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Original Article

Genetic marking of farmed Atlantic cod (*Gadus morhua* l.) and detection of escapes from a commercial cod farm

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A genetically marked Atlantic cod (*Gadus morhua* L.) strain was used to identify escapes from commercial cod farms, and to investigate the potential interbreeding between farmed and wild cod. This farmed cod was homozygote for a rare allele (*30*) in the *GPI-1* locus expressed in white muscle tissue. Juveniles were produced from this strain in 2007 and 2008, and 500 000 individuals of each year class were transported to a cod farm in western Norway, where they were raised under commercial conditions. A monitoring fishing program was established from spring 2007 to detect escapees during the farming period. The first farmed cod escapees, identified to the 2007 year class through the genetic mark, age and body size, were detected during the fishing survey in November 2008. The second escape of the same year class was detected during the natural spawning season in early April 2009. A third escape was detected in November 2009, and this time the farmed cod were identified to the 2008 year class. The escapees were spreading through the whole fjord system, including local spawning sites for wild cod. Detailed examination of the escaped cod revealed a substantial degree of sexual maturation, and nearly 1000 cod larvae and early juveniles were therefore collected through spring 2009. The genetic analyses identified eight of these as genetically marked, demonstrating successful reproduction either in the cage or after escape. Interbreeding between escaped and wild cod may also have occurred, but cannot be proven from our material. In all years after the three identified escapes, genetically marked cod were found in the fjord area. In addition, several specimens were observed in adjacent fjord systems, demonstrating long-term survival in the local spawning areas as well as substantial spread over larger distances.

Keywords: aquaculture, Atlantic cod, escape, introgression.

Introduction

Escaped fish from the aquaculture industry may breed with wild fish. Such interbreeding between wild fish and escaped farmed fish (or deliberate releases in stock enhancement or sea ranching programs) could result in genetic changes in the wild populations and reduced fitness (Utter *et al.*, 1993; Utter, 1998). Although this problem has been discussed for a long time (Skaala *et al.*, 1990; Hindar *et al.*, 1991; Hutchinson, 1997 and references within), experimental evidence of harmful effects from interbreeding between cultured and wild fish stocks has been limited. However, for Atlantic salmon, *Salmo salar* (McGinnity *et al.*, 1997; Fleming *et al.*, 2000; McGinnity *et al.*, 2003; Skaala *et al.*, 2012) a reduction in survival of the offspring of farmed and hybrids compared with wild salmon offspring has been demonstrated.

The cod farming industry in Norway has an annual production capacity of about 300 000 t distributed among 507 production licences (Directorate of Fisheries, Bergen, Norway, www.fiskeridir.no). Production has been much lower, and had a maximum of approximately 21 000 t in 2010 (Directorate of Fisheries, Bergen, Norway, www.fiskeridir.no). However, due to low profitability, the interest in cod farming has declined dramatically.

In 2008, 304 000 escaped cod were reported compared with 111 000 escaped salmon; production of salmon was 45 times larger than for cod in that year (Directorate of Fisheries, Bergen,

Norway, www.fiskeridir.no). In contrast with salmon, cod displays more net biting and actively searches for holes in the cage wall. These behavioural traits for cod are reported to be a major cause of the differences in escape rate between cod and salmon (Jensen *et al.*, 2010). Nevertheless, commercial cod farming represents a potential genetic threat to wild cod populations (Jensen *et al.*, 2010). Further, escape episodes of farmed fish may occur without reporting to the fishery authorities occurring. In such cases, molecular genetic methods such as microsatellite DNA analyses have been used to trace the escapees to their farm origin. This approach has been successfully demonstrated in several species, including cod (Glover *et al.*, 2011). However, the method has some limitations for farmed cod, which is less domesticated and therefore more similar to its wild counterpart compared with salmon (Glover *et al.*, 2010, 2011).

Youngson *et al.* (2001) evaluated the situation in Europe with respect to new marine species in aquaculture. They discussed the importance of obtaining detailed information on the population structure of the species in question, and noted that various aquaculture approaches may have different objectives and thus the genetic problems associated must be carefully considered. Utter and Epifanio (2002) reviewed a number of aquaculture models and discussed the potential genetic problems. Their recommendations focused on minimizing adverse genetic effects on natural populations. It is of particular importance here to incorporate genetic knowledge of broodstock, as discussed by Taniguchi (2003). Large negative reduction (40%) in lifetime reproductive success was found in steelhead trout, *Oncorhynchus mykiss*, when subjected to an enhancement program (Araki *et al.*, 2007), while minor genetic changes were detected in a Japanese marine enhancement program for red sea bream, *Pagrus major* (Kitada *et al.*, 2009). In a recent review Araki and Schmid (2010) found clear signs of negative effects such as lower survival and reproductive fitness, and reduced genetic variation in many of the studies conducted on hatchery stocks and their effects on stock enhancement. They also pointed to our limited knowledge of the influence of hatchery fish on wild stocks and the importance of genetic methods in future investigations.

In an early review about the potential use of genetic methods in fishery research, Utter *et al.* (1974) discussed the application of genetic marking of fish populations by manipulating allelle frequencies. These aspects have been evaluated both from a general point of view, but also in connection with aquaculture (Hedgecock *et al.*, 1976) and have been actively used in breeding programmes (Moav *et al.*, 1976). In the last-mentioned work, the purpose was to incorporate a genetic marker in the cultured strain as an internal control. The principles of genetic marking have been evaluated by Gharrett and Seeb (1990), and early work was based mainly on protein variation (Utter and Seeb, 1990). There are, however, relatively few empirical studies that have implemented genetic marking. Some examples have been published, such as Alaskan pink salmon, *Oncorhynchus gorbuscha* (Lane *et al.*, 1990), chum salmon, *Oncorhynchus keta* (Seeb *et al.*, 1990), brown trout, *Salmo trutta trutta* (Taggart and Ferguson, 1984) and mud crab (Obata *et al.*, 2006).

