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Offspring from farmed cod (*Gadus morhua* L.) spawning in net pens: documentation of larval survival, recruitment to spawning stock, and successful reproduction.

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ABSTRACT

Marine fish in culture, such as Atlantic cod, will mature in the seawater cage farms and thereby spawn and spread fertilized eggs into the surrounding water. The present study investigates the fate of such eggs originating from coastal Atlantic cod, kept in net pens during the spawning season in the small fjord system Heimarkspollen in Austevoll on the west coast of Norway. The fjord is known to be a spawning ground for wild cod. Use of a genetically marked farmed cod strain made it possible to identify the origin of collected cod larvae, juveniles, and adults in the wild environment. Eggs were released in the spawning seasons from 2006 to 2008 in Heimarkspollen, and a sampling program was initiated to monitor the occurrence of genetic marked cod at various life stages. Cod larger than 30 cm were tagged by T-Floy after muscle biopsy sampling, and then released back to the fjord. Substantial fractions of genetically marked cod larvae (6-36%) were collected in Heimarkspollen during the three spawning seasons. However, recovery of genetically marked juvenile cod within the expected size ranges was low (0.4-5.8% of catches). Largest genetically marked cod caught from Heimarkspollen in 2011 was 62 cm, which is well into the size of mature coastal cod. In the spawning season 2011, genetically marked cod larvae (2.4%) were again found, documenting recruitment to the spawning population in the area and successful reproduction. Interbreeding between cod of farmed origin and wild cod has so far not been indicated.

Keywords: Genetic interactions, spawning stock recruitment, successful reproduction, Atlantic cod

INTRODUCTION

Farming of Atlantic cod (*Gadus morhua* L.) has gained increasing attention in Norway the last decade, with a top production of 20 000 metric tonnes in 2010 (Norwegian Fishery Directorate www.fiskeridir.no). As for Atlantic salmon (*Salmo salar* L.), cod escapees may have negative influence on the genetic diversity of the wild cod stocks, and the official escape rate has been reported to be from 0.5 to 1.9% of total standing stock numbers in the cage farms during the period from 2006 to 2010 (www.fiskeridir.no). In contrast to anadrom fish like the salmonids, cod is a marine species that readily spawns in the net pens. So far, control of maturation in farmed cod has been more difficult than in salmon, and use of continuous light in the cages has only delayed maturation in cod by 3 to 5 month (Taranger *et al*. 2006, Skulstad *et al*. 2012). Therefore, fertilized cod eggs are readily produced, drift easily through the nets, and hatch to apparently viable larvae in the surroundings of the cage farms (Jørstad *et al*. 2008). Thus, the question is if these larvae will survive to adulthood, mature and spawn with wild cod? This scenario opens for escapement of genetic material from selectively breed cod in captivity, with possible genetic interactions between farmed and wild cod. The present work describes an ongoing study since 2006 of how spawning in net pens among farmed cod may affect the wild cod in a natural fjord system. By use of a unique genetic marker, survival, maturation, and spawning among of cod originating from spawning in pens have been investigated.

MATERIALS & METHODS

The study was carried out in Heimarkspollen, a land-locked fjord system in Austevoll on the west coast of Norway (Fig. 1). Heimarkspollen has a volume of about 80 million m^3, is 121 m deep, and has one narrow inlet (ca 30 m wide and 2.5 m deep) and three additional small tidal canals a few meters wide and less than 1 m deep. Spawning grounds for wild coastal cod are located in Heimarkspollen, which also is a nursery area for young coastal cod.

Broodstock of genetically marked farmed cod, located at the Institute of Marine Research (IMR) in Austevoll, were transferred to net pens in Heimarkspollen in 2006 and 2007 (Table 1), while in 2008 fertilised eggs were obtained from tanks and plastic bag pens with spawning cod at IMR-Austevoll before transported by car and released at exact same location and depth as the net pens the two previous years through a 200 m long PEH plastic tube of 32 mm diameter.

Table 1. Details of setup used in the spawning experiments.

Year:	2006	2007	2008
No. of net pens:	1	2	-
Net pen size:	125 m^3	350 m^3	-
Total number of farmed cod in broodstock:	1000	4000	600
Biomass of pre-spawned female cod:	1000 kg	7000 kg	1250 kg
Number of fertilized eggs released:	unknown	unknown	1.3 billion

