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Mating competition between farmed and wild cod *Gadus morhua*

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ABSTRACT: Increasing numbers of hatchery-produced fish entering marine environments has caused concern over potential fitness depressions in wild populations, yet no study has addressed the likelihood of hybridisation between farmed and wild marine fish. Escape rates of Atlantic cod *Gadus morhua* L. from commercial net pens have been substantial and there is a risk of interbreeding between depleted local coastal populations and escapees. We studied mating competition between farmed and wild cod in 2 mixed spawning groups. In addition to detailed behavioural analysis, we examined a suite of individual male characteristics thought to be associated with male reproductive success, including, for the first time in any 'naturally' spawning teleost, sperm motility traits. We found that the expression of reproductive behaviours was similar for both male types (farmed and wild). Males 'courted' both sexes, but courtships lasted longer with a female recipient. Both farmed and wild males also directed most female courtships towards farmed females. The frequency of male displays was linked to their steroid levels. Wild males sired 75% of eggs spawned by wild females, but only 48 to 67% of eggs spawned by farmed females. It is likely that wild females rejected farmed males and chose among the wild males based primarily on behavioural cues. Female choice thus appears to be an integral part of the cod mating system. Sperm traits were not associated with overall reproductive success. Our results suggest that hybridisation between farmed escapees and wild cod is likely and that farmed cod may interfere with the natural spawning behaviour of cod.

KEY WORDS: Genetic introgression · Leks · Female choice · Sperm traits · *Gadus morhua*

INTRODUCTION

Declining wild stocks have provided the impetus for developing mariculture, culture-based fisheries and restocking (Brown & Laland 2001, Pauly et al. 2002). This has led to a worldwide increase in the number of hatchery-produced fish entering marine environments, from both intentional releases and escapes from farms (Born et al. 2004, Naylor et al. 2005). There is a concern about the potential for these farmed fish to impact on the genetic integrity of wild populations, for example by introgression of farmed genes (e.g. Bekkevold et al. 2006). Numerous studies have addressed this issue in anadromous salmonids; generally, farmed males are inferior to their wild counterparts and females are the main vector for farmed genes into wild populations (Fleming et al. 1996, Weir et al. 2004). In contrast, we are aware of no study that has examined the outcome of mating competition between farmed and wild marine fish.

The chance of hybridisation is likely to be linked to the mating system of the species in question. In lekking species, males form localised groups that females visit to choose a mate (Höglund & Alatalo 1996). Although no non-genetic resources are gained, the male reproductive skew is usually very high (Kirkpatrick & Ryan 1991, Neff & Pitcher 2008). In fish, there are comparatively few descriptions of leks. One example

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is African cichlids where males can attract females by building bowers, which females subsequently use to assess males (e.g. McKaye 1983). The batch-spawning Atlantic cod *Gadus morhua* L. also possess a complex lek-like mating system (e.g. Brawn 1961a, Hutchings et al. 1999, Nordeide & Folstad 2000), and are one of the few cultured marine species for which reproductive behaviour has been studied in detail. Males act aggressively towards other males, court females with acoustic and behavioural displays, and broadcast spawning occurs when a female has been ventrally mounted by a male (Brawn 1961a, Hutchings et al. 1999, Rowe & Hutchings 2006). After gamete release by the mated pair, other males commonly rush in and release sperm (Hutchings et al. 1999), imposing sperm competition (Birkhead & Møller 1998).

With declining wild cod populations (Hutchings & Baum 2005), cod farming is now a rapidly developing industry in coastal areas used as spawning sites by cod in the North Atlantic (Kjesbu et al. 2006). The number of escapes from net-pens has, to date, been surprisingly high (Moe et al. 2007), and experimentally released farmed cod are known to navigate to local spawning grounds (Svåsand et al. 1990, Uglem et al. 2008) and join spawning shoals (Meager et al. 2009). In salmonids, hybridisation between escaped farmed and wild fish has resulted in genetic introgression and fitness depression in wild populations (McGinnity et al. 2003, Araki et al. 2007). The highly localised genetic population structure of coastal cod populations (e.g. Sarvas & Fevolden 2005) and possible local adaptation (Olsen et al. 2008) suggests the potential for farmed genotypes to swamp local genetic structure leading to the breakdown of co-adapted gene complexes (Ward 2006, Darwish & Hutchings 2009). It is therefore paramount to assess the likelihood of interbreeding between escapees and wild cod.

Farming and domestication typically affect traits associated with mate choice and reproductive success, such as morphology, physiology and reproductive behaviour (e.g. Huntingford 2004, Jonsson & Jonsson 2006). For example, farmed male Atlantic salmon are outcompeted by wild males, due to wild behavioural dominance and failure of farmed males to release sperm concurrently with female egg release (Fleming et al. 1996, Weir et al. 2004). In captive groups of wild cod, male reproductive success is positively associated with body size and reproductive behaviour (Rowe et al. 2008). Thus, examining the reproductive success of farmed cod in mating competition with wild cod will not only indicate the risk of genetic introgression, but illuminate further the link between male morphology, behaviour and reproductive success in a lekking marine broadcast spawner.

We therefore studied mating interactions between farmed and wild cod in mixed groups. To determine the causal mechanism for the observed patterns of reproductive success, we examined a suite of behavioural, physiological and morphological traits, including, for the first time in any 'naturally' spawning teleost, sperm motility.

MATERIALS AND METHODS

History of fish. All experimental cod originated from local coastal cod caught in the vicinity of Bergen, Norway. Wild coastal cod were caught by hook and line, and fish traps (6 to 20 m depth) in the Herdla-Øygarden area: 51 fish were caught at 60° 29′ N, 4° 53′ E, and 24 fish were caught at 60° 34′ N, 4° 56′ E between November and January 2006. Fish were kept at the site of capture until transfer to the Institute of Marine Research (IMR) facility at Austevoll (60° 05′ N, 5° 15′ E) in late January, where the fish were placed in a 28 m^3 holding tank. Wild cod were fed a mixture of shrimp and fish prior to the experiment.

