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Wild Atlantic cod sperm motility is negatively affected by ovarian fluid of farmed females

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ABSTRACT: Atlantic cod escape from fish farms at higher rates than commonly cultured marine species, and escapees have been observed to interact with wild fish in mating aggregations. Previous research suggests that potential interbreeding is mediated largely by the likelihood of wild males spawning with escaped females, and as such, the egg and ovarian fluid characteristics of these females could affect fertilization success and the likelihood of hybridization. Farmed cod have been noted to have poor egg quality compared to wild individuals, and some of this difference may be due to the ovarian fluid, which can affect key sperm-motility parameters related to fertilization success. We tested the hypothesis that the ovarian fluid of farmed females negatively affects the sperm performance of wild males. Sperm-motility parameters and fertilization capacity of wild male sperm were analyzed in the presence of both farmed and wild female ovarian fluid. Sperm performed similarly in the presence of wild female ovarian fluid and a seawater control. Ovarian fluid of farmed females negatively affected sperm swimming and the capacity to fertilize eggs. These differences may be related to nutritional deficiencies of farmed individuals. Although it has been demonstrated that wild males actively court farmed females, our results indicate that their ovarian fluid quality can inhibit fertilization success.

KEY WORDS: Aquaculture escapes · *Gadus morhua* · Interbreeding · Sperm performance · Maternal effects · Egg quality · Hybridization

INTRODUCTION

Aquaculture escapes represent a threat to both the genetic integrity and ecology of wild fish populations. In the relatively well-studied Atlantic salmon *Salmo salar*, most escape events are related to structural failures of equipment, either due to human error or harsh weather conditions (Jensen et al. 2010). In Atlantic cod *Gadus morhua*, the same problems exist, but exploratory behaviour and net biting are important additional drivers of escape events (Moe et al. 2007, Damsgård et al. 2012, Zimmermann et al. 2012), contributing to 10- to 20-fold greater rates of escape than observed in salmonids (Moe et al. 2007). Unmeasured escapes of cod embryos resulting from spawning within sea cages contribute even further to escape rates (see references within Puckrin et al. 2013).

Aquaculture strains often differ genetically from conspecific populations of wild fish that live around farms due to 3 mechanisms. First, although farmed fish are derived from wild populations, they undergo intentional and unintentional selection in captivity that leads to domestication and potentially maladaptation to the wild. Second, cultured strains are usually derived from populations foreign to those locally adapted to the farm area. Finally, reduced genetic diversity among individuals can arise due to

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founder effects, small effective population sizes and genetic drift (see Bekkevold et al. 2006, Hutchings & Fraser 2008 for reviews and Pampoulie et al. 2006, Glover et al. 2010, 2011 for examples in cod). In this context, one of the main ecological concerns is that escapee fish hybridize with wild populations leading to introgression of farmed genetic material into the wild pool (as observed in some Norwegian Atlantic salmon populations by Glover et al. 2012, 2013), potentially lowering wild stock fitness and/or causing the extinction of the original wild genotypes (reviewed by Fleming 1995, Weir & Grant 2005, Ferguson et al. 2007, Hutchings & Fraser 2008; see Araki et al. 2009 for a study in a fish population). As an example, in Atlantic salmon, interbreeding and competition between farmed and wild individuals causes lower fitness and productivity of wild populations (Fleming et al. 2000, McGinnity et al. 2003), even though this effect seems to be family specific (Skaala et al. 2012). In Atlantic cod, several populations are under a threat of extinction (COSEWIC 2010, Hutchings & Rangeley 2011) and may be particularly sensitive.

Telemetry studies of simulated Atlantic cod escapes have indicated that farmed fish distribute over large areas (Serra-Llinares et al. 2013), often matching the habitat of their wild counterparts (Zimmermann et al. 2013) and, most worryingly, mixing with wild fish on the spawning grounds (Uglem et al. 2008). In addition, genetic markers demonstrate that escaped cod are able to spawn in the wild and contribute to recruitment (Jørstad et al. 2014). At spawning sites, however, farmed males have been observed to occupy different positions in the water column relative to wild cod (Meager et al. 2009, 2010) and are more submissive when in direct contests (Sverdrup et al. 2011). These behaviours, along with lower quality sperm in farmed cod (Skjæraasen et al. 2009), likely limit reproductive success during sperm competition with wild males and reduce the likelihood of introgression. Farmed females, however, readily enter spawning aggregations (Meager et al. 2009, 2010) and interact with males indiscriminately of the males' wild or farm origin (Meager et al. 2010). Moreover, Skjæraasen et al. (2010) observed a higher rate of courtship by wild males toward farmed than wild females. Thus, spawning between farmed females and wild males may be an important route of introgression.