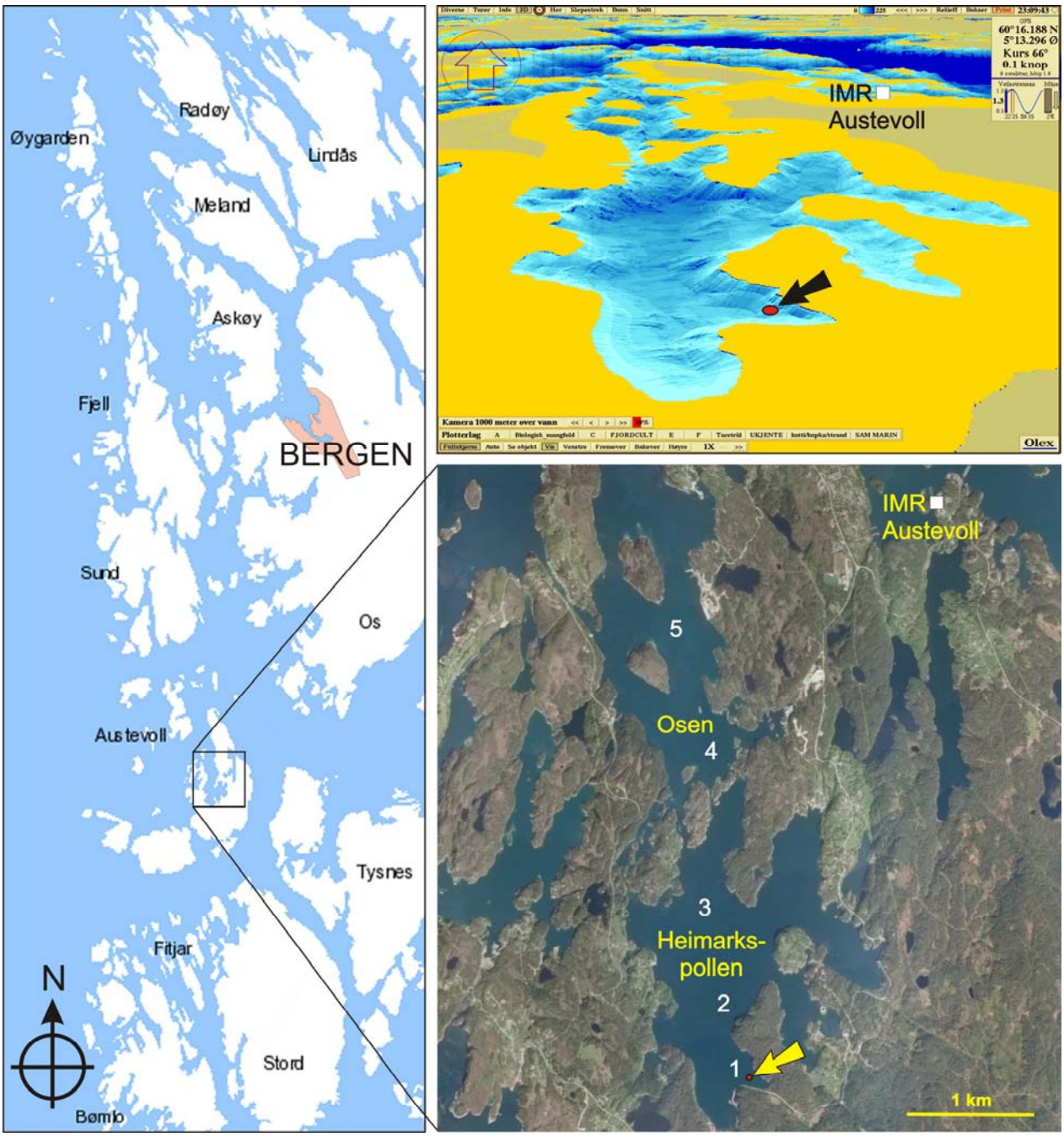

Figure 1. Map of study area, showing the release site of eggs in Heimarkspollen (arrows) and the IMR Austevoll Research station. Numbers denote egg-sampling stations.

Hydrography was monitored by using a CTD-multifunction meter, a MINI STD/CTD model SD-202 with depth (pressure), temperature, conductivity and oxygen sensors, (SAIV AS, Bergen, Norway).

The experiment was based on genetically (*GPI-1*30*) marked cod from the 2003-year-class raised at IMR Austevoll (Jørstad *et al*. 1991, 2008). The allozyme locus (*GPI-1**) is expressed in white muscle tissue and can reliably be detected as early as the yolk sac stage in cod (Jørstad *et al*. 1980). Weekly egg surveys were carried out yearly during the spawning season from February

to April by means of vertical hauls from 40 m depth with a 375 µm Juday plankton net of 80 cm diameter opening. To remove large and small plankton, the samples were sieved through 2000 and 1000 µm meshes, respectively. Additional plankton was removed by flotation using tiny air bubbles (Eltink 2007). The Juday net was also used for collecting larval cod in the period between late March and early June, towing the net up to 15 min at 1-2 knots at 2-5 m depth. From 2009 to 2012, the larval surveys were carried out 1-2 times a week. The collected eggs were photographed and diameter measured from the photographs. Eggs were assigned as cod eggs from diameter (1.2-1.5mm) and appearance. The cod larvae were immediately after collection frozen and later analysed for the *GPI-1*30* enzyme loci (see Jørstad *et al*. 2008 for more details).