Farmed F1-generation cod were obtained from a population maintained under commercial conditions typical for farming at IMR. These cod were the progeny of local wild cod again caught at Øygarden (60° 37′ N, 4° 48′ E) and hatched in spring 2003 and 2004. Further details on the pre-experimental protocol are given in Skjæraasen et al. (2008).

Experimental fish. Cod were selected for the experiment on February 20 and 22. Fish were first sedated with metacaine (0.5 g l^{-1}) and then examined with ultrasound to determine the sex of each fish (Karlsen & Holm 1994). All experimental males released milt following the application of gentle pressure on their ventral side. Milt samples were collected into 50 ml vials for subsequent analyses of male sperm traits. Similarly, all experimental females were either running with eggs, or based on the ultrasound image, thought to be close to ovulation. Fish were then measured for total length (±1 cm) and whole body weight (±1 g). In addition, vernier calipers were used to measure pelvic fin length, a secondary sexual characteristic (Skjæraasen et al. 2006), from the base of the pelvic fin to the tip of the longest pelvic fin ray (±0.1 cm). A blood sample was withdrawn from the caudal vein for subsequent measurement of sex steroid levels. All fish were then individually tagged with 2 white, 7 cm Floy T-bar tags following the procedure of Hutchings et al. (1999) and distributed between 2 mating arenas at equal ratios of males to females, and wild to farmed cod. We kept 40 cod in Tank 1 (7 m diameter, 1.4 m depth, volume 53.9 m^3) and 24 cod in Tank 2 (5 m diameter, 1.4 m depth, volume 27.5 m^3). An egg collector skimmed

eggs from the surface waters of each tank. Fish were allowed 1 to 2 d to recover after sampling before the behavioural observations commenced on February 23.

All fish were euthanised by an overdose of anaesthetic on March 26. Drumming muscles (Brawn 1961b) were then removed using forceps and dried at 60°C for 3 d to obtain dry weight to the nearest 0.0001 g. Otoliths were also removed from all wild fish for age determinations. During the course of the experiment, 3 farmed and 2 wild females died in Tank 2 on March 8, 16, 27, 12 and 19, respectively, whereas 1 farmed female died in Tank 1 on March 25 and, unfortunately, was disposed of before a tissue sample was obtained. Only 39 fish were therefore fingerprinted in Tank 1.

Hormonal analyses. Plasma concentrations of the main sex steroids for cod (e.g. Dahle et al. 2003): testosterone (T, both sexes), 11-ketotestosterone (11-KT, males) and 17β-oestradiol (E2, females), were measured by radioimmunoassay following the procedure of Schulz (1985). In brief, steroids were extracted from 200 µl plasma with 4 ml diethylether. The aqueous phase was frozen on dry ice, after which the organic phase was transferred to a glass tube, evaporated in a water bath, and reconstituted with 600 µl assay buffer. Samples were assayed in duplicate.

Sperm analyses. Sperm motility was analysed following the procedure of Rudolfsen et al. (2005). The parameters included in the present study were seawater measurements of: mean average path velocity (VAP, µm s^{-1}), mean straight line velocity (VSL, µm s^{-1}), mean curvilinear velocity (VCL, µm s^{-1}), percentage of motile cells (MOT) and percentage of progressive sperm (PPC, the percentage of all sperm that moved with straightness (STR) > 80 and VAP > 25 µm s^{-1}; STR = VSL/VAP). We also determined the spermatocrit values of each male, as this correlates to sperm concentration in cod milt (Rakitin et al. 1999). To achieve this, milt was collected in duplicate 75 mm capillary tubes. Tubes were sealed with Sigillum wax and spun for 2 min in a haematocrit centrifuge. The spermatocrit value was then determined as the percentage volume sperm cells in relation to total milt volume. Further details are given in Skjæraasen et al. (2009).

Behavioural observations. Both tanks were filmed using overhead CCTV cameras (Panasonic WV BP550), but the large size of Tank 1 prevented us from identifying all 40 fish simultaneously in the entire tank. In Tank 2 we had very good coverage of all 24 fish simultaneously. In this tank, we therefore recorded daily between 10:30 and 18:30 h for 31 d (February 23 to March 25) and subsequently analysed 15 min of every recorded hour in detail by scoring the frequency of reproductive behaviours (Table 1) and the identity of fish involved. Initially, we only recorded on an analogue VHS recorder, whereas from February 4 the signal from the camera in Tank 2 was split and recorded onto both a digital DV recorder and the analogue VCR. For time periods recorded on digital tape, we also recorded behaviour duration.

Egg collection and preservation of larvae. The egg collectors were emptied around 10:00 h daily and eggs were put into measuring columns and allowed 20 min to settle into a floating and sinking fraction. The volume of each fraction was then noted and a subsample of 100 eggs from each fraction was examined under a microscope to back-calculate approximate spawning times based on cell stages (e.g. Kjesbu 1989) and the total ratio of fertilised eggs. In both tanks, ~80% of the eggs had been fertilised within the previous 24 h (i.e. <256 cells), with the remaining being no more than 48 h old (i.e. 256 cells).

Table 1. *Gadus morhua*. Behaviours scored in the present study

Behaviour	Source	Operational definition in present study
Courtship		
Ventral mount	Brawn (1961a)	A cod slides down the side of another cod, ending up stomach to stomach with genital pores close together
Paired swim	Brawn (1961a)	The cod are <1 body length apart and the movement involves ≤2 alterations of direction (circles) and/or ends in attempted ventral mount
Circling	Hutchings et al. (1999)	A cod rests on the bottom, while another cod swims in circles above
Lateral display	Nilsson (2004)	A cod approaches another cod and freezes in mid-water, flexing its pectoral and pelvic fins. The behaviour lasts a minimum of 2 s, and the focal cod is <1 body length away from the target cod
Aggression		
Approach	Hutchings et al. (1999)	A cod approaches another cod at a speed of >2 body lengths s^{-1}, ending up >1 body length away from the target
Chase	Brawn (1961a)	A cod approaches another cod at a speed of >2 body lengths s^{-1}, ending up <1 body length away from the target
Prod	Hutchings et al. (1999)	A cod swims into the side of another cod, making contact with its snout
Nip/bite	Hutchings et al. (1999)	A cod bites or attempts to bite another cod

Every day, ~300 live eggs were taken from the floating fraction and pipetted into two 0.4 l seawater filled containers. These containers were left overnight in a constant-temperature cabinet maintained at 10°C, and then floating eggs were siphoned out and put in a new container until hatching. Dead eggs were siphoned out daily and water exchanged every third day to avoid bacterial growth. After hatching, larvae <24 h post-hatch were preserved in ethanol for later pedigree analyses.