Sperm swimming characteristics, such as velocity and straightness, are known to influence fertilization success in several species (e.g. Beirão et al. 2011), including Atlantic cod (Rudolfsen et al. 2008, Skjæraasen et al. 2009). Ovarian fluid surrounds externally spawned eggs and can affect sperm swimming (Turner & Montgomerie 2002, Elofsson et al. 2003, Rosengrave et al. 2009a, Diogo et al. 2010, Kanuga et al. 2012). After sperm ejaculation, this fluid provides close contact between gametes, reducing dispersion and maintaining an ionic concentration that creates an enriched and stabilized fertilization microenvironment (Lahnsteiner 2002, Rosengrave et al. 2009b). The characteristics of ovarian fluid that have the most effect on sperm swimming seem to be pH and the inorganic and protein composition (Lahnsteiner 2002, Elofsson et al. 2006, Wojtczak et al. 2007, Rosengrave et al. 2009b). In Atlantic cod, sperm velocity and longevity were observed to be influenced positively by ovarian fluid (Litvak & Trippel 1998), and Rakitin et al. (1999a) suggested that ovarian fluid characteristics could be one of the reasons for differential male fertilization success. Rudolfsen et al. (2005) observed a significant female × male interaction on offspring survival, and because they did not measure fertilization success, they suggested that the ovarian fluid could be one of the causes underlying this interaction.

The eggs of farmed cod have poorer fertilization success than those of wild fish (Salze et al. 2005). We hypothesized that some of this effect is due to differences in ovarian fluid composition and its effect on sperm performance. Such a mechanism could reduce the possibility of hybridization between wild males and escaped farm females. To test this hypothesis, we compared the percentage of sperm that were motile, the swimming characteristics of motile cells and the fertilization capacity of sperm from wild males in the presence of ovarian fluid from either wild or farmed females, originating from the same population, using a split-ejaculate design.

MATERIALS AND METHODS

Fish origin

Wild Atlantic cod (mean total length ± SEM; males = 65.4 ± 3.4 cm, females = 70.0 ± 4.7 cm, n = 44, sex ratio unknown because it was not possible to determine the sex of 28 individuals) were captured in December 2011 in Smith Sound, Trinity Bay, Newfoundland, and transported to the Ocean Sciences Centre at Memorial University, where they were housed in an indoor flow-through tank (21.3 m^3). The farmed females (64.2 ± 0.6 cm) were spawned in 2008 from fish captured in Smith Sound, cultured until 1 yr

of age in a hatchery and then raised in sea cages in southern Newfoundland on a diet of commercial pellets. Four months prior to the beginning of the experiment, individuals were transported (December 2011) to the Ocean Sciences Centre and placed in a separate indoor flow-through tank (25.5 m^3) together with males of the same origin and other farmed fish (171 fish in total in a male:female sex ratio of 1.3:1). We were required to keep farmed and wild fish in separate tanks as a quarantine procedure of the facility. Both wild and farmed individuals were fed a forage diet (herring *Clupea harengus*, mackerel *Scomber scombrus* and squid *Illex* spp.) to mimic the natural diet that fish might experience after escape. Tanks were provided with aeration, and temperature remained at 5.3 ± 0.1°C; water exchange was of 27% h^{-1} and 15% h^{-1} for the farmed and wild tanks, respectively. Conditions followed protocol 12-09-IF approved by the Memorial University Animal Care Committee and followed the regulations of the Canadian Council on Animal Care.

Experiment 1: sperm motility parameters in farmed vs. wild ovarian fluid

Split-ejaculate experimental design

Semen samples from 6 wild males were assessed for percentage of motile cells (%MOT) and swimming characteristics of the motile cells in the presence of ovarian fluid from 5 farmed and 4 wild females in a full factorial design. Each ovarian fluid was tested after pre-dilution at 2 concentrations (5 and 25%) in seawater (6 males × 9 females × 2 concentrations). We used sperm activated in the presence of seawater without ovarian fluid as a control.

Sperm and ovarian fluid sampling

Gametes were collected when the fish were naturally spawning in the tank from randomly selected individuals that released gametes when gentle pressure was applied to the abdomen. Eggs were extruded directly into plastic beakers and kept on ice until arrival at the laboratory (<2 h). Eggs (between 28 and 180 ml) were poured onto a 1 mm mesh to collect drained ovarian fluid (10 to 20% of the total egg volume), which was then centrifuged at 5000 × *g* at 2°C for 10 min to remove suspended particles that could interfere with the sperm analyses. Because it was not possible to collect all the sperm and ovarian fluid samples on the same day, the ovarian fluid samples were collected between the middle of May and beginning of June and stored individually at −80°C until use. Each ovarian fluid sample was stored in at least five 1.5 ml microcentrifuge tubes to avoid the need to defrost and re-freeze the samples. The sperm samples were collected in June after all the ovarian fluids. For sperm sampling, the urogenital papilla was carefully cleaned and dried, and the semen was collected with the help of a syringe after applying slight pressure on the male's abdomen. The first 1 ml of ejaculate was discarded to avoid seawater, urine and faecal contamination. Samples were kept in 2 ml syringes at 3 to 6°C until arrival to the laboratory (<2 h).