Further, fishing was initiated to identify genetically tagged cod in the nursing area located in the vicinity of the spawning areas in Heimarkspollen. In addition to own fishing surveys, local fishermen were used to obtain samples of young cod of sizes between 30 and 60 cm total length. The fishing gears used were trammel nets and eel creels. The catches by local fishermen were kept alive, sampled by muscle biopsy after anaesthetised, and marked with a T-floy plastic tag before released back to the sea. All white muscle samples were analysed by starch gel electrophoreses (Jørstad *et al*. 1991) to identify offspring from the net pen spawning.

To trace potential crosses between offspring of the farmed cod and wild cod, the broodstock of farmed cod was analysed using microsatellite markers. Cod larvae caught in Heimarkpollen and Osen from the 2011 and 2012 seasons, that displayed the *GPI-1*30* genetic marker, were prepared for genotyping with 15 microsatellites (Gmo2m Gmo3, Gmo8, Gmo19, Gmo34, Gmo35, Gmo37, GmoC18, GmoC20, GmoG13, GmoG18, Gmog40, GmoG43, Tch11, Tch22). DNA was isolated using the HotSHOT method (Truett *et al*. 2000). The method is based on heating (at 95°C) the eye of a larvae in sodium hydroxid (25mM) for 30 minutes, neutralizing the sample with Tris buffer, and then use 2-4 ul of the solution directly for the PCR reaction. Genotyped larvae were used to perform direct assignment to a sample of relatives from the broodstock line of farmed genetically tagged cod, using the GenClass2 software (Piry *et al*. 2004). GenClass2 permits calculation of exclusion (i.e. rejecting individuals from the samples of relatives at different significance levels).

RESULTS

Hydrography

Details of typical hydrographical profiles for the fjord system are given in Jørstad et al. (2008). It was found that Heimarkspollen despite its large depth (121 m) is unique compared to the seaward water areas outside the narrow inlet. The areas outside Heimarkspollen have several 20 to 30 m deep sills allowing high-saline oceanic water to occasionally flow in and exchange bottom layers of the deeper basins of 65 to 85 m depth, during winter and spring seasons. During summer and

autumn, this bottom water frequently got extinct in oxygen. However, oxygen depletion was observed much less frequent in the landlocked Heimarkspollen, despite the depth is greater and sill is much shallower (only 3 m deep) than the water system outside. Salinity in Heimarkspollen was between 32 and 33 ppt, with some stratification building up during the summer as surface water became warmer. Periodic rainfalls also decreased the salinity of the surface layers.

Spawning season and egg distribution

Two spawning grounds were identified in the research area, one in the inner part of Heimarkspollen, and one in Osen, the area outside the narrow inlet to Heimarkspollen (Fig. 1). Documentation of these spawning grounds is given in Figure 2 which shows the location of the egg abundances among the sampling stations (Fig. 1).

