci analyzed.

Locus	k	N	HObs	HExp	PIC	NE-1P	NE-2P	NE-PP	NE-I	NE-SI	HW	F(Null)
HhiA44	6	13	0.923	0.815	0.754	0.594	0.414	0.228	0.076	0.377	ND	-0.0828
HhiC17	4	13	0.615	0.680	0.601	0.766	0.600	0.425	0.173	0.466	ND	+0.0172
HhiD34	3	13	0.538	0.526	0.448	0.872	0.737	0.594	0.302	0.573	ND	-0.0550
HhiI29	7	13	0.923	0.837	0.777	0.562	0.386	0.205	0.066	0.364	ND	-0.0719
HhiJ42	6	13	0.692	0.775	0.708	0.651	0.472	0.283	0.102	0.403	ND	+0.0328
Hhi3	9	13	0.923	0.862	0.809	0.502	0.331	0.152	0.049	0.348	ND	-0.0540
Hhi52	5	13	0.923	0.729	0.660	0.706	0.527	0.337	0.130	0.432	ND	-0.1577
Hhi53	7	13	0.846	0.782	0.717	0.637	0.458	0.265	0.096	0.398	ND	-0.0579
Hhi57	5	13	0.846	0.698	0.618	0.744	0.577	0.397	0.161	0.454	ND	-0.1382
Hhi60	8	13	0.846	0.849	0.794	0.530	0.355	0.174	0.056	0.356	ND	-0.0112

Number of individuals:……13

Number of loci:…… 10

Mean number of alleles per locus:…….. 6.00

Mean proportion of individuals typed:……. 1.0000

Mean expected heterozygosity:…….. 0.7554

Mean polymorphic information content (PIC):……... 0.6887

Combined non-exclusion probability (first parent):…… 0.01293333

Combined non-exclusion probability (second parent):…… 0.00054637

Combined non-exclusion probability (parent pair):…… 0.00000313

Combined non-exclusion probability (identity):……... 1.48E-0010

Combined non-exclusion probability (sib identity):…… 0.00014291

4. DISCUSSION

Indirect sex reversal is a standard and accepted method for the production of monosex populations in aquaculture (Pandian & Kirankumar, 2003; Piferrer, 2001). Based on a published protocol by Hendry et al. (2003), this study has demonstrated the effective direct masculinisation of Atlantic halibut for the production of a UK neomale population which can be used as future broodstock to generate monosex female populations, with a 5ppm treatment for 6 weeks being the most effective. This supports the study by Hendry et al. (2003) and confirms that hormone treatment can be conducted when the halibut move from weaning onto the first feeding diet (Pandian & Sheela, 1995). In our study, hormone treatments commenced with halibut at a size of 40 mm, which was later than planned due to difficulties with facility access, according to Hendry et al. (2002) ovarian cavity formation has begun by 38 mm. However, this did not appear to affect our results as we effectively generated a male skewed population under both hormone treatments. It is clearly not necessary to treat the halibut immediately from first feeding and this is true for other species with research being focussed on identifying the most sensitive period for steroid action prior to morphological sex differentiation (Kavumpurath & Pandian, 1993). Interestingly, the shortened 10 ppm treatment was less effective, this may suggest that a longer period of treatment (~ 6 weeks) is required to fully induce masculinisation in the entire population. Alternatively it may just be a reflection of the intra-population variability in developmental stage. It is unlikely that all individuals within the population will be at a uniform stage of development therefore it is understandable that a minimum period of hormone treatment is required to effectively sex reverse the majority of target individuals.