Parentage analyses. DNA was extracted from whole larvae. We added a mixture of 3 µl of Proteinase K (20 mg ml^{-1}) and 100 µl of a 10% Chelex solution (Tris, 50 mM EDTA, 2% SDS) to each larva and left the mixture overnight at 55°C. The proteinase was then inactivated by putting the mixture at 95°C for 15 min. The number of genotyped offspring from each day averaged 36.1 ± 12.5 (SD) (range: 10 to 93) across tanks. All parents were first genotyped twice with available microsatellites to ensure correct genotyping (error rate 0.79%). Then, a set of microsatellites was chosen based on a predictive analysis in FAP 3.6 (Family Assignment Program) (Taggart 2007). In Tank 2, larvae were analysed with 8 microsatellites: Gmo2, Gmo3, Gmo8, Gmo19, Gmo35, Gmo37, Gmo132 and Tch11 (Brooker et al. 1994, Miller et al. 2000, O'Reilly et al. 2000). Two additional microsatellites (Gmo16 and Gmo18; Wesmajervi et al. 2007) were used in Tank 1 because of the larger number of possible parental combinations (380) compared to Tank 2 (144). All loci were amplified in 3 (Tank 2) or 4 (Tank 1) multiplexes, run on an ABI3730 sequencer, and alleles were determined with the Genemapper program (Applied Biosystems). Due to the nature of PCR amplifications, i.e. occasionally no fragments produced, ~7% of the offspring could not be assigned to a specific family in either tank. The putative parental genotypes were simulated with FAP 3.6 to predict the resolving power of the specific parental data set for unambiguous discriminating among families. For Tank 1 the predicted assignment was 81.2%, with no significant difference between the possible family combinations (308), and for Tank 2 the predicted assignment was 100%. Thus, there was no indication that the probability of identifying both parents depended on the dyadic combination, i.e. F♂ W♀ (farmed female, wild male), F♂ F♀, etc., in either tank. In total, 71.3% ($n_{identified}$ = 1040) and 93.0% ($n_{identified}$ = 910) of the genotyped larvae could be identified to both parents in Tanks 1 and 2, respectively. The non-fingerprinted fish in Tank 1 would thus have very little, if any, effect on the number of offspring identified to both parents. Offspring for which the pedigree could not be unambiguously established were excluded from further analyses.

Data analyses. ***Behavioural displays of farmed and wild cod:*** We explored potential differences in reproductive behaviour between fish types from the detailed individual observations obtained in Tank 2. We first examined if there was a difference between farmed and wild males in (1) the ratio of aggressive to courtship behaviours and (2) the proportion of aggressive displays towards males of the other type, i.e. we sought to discover if there was a difference between which fish type wild and farmed males directed their aggressive displays towards and if this ratio differed from a random distribution. We also examined if the ratio of ventral mounts and paired swims directed towards females differed between male types — both Brawn (1961a) and Rowe & Hutchings (2006) found that males 'court' both males and females — and if the observed ratio was different from a random distribution of displays towards males and females. We compared the proportion of experimental days farmed and wild females received courtships with a *t*-test. All ratios were converted to proportions and then normalised by an arcsine transformation before conducting the analyses. Each data point was weighted by the total number of individual displays relevant to each test.

We then examined if there was a difference in the duration of paired swims and ventral mounts (Table 1) between farmed and wild males and whether this depended on recipient sex. For paired swims this was done with a linear mixed-effects model (LME, nlme library of R v. 2.9.2; R Development Core Team), where the duration of paired swims was the dependent variable, and initiator type and recipient sex plus their interaction were modelled as categorical variables. Repeated measures for the initiator were treated as a random effect. As we had scored fewer durations for ventral mounts, we only included the main effects for this analysis. For both models, we used a hierarchical approach and log-likelihood ratios to arrive at the most parsimonious model.

Finally, we examined if male steroid levels were correlated to initiated displays, using linear regressions where the initial models allowed farmed and wild males to have different intercepts and slope values. All values were log-transformed and simplified as described in the previous paragraph.

Reproductive success: We tested if there was a difference in reproductive success between wild and farmed males and whether this depended on female type. We first calculated the total volume of fertilised eggs for a given day in each tank. From the DNA fingerprinting analyses we then calculated the fraction of eggs sired by a given male-female combination. This fraction was then multiplied by the volume of fertilised eggs for the day in question and then summed for individual males and females for the entire experimental period. We assumed that all fertilised eggs had a similar probability of hatching. We then used a LME model

where the response variable was the volume of eggs sired by a given male with (1) wild and (2) farmed females. Male and female type and their interaction were treated as fixed effects. A nested random-blocks effect accounted for variation associated with differences between individual males in each tank and between tanks.

Correlates of reproductive success: We examined if male behavioural (Tank 2 only), physiological, morphological and sperm traits were correlated to male reproductive success. The response variable was the arcsine-transformed proportion of eggs sired by individual males. The independent variable was either male weight, length, residual condition (i.e. the residuals from a ln-transformed regression of male length against body mass), steroid levels, residual pelvic fin length (i.e. the residuals from a ln-transformed regression of male length against pelvic fin length), and residual drumming muscle mass (i.e. the residuals from a ln-transformed regression of body mass against drumming muscle mass). Initially female type, i.e. farmed or wild, was allowed to affect both the intercept and slope of the LME regression. Repeated measures for individual males were treated as a random effect. Analyses were performed separately for farmed and wild males in each tank. Log-likelihood ratios were used to arrive at the final model.