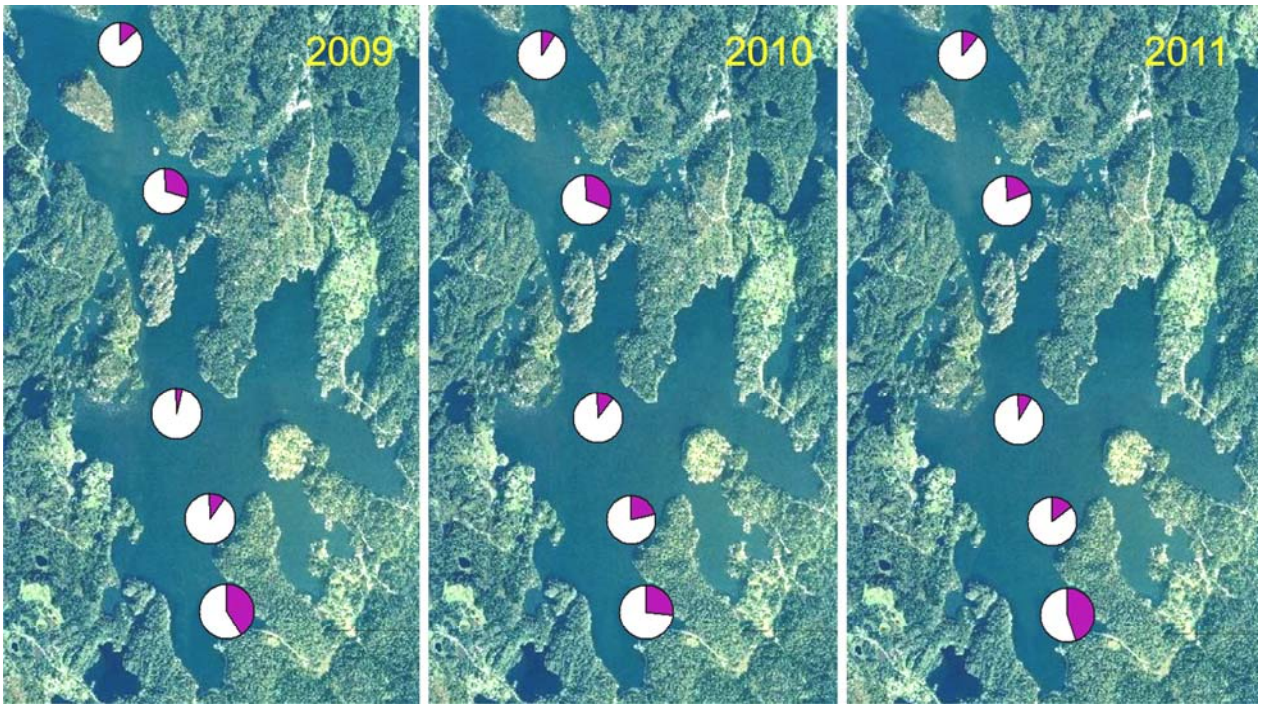

Figure 2. Egg abundance as fractions (purple pie pieces) of total egg catch during the whole spawning season along the sampling stations from each of the years 2009 to 2011 when no eggs of farmed cod were supplied to Heimarkspollen.

Total amounts of eggs from spawning in net pens was not determined except for in 2008 where 1.3 billion fertilised eggs was collected from the spawning bags and released in Heimarkspollen. During the six years of egg surveys, both amount of cod eggs and time of peak spawning varied. Time of peak spawning (Fig.3) occurred generally in the last week of March, but the time difference between 2009 and 2011 was nearly 3 weeks. Total amount of eggs sampled during the whole spawning season, standardised as egg catches per haul per sampling station, was highest in