In terms of growth, no differences in length and weight were found between treatments during the in-feed hormone period. By April 2010, maturing males were evident in both treatments and these clearly showed a lower mean weight in comparison to immature individuals. This was expected since as males enter maturation, resources are diverted into

reproduction. Hagen et al. (2006) reported a cessation in muscle fibre recruitment from November to April in male halibut undergoing maturation in the same spring. Interestingly in November and April 2010, immature individuals in the 5 ppm treatment consistently showed lower weight than the control individuals. This can not be attributed to maturation and is more likely due to body size dimorphism with a higher female proportion in the control population (circa 50 % female) as compared to the 5 ppm population (97% phenotypic male). Studies with 1-2 kg halibut have shown that females have a 1.9 fold higher fast myotomal muscle fiber number than males (Hagen et al., 2006, 2008) reflecting the larger ultimate body size in females. The coefficient of variation however was not different between treatments suggesting that there didn't appear to be distinct population differences between the 5 ppm and control population at this stage. Furthermore although the muscle fiber recruitment rates of males and females appear to differ, the sexual dimorphism in weight between gender generally only became apparent at the start of male maturation at 3 years of age (Hagen et al., 2006). The precise physiological mechanisms regulating differences in muscle fiber recruitment between male and female fish showing a sexual dimorphism in body size are unknown (Hagen et al., 2008), thus weight differences cannot be attributed to genetics or phenotype alone. The SGR of tagged immature fish did not vary between treatments, as expected. Halibut growth is generally calculated on the basis of yearly weight gain due to the seasonal changes associated with sexual maturation (Imsland and Jonassen, 2005), furthermore since SGR reduces as weight increases, it makes the evaluation of growth difference difficult (Bjornsson, 1995), yearly weight gain will be calculated in the future after the halibut have been monitored for greater then 3 years

In order to determine neomales within our 5 ppm treatment, as a pilot study, fertilisation crosses have been performed with seven of the early maturing 5 ppm putative neomales (spring 2010) and sex determination (identification of monosex female progeny) was conducted on the progeny once they have obtained a suitable size (~50 g) (February

2011). Only crosses from four of the crosses (out of seven) could be progeny tested due to poor survival during larval development in three of the batches. Histological results indicated that 2 out of the 4 males tested gave 100% female progeny. This confirmed that the strategy worked very efficiently. As sexual dimorphic growth is not apparent until 3 years of age in halibut differences in size between sexes and treatments is most likely a reflection of genetic variability in growth in combination with a limited sample size. Large scale fertilisation crosses will take place when halibut have reached four years of age (Spring 2011) at which point a greater percentage of males should be maturing (Jákupsstovu & Haug, 1998) and from this, broodstock for future monosex production (first commercial batch schedule for spring 2012) will be selected. In order to promote the chances of selecting a neomale, as many individuals as possible will be tested (at least 20) and the ability to pool progeny from different putative neomales would greatly help in this respect. The present work has therefore also been directed into investigating genetic marker (microsatellites) variation between potential male broodstock (taking into account the females to which they are crossed) with the aim to identify their offspring within pooled progeny. The use of parentage analyses based on a set of 10 microsatellites has been validated with a very good exclusion probability (99.997% if both parents are known in theory which equated to 98% in practice due to the "relatedness" of the parents). Furthermore, their efficiency was further validated by blind testing them in samples from the four crossed test families produced in 2010 which have been held in isolation and thus parentage can be assured. Results are very promising towards reducing costs by pooling moderate amounts of breeders into common tanks, especially if we can rely on sampling all parents in future experiments. In any case, the option of adding additional microsatellite loci would be convenient for several reasons. First, additional microsatellite loci are easily available in the literature. Second, also a reliable genetic map exists for this species, enabling us to select unlinked markers. Finally, a good behaviour of an

additional microsatellite locus can be inferred from the observed behaviour of the 10 microsatellite loci used in this study.

Additional work is ongoing in parallel to this project to identify sex-specific or sex-linked markers to determine the genetic sex of halibut which could ultimately fast-track the process of monosex production. Neomales could be identified when large enough to non-destructively sample i.e. at about 1 year of age which will preclude the requirement for progeny testing which will allow neomales to be used for commercial production as soon as they reach maturity.

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