Motility analyses

As cod sperm are greatly affected by temperature (Purchase et al. 2010), the percentage of motile sperm and swimming characteristics of motile cells were analyzed at a controlled 9°C within 10 h of collection following the protocol described by Beirão et al. (2014). The seawater (31.7 psu and 964 mOsm kg^{-1}) was filtered and UV sterilized. A sample of 1 µl of pre-diluted semen (1:20 in a non-activating solution [2/3 freshwater and 1/3 seawater]) was activated with 15 µl of seawater plus 5 or 25% ovarian fluid in a single well (8 mm) of a 10 well multitest slide (MP Biomedicals) and immediately covered with a coverslip. Videos were captured at 100 fps and were analyzed at 10 s post-activation with the ImageJ (http://rsb.info.nih.gov/ij/) CASA (computer assisted sperm analysis) plugin developed by Wilson-Leedy & Ingermann (2007) and modified by Purchase & Earle (2012), available at www.ucs.mun.ca/~cfpurchase/CASA_automated-files.zip. Input parameters were chosen so the software could differentiate drifting from motile sperm and are described in Table S1 in the Supplement at www.int-res.com/articles/suppl/q005p061_supp.pdf. In addition to percentage motility (%MOT), the following sperm swimming characteristics obtained with the CASA plugin were analyzed: VCL (curvilinear velocity, velocity according to the actual path; µm s^{-1}), VSL (straight line velocity, velocity according to the straight path, i.e. displacement; µm s^{-1}), VAP (velocity according to the smoothed path; µm s^{-1}), WOB (wobble; % calculated from VAP/VCL) and LIN (linearity; % calculated from VSL/VAP). Each semen sample was tested 3 times in each ovarian fluid, and the replicates were averaged.

Ovarian fluid analyses

Ovarian fluid osmolality was analyzed with a model 3320 Osmometer (Advanced Instruments) and pH with a multiparameter meter (Accumet XL50). Total protein concentration was measured with the DC Protein Assay (Bio-Rad) in a plate reader (Powerwave XS, Biotek), setting the absorbance at 750 nm; bovine albumin was used as the standard. The proportion of proteins based on their molecular masses was determined by SDS-PAGE electrophoresis. Ovarian fluid was pre-diluted (5- or 10-fold depending on the initial protein concentration), mixed with Laemmli buffer 1:3 and loaded into the wells together with a protein ladder (Prestained Protein Marker, Broad Range [7 to 175 kDa], BioLabs). Gel (10 × 8 cm) electrophoresis was conducted for 85 min at 100 V in a vertical electrophoresis system (MGV-402, CBS Scientific). Gels were then stained with 0.25% Coomassie Blue for 2 h. The molecular weights of the protein bands were estimated by interpolation based on the known weights of the protein ladder. The proportion of detected proteins of each mass was calculated based on the sum of all protein bands. Ovarian fluid ionic composition (Na^+, K^+ and Ca^{2+}) was measured by flame photometry (model PFP7, Jenway), using propane as fuel. Each sample was measured 3 times, and a calibration curve was run after every 10 measurements.

Data analyses

Statistical tests were conducted using R 2.15.1 (R Development Core Team 2012). Sperm motility parameters (%MOT and swimming characteristics of motile cells) were first standardized to the seawater control of that male (with a value of 0 if the behaviour in ovarian fluid and seawater were identical). Differences between the treatments in terms of %MOT were analyzed with repeated-measures ANOVA considering male as a random factor exposed to different conditions (ovarian fluid origin and concentration), using the 'aov' function of the R stats package and after normalizing the percentile data through arcsine transformation. The 5 swimming velocity and straightness parameters of motile cells were reduced by principal component analysis to 1 parameter (denoted 'PC') using the 'prcomp' function of the R stats package and calculated from the covariance matrix, which explained 76% of the variation and is described in Table S2 in the Supplement. As for %MOT, differences in PC among treatments were also analyzed with a repeated-measures ANOVA. Both ANOVAs were first analyzed as saturated models, and then when appropriate, the models were reanalyzed with non-significant interactions removed. As we were also interested in potential differences in individuals' responses, we included the interactions between male ID and the fixed factors. In both ANOVAs, there was a significant interaction between the ovarian fluid origin and male ID. In the PC model, there was also a significant interaction between ovarian fluid origin and concentration. The ANOVA models were thus simplified post hoc by creating 1 ANOVA for each male. For biological interpretation and to understand how the spermatozoa behave in the presence of the ovarian fluids, we visually inspected 2 sperm swimming characteristics, one related to velocity (VAP) and significantly correlated with VCL, VSL and wobble (Pearson correlation r at least 0.73, $n = 108$, $p < 0.0001$) and another related to path straightness (linearity).