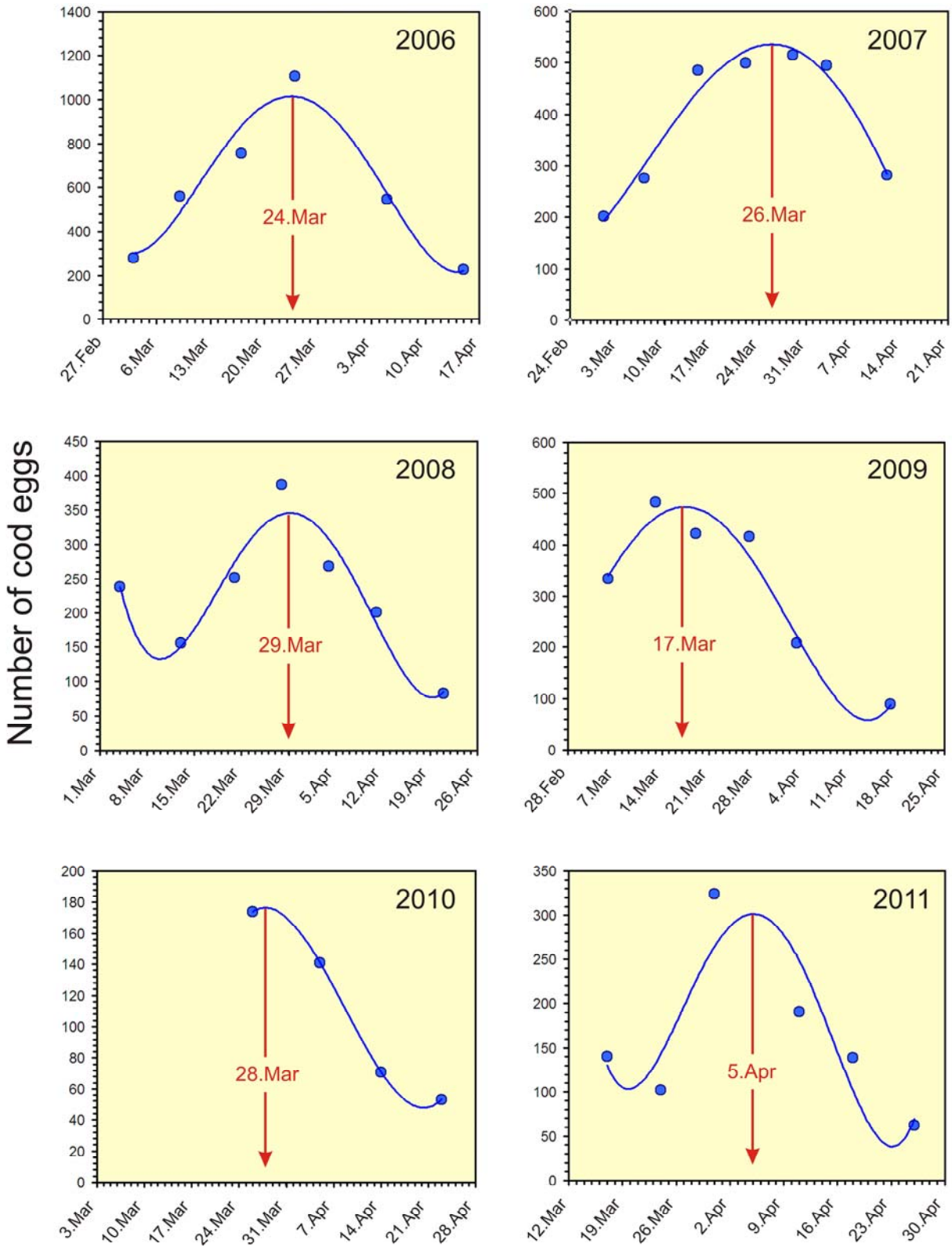

Figure 3. Estimated spawning curves for Heimarkspollen and Osen combined. The red arrows indicate dates for peak spawning, and spawning curves are fitted total egg catches for singe surveys (blue circles) as polynomial functions.

Heimarkspollen, during the first year of the study, and thereafter declining with the exception of 2009 (Fig.4). Considering the eggs collected during the survey closest to peak spawning, a similar trend was observed. However, this was not seen in Osen outside Heimarkspollen, which showed variation of 5 times in magnitude between years at peak of spawning (Fig. 4). Regarding egg data from all surveys, egg abundance was significantly higher in Heimarkspollen during the period 2006-2008 when eggs were released from farmed cod to the system compared to the period 2009-2011 without contribution from farmed cod (Student t-test, $p = 0.0475$). A similar difference was not found for the eggs collected from stations 4 and 5 in the vicinity of the spawning ground in Osen (Student t-test, $p = 0.3625$).