RESULTS

Fish sizes were matched as closely as possible (Tables 2 & 3). In Tank 1, weight did not significantly differ between farmed or wild fish for either males or females (Student's *t*-test: df = 18, all p-values > 0.13) (Tables 2 & 3). However, female wild cod were longer than female farmed cod ($t = -4.24$, df = 18, $p < 0.001$) (Table 2) and wild males were slightly, but significantly,

Table 2. *Gadus morhua*. Female cod data. 1FW1 denotes Tank 1, wild female 1; 2FF1 denotes Tank 2, farmed female 1; etc. T: testosterone concentration; E2: 17β-oestradiol concentration; Drum dry: drumming muscle dry weight; Court rec.: no of courtships received; Volume spawned: the calculated volume spawned that led to hatchlings (i.e. the ratio of fingerprinted larvae from an individual female for a given sampling day multiplied by the volume of fertilised eggs for the day in question and then summed up for the total experimental period). Data on pelvic fin length and drumming muscle mass have previously been used in Skjæraasen et al. (2008). –: data not determined or missing

Fish	Total length (cm)	Pelvic fin length (cm)	Total weight (g)	T (ng ml^{-1})	E2 (ng ml^{-1})	Age (yr)	Drum dry (g)	Court rec. (n)	Volume spawned (ml)
1FW1	62	6.7	3072	1.3	1.7	4	0.2808	–	2108
1FW2	63	7.2	2144	2.1	1.6	5	0.1451	–	466
1FW3	60	6.0	2143	1.6	2.3	3	0.1802	–	1933
1FW4	61	6.9	1936	1.9	2.7	4	0.0971	–	687
1FW5	58	6.0	2320	2.8	5.9	4	0.1065	–	3222
1FW6	57	5.6	1923	2.5	5.3	4	0.1366	–	2056
1FW7	55	6.2	1956	2.1	7.5	3	0.2437	–	1686
1FW8	65	6.9	2476	1.8	3.3	4	0.2352	–	2455
1FW9	61	6.6	2132	3.7	3.8	4	0.1589		4810
1FW10	65	6.5	3204	1.5	3.2	4	0.2144	–	3964
1FF1	53	5.1	2478	2.1	7.7	2	0.028	–	461
1FF2	58	5.5	2920	1.8	9.2	2	0.169	–	302
1FF3	56	4.6	2542	2.3	5.7	2	0.0171	–	870
1FF4	52	5.1	2322	2.2	8.6	2	0.0812	–	896
1FF5	56	5.6	2846	1.9	11.1	2	–	–	–
1FF6	54	5.5	2520	1.5	7.7	2	0.0474	–	1181
1FF7	57	5.2	2933	1.7	10.5	2	0.0198	–	2532
1FF8	59	6.2	3846	2.2	7.3	2	0.2028	–	48
1FF9	55	5.3	2356	2.3	7.9	2	0.0484	–	357
1FF10	52	4.1	1976	1.6	9.9	2	0.0503	–	516
2FW1	66	7.0	3158	1.5	4.2	4	0.2458	2	613
2FW2	63	6.3	2640	1.1	5.6	4	0.1342	6	2942
2FW3	64	6.3	2890	1.3	4.8	4	0.304	17	1995
2FW4	65	7.6	2976	1.5	6.9	5	–	18	775
2FW5	67	6.2	3198	1.3	4.8	4	0.1496	12	232
2FW6	63	6.7	2938	1.2	3.5	4	0.224	9	4210
2FF1	67	6.2	4262	1.2	4.1	3	0.2518	14	1687
2FF2	65	5.3	3528	1.5	5.7	3	0.2329	36	111
2FF3	66	7.2	4114	1	3	3	0.0786	37	1195
2FF4	66	5.9	3938	1.2	3.9	3	–	72	765
2FF5	64	5.7	3970	1.7	10.1	3	0.124	51	1048
2FF6	63	5.4	3152	1.3	1.6	3	0.2102	51	914

Table 3. *Gadus morhua*. Male cod data. 1MF1 denotes Tank 1, farmed male 1; 2MW1 denotes Tank 2, wild male 1; etc. T: testosterone concentration; 11-KT: 11-ketotestosterone concentration; VCL: curvilinear velocity; MOT: percentage of motile cells; PPC: percentage of progressive cells; Scrit: spermatocrit (sperm density); Sp exp: spawning experience; Rec: recruit spawner; Rep: repeat spawner; Drum dry: drumming muscle dry weight; A Ini.: no. of aggressions initiated; FC Ini.: no. of courtships directed at females; MC Ini.: no. of courtships directed at males; Volume sired: egg volume sired (i.e. the ratio of fingerprinted larvae sired by an individual male for a given sampling day multiplied by the volume of fertilised eggs for the day in question and then summed up for the total experimental period). Data on pelvic fin length and drumming muscle mass have also been used in Skjæraasen et al. (2008, 2009). –: data not determined or missing