Differences between the control (sperm activated with seawater without ovarian fluid, standardized as 0 for each male in each case) and each of the 4 treatment combinations (ovarian fluid origin and concentration) were analyzed with 4 one-sample *t*-tests using the standardized values of the %MOT, VAP and linearity and considering the 6 males × 4–5 ovarian fluids as replicates (n = 24 or 30).

Differences in the pH, osmolality, ion concentration (Na^+, K^+ and Ca^{2+}), total protein concentration and protein bands between the wild and the farmed ovarian fluids were analyzed with 2 MANOVAs, one for the ion-related parameters (pH, osmolality and concentration of ions) and another for the protein parameters (total protein concentration and the protein bands) using the 'manova' function of the R stats package. For all the statistical analyses, values were considered significantly different at $p < 0.05$. Numeric results are expressed as means ± SEM. We checked the underlying assumptions of our parametric tests with the R stats package with the 'bartlett.test' function for homogeneity of variances and the 'shapiro.test' for residual normality.

Experiment 2: sperm fertilization capacity in farmed vs. wild ovarian fluid

Experimental design

This experiment was performed on 2 different days, using 2 batches of eggs, 1 each day, from 2 different wild females. For each batch, we used sperm

from 2 different males (4 males in total) and conducted each treatment in duplicate (Fig. 1). For each egg batch, the ovarian fluid was removed according to the procedure described in the first experiment, and the eggs were rinsed with 2 ml of Cortland solution (124.1 mM NaCl, 5.1 mM KCl, 1.0 mM $MgSO_4 \times 7H_2O$, 1.6 mM $CaCl_2 \times 2H_2O$ and 5.6 mM glucose) adapted for fish eggs following Goetz & Coffman (2000). Broods of eggs with ovarian fluid removed were split into 24 groups (2 males × 6 treatments × 2 replicates) of 500 µl and placed in 50 mm petri dishes above ice packs. For each group, the ovarian fluid was replaced by 100 µl of one of the following solutions: (1) artificial Cortland solution, (2) source female (egg donor female) ovarian fluid to control for negative effects of the ovarian fluid removal procedure, (3 and 4) ovarian fluid of 2 different wild females and (5 and 6) ovarian fluid of 2 different farmed females (Fig. 1). The ovarian fluid removal technique did not have an effect, as was also the case in a study in salmonids applying a similar fluid replacement technique (Yeates et al. 2013). As sperm concentration affects fertilization success in cod (Rakitin et al. 1999a), sample concentration was checked with a Neubaur counting chamber, and the semen volume from each male was adjusted. To each group of eggs, a volume corresponding to 1×10^5 spermatozoa per egg was added and carefully mixed. This low concentration was chosen because at higher densities variation in sperm motility does not affect fertilization success (Rakitin et al. 1999b). Then, 10 ml of seawater was added to the egg/sperm mixture to activate sperm and left for 45 s. This short resting time was chosen because an increase in sperm–egg contact time can increase the percentage of fertilization in cod (Butts et al. 2009), and we wanted to limit fertilization success to allow comparisons among samples. After 45 s, eggs were rinsed with seawater through a 1 mm mesh to remove excess sperm and placed in 90 mm petri dishes with 25 ml of seawater at 6°C for incubation. The next day, 15 to 20 h into incubation, we evaluated fertilization success by counting the number of unfertilized and fertilized eggs (32- and 64-cell stage).

Data analysis

Statistical tests were conducted using R 2.15.1 (R Development Core Team 2012). Fertilization success was normalized through arcsine transformation. Variation in success in the presence of the different ovarian fluid origins (fixed factor) was analyzed with a mixed model ANOVA, where each male (random factor) was considered nested inside of an egg batch. The ANOVA was first analyzed as a saturated model. Also, in this case, we included interactions between male ID and the fixed factors. In no instances were there significant interactions between ovarian fluid origin, batch and male ID, and therefore, the model was reanalyzed with the interactions removed. Differences were considered significant for $p < 0.05$. Duncan's test was used for multiple comparisons to detect differences between ovarian fluid treatments using the 'duncan.test' function from the 'agricolae' R package (de Mendiburu 2012). Numeric results are expressed as means ± SEM.