In Norway, a genetically marked cod strain was developed in connection with the large-scale enhancement experiments carried out about 1985–1997 (Svåsand *et al.*, 2000). All the fish in the marked broodstock were homozygous for a rare allele in the polymorphic enzyme phosphoglucose isomerase (*GPI-1*30*) expressed in white muscle (Jørstad *et al.*, 1991). Offspring of this cod have been used in a number of studies, including early larval comparison (Blom *et al.*, 1994; van der Meeren *et al.*, 1994; Kristiansen *et al.*, 1997; van der Meeren and Jørstad, 2001) and several release experiments (Jørstad *et al.*, 1994; Otterå *et al.*, 1999a). The release activities and genetic aspects were evaluated by Jørstad *et al.* (1999) and Jørstad (2004).

As mentioned earlier, the development of Atlantic cod farming recently raised a number of questions connected with interaction between cultured and wild cod populations. For marine fish, spawning in net pens has been indicated (Dimitriou *et al.*, 2007) and recently observed (Somarakis *et al.*, 2013). The re-establishment in 2003–2004 of the genetically marked strain in Atlantic cod (Jørstad *et al.*, 2008) enabled new studies focused on interactions between escaped cod and wild populations. Based on this, successful spawning in cages and spread of viable offspring to the natural environment were documented for cod (Jørstad *et al.*, 2008).

In the present study, we describe a novel large-scale case study where a full-size cod farm raised genetically marked cod under commercial conditions, and where the surrounding area was monitored for several years in order to reveal possible escapes and interbreeding of escaped cod with local wild cod.

Material and methods

The marker allele *GPI-1*30* occurs naturally at very low frequencies (<0.03) in wild cod populations in the Northeast Atlantic (Svåsand *et al.*, 1990; Jørstad *et al.*, 1991; Mork and Giæver, 1999), and this allele is almost exclusively detected as heterozygotes, genotype *GPI-1*30/100* or *GPI-1*30/150*. During the last 30 years, nearly 30 000 wild cod have been screened, and only 2–3 fish have been identified to be of genotype *GPI-1*30/30* (K. Jørstad, unpublished data).

This situation allowed the development of a genetically marked (GM) farmed strain in the 1980s, being homozygous, genotype *GPI-1*30/30*, for this rare allele (Jørstad *et al.*, 1991).

Production of genetically marked juvenile cod

The production of the genetically marked juvenile cod that was used in the present study involved two major steps: establishing a broodstock with the marker, and the production of juveniles from that broodstock. The broodstock was established in 2003, based on wild cod caught in the Øygarden (N60 37 E4 47) and Austevoll (N60 4 E5 13) regions, on the coast of western Norway. These fish were possibly offspring from the large-scale releases of GM cod conducted in the late 1980s and early 1990s in those regions (Jørstad *et al.*, 1994; Otterå *et al.*, 1999b; Svåsand *et al.*, 2000). The first year class of cod possessing the genetic marker reached maturity in 2006, and was first used in a net pen spawning experiment (Jørstad *et al.*, 2008).

The GM juveniles for the current experiment were produced in 2007 and 2008. Two large tanks were used at the research station of the Institute of Marine Research (IMR) in Austevoll (Figure 1), allowing the GM broodstock to spawn naturally. All spawners were tagged by Passive Integrated Transponder (PIT) implants (Trovan Ltd, Germany), and samples were taken for microsatellite analyses. Based on the individual DNA profile for each fish, their family relationships were estimated (Taggart, 2007), and the spawners were sorted into two tanks in order to minimize potential inbreeding between relatives. Fertilized eggs were collected daily during the spawning season, and egg groups with a high fertilization rate and normal morphology were shipped for 3 h to the IMR extensive lagoon facility at Parisvatnet in Øygarden (Figure 1) where they were incubated.

After hatching in mid March, the yolk-sac larvae were carefully released into a large seawater lagoon at IMR-Øygarden (Blom *et al.*, 1991) where they fed on natural zooplankton during the