Fish	Total length (cm)	Pelvic fin length (cm)	Total weight (g)	T (ng ml^{-1})	11-KT (ng ml^{-1})	VCL (µm s^{-1})	MOT (%)	PPC (%)	Scrit (%)	Age (yr)	Sp exp	Drum dry (g)	A Ini. (n)	FC Ini. (n)	MC Ini. (n)	Volume sired (ml)
1MF1	55.5	5.6	2158	7.8	14	111.8	71.5	63	22.5	2	Rec	0.2566	–	–	–	1584
1MF2	54	5.9	2090	4	8.5	98.4	82.5	67.5	18.4	2	Rec	0.4475	–	–	–	1030
1MF3	53	5.9	2042	7.3	17.2	95.4	54.5	43.5	10.1	2	Rec	0.2274	–	–	–	1773
1MF4	52	5.4	1901	5.4	5.9	99.9	79	61	41.4	2	Rec	0.1767	–	–	–	803
1MF5	56	5.4	2262	4.1	13.4	104.4	83.5	61	12.7	2	Rec	0.043	–	–	–	789
1MF6	55	5.6	2118	4.8	6.1	75.5	56	44.5	18.0	2	Rec	0.2289	–	–	–	699
1MF7	55	5.8	1733	3.8	8.6	94.9	52	47	4.8	2	Rec	0.2102	–	–	–	136
1MF8	54	5.8	1914	4.7	6	100.8	57.5	49	15.0	2	Rec	0.1284	–	–	–	1202
1MF9	54	5.9	2047	3.3	4.1	66.6	16.5	14.5	15.0	2	Rec	0.3371	–	–	–	369
1MF10	52	5.1	2074	7	8.7	84.5	44.5	41.5	11.3	2	Rec	0.2251	–	–	–	947
1MW1	54	5.3	1665	3.3	8	104.7	64	56	11.8	3	Rec	0.2508	–	–	–	3789
1MW2	51	6.4	1682	3.7	6.5	104.6	88	80	7.9	4	Rep	0.1311	–	–	–	1671
1MW3	54	6.6	1592	3.5	7.6	115.65	91	69	28.9	4	Rep	0.2728	–	–	–	1074
1MW4	61	7.2	2256	6.8	19.4	105.6	72	65	30.0	4	Rep	0.2195	–	–	–	887
1MW5	58	6.7	2210	11.6	34.2	101.5	88	73.5	20.8	4	Rep	0.2712	–	–	–	3132
1MW6	60	6.6	2108	4.8	10.5	107	92	78	26.4	4	Rec	0.3443	–	–	–	3780
1MW7	59	7.0	2034	8.6	22.9	103.3	78.5	68	40.2	4	Rep	0.2531	–	–	–	1488
1MW8	55	5.5	1676	5.7	17.5	103.3	89.5	81	24.8	5	Rec	0.2804	–	–	–	2414
1MW9	60	6.4	2280	10.8	25	112.6	95.5	74.5	43.5	4	Rec	0.6577	–	–	–	1003
1MW10	63	7.8	2374	3.4	5.2	106.3	95	80	11.9	4	Rec	0.4458	–	–	–	523
2MW1	68	7.6	3418	1.7	3.2	102.7	92.5	69.5	87.8	4	Rec	0.7521	11	4	26	825
2MW2	64	7.6	2798	6.3	13.2	80.6	85.5	73.5	89.2	3	Rec	0.526	2	33	64	2511
2MW3	62	6.0	2650	4	9.9	121.3	96	75.5	25.6	3	Rec	0.3765	1	29	28	904
2MW4	66	7.1	3400	4.9	10.1	130.4	93.5	64.5	61.4	4	Rep	0.4521	17	12	53	2331
2MW5	63	7.6	2604	3.5	5.4	87.5	84	73.5	21.8	5	Rep	0.5015	2	14	11	2130
2MW6	66	8.1	2826	3.3	7.5	112.7	93	75.5	52.0	4	Rec	0.6915	1	81	24	3232
2MF1	62	6.1	3170	7.3	12.2	102.9	93.5	75.5	3.8	3	Rep	0.5903	9	28	68	160
2MF2	65	6.3	3170	3.1	4	92.2	93.5	84.5	42.5	3	Rep	0.5114	33	18	10	1332
2MF3	63	5.7	3208	6.4	13.5	70	60	45.5	11.7	3	Rep	0.7662	110	77	246	1122
2MF4	67	6.4	3466	4.1	6.8	116	98	69.5	41.7	3	Rep	0.7231	3	12	18	447
2MF5	59	5.9	2630	5.2	6.3	104.3	95	77.5	21.7	3	Rep	0.5545	78	41	43	650
2MF6	67	6.1	3402	4.3	5.1	103.9	85.5	71.5	26.8	3	Rep	1.0885	1	28	53	843

longer than farmed males ($t = -2.65$, df = 18, p = 0.016) (Table 3). In Tank 2, neither males nor females differed significantly in length (df = 10, p > 0.54) (Tables 2 & 3). Males were of similar weight ($t = 0.27$, df = 10, p = 0.27) (Table 3), but farmed females were significantly heavier than wild females ($t = 4.60$, df = 10, p = 0.001) (Table 2). Farmed cod had significantly higher condition than wild cod in both tanks (*t*-tests on Fulton's *K*, all p-values < 0.001). Macroscopic gonad examination revealed that all cod were nearly spent or well into their spawning period at sacrifice. In Tank 1, farmed females had higher plasma levels of E2 than wild females ($t = 6.031$, df = 18, p < 0.001) (Table 2). All other hormonal levels were similar between farmed and wild fish of either sex in both tanks (all p-values > 0.14) (Tables 2 & 3).

Reproductive behaviour of farmed and wild cod

There was no difference in the total amount of displays initiated by farmed or wild males ($t = 1.26$, df = 10, p = 0.24) (Table 3). We found no difference between farmed and wild males in any of the variables tested, i.e. the ratio of aggressions to courtships, to which type of males aggression was directed, the proportion of ventral mounts and paired swims directed at females compared to males, and to which type of female males directed their courtships (all p-values > 0.063). Interestingly, the proportion of paired swims and ventral mounts directed at females was less than expected based on a random distribution (*t*-tests, Tank 2: both p-values < 0.01) (Fig. 1A). Males also