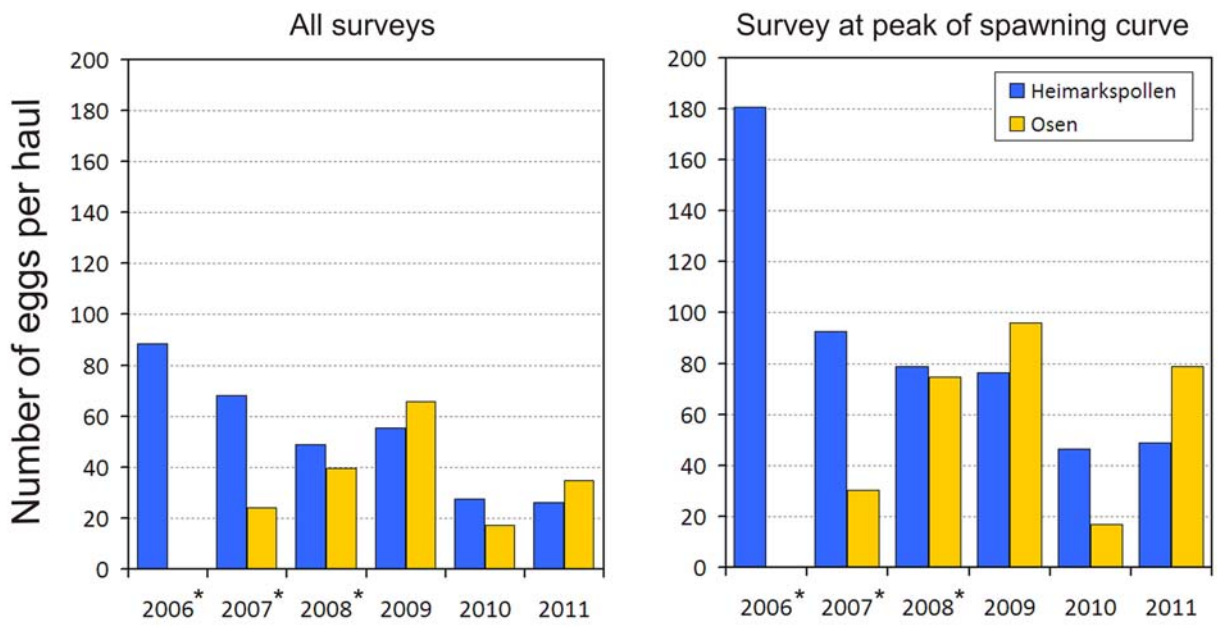

Figure 4. Standardised catches of cod eggs (number of cod eggs per haul) with a Juday net during surveys from 2006 to 2011, averaged for the whole spawning season (left panel) and for the survey closest to the peak of spawning (right panel). Data is calculated from three stations in Heimarkspollen (Fig.1: Station 1 to 3) and two stations in Osen outside Heimarkspollen (Fig.1: Station 4 and 5). The asterix (*) denotes years with spawning among farmed cod in net pens (2006-2007) or release of eggs from farmed cod spawning in plastic bags (2008). No hauls were taken in Osen in 2006.

Genetic marker in larval cod

During first three years when eggs from the farmed cod were released to Heimarkspollen, cod larvae collected from the surveys in Heimarkspollen and Osen were found to be homozygous (in the genetic marker *GPI-1*30/30*) in rates between 19.7% (2006) to 4.3% (2008) as calculated from total larval catches over the spawning season. During this period, 7.0 (2006) and 7.4% (2008) of the cod larvae were heterozygous in the genetic marker as (*GPI-1*30/X*), while it was fond to be as high as 12.6% in 2007 due to an increased share of heterozygotes among the