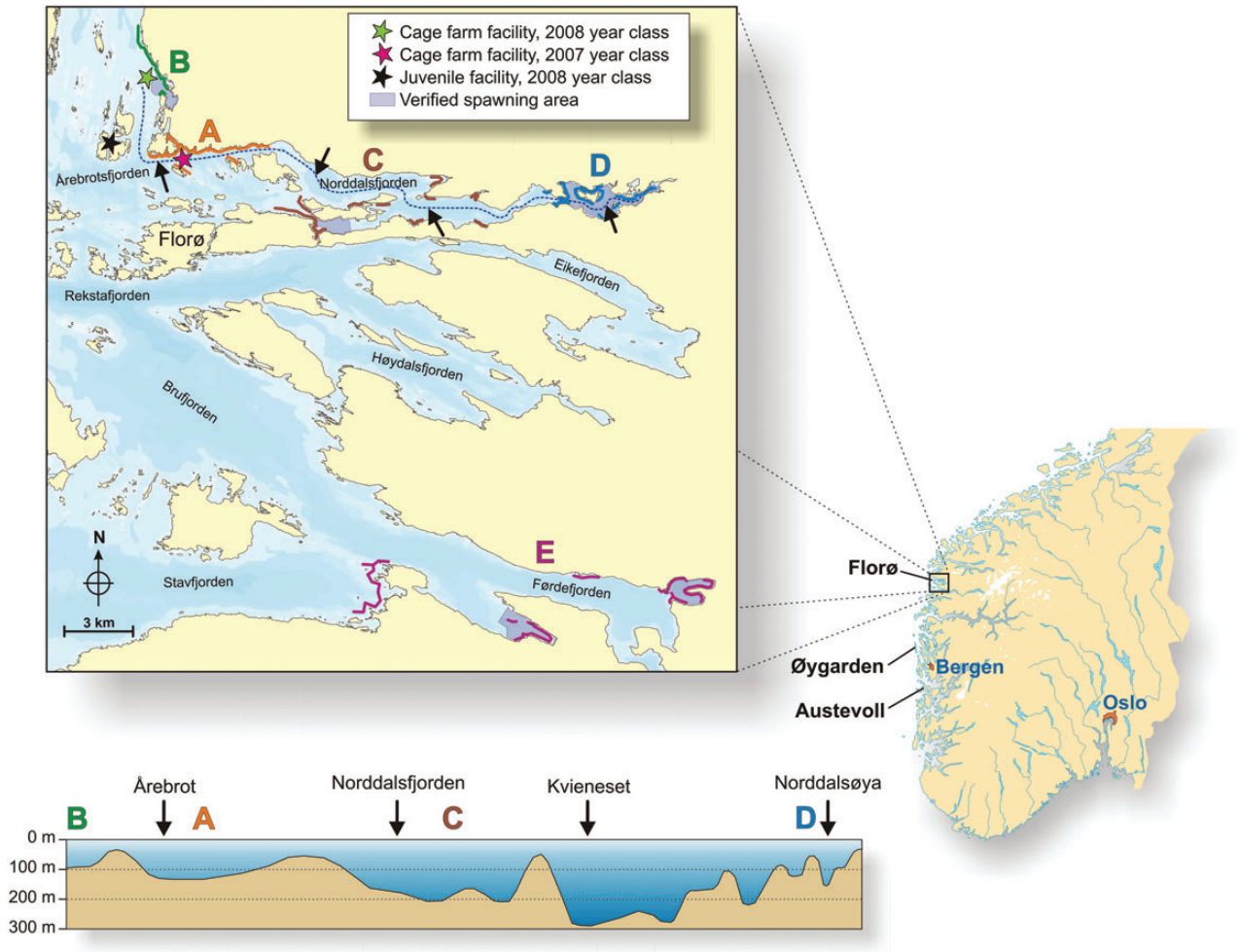

Figure 1. Geographical map of the Florø region with the different monitoring fishing Areas A to E (coloured lines). Lower panel shows depth profile of the main investigation area (Norddalsfjorden) along the dotted blue line in the upper left chart panel. Black arrows are hydrography locations given with DMM positions: Årebrot N61 37.43 E05 00.00, Norddalsfjorden N61 37.64 E05 08.10, Kvieneset N61 37.31 E05 13.44, and Norddalsøya N61 37.93 E05 22.12.

larval period. Reaching the early juvenile stages, commercial formulated feed was added for weaning and replacement of the zooplankton that became very scarce due to heavy grazing by the young cod. The juveniles were harvested during late May and early June by feed attraction over a lift-net system, and kept in net pens with automatic feeding before they were delivered to commercial cod farms in June for ongrowing to marked size. The production cycle for cod is described by Svåsand *et al.* (2004).

Transfer to a commercial cod farm

To be able to monitor potential escapes under realistic conditions the GM juveniles were placed in a commercial cod farm and treated like any other fish in the farm, with no intention of deliberate releases.

The first year class (2007), consisting of approximately 500 000 GM cod juveniles, were transported by well-boat in late June 2007 from Øygarden to the cod farm in the Florø region, 115 km further north (Figure 1). After an acclimatization period the fish were held in two net pens (Figure 1; farming site in Area A) and grown to marked size under strict farming conditions. This year class was kept through the whole production cycle and slaughtered during July 2009.

The second year class (2008), also consisting of 500 000 GM juveniles, were similarly transported one year later. This year class was first placed in a juvenile cod facility (Figure 1) before they were transported to an ongrowing farm facility (Figure 1; farming site in Area B) in July 2009. This farming site was closed down late in 2009, before the fish reached market size. A small sample of fish ($n = 20–40$) was taken from both year classes at various times during the ongrowing period for length and weight measurements (data not reported here).

The farm also had other groups of cod, bought from commercial hatcheries that produced juvenile cod by the intensive method (van der Meeren and Naas, 1997; Svåsand *et al.*, 2004). Artificial light was used in the farm to prevent sexual maturation, although this method would only delay maturation to some extent (Taranger *et al.*, 2006).

Monitoring fishery and detection of cod escapees

As shown in Figure 1, the two farming sites (Areas A and B) are located in the outer part of the larger fjord system, Norddalsfjorden. According to local fishermen there is one spawning site for wild cod in Area B and another spawning site in the bottom of the fjord system, Area D (Figure 1). The fishing approach was therefore to monitor wild cod in four parts of the fjord system, including the

two outer areas (with farming facilities), one middle fjord area (C) and the inner part (D). In the latter period of the study more distant areas were also incorporated into the investigation, as parts of Førdefjorden (south of Florø) were included (Figure 1, Area E) due to a suspicion of escaped farmed cod in that area.

Ideally, three fishing periods were planned for each year. These included collection of cod samples during the spawning season (February–April), and sampling of juveniles and adults in June as well as in October–November. Most of the samples from the spawning season were taken by selected local fishermen (using125–179 mm mesh size nets), while the summer and autumn fishery was carried out by IMR staff using eel traps and trammel nets of 89–125 mm mesh size in shallower waters of the areas, as indicated by the coloured lines in Figure 1.

For most of the captured cod, length, round weight, liver weight and gonad weight was measured, as well as registration of sex, maturation status and parasite load. In addition, various samples like otoliths, white muscle tissue for the genetic marker, and fin or gill tissue for DNA analysis were collected in accordance with the sampling protocol for fish in IMR surveys. For a fraction of the specimens collected by fishermen, only part of these samples (otoliths, white muscle tissue, and gill tissue) could be taken, because only heads along with length measurements, catch location, and catch date were delivered. The white muscle samples were immediately frozen at − 20°C on the research vessel and later analysed on board using starch gel electrophoreses. Escaped farmed cod possessing the genetic marker were identified by their specific gel banding patterns, as described by Jørstad *et al.* (2008), which correspond to homozygotes (*30/30*) at the *GPI-1* locus. Body shape and characteristics were examined for deformities such as those described and reported for intensively farmed cod (Fjelldal *et al.*, 2009). Among others, these included morphological characteristics such as erosion of the first dorsal fin, various curvatures of the spine, neck bending ("stargazer"), pughead, and lower jaw deformities. Cod with these characteristics and without the genetic marker were classified as escaped farmed cod from other cages, and excluded from the data material if not otherwise stated.