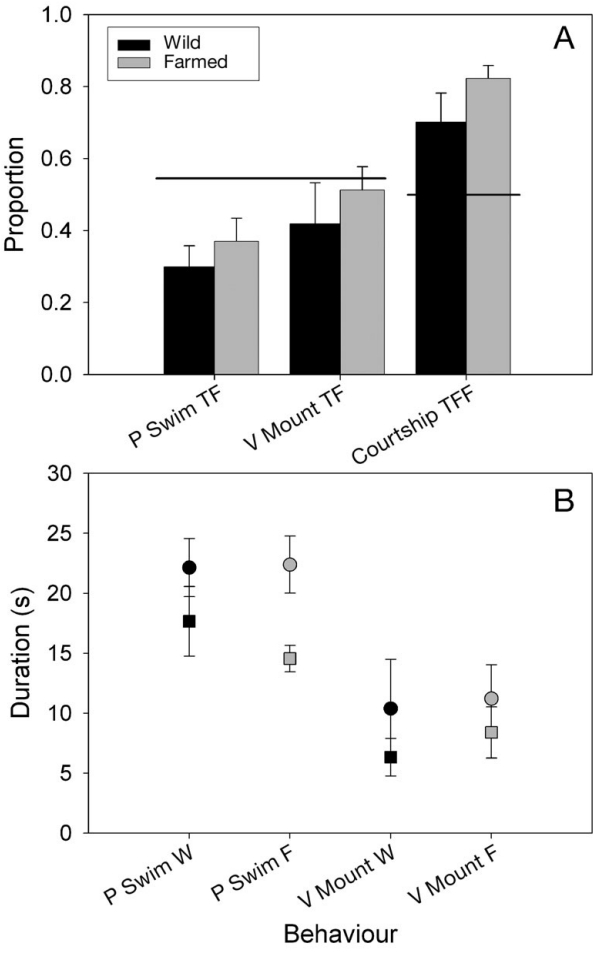

Fig. 1. *Gadus morhua*. Behavioural patterns of farmed (grey) and wild (black) males in Tank 2. (A) Proportion of the total number of observations for the behaviour in question. P Swim: paired swim, V Mount: ventral mount, TF: towards females, TFF: towards farmed females. Horizontal line above or through bars depicts the expected value for the behaviour in question if it was distributed randomly against the potential target fish. (B) Duration of paired swims and ventral mounts initiated by wild (W) and farmed (F) males towards female (●,○) and male (■,□) recipients. Error bars = SE in average duration between different males

directed significantly more courtships towards farmed females (*t*-test, Tank 2: $t = 6.82$, df = 11, $p < 0.001$) (Fig. 1A) and courted these females on proportionally more days than wild females (Tank 2: average proportion 0.62 vs. 0.27; *t*-test: df = 10, $t = 2.94$, $p < 0.05$) (data not shown). Males initiated 99% of all aggressions (n = 302), and where both fish could be identified (n = 260) the recipient was male in 99.6% of the cases.

Paired swims lasted longer when the recipient was a female than a male (LME model: df = 295, $t = -2.47$, $p < 0.05$; n = 308) (Fig. 1B), which was the only significant explanatory variable of paired swim duration. A similar non-significant tendency was observed for the fewer recordings of ventral mounts (n = 112). Male steroid values around the onset of spawning tended to be positively associated with the number of displays initiated (simple regressions: Tank 2: df = 10; T: $t = 2.41$, $p < 0.05$; 11-KT: $t = 2.21$, $p = 0.052$) (Fig. 2). Male type did not influence the intercept or the slope of these regressions (Tank 2: df = 8, all p-values > 0.16).

Reproductive success

All cod were identified in the pedigree analyses (Tables 2 & 3). More genotyped eggs were spawned by wild than farmed females (LME model: df = 30, $t = 4.58$, $p < 0.001$) (Fig. 3). Overall, wild males sired more eggs than farmed males (df = 29, $t = 3.74$, $p < 0.001$) (Fig. 3), but there was also a significant interaction between male and female type (df = 30, $t = 3.31$; $p < 0.001$). Wild males sired around 75% of the eggs released by wild females in both tanks, whereas the corresponding number for farmed females varied between 48 and 67% (Fig. 3).

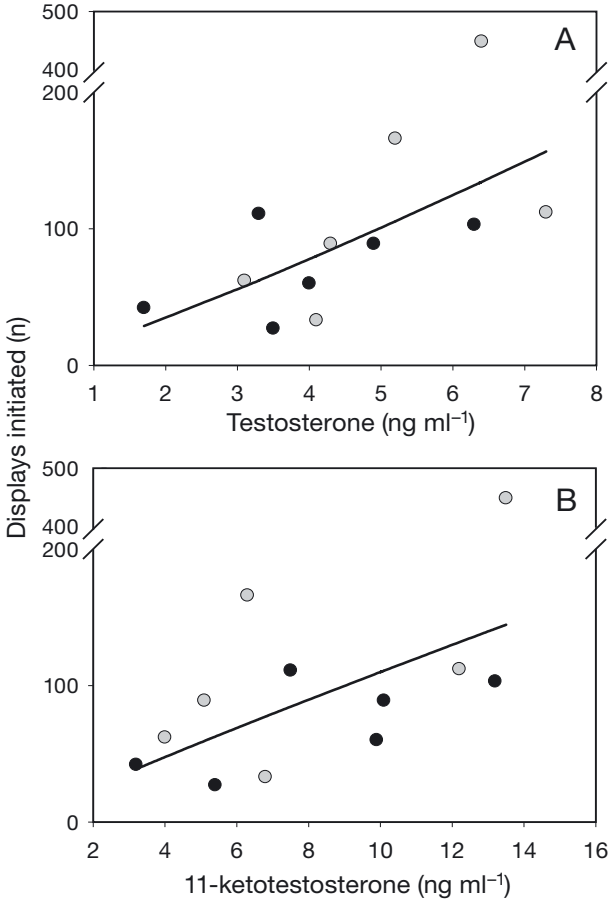

Fig. 2. *Gadus morhua*. Relationship between the number of displays initiated and (A) testosterone and (B) 11-ketotestosterone concentrations for wild (●) and farmed (○) males. Lines are the fitted regression lines

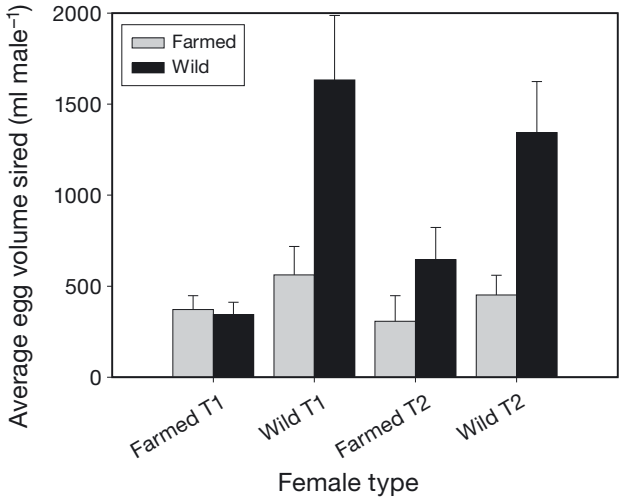

Fig. 3. *Gadus morhua*. Average male reproductive success given as the average volume of eggs fertilised by wild (black bar) and farmed (grey bar) males, in Tanks 1 (T1) and 2 (T2), separated into eggs spawned by farmed and wild females. Error bars: + SE