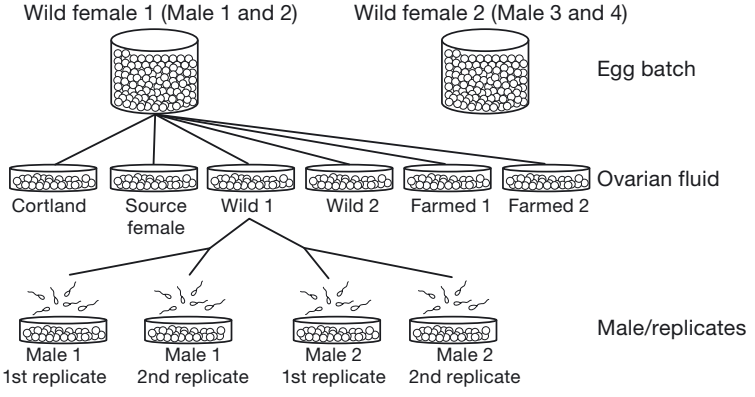

Fig. 1. Schematic representation of the experimental design of the sperm fertilization-capacity experiment (Expt 2); the top row represents the 2 batches of eggs collected on different days, the middle row represents the 6 treatments (egg-source ovarian fluid replaced by different origin ovarian fluid or Cortland solution), and the bottom row represents the males and replicates used in each egg batch and ovarian fluid treatment

RESULTS

In Expt 1, there was a significant interaction between the ovarian fluid origin (wild vs. farmed female) and male ID for %MOT ($F_{5,94} = 2.982$, $p = 0.015$). The subsequent 2-way ANOVAs for each male only detected a significant effect of ovarian fluid origin for the third male ($F_{1,14} = 15.081$, $p = 0.002$), with higher %MOT in the presence of wild ovarian fluid (Fig. 2). The ovarian fluid concentration did not affect %MOT (Fig. 2). In contrast, sperm swum in both 5 and 25% farmed female ovarian fluid had significantly lower %MOT compared to sperm in seawater without ovarian fluid, while the

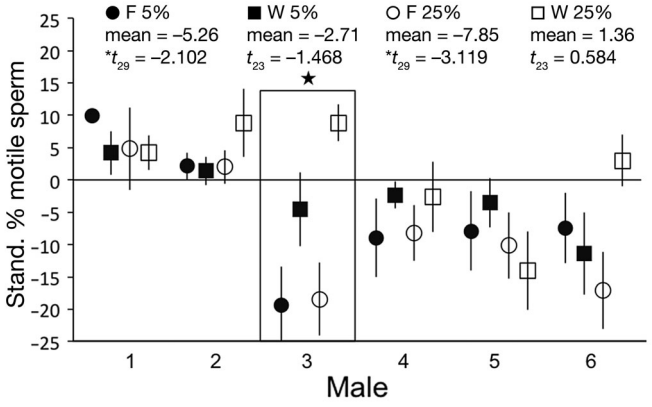

Fig. 2. Percentage of motile sperm 10 s after motility activation, standardized to the seawater control (Expt 1). Values are means (±1 SEM) of the ovarian fluids for the 5 farmed or 4 wild females. The symbols are sperm activated in the presence of the ovarian fluid of wild (squares) or farmed (circles) females. The closed and open symbols stand for 5% and 25% dilution of ovarian fluid in seawater, respectively. The significant difference in the % of motile cells in wild versus farmed ovarian fluid for the third male is indicated by ★. Below each symbol legend is the mean for the 6 males and the *t* value of the 1-sample *t*-test; *results differing significantly from 0 (control)