Verification of spawning grounds

Egg surveys were carried out during the spawning seasons of 2010 and 2011. Vertical hauls from 40 m depth with an 80 cm diameter Juday net of 375 μm mesh size were used to collect the planktonic cod eggs throughout the fjord systems investigated (Jørstad *et al.*, 2008). The eggs were separated from the plankton by the "spray technique", modified from Eltink (2007). Fine air bubbles were produced by pumping seawater of ∼22 ppt salinity vigorously into the egg separation container through a tiny elongated 15 mm long slit at the end of a PVC tube, before quickly adding the sample with eggs and plankton. Separation took place because the plankton was floating due to attachments of tiny air bubbles, while the eggs were sinking. The eggs were counted and measured under a × 6–40 magnification binocular. Cod eggs were identified by egg diameter (1.2–1.5 mm) and appearance. Areas with an abundance of eggs (>20 eggs per haul) were assigned as "spawning areas".

Sampling and genetic analyses of cod larvae and early juveniles

Escaped farmed cod possessing the genetic marker may spawn and thereby transfer the marker to the wild offspring. In addition to sampling of juveniles, adults and spawners, it was important to also investigate pelagic cod larvae and early juveniles, which were less dispersed geographically than older stages. Young larval cod were collected on several surveys with the boat of a local fisherman (from 1 April to 16 June 2009). The larvae were sampled by means of horizontal tows by the same Juday net used for collection of cod eggs (Jørstad *et al.*, 2008), towed 2–5 m below the surface at 1–2 knots for 10–20 min. Older cod larvae and juveniles were collected on IMR research vessel surveys, applying an MIK plankton trawl in early June 2008, mid June 2009, and late May to early June 2010. In one occasion, newly settled cod juveniles were caught in the shallowest part of an eel trap among the rockweed (*Ascophyllum nodosum*) at 0–2 m depth within the spawning location in Area D (Figure 1). The collected cod larvae were mostly dead on arrival from the Juday net or died quickly after separation from the plankton. The fresh larvae were immediately placed on filter paper strips and frozen, and except for the larger cod larvae and young juveniles the head was removed before putting on the filter paper and frozen. The genetic analyses were carried out as soon as possible, and specimens identified through the specific banding pattern as mentioned above.

Hydrography

As the hydrographical conditions in a fjord system is of importance for the distribution of eggs and larvae of pelagic spawners like cod, hydrographical parameters were monitored during the fishing periods and egg and larval surveys. One to six times a year a CTD-multifunction meter (MINI STD/CTD model SD-204, SAIVAS, Bergen, Norway) with depth (pressure), temperature, conductivity and oxygen sensors was used to collect hydrographical data from surface to bottom at fixed locations (Figure 1) throughout the Norddalsfjorden fjord system.

Statistical analyses

Samples collected from a mixture of individuals from two to several genetically different populations will usually depart from Hardy–Weinberg (HW) genetic equilibrium. For this reason, statistical testing for deviation from HW was carried out, using Fisher's exact test, as implemented in Genpop 4.2 (Raymond and Rousset, 1995).

Results

Hydrography

The Norddalsfjorden fjord system is a 23 km long complex structure with several sills and basins, with the greatest depth being 290 m, and entrance of freshwater from a river system at the most distal part to the east (Figure 1). Figure 2 shows the hydrographical data for 2009, which also are representative for the other years of investigation. The hydrographical data showed seasonal patterns with a more mixed water column at the fjord entrance and a more distinct stratification at the inner parts of the fjord system. Throughout the whole fjord system, temperature increased in the upper 80 m from spring to autumn, while salinity changed in accordance with inflow of water along the bottom from the coastal region, particularly in late May and early June (Figure 2). Thus, the halocline rose from 85 to 35 m depth between 13 May and 16 June 2009 at the fjord entrance, and from 60 to 35 m depth in the most distal part of the fjord. During this same period, the oxygen data revealed the initiation of a complete renewal of the bottom water in the whole fjord system, from almost anoxic conditions at the bottom inside the 50 m sill between fishing Areas A and C to about 50% saturation or more, even at the deepest location, and further at most inner parts (Area E, 154 m deep) of the fjord.