Correlates of reproductive success

No correlate of male reproductive success was found for either male type in Tank 1. In Tank 2 the relationship between displays initiated and reproductive success for wild males was strongly influenced by a single wild fish, which had a sired a relatively high proportion of eggs compared to the number of displays initiated. When we excluded this fish from the analyses there was a clear positive association for displays initiated and male reproductive success for wild males (df = 3, $t = 4.56$, $p < 0.05$) (Fig. 4). The interactive effect between MOT and female type also came out significant in Tank 2 (df = 4, $t = -3.98$, $p < 0.05$), but this was caused by a tendency for male MOT to be weakly positively correlated with reproductive success with farmed females and negatively correlated with reproductive success with wild females. Looking at the regression lines separately, neither relationship was significant ($r^2 = 0.09$, $p = 0.55$ and $r^2 = 0.48$, $p = 0.13$) (data not shown). No other association between sperm traits and reproductive success was found in either tank.

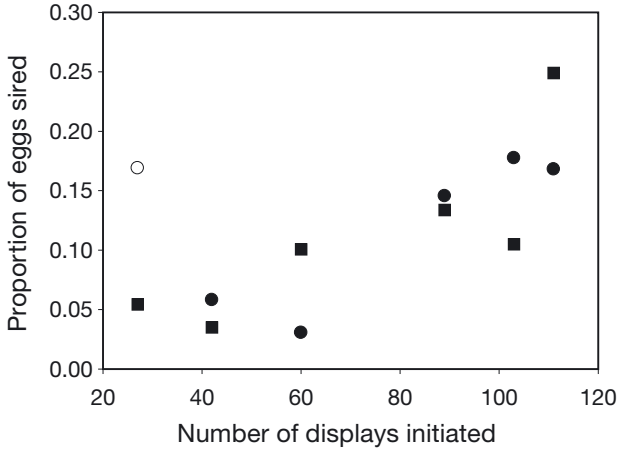

Fig. 4. *Gadus morhua*. Number of displays initiated by wild males and their relative reproductive success with wild (●) and farmed (■) females in Tank 2. The 'behavioural outlier' (○) is the fish that was excluded in the final analysis

DISCUSSION

Our results provide compelling evidence for the risk of hybridisation between fish that have spent their entire lives in captivity and their wild marine counterparts. We also shed light on the complexity of the cod mating system through examining reproductive success in relation to simultaneous measurements of morphology, physiology and sperm traits in addition to detailed analysis of behaviour.

Reproductive behaviour of farmed and wild males and females

This is, to our knowledge, the first study to investigate the outcome of mating competition between farmed and wild fish in a marine broadcast spawner. Earlier studies have primarily focused on salmonids that have a long history of domestication (see Huntingford 2004). Farmed male salmonids generally do poorly in spawning competition with wild males; they do not establish proper dominance hierarchies and they court females in bigger groups and fail to release sperm upon female egg release (Fleming et al. 1996, Weir et al. 2004). In contrast, the behaviour of farmed and wild male cod appeared similar in terms of the absolute numbers of reproductive behaviours initiated, the relative proportion of aggressions and courtships, whom they directed their displays towards and the duration of specific behaviours (Fig. 1). As we observed no spawnings where we could identify all individuals involved, we cannot comment on the possibility of differential sperm release by farmed and wild males.

The higher rates of courtships received by farmed females may either be linked to male preference or female behaviour. Although we cannot distinguish between these explanations, it is worth noting that in a recent field study the behaviour of wild females suggested that they avoided male spawning aggregations during the interval between spawning, whereas farmed females regularly visited the male aggregation (Meager et al. 2009, 2010).

Interestingly, males in the present study directed significantly more 'courtship' towards males than fe-

males (Fig. 1). We suggest that males court fish in their immediate vicinity. Within our tanks males were mainly associated with the bottom, whereas females stayed closer to the surface (Meager et al. 2009). This situation is likely reciprocated in the field (Nordeide & Folstad 2000, Meager et al. 2009). Male-male courtships in the *Oreochromis mossambicus* cichlid likely occur because of low sex discrimination by territorial fish (Oliveira & Almada 1998). As aggression only occurred between males in the present study, male cod clearly can distinguish between the sexes. In hissing cockroach *Gromphadorhina portentosa*, a positive relationship between courtships directed at males and females in a male-male and male-female context, termed 'libido', was positively associated with reproductive success (Logue et al. 2009). This 'libido' syndrome coupled with sexual selection favouring high courtship intensity was suggested to explain the persistence of male-male courtships. Similar mechanisms may apply for cod. The generally longer duration of male-female courtships suggest that male recipients break off courtships faster than females or that initiator males are more persistent with a female recipient. Given the limited number of males in Tank 2, we encourage further studies on the reproductive behaviour of farmed and wild cod.

The positive association between steroid levels and the frequency of initiated displays is a new result for cod, but is commonly observed in other teleosts (Borg 1994, Rudolfsen et al. 2006). Folstad & Karter (1992) suggested that increased hormonal levels suppress immune function, and the link to male displays may therefore function as an honest signal of male quality. This link has not been unequivocally demonstrated in fish, but indirect support for this theory exists (Kurtz et al. 2007).

Reproductive success of farmed and wild cod and the risk of genetic introgression

The present study demonstrates that both male and female farmed cod interbreed with their wild counterparts in mixed spawning shoals. Numerous hybridisations occurred in both tanks through both farmed sexes, although wild males had overall higher reproductive success than farmed males, and farmed females produced fewer offspring than wild females (Fig. 3). The latter result is likely caused by the commonly observed lower fertilisation success in farmed females (Ø. Karlsen pers. obs.) and not a mismatch in the spawning period as farmed female offspring were identified throughout the study period in both tanks.