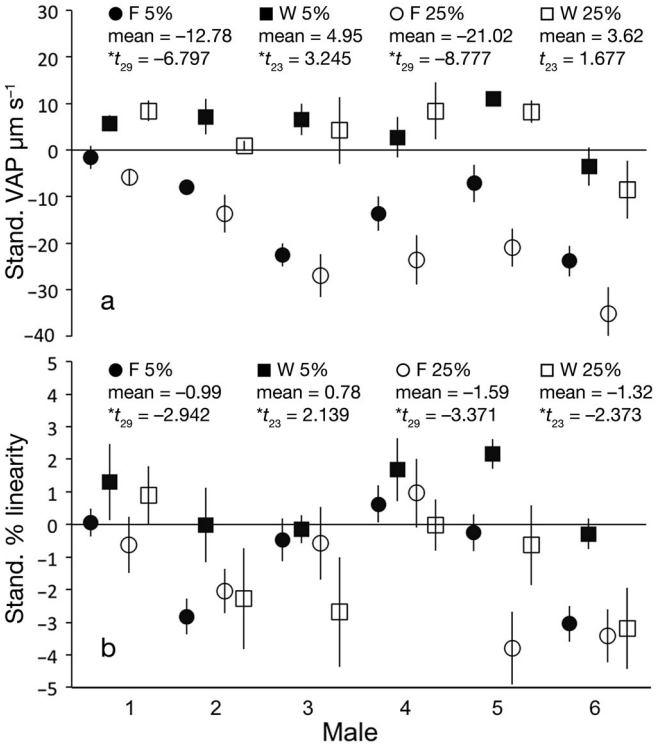

Fig. 3. Mean (a) sperm velocity (VAP) and (b) linearity 10 s after motility activation standardized to the seawater control (Expt 1). Values are means (±1 SEM) of the ovarian fluids for the 5 farmed or 4 wild females. The symbols are sperm activated in the presence of the ovarian fluid of wild (squares) or farmed (circles) females. The closed and open symbols stand for 5 and 25% dilution of ovarian fluid in seawater, respectively. Below each symbol legend is the mean for the 6 males and the *t* values of the 1-sample *t*-tests; *results differing significantly from 0 (control)

sperm swum in both 5 and 25% wild female ovarian fluid had statistically similar %MOT to that in seawater (Fig. 2).

There was a significant interaction between ovarian fluid origin and male ID for the PC of sperm swimming characteristics of motile cells ($F_{5,4}$ = 2.992, p = 0.015). In the 6 subsequent 2-way ANOVAs, there was a significant effect of the ovarian fluid origin for all individual males ($F_{1,14}$ > 22.807, p < 0.001), but only for the fifth male did the ovarian fluid concentration significantly affect the PC value ($F_{1,14}$ = 5.882, p = 0.029). Velocity (VAP) was higher in the presence of wild than farmed ovarian fluid, and this difference was generally more pronounced in 25% than in 5% ovarian fluid (Fig. 3a). Linearity was also generally higher in the presence of wild ovarian fluid (Fig. 3b) but was variable among males. Compared to the seawater control, sperm velocity was higher in the presence of 5% but not 25% wild ovarian fluid and lower in the presence of both 5 and 25% farmed female ovarian fluid. Linearity was higher in the presence of 5% wild female ovarian fluid but lower in either 25% wild and both 5% and 25% farmed female ovarian fluid.

For the ions we measured, the MANOVA failed to detect significant differences between farmed and wild female ovarian fluid composition (Pillai's = 0.807, $F_{5,3}$ = 2.524, p = 0.238) (Table 1). Only the protein bands present in all ovarian fluids (which included 80, 60.1, 58, 25 and 23 kDa, as estimated by the protein ladder molecular weights) were used in the statistical analysis, which is why the proportions do not sum to 1. For these protein-related parameters, the MANOVA failed to detect significant differences between farmed and wild female ovarian fluid (Pillai's = 0.848, $F_{6,2}$ = 1.857, p = 0.391).

In Expt 2, the ovarian fluid origin ($F_{5,39}$ = 3.254, p = 0.015), batch ($F_{1,39}$ = 4.558, p = 0.039) and male ($F_{2,39}$ = 6.205, p = 0.004) significantly affected the percentage of fertilized eggs (Fig. 4). Fertilization success in the presence of ovarian fluid from different wild females (between 50.6 ± 0.4% and 48.9 ± 4.2%) was statistically similar to that from the source female (52.3 ± 1.3%), but in the presence of ovarian fluid from farmed females, there was a significant decrease (42.3 ± 0.8% and 37.1 ± 4.7%). The percentage of fertilized eggs in the presence of wild female ovarian fluid, however, did not differ significantly from that in the saline solution (Cortland solution) (Fig. 4).

Table 1. pH, osmolality, ion concentrations (Na^+, K^+ and Ca^{2+}), total protein concentrations and the proportion of the main protein bands for the ovarian fluids of wild and farmed females. Values are expressed as mean ± SEM

Parameter	Ovarian fluid origin	
	Farmed	Wild
pH	7.26 ± 0.04	7.00 ± 0.28
Osmolality (mOsm/kg)	343.4 ± 8.1	339.0 ± 8.7
Na^+ (mmol l^{-1})	200.0 ± 8.9	174.6 ± 19.6
K^+ (mmol l^{-1})	8.04 ± 1.88	11.87 ± 2.99
Ca^{2+} (mmol l^{-1})	2.22 ± 0.04	2.31 ± 0.24
Total protein (mg 100 ml^{-1})	775.5 ± 132.0	826.0 ± 233.2
Protein bands (kDa)		
80	0.676 ± 0.046	0.567 ± 0.139
60.1	0.069 ± 0.015	0.153 ± 0.075
58	0.046 ± 0.006	0.050 ± 0.011
25	0.057 ± 0.009	0.088 ± 0.043
23	0.092 ± 0.010	0.066 ± 0.015

