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Inter-population ovarian fluid variation differentially modulates sperm motility in Atlantic cod *Gadus morhua*

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This study tested the hypothesis that the effects of Atlantic cod *Gadus morhua* ovarian fluid on sperm motility variables are population specific. Sperm from a northern *G. morhua* population were activated in the presence of ovarian fluid from either northern or southern *G. morhua* at different concentrations. Ovarian fluid acted as a filter, in some cases reducing sperm swimming performance compared with seawater. Fluid from females foreign in population (southern) to the males (northern) had a greater inhibiting effect than those from the native population. Follow-up analysis indicated that the ovarian fluids had lower Ca^{2+} concentration in northern than southern *G. morhua*, which could be the causative mechanism. If widespread, such cryptic female choice could reduce the incidence of intraspecific hybridization among diverged populations and contribute to reproductive isolation.

Key words: Ca^{2+} concentration; cryptic female choice; local adaptation; reproductive isolation.

INTRODUCTION

Genetically distinct populations of Atlantic cod *Gadus morhua* L. 1758 occur throughout their range (Ruzzante *et al.*, 1998; Beacham *et al.*, 2002; Skarstein *et al.*, 2007; Nielsen *et al.*, 2009), and have been attributed to spawning site fidelity (Ruzzante *et al.*, 1998, 1999) and local adaptation (Skarstein *et al.*, 2007; Bradbury *et al.*, 2010). In the north-west Atlantic Ocean, six evolutionary distinct designable units (DU) are considered to be present in Canadian waters (COSEWIC, 2010). When reared under common environmental conditions, genetic differences among these populations have been observed in several traits such as growth rate (Purchase & Brown, 2000; Dutil *et al.*, 2008), juvenile food conversion efficiency (Purchase & Brown, 2000, 2001), morphology and allometry (Marcil *et al.*, 2006) and metabolism (Sylvestre *et al.*, 2007). Although traits most directly related to reproduction have been less studied, there is evidence that fecundity varies among these populations in the wild (McIntyre & Hutchings, 2003), and that under common conditions there are intrinsic differences in thermal plasticity of sperm swimming ability (Beirão *et al.*, 2014*a*). An important open question is whether population differences in reproductive traits constitute a mechanism for reproductive isolation. This question gains more relevance in populations that

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have suffered a tremendous collapse and are well below the limit reference points; such is the case of north-west *G. morhua* (Hutchings & Rangeley, 2011).

Many species reproduce under sperm competition (Parker *et al.*, 2013), including *G. morhua* (Hutchings *et al.*, 1999). In such scenarios, sperm motility variables such as swimming speed or straightness often influence fertilization success (Gage *et al.*, 2004; Skjaeraasen *et al.*, 2009). Fish eggs at release are surrounded by an extracellular matrix referred to as ovarian fluid. The composition of which has been observed to affect sperm motility variables, including speed, percentage of motile cells and motility duration (Turner & Montgomerie, 2002; Elofsson *et al.*, 2006; Diogo *et al.*, 2010; Kanuga *et al.*, 2012). For example, the ovarian fluid in three-spined stickleback *Gasterosteus aculeatus* L. 1758 is crucial for extending sperm motility long enough for fertilization (Elofsson *et al.*, 2006), in Pacific herring *Clupea pallasii* (Valenciennes 1847) for the sperm motility initiation and guidance to the micropylar region (Cherr *et al.*, 2008) and in several salmonids its chemistry affects fertilization rates (Lahnsteiner, 2002; Hatef *et al.*, 2009; Evans *et al.*, 2013; Yeates *et al.*, 2013). Furthermore, in some cases, ovarian fluid has been associated with post-copulatory cryptic female mate choice by selecting for a particular male's sperm (Urbach *et al.*, 2005; Rosengrave *et al.*, 2008; Butts *et al.*, 2012), and can be more influential in paternity than egg species (Yeates *et al.*, 2013). This effect has been associated with relatedness between the female and male, sometimes favouring unrelated males, reducing inbreeding (Gasparini & Pilastro, 2011) and other times benefiting closely related males, potentially as a means of outbreeding avoidance between locally adapted populations (Butts *et al.*, 2012).

Exactly how ovarian fluid affects sperm motility appears to vary among species. For example, in salmonids, the pH (Wojtczak *et al.*, 2007), inorganic ions (Lahnsteiner, 2002; Rosengrave *et al.*, 2009) and protein composition (Lahnsteiner, 2002) of ovarian fluid were proposed to be responsible for its effect on sperm swimming, while for *G. aculeatus* the effects were attributed solely to inorganic composition (Elofsson *et al.*, 2006). In *G. morhua*, Litvak & Trippel (1998) observed a general improvement in sperm speed and longevity in the presence of ovarian fluid; nonetheless, in the study of Beirão *et al.* (2014*b*), this effect appeared to be influenced greatly by ovarian fluid quality and varied among males. Furthermore, fertilization success may vary among male–female pairings (Rakitin *et al.*, 1999), all of which support the notion of some mechanism of cryptic female choice.

This study tested the hypothesis that sperm motility variables are modulated by inter-population differences in ovarian fluid in *G. morhua*, thereby providing a potential mechanism contributing to reproductive isolation. A common garden approach was used, including a split-ejaculate experiment, to determine sperm reactions to ovarian fluid from native and foreign females.