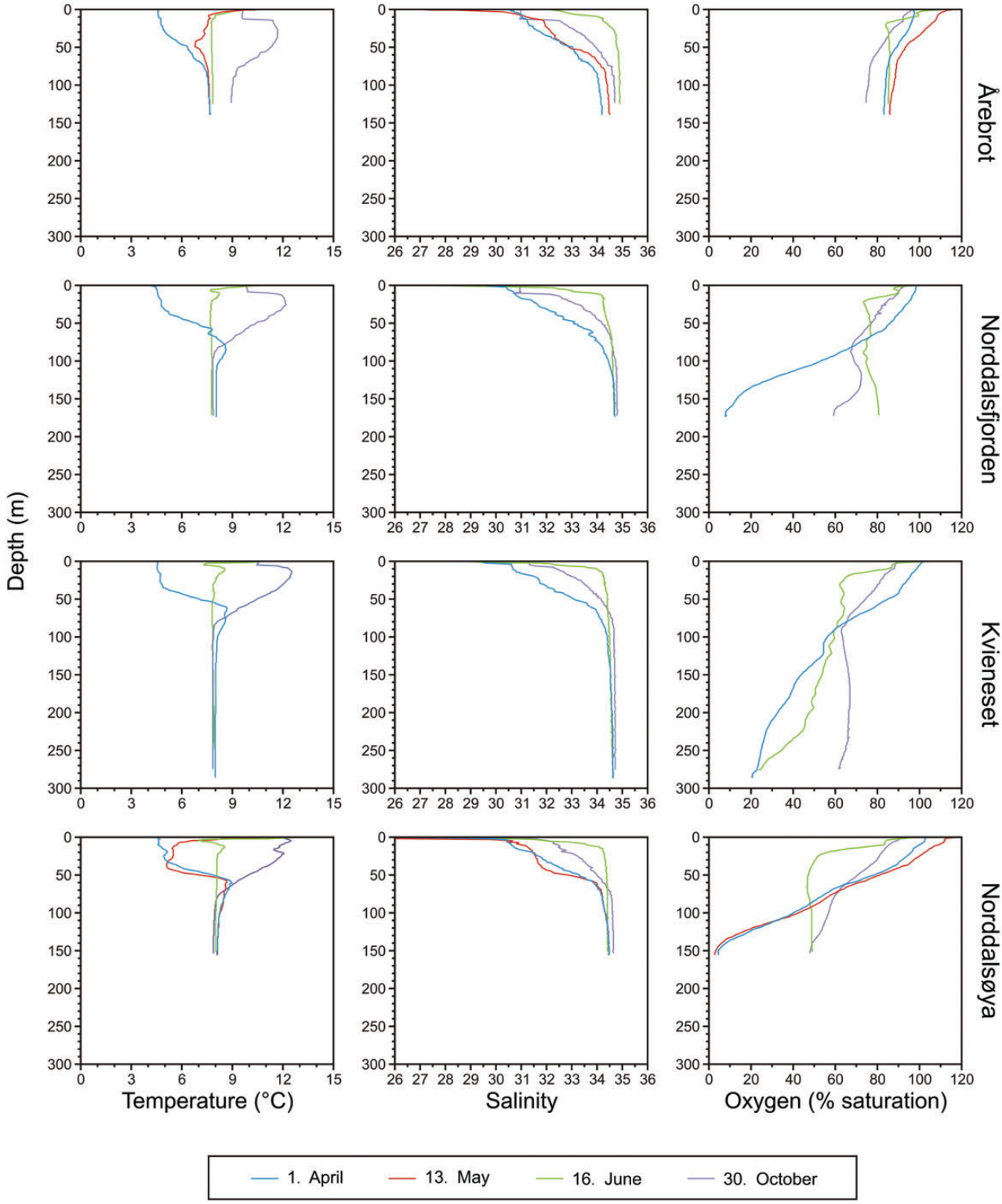

Figure 2. Depth profiles of hydrographical data throughout 2009 at four fixed locations in the study area (see black arrows in Figure. 1).

Detection of escape episodes and geographical spreading

During the experimental fishery 2008–2012 we captured a total of approximately 2900 cod in the Florø area (Figure 1, Areas A–D). The capture was dominated by the younger age classes, particularly 1-, 2- and 3-year-old cod, but age classes from 0–12 years were all represented in the catches (Figure 3).

The genetic analyses of the sample taken before the GM marked cod was introduced to the area (Table 1, A1) confirm the low frequency of the *GPI-1*30* allele here, exclusively found as *GPI-1*30/100* and *GPI-1*30/150*, and this confirms the applicability of this allele as a marker. A similar distribution of *GPI-1* genotypes was found in samples collected around the farming site in

April and June 2008 (Table 1, A2 and A3). Based on morphological characteristics (see MM), however, escaped farmed cod that were not GM were found in high frequencies in all those three samples ($n = 19$, 5 and 48, respectively, not included in the data analysis).

Based on our genetic analysis of the captured cod, and combined with age readings from otoliths, we identified two escape episodes from the 2007 year class (Area A) and one from the 2008 year class (Area B).

The first sign of escaped GM cod were found in our sample taken October 2008 in Area A, where seven cod with the genotype *GPI-1*30/30* were identified among 34 cod (Table 2, sample A4). An additional 104 cod were categorized as escaped cod from other cages or farms not included in the present study, and were therefore excluded from the material. These seven GM cod constituted 35% of the 2007 year class of cod found in that sample, and provide evidence of the first escape episode from farming site A, as indicated in Figure 4. Thus, the GM cod had possibly escaped August–September 2008 and had time to spread over larger areas before actually being detected in October of the same year. Spreading of the fish was confirmed by genetic analyses of the samples collected from both the outer and inner fishing areas (Figure 4; Table 2, B1, D1, D2).

According to the local fishermen, Area A is not a natural spawning site for wild cod. Natural spawning usually takes place in Area B (outer part) and in the bottom of the fjord system (Area D). These spawning grounds were verified from the egg surveys in March 2010 and 2011, which also identified a spawning ground in Area C and two spawning grounds in Area E (Figure 1). In the spawning sites in Areas B and D, cod with the genetic marker was found in the spawning season of 2009, and the frequency of these fish was as high as 26% in the inner spawning site (Table 2, D2), or 100% if only the 2007 year class was included. Since the fishing for cod spawners was mainly carried out in Areas B and D, it was a great surprise for the local fisherman that in early April 2009 he caught lots of equal-sized cod in Area A. Samples of this group were collected, and the genetic analyses demonstrated a second escape episode of the 2007 GM group, separated from the first one by the larger size of the fish (Figure 4). The size of these GM cod corresponds closely to the size measured in the cage in April 2009 (data not shown here). Most of the GM cod captured in April 2009 were sexually mature.

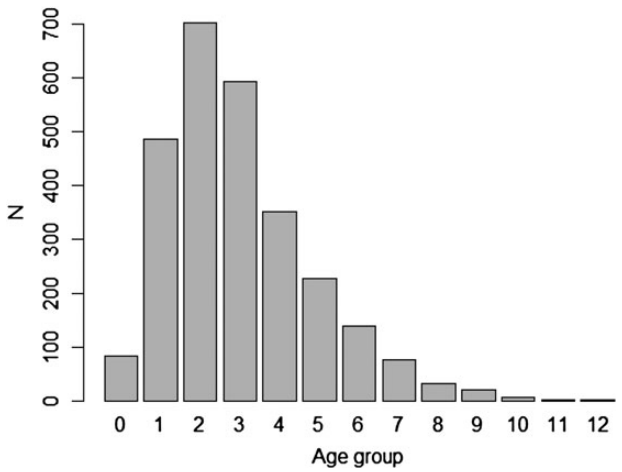

Figure 3. Total number of cod captured in the main monitoring area (A–D) per age group. Age group was determined from otolith readings.

In the fishing surveys of 2009, a spreading of the escaped GM cod was observed, especially in the inner part of the fjord (samples D3 and D4). Only one escaped GM cod was detected in the first part of 2009 in the outer area (sample B1). As pointed out in the M&M section, the 2008 year class of genetically marked cod was transferred to net pens in Area B in July 2009. In October 2009 the fishery in this region caught 41 fish, and 17 (41%) of these were of genotype *GPI-1*30/30* and thus classified as escaped farmed cod from our experiment (B3; Table 1). This identified a third escape episode (Figure 4). This escape involved the 2008 year class of GM farmed cod, as confirmed by the age-readings of otoliths and size compared with the cage measurements.