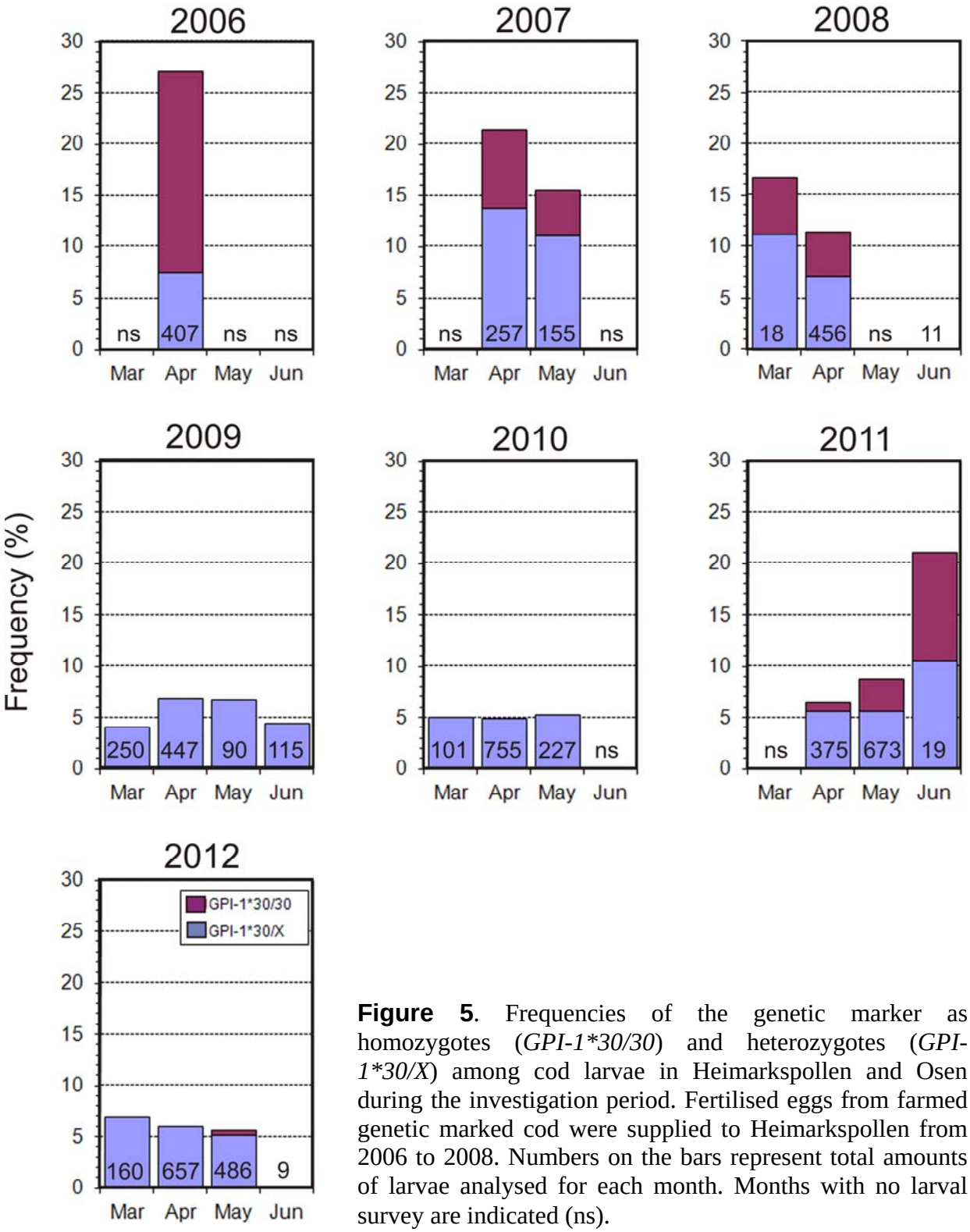

Figure 5. Frequencies of the genetic marker as homozygotes (*GPI-1*30/30*) and heterozygotes (*GPI-1*30/X*) among cod larvae in Heimarkspollen and Osen during the investigation period. Fertilised eggs from farmed genetic marked cod were supplied to Heimarkspollen from 2006 to 2008. Numbers on the bars represent total amounts of larvae analysed for each month. Months with no larval survey are indicated (ns).

broodstock cod that year. In the period from 2009 to 2012, the genetic marker as heterozygotes remained at a level between 5.0 and 5.7%. The first reappearance of the genetic marker as homozygotes was found in 2011 (2.4% of all sampled cod larvae), with another occurrence of

0.2% in 2012. Figure 5 gives the details of the allele frequencies among sampled cod larvae from March to June in each of the years from 2006 to 2012.

A preliminary test on 48 cod larvae from the 2011 season showed that DNA typing on samples originally prepared for analysis of the *GPI-1*30* genetic marker, is possible. DNA typing of all larvae with the genetic marker recovered from Heimarkspollen and Osen in 2011 and 2012 are now in progress.

Genetic marker in juvenile and adult cod

Altogether 1635 cod in the size range from 9 to 70 cm total length were caught and sampled in Heimarkspollen and Osen for analysis of the genetic marker. Of these, 1175 cod were released back to Heimarkspollen after sampling, and the rate of first recapture is between 29.8 and 47.3% (Table 2). Cod, which was homozygote in the genetic marker, were found in the range of 0 to 5.8%, while heterozygotes occurred between 2.3 and 9.4% among the captured cod. The largest recovered cod with the genetic marker was 62 cm in January 2011.

Table 2. Analyses of the genetic marker in juvenile and mature cod caught in Heimarkspollen and Osen from October 2007 to May 2011.

Fishing period	Cod material	Fish size range	Number of fish			Fraction (%)		Rate of first recapture[8] (%)
			Total (N)	GPI-30/30[6]	GPI-30/X[7]	GPI-30/30[6]	GPI-30/X[7]	
Oct - Nov 07	Juvenile & adult[1]	11-53 cm	85	1	8	1.2	9.4	-
Feb - Oct 08	Juvenile & adult[1]	9-70 cm	98	0	4	0	4.1	-
Des 08 - May 09	Juvenile[2, 3]	30-43 cm	154	9	13	5.8	8.4	42.9
Des 08 - May 09	Adult[2]	44-53 cm	129	0	3	0	2.3	41.7
Des 08 - May 09	Juvenile & adult[1]	11-69 cm	277	2	16	0.7	5.8	-
Des 09 - May 10	Juvenile & adult[2, 4]	31-60 cm	315	1*	22	0.3	7.0	47.3
Des 10 - May 11	Juvenile & adult[2, 5]	28-69 cm	577	2	40	0.3	6.9	29.8

[1] Cod collected for full sampling, including genetics, maturation, otoliths, and some common external parasites.
[2] Kept alive, measured, sampled (muscle biopsy for genetic analyses), tagged (T-floy), and released.
[3] Corresponding to fish lengths of genetically marked (GM) cod which originate from the 2006 year class.
[4] Size range of cod which both the GM 2006 and 2007 year class might occur.
[5] Size range of cod which the GM 2006, 2007 and 2008 year class might occur.
[6] GM cod with the rare PGI-30 allele in homozygote form.
[7] The rare PGI-30 allele in heterozygote form which can be potential offspring of crosses between GM cod and wild cod.
[8] Status at 1. January 2012. In Heimark most fish was released again for additional recaptures.
* Two more GM cod were caught, but considered recaptures of the T-floy-tagged GM cod from the Des 08 - May 09 catch.