Whether or not hybridisation occurs in nature following a sea-pen escape depends on the ability of farmed cod to (1) navigate to spawning grounds and (2) enter the spawning shoal. While earlier studies have established (1) in a number of regions (e.g. Svåsand et al. 1990, Uglem et al. 2008), the results of a recent study suggest that farmed females but not farmed males enter spawning shoals (Meager et al. 2009, 2010). Field telemetry of the same putative population as we used in the present study indicated that farmed males were less likely to remain on the spawning ground, and those that did maintained positions above the spawning shoal (Meager et al. 2009, 2010).

Collectively, these results indicate that farmed females are the main vector for the introduction of farmed genes into wild populations, which is the same sexual bias in gene flow as found in salmon (Fleming et al. 1996, 2000, Weir et al. 2004). Nevertheless, the reproductive success of farmed males over the longer term is unclear, and we encourage further studies on other farmed and wild cod populations to test the generality of our results.

We have calculated reproductive success under the assumption that all fertilised eggs had equal probability of hatching. In reality, maternal and paternal effects may cause differential survival of early embryonic stages (e.g. Trippel et al. 2005). If, for example, crosses between farmed males and females consistently had lower survival pre-hatching than other type combinations, this could cause the patterns of reproductive success we observe, rather than behavioural mechanisms and female choice. We have no *a priori* reason to believe this is the case, but to test for this possibility it would be beneficial if future studies on reproductive interactions between farmed and wild cod estimate reproductive success both at the earliest egg stage possible and at later larval stages.

Interbreeding between artificially selected and wild organisms typically results in offspring with reduced fitness (Ellstrand 2003, Hails & Morley 2005). For Atlantic salmon, reduced fitness in wild populations has been documented even at low levels of interbreeding (McGinnity et al. 2003). Even when using local broodstock, the genotype of farmed and wild fish rapidly diverges because of founder effects and genetic drift (Bekkevold et al. 2006). At the time of writing, >500 commercial cod-farming licenses have been issued in Norway, representing a total production capacity of 300 000 t yr^{-1}. In 2008, 304 000 cod escapees were reported in Norway (Norwegian Directorate of Fisheries 2010). Interactions between farmed and wild stocks are expected to increase with continued expansion of the industry and with it the potential for negative impacts on the local coastal cod populations (Bekkevold et al. 2006, Jørstad et al. 2008, Uglem et al. 2008). The spawning stock biomass of the Norwegian Coastal cod population north of 62° N is estimated to be only 57 000 t in 2007. The negative effects of inter-

breeding and genetic introgression could thus be substantial in the near future. Maximum effort should therefore be focused on preventing escapes.

The cod mating system

Our results indicate a major element of female choice in the natural cod mating system. If male reproductive success was purely mediated through some male trait, like behaviour or sperm motility, we would expect the same patterns of male reproductive success regardless of female type. Instead, wild females reproduced principally with wild males, whereas farmed females spawned more or less equally with both male types (Fig. 3). This complexity concurs with previous studies (Hutchings et al. 1999, Rowe et al. 2007, 2008). In comparison, farmed and wild salmonids generally show wild male dominance, but the same patterns of reproductive success regardless of female origin (Fleming et al. 1996, Weir et al. 2004). As both male cod types behaved similarly, a plausible mechanism is that wild females rejected the farmed males based on their physical appearance, i.e. higher condition and their smaller pelvic fins (see Skjæraasen et al. 2008) or potentially, unrecorded cues such as pheromones, when choosing among the wild males based on their behaviour (Fig. 4). Whether the ultimate cause of this is phenotypic plasticity or genetic differences between farmed and wild fish is beyond the scope of the present study. Behaviour and fish size are the main determinants of reproductive success in wild cod populations (Rowe et al. 2008). The low size variation within our groups likely explains the lack of the latter association (see Bekkevold 2006). Age and spawning experience may also increase male reproductive success (Liley et al. 2002). Given that farmed fish grow faster and mature at earlier age than wild fish, it is not possible to match fish by size and age without introducing substantial biases, i.e. by starving farmed fish. Arguably, this could thus have relevance to our study. Nevertheless, repeat-spawning farmed males in Tank 2 did not do better than the recruit spawners in Tank 1.

This is, to our knowledge, the first study to examine the power of sperm traits as explanatory variables of male reproductive success in a freely breeding teleost. Using sperm collected from the males in Tanks 1 and 2 we earlier found that the main determinant of *in vitro* sperm competition was VCL in seawater, which positively correlated to male reproductive success (Skjæraasen et al. 2009). The same has been found in domestic fowl *Gallus gallus* (Birkhead et al. 1999) as well as other externally fertilising species (Gage et al. 2004, Casselman et al. 2006). In the present study, sperm traits were not associated with reproductive success. This does not mean that sperm competition is absent given the frequently observed multiple paternity in single batches (e.g. Bekkevold et al. 2002 and the present study). Sperm traits may also change during the spawning season (Rakitin et al. 1999) and can be modified with female presence (Gasparini et al. 2009). Factors like ejaculate volume, although impossible to measure in freely spawning fish, are likely also important. Nevertheless, while numerous studies have inferred the importance of sperm traits from *in vitro* competition only, ours is the first to assess the importance of sperm traits for reproductive success in freely spawning fish. In agreement with the distinct reproductive behaviours observed in cod spawning groups (Brawn 1961a, Hutchings et al. 1999), overall male reproductive success was not associated with sperm motility.

Concluding remarks

The present study simulates the very real scenario of male and female escapee cod entering a natural spawning ground (Svåsand et al. 1990). In this context, we showed that both male and female farmed cod are likely to hybridise with wild fish. Together with the results from earlier field work (Meager et al. 2009, 2010), the present results indicated that introgression of farmed genes into wild gene pools is likely, particularly from farmed females. Even so, gene flow is not the only risk imposed by farmed fish to wild stocks (Bekkevold et al. 2006). In fishes with complex mating systems such as cod, influx of large numbers of escapees on spawning grounds may interfere with the natural mating system. The propensity of wild males to court farmed over wild females indicates that this is a salient risk. The ecological and genetic consequences of farmed fish in marine environments should be a priority for future work.

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