Fish from the two escape episodes at site A (2007 year class) were found in that area even 3 years after the escapes (Figure 4), and they constituted a large proportion of that specific year class. They were also occasionally found in the other sampling areas for a prolonged period. Contrary to this, the cod from the single escape episode in Area B (2008 year class) were mainly found within that area, with less signs of spreading in time and space (Figure 4). For both year classes, the escaped cod were generally larger than their wild conspecifics (Figure 4), with a tendency to maintain their position in the upper part of the size distribution after escape.

In addition to the escaped GM cod captured in the main study areas (A–D), three escaped GM cod from the 2007 year class were recaptured in Area E (March 2009, 52 cm/1760 g; March 2010, 60 cm/2210 g; March 2011, 54 cm/1800 g). Thus, the escaped GM cod spread 21 km into the fjord system of the main study area (A–D), and 32 km into an adjacent fjord system (Area E).

Spawning success of escaped GM cod

The results from the larval and early juvenile surveys are given in Table 3, and for comparison, two samples of adult fish taken at the spawning grounds in spring 2012 (when the cod born in 2009 should have reached maturation) is also included. As expected, no larvae or juveniles with the genetic marker (*30/30*) were found in 2008. Offspring identified as the *30/30* genotype were, however, detected in late April 2009 (Table 3, sample no. 5) at the inner spawning site and also later in May (no. 6 and 7) and June (no.

Table 1. *GPI-1** genotype distribution, allele frequency of the *30* allele and test for Hardy–Weinberg equilibrium of the adult fish.

		***GPI-1* locus**								
		Genotypes							**allele frequency**	**HW test**
Sample/date	***n***	***30/30***	***30/100***	***30/150***	***70/150***	***100/100***	***100/150***	***150/150***	***30***	***p***
A1 07-02	90	0	8	0	0	38	37	7	0.04	0.28
A2 08-04	54	0	2	1	0	26	23	2	0.03	0.58
A3 08-06	30	0	3	0	0	12	15	0	0.05	0.17

A1–A3 refer to sample numbers in Table 2. Date is given as year-month.

Table 2. Overview of the samples taken in the different areas during the experiment.

Area/No	Date	*n*	*n* GM	Area/No	Date	*n*	*n* GM
A1	07–02	90	0	D1	08–10	104	1
A2	08–04	54	0	D2	09–03	31	8
A3	08–06	30	0	D3	09–06	53	4
A4	08–10	34	7	D4	09–10	80	3
A5	08–04	48	33	D5	10–03	38	0
A6	09–06	56	10	D6	10–10	98	1
A7	09–10	55	6	D7	11–03	30	0
A8	10–03	43	2	D8	11–10	81	1
A9	10–10	55	4	D9	12–03	80	1
A 10	10–11	39	0	D10	12–10	72	0
A11	11–03	39	1				
A12	11–10	65	0	E1	09–03	96	1
A13	12–02	21	1	E2	10–03	52	0
A14	12–10	73	1	E3	10–03	5	1
A15	12–10	141	0	E4	10–03	61	0
				E5	10–06	39	0
B1	09–03	82	1	E6	10–10	32	0
B2	09–06	36	0	E7	10–10	112	0
B3	09–10	41	17	E8	10–10	77	0
B4	10–03	37	0	E9	11–03	200	1
B5	10–04	115	0	E10	11–03	46	0
B6	10–10	9	0	E11	11–03	10	0
B7	11–03	32	0	E12	11–10	62	0
B8	12–03	158	0	E13	11–10	106	0
				E14	11–10	74	1
C1	08–10	36	0				
C2	09–06	34	0				
C3	09–10	80	0				
C4	10–03	32	0				
C5	10–10	108	0				
C6	11–03	44	1				
C7	11–10	89	1				
C8	12–10	163	0				

Samples are numbered chronologically (year-month) within each area (A–E). Total number of cod captured and number of GM cod detected is indicated.

10, 11 and 12) at all spawning grounds. As is also shown in Table 3, there is some variation in the *GPI-1*30* allele frequency between the different samples, and significant deviation from Hardy–Weinberg equilibrium within three of the samples.

Egg surveys carried out in June–August 2007, when the GM cod still were in the cages gave no indication of delayed spawning (data not shown).

Three individuals of *30/30* cod were captured during the latter part of the monitoring program (Table 2, A14, C7, E14). Based on size at capture and otolith readings, these were not escaped GM cod. Two were captured in October 2011 as a result of the 2009 spawning (Area C, 35 cm/335 g and Area E, 46 cm/888 g), and one was captured in October 2012 (Area A, 39 cm/510g) as a result of the spawning in 2010.

Discussion

The present study is the first comprehensive investigation carried out on a marine fish species under realistic commercial farming conditions by using genetically marked individuals. Three unnoticed escape episodes were identified through detecting escaped cod possessing the genetic marker. A relatively short time after the two first escapes, involving the 2007 year class, the fish were detected over the whole fjord system inside the Florø region, including on local spawning sites. Spreading of the escaped cod was also detected in adjacent fjord systems, such as Førdefjorden, providing convincing evidence for movements over longer distances. Thus, all our data suggest that escaped farmed cod can survive and grow in the wild.

Several studies have demonstrated inferior fitness of escaped farmed fish in different species, compared with their wild conspecifics (see *Introduction*). The first data on growth and survival of Atlantic cod in a controlled breeding program were published by Gjerde *et al.* (2004). The Norwegian national breeding programme for Atlantic cod was initiated in 2002 (Bangera *et al.*, 2011), and we should bear in mind that little genetic change is to be expected so far. The broodstock used in this experiment was based on partly domesticated fish; it had been kept in captivity for several generations but without any deliberate selection, and had lived for periods in the wild with subsequent recapture (see Introduction). At this stage of the domestication of cod, therefore, rather small differences in fitness between wild and escaped fish are to be expected.