MATERIALS AND METHODS

FISH ORIGIN AND REARING

The fish used in the experiments were from two evolutionarily significant distinct groups of north-west *G. morhua*: (1) the Newfoundland and Labrador (Canada) population and (2) the southern population (as described in COSEWIC, 2010), and derived from wild parents and grandparents that had been spawned in captivity. The Newfoundland and Labrador

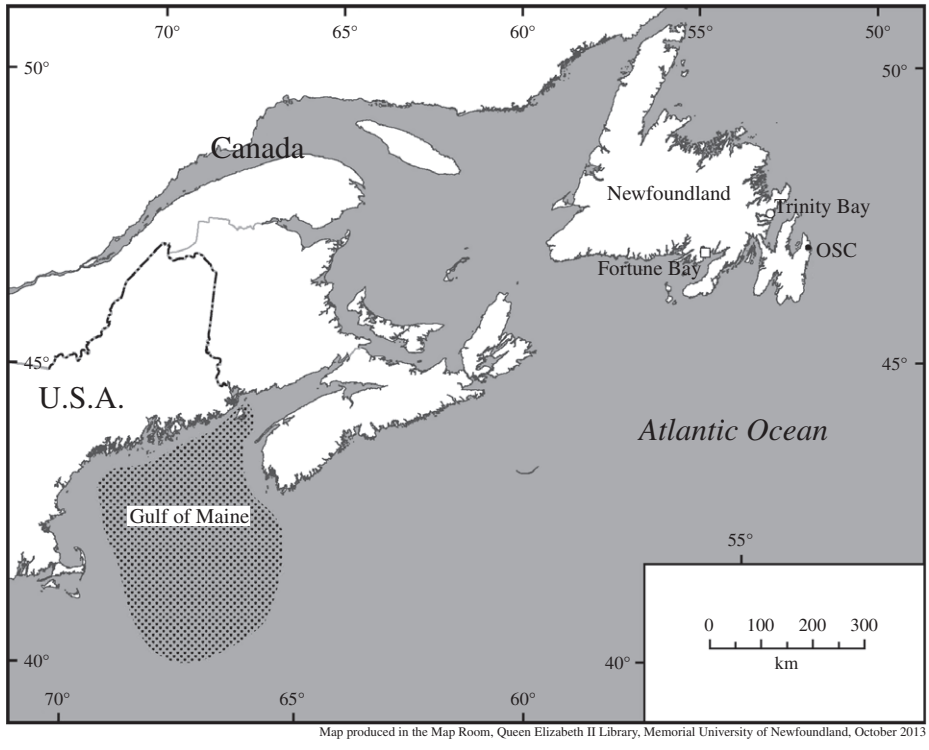

FIG. 1. Map showing the two *Gadus morhua* population origins (○, northern, Newfoundland–Labrador; ⚄, southern) and where they were kept. Fish were initially held in sea cages (□) and transported to indoor tanks in the Ocean Science Centre (OSC, ●) in December 2011.

group (mean ± S.E. total length, L_T, and mass, M, males = 63·2 ± 1·0 cm, 2·9 ± 0·2 kg; females = 62·4 ± 1·4 cm, 2·9 ± 0·1 kg) (northern population) were spawned in 2008 from fish captured in Smith Sound, Trinity Bay, Newfoundland (Fig. 1). In 2009, these fish were placed in sea cages in Fortune Bay, Newfoundland (Fig. 1) and fed commercial aquaculture pellets. The southern group (females = 53·4 ± 1·5 cm, 2·2 ± 0·1 kg) (southern population) were spawned in 2009 from fish captured in the Gulf of Maine (Fig. 1) and placed in cages in 2010 directly adjacent to the northern population group and fed the same pellets. In December 2011, both groups were transported to the Ocean Sciences Centre at Memorial University of Newfoundland, passive integrated transponder (PIT)-tagged and placed in an indoor flow-through tank (25·5 m^3) and fed a forage diet (dead herring *Clupea harengus* L. 1758, mackerel *Scomber scombrus* L. 1758 and squid *Illex* spp.). Fish lived under these conditions for 3 months before the first eggs were collected. The tank was provided with constant aeration and water exchange of 27% h^{-1}, and the temperature remained constant at 5·3° C range ± 0·1° C. The photoperiod varied according to the location latitude (see Fig. 1) and the lights faded in and out with sunrise and sunset. Rearing and experimentation at the Ocean Sciences Centre were conducted in accordance to the protocol 12-09-IF approved by the Memorial University Animal Care Committee following the regulations of the Canadian Council on Animal Care for the treatment and welfare of animals.

SEMEN AND OVARIAN FLUID SAMPLING

Eggs were collected by abdominal massage and the ovarian fluid was separated using a 1 mm mesh. Ovarian fluid (10–20% of the total egg collection volume) was then centrifuged

at 5000 *g* at 2° C for 10 min to remove suspended particles that could interfere with the sperm motility analyses. Individual ovarian fluid samples were stored at −80° C until analysed. Samples were obtained from 10 northern population fish and six southern population fish.

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 abdomen. The