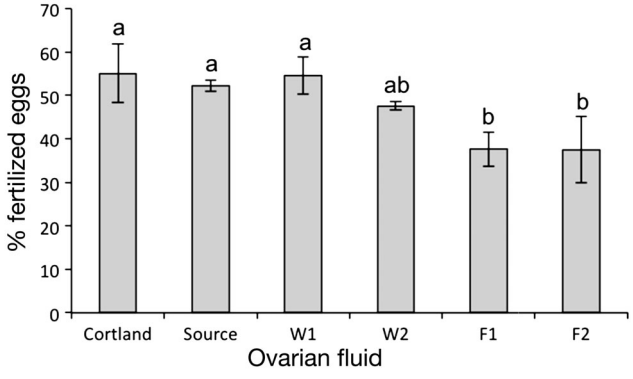

Fig. 4. Percentage of fertilized eggs in the presence of Cortland solution and ovarian fluids of the egg source female and that of 2 wild (W1, W2) and 2 farmed (F1, F2) non-egg source females (Expt 2). Different letters indicate significant differences between the groups as detected by Duncan's multiple comparison test. Values are means for the 2 batches ± SEM

DISCUSSION

Concern exists about the potential for hybridization between farmed and wild Atlantic cod (e.g. Meager et al. 2010, Puckrin et al. 2013, Zimmermann et al. 2013) given the high percentage of escapees from net pens (Moe et al. 2007, Jensen et al. 2010). While hybridization between newly escaped males and wild females may be strongly constrained by behaviour, that between escaped females and wild males is less so (Skjæraasen et al. 2009, Meager et al. 2010). Our results, however, indicate that the low gamete quality (as reflected by the ovarian fluid) of farmed females, similar to that observed in recently escaped males (Skjæraasen et al. 2009), reduces fertilization success. The reduced ovarian fluid quality relative to that of wild females decreases the swimming characteristics and fertilization capacity of the sperm of wild males. This effect will reduce the likelihood of hybridization between wild males and escaped farmed females, at least among those that have recently escaped.

Positive effects of ovarian fluid on sperm motility parameters have been documented in several species, from salmonids (Lahnsteiner 2002, Turner & Montgomerie 2002, Rosengrave et al. 2009a, Kanuga et al. 2012) to *Gasterosteus aculeatus* (Elofsson et al. 2006) and *Solea senegalensis* (Diogo et al. 2010). Although the intensity of these effects varies, from improving sperm velocity and longevity in *Salvelinus alpinus* (Turner & Montgomerie 2002) to being an essential factor in motility activation in *Clupea pallasi* (Cherr et al. 2008), potential negative effects are normally only seen at very high concentrations of ovarian fluid which arrest the osmotic mechanisms needed for sperm motility activation (e.g. Diogo et al. 2010). In our study, there was a clear negative effect of farmed female ovarian fluid on sperm swimming characteristics, which negatively affected fertilization success. In contrast, sperm tested in wild ovarian fluid performed similarly to those in the seawater control, and the fertilization success was similar in both wild ovarian fluid and saline solution.

In general, the different ovarian fluid concentrations had similar affects on the sperm motility parameters. Nonetheless, concentration effects of ovarian fluid seem to be species-specific. For instance, in *S. alpinus*, the ovarian fluid concentration that resulted in the greatest sperm motility parameters was 50% (Turner & Montgomerie 2002), and in *S. senegalensis*, it was 25% (Diogo et al. 2010), with higher concentrations slowing down the sperm. In our work, low concentrations of wild ovarian fluid increased both velocity and linearity relative to that in seawater but decreased or had no effect on these parameters at the higher concentration, while both concentrations of farmed ovarian fluid decreased velocity and linearity. This suggests that the decrease in sperm swimming characteristics was caused by both ovarian fluid quality and concentration in the case of farmed females. Concentration effects most likely result from increased viscosity, as has been observed in *S. alpinus* and *Oncorhynchus tshawytscha* (Turner & Montgomerie 2002, Rosengrave et al. 2009b).

Our results contradict the observations of Litvak & Trippel (1998), who reported a general increase in the percentage of motile sperm and swimming velocity in the presence of ovarian fluid for wild Atlantic cod. The differing results might reflect the different

methodologies used to collect the sperm motility data and measurements taken 30 s after motility activation instead of 10 s (present study). Although the measurements taken by Litvak & Trippel (1998) were not automated, they used a repeatable technique, following the sperm displacement along video frames, and thus, the differences between their findings and ours remain to be clarified. Also, we used previously frozen (–80°C) ovarian fluid samples to be able to test the same wild and farmed ovarian fluids against the same sperm samples, while Litvak & Trippel (1998) used freshly collected ovarian fluid samples. Though other authors have used previously frozen ovarian fluid when studying its effect on sperm motility (Elofsson et al. 2006, Diogo et al. 2010), we are unaware of any work comparing the effects on the motility or differences in the composition between fresh and previously frozen ovarian fluid.