We observed rather large differences between the two year classes studied (2007 and 2008) in terms of distribution and recapture. The most striking difference between them may be size at the time of escape (40–60 cm vs. 40 cm, respectively), which could explain the higher recapture among the 2007 year class. From the large-scale Norwegian enhancement program of cod (PUSH) carried out in the 1990s, we know that the size of juveniles at release is very important with respect to their survival in the wild and recapture rate (Kristiansen *et al.*, 2000; Svåsand *et al.*, 2000). As demonstrated by Otterå *et al.* (1999a), recapture rates of cod juveniles in the size range 25–30 cm, are estimated to be from 4–6%, while releasing larger-sized juveniles (35–40 cm) increases the recapture rate to ~16%. In a recent behavioural experiment, simulating escape of cod from net pens, very high predation pressure was observed, especially close to the cages and for the smallest cod (Serra-Llinares *et al.*, 2013). Furthermore, Area B (2008 year class) that gave rise to fewer recaptures was closer to the open ocean, suggesting that some of the escaped cod may have migrated to areas outside the monitoring area. Finally, we do not know how many fish escaped each episode, which obviously may explain the differences in numbers recaptured between the two year classes of escaped GM cod.

Indirectly, we can get an idea of the magnitude of the escape episodes by comparing the number of GM cod captured with the number of wild cod captured from the same year class. In several cod enhancement experiments in western Norway, in habitats relatively similar to ours, the number of wild cod per year class was coarsely estimated at 3000–8000 individuals per km^2 as early 1-group (Svåsand *et al.*, 1990; Salvanes and Ulltang, 1992; Otterå *et al.*, 1999b). The total instantaneous rate of mortality (Z) of 1-group cod may be as high as $Z = 2$ per year (Kristiansen *et al.*, 2000), suggesting that the number of wild cod per year class and km^2 at the time of the first escape (third quarter) would be of the magnitude of 700–1800 individuals. The high ratio of GM cod to wild cod (Figure 4) suggests that the number that escaped in the first episode was a few thousand individuals. The local fishermen carried out intensive fishing in the A-region after the second escape episode in April 2009, and claimed that about 2000 mature cod were caught.

The finding of larvae and young pelagic juveniles possessing the *GPI-1*30/30* allele during the spring and early summer of 2009 in large parts of the monitoring area demonstrates that spawning among GM cod had occurred, either in the cages where they stayed until slaughter late 2009, or after escape. In support of the latter possibility is the fact that after escape cod would no longer be under the influence of the continuous light used to prevent

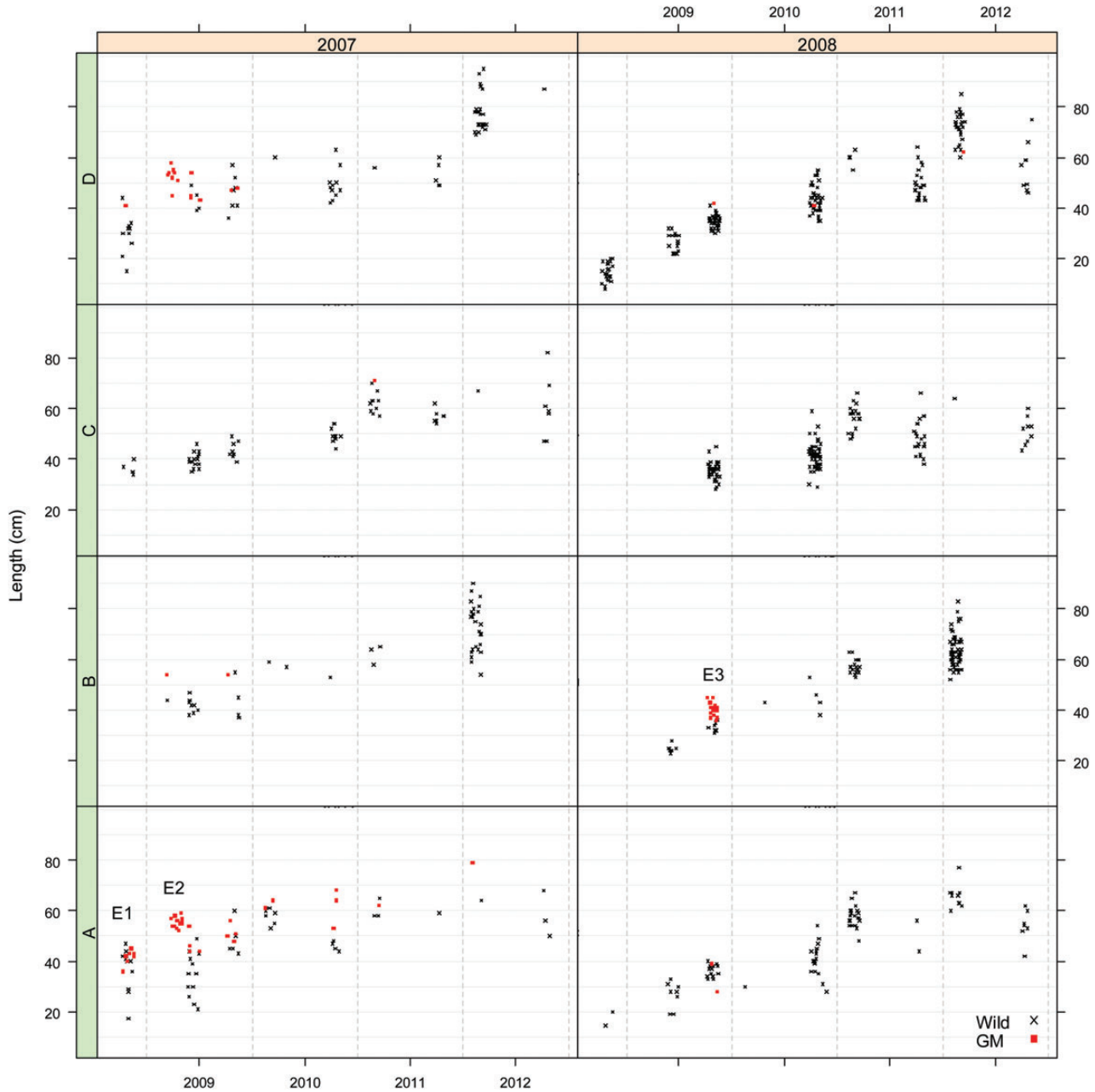

Figure 4. Length of the captured fish (*y*-axis) plotted against time of sampling (*x*-axis). Only the 2007 (left panel) and 2008 year classes (right panel) are included, as backcalculated from the otoliths. Different colours and symbols differentiate between the types of cod captured, escaped or wild. The three escapes identified are noted by E1 – E3 in the graphs. "Jitter" has been added to the *x*-coordinate of the observations to increase readability.