DISCUSSION

Heimarkspollen displayed a unique and unexpected hydrography due to the shallow sill. The 3 m deep narrow inlet, only allow less saline surface water entered Heimarkspollen, which was observed with a much more uniform salinity (32 to 33 ppt) through the whole water column, compared to the water on the outside of the narrow inlet (Jørstad et al. 2008). Winter cooling of surface layers frequently caused completely vertical turnover in Heimarkspollen, resulting in oxygen concentrations in the bottom layers above 60% saturation. This enabled a frequent renewal of the bottom water inside the much deeper Heimarkspollen, compared to the bottom water of the adjacent seaward fjord system (e.g. in Osen) which had high-saline (34.5 ppt) and warmer (8°C) characteristics resembling more the Atlantic water. This mechanism may explain why the landlocked Heimarkspollen seems to be a highly productive system, with stocks of cod, herring (*Clupea harengus* L.), sprat (*Clupea sprattus* L.), hake (*Merluccius merluccius* L.), American plaice (*Hippoglossoides platessoides* Fabricius, 1780), and Norway pout (*Trisopterus esmarkii* Nilsson, 1855), among others. Such hydrographical situation in combination with the depth of Heimarkspollen may enable high production of zooplankton, which includes both species with different over-wintering strategies like resting eggs (e.g. *Acartia* sp.) or adult diapauses (e.g. *Calanus* sp.). Such zooplankton is the main food source for marine larval fish.

Nevertheless, two spawning areas for cod were identified, both in Osen and in Heimarkspollen, with exchange of cod eggs and larvae between these two localities through the tidal canals. Thus, both these spawning areas were included in the monitoring of cod eggs and larvae. Spawning season lasted from late February to beyond mid-April. Peak of spawning was concentrated to the last week of March, although shifts of almost 3 weeks apart were observed (2009 vs 2011, Fig. 3). Such shifts may be explained by differences in temperature among years, as 2009 had a warm winter with temperatures between 5.5 and 6.0°C through the water column in Heimarkspollen in late February, and 2011 on the contrary was cold with temperatures between 3.6-4.2°C in Heimarkspollen in early March.

Egg production was higher in Heimarkspollen during the years of releases of eggs from the farmed cod, but not in the Osen spawning ground outside Heimarkspollen, This confirm that the contribution from the farmed cod was detectable despite the small scale of the setup. The amount of eggs released from spawning in net pens the two first years may however be estimated from fecundity calculated from the 2008 spawning or from the more conservative data on fecundity in captive cod given by van der Meeren and Ivannikov (2006). This would give estimates in the ranges of 0.5-1.0 billion eggs for 2006 and 3.5-7.3 billion eggs for 2007. Unfortunately, problems with the net cage in 2007 caused increased mortality among the broodstock cod, and egg production may therefore have been considerable lower this year than expected, as indicated from the egg surveys (Fig. 4).

The contribution of the spawning from farmed cod was clearly observed as substantial fractions of cod larvae with the genetic marker, particularly in 2006 when 19.7% of all larvae from Heimarkspollen and Osen displayed the genetic marker as homozygotes (*GPI-1*30/30*). In 2007, application of more broodstock fish which was heterozygote (*GPI-1*30/X*) in the genetic marker loci made it more difficult to evaluate the contribution of farmed fish to the larval stock. It is reasonable to use a fraction of 3 to 5% heterozygotes in the contribution from the wild cod, which then implies a contribution from the farmed cod between 14.2 and 16.2% of all cod larvae on the spawning grounds in Osen and Heimarkspollen. In 2008, the contribution from farmed cod was considerable lower than the two previous years. This may be due to handling stress when the newly fertilised eggs were transported for about 10 minutes in a tank on a small truck before released through a 200 m tube to Heimarkspollen. During egg sampling and counting, transparent eggs were observed to become opaque and die due to handling. Thus, reduced survival among the transported eggs may explain an overall contribution of only 4.3% from the farmed cod to the larval population in 2008.

Despite high input of cod larvae from the farmed cod the two first years, the magnitude of this signal decreased substantially in the samples collected from juveniles and immature cod later in the period. Still, the investigation clearly demonstrates that offspring from cod net pen spawning do survive in the wild environment. Considering the expected size ranges of cod with origin form the spawning in pens, the frequencies of the genetic marker reached a maximum of 5.8 for homozygotes and 9.4% for heterozygotes during winter 2008-2009 and autumn 2007, respectively. However, the data indicate that some fish of farmed origin survived and stayed in Heimarkspollen and Osen, but it has not been verified whether the decrease in the signal is due to selective mortality or migration out of the area.

In 2011, offspring from cod originating from spawning in pens was detected. This is proof of reaching maturation and new contribution to the wild cod in the area. It is also intriguing that this contribution seemed to happen among cod that came from spawning in net pens only, as crosses between cod of farmed and wild origin still remain to be verified. The occurrence of cod larvae homozygous in the genetic marker in 2011 indicates that cod somehow may have the ability to recognize their affiliation which in next turn may be an important mechanism in how genetic distinct populations can be maintained. Such behavioural mechanisms may work in addition to physical factors like e.g. retention of eggs and larvae in fjord systems.

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