Our analyses of the biochemical composition of farmed and wild ovarian fluid did not detect differences that might explain their differing effects on swimming characteristics as well as fertilization capacity. Our wild and farmed females had ovarian fluids with similar osmolality (324 to 361 mOsm kg^{-1} vs. 322 to 419 mOsm kg^{-1}) but lower pH (6.5 to 7.4 vs. 7.5 to 7.8) than that described by Litvak & Trippel (1998). According to Wojtczak et al. (2007), the pH of the ovarian fluid is a determinant of sperm motility in *Oncorhynchus mykiss*. The ion composition of ovarian fluid is also believed to influence sperm motility (Lahnsteiner 2002, Elofsson et al. 2006, Hatef et al. 2009, Rosengrave et al. 2009b, Kanuga et al. 2012), which in our cod was comparatively closer to that of marine *G. aculeatus* (Elofsson et al. 2006) than to salmonids (Lahnsteiner et al. 1995, Hatef et al. 2009, Rosengrave et al. 2009b). This is likely related to the higher ionic concentration of the marine environment. Of course, other unmeasured chemical characteristics may be responsible for the differences in sperm performance in wild versus farmed ovarian fluid. Mg^{2+} was found by Rosengrave et al. (2009b) to significantly affect the percentage of motile sperm in *O. tshawytscha*, and proteins (that we might have failed to detect) are known to be critical for sperm motility activation (e.g. *Clupea pallasi*; Cherr et al. 2008) or affect the capacity of ovarian fluid to improve fertilization success (e.g. *Salmo trutta*; Lahnsteiner 2002). Nevertheless, we observed that the sperm fertilization capacity was negatively affected by the farmed females' ovarian fluid but not by the replacement of a saline solution (Cortland solution). Similar results were observed in *S. trutta caspius* (Hatef et al. 2009) and in *G. aculeatus* (Elofsson et al. 2006), where beneficial effects on sperm motility and fertilizing capacity occurred in the presence of both the ovarian fluid and a specific saline solution mimicking its ionic composition. These results provide some evidence that the ionic composition of the ovarian fluid is, at least partly, responsible for its effects on sperm fertilization capacity.

In any case, the differences between the wild and farmed ovarian fluid could result from some form of reproductive dysfunction caused by the inhibition of behaviour and social structure under culture conditions, which has been shown to affect gamete quality, especially in new aquaculture species (reviewed by Bobe & Labbé 2010). In cod, farmed females often have difficulty releasing eggs and can become egg bound, resulting in egg aging, which in salmonids has been described to result in poor egg quality and to affect the composition of the ovarian fluid (Rime et al. 2004). Another possible explanation extends from diet: as described by Burton et al. (1997), spring-spawning cod physiologically begin the initial stages prior to vitellogenesis in the late fall, up to 7 mo before spawning — before we placed our farmed cod on forage diets, which was 4 mo before the beginning of the experiment. Therefore, historical nutritional deficiencies (reviewed by Izquierdo et al. 2001) may help explain the differences in ovarian fluid between farmed and wild females. Nevertheless, because escaped cod have been shown to survive for several years in spawning areas (Jørstad et al. 2014), the quality of ovarian fluid of escaped farmed fish should at least partially improve in the subsequent years following the escape event. Therefore, escapee fish that survive several years may have ovarian fluid that is nearly identical to that of wild fish and thus would not exhibit the same barriers for introgression. Future works should focus on ovarian fluid quality between females kept on a pellet diet and those fed with natural diets for different time periods to try to understand how ovarian fluid quality may change after an escape situation. Furthermore, our fertilization results were obtained using a limiting amount of sperm; in natural conditions, sperm is in excess, and thus, unless under sperm competition, these results could differ.

Although it is well known that both male and female gametes of farmed fish are often of lower quality than those of wild fish, we report here that the effects extend to the ovarian fluid and affect fertilization success. These negative effects of farmed ovarian fluid may explain the observations by Skjæraasen et al. (2010) that although wild males interacted more with farmed than wild females, they sired more eggs with wild than with farmed fish. While this effect may

inhibit hybridization, it may also lead to energetic and mating opportunity losses by wild males. Nonetheless, at least for the first months after escaping, the low ovarian-fluid quality of farmed females will partially inhibit fertilization success and buffer the risk of genetic introgression from escapees.

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