maturation at the farm site. In view of this, it is intriguing that most of the genetically marked larvae and young juveniles were found in the close vicinity of the spawning grounds in Areas C and D, which is at some distance from the farming site. It has previously been reported that farmed cod disperse rapidly after an escape, and further that they may be found among wild cod on the spawning grounds during the spawning season (Uglem *et al.*, 2008). Most larvae were also recovered in the vicinity of the spawning areas, indicating some retention in those areas despite a major exchange of the fjord water during late May and early June of that year. In a modelling study, Myksvoll *et al.* (2011) suggested that cod eggs spawned in a fjord area were likely to be trapped in that fjord. However, although the sampled cod remaining in the farm showed some delay in maturation, maturing individuals were also clearly observed. Cod in aquaculture generally mature at the age of two years (Karlsen *et al.*, 1995) compared with age 3–5 years for wild cod from Western Norway (Svåsand *et al.*, 1990). The use of artificial light in the cages will postpone the maturation for some months, but the method is not 100% effective (Taranger *et al.*, 2006). This implies that spawning in the net pens as previously demonstrated (Jørstad *et al.*, 2008) is another plausible source of the *GPI-1*30/30* larvae observed.

Interbreeding between the GM cod and wild, local cod would produce offspring that would be heterozygous for the *GPI-1* locus

Table 3. *GPI-1** genotype distribution, allele frequency of the *30* allele and test for Hardy–Weinberg equilibrium of larvae and juvenile fish.

			***GPI-1* locus**								
			Genotypes							**allele frequency**	**HW test**
# Area	**Date**	***n***	***30/30***	***30/100***	***30/150***	***70/150***	***100/100***	***100/150***	***150/150***	***30***	***p***
1A–D	08–06	74	0	5	4	0	43	20	2	0.06	0.304
2C	09–04	30	0	0	0	0	14	11	2	0.05	0.848
3D	09–04	23	0	6	0	0	10	5	2	0.13	0.286
4A–B	09–04	63	0	2	0	0	33	25	3	0.02	0.854
5D	09–04	87	1	6	0	0	51	24	5	0.05	0.104
6B	09–05	24	1	1	0	0	13	8	1	0.06	0.114
7D	09–05	185	1	9	1	0	102	63	9	0.03	0.250
8A	09–06	106	0	5	3	0	52	31	15	0.04	**0.046**
9D	09–06	73	0	4	5	0	40	20	4	0.06	0.107
10C	09–06	95	3	5	2	0	48	29	8	0.07	**0.006**
11C	09–06	66	1	4	3	0	35	14	9	0.07	**0.047**
12D	09–06	83	1	8	3	0	35	32	4	0.08	0.569
13C	09–06	39	0	3	1	0	25	9	1	0.05	0.803
14A–D	10–05	52	0	1	1	1	27	16	6	0.02	0.131
Adult fish, spring 2012											
B8		158	0	13	6	1	71	52	15	0.06	0.437
D9		80	1	5	1	0	39	29	5	0.05	0.331

Two samples of adult fish, taken at the spawning grounds spring 2012 are also included. Date is given as year-month.

(*30/100* or *30/150*). This could potentially be observed in the larvae and juvenile data from the spawning season of 2009, but also from the spawning data of spring 2012, when those born in 2009 should be sexual mature. Our data (Table 3) may indicate such an increase in heterozygotes, but is not conclusive due to the high variability and low sample size. The frequency of heterozygotes is higher than those reported by Jørstad *et al.* (1991) but similar to those we observed before the escapes (Table 1).

The finding of *30/30* larvae and young juveniles in late June 2009 suggests that the escaped GM cod spawned later in the season than the wild cod. The GM cod made up nearly 25% of the adult cod captured at spawning site D during the spawning period of 2009 (8 of 34 cod). However, the GM cod were all around 2 kg, while the wild cod ranged in size from 2–12 kg. Thus, there are indications of segregation both in spawning time and size. Potential differences in spawning time between escaped farmed cod and wild cod, due to light manipulation while in the cages (Taranger *et al.*, 2006) or genetic differences (Otterå *et al.*, 2012) may be persistent and cause segregation in spawning for several years. There is also some indication that behaviourally induced segregation between farmed and wild groups of cod may occur (Meager *et al.*, 2010). In another experiment with cod spawning in net pens, a similar indication of late spawning and interbreeding between individuals originating from spawning in net pens has been indicated (van der Meeren *et al.*, 2012).

The finding of three cod with the *30/30* allele late in the monitoring period, which were not GM but born in the wild (2009 and 2010), is most likely a result of interbreeding between escapees, rather than between escapees and wild cod, due to the low occurrence of the *30/30* genotype in the wild. This is a strong indication that genes from farmed cod are carried on in new generations.

Genetic marking of cultured organisms through a selective breeding approach was suggested more than 30 years ago (Utter *et al.*, 1974; Moav *et al.*, 1976; Hedgecock *et al.*, 1976), based mainly on genetic markers detectable by starch gel electrophoresis. There are, however, few empirical studies that have been conducted (see *Introduction*; Utter and Seeb, 1990. In the case of Atlantic cod, 3–4 years were needed to develop a broodstock possessing the genetic marker, and this was a prerequisite for conducting the full-scale cod escapement study. The genetic approach used was based on an allozyme marker, but the rapid development of DNA technology during recent decades has provided a large number of new markers and opportunities. Since commercial breeding programs have been established for cultured species like Atlantic cod and Atlantic salmon, new markers could easily be incorporated to develop diagnostic genetically marked commercial strains.

Concluding remarks

A large-scale and long-term investigation has been carried out to study the escape of genetically marked cod from a commercial farm. The monitoring fishery in the farm region revealed three different escape episodes. The escaped cod were spreading through the total fjord system, constituting a significant part of the actual year classes and were also found in another fjord system south of the farm region. The genetic marker was found in the offspring, providing evidence for successful spawning and recruitment. Cod is a multiple spawner with several year classes contributing to the offspring production. Together with the in- and outflux of cod from other areas, this implies that one or a few such escapes are unlikely to alter the genetic structure of the local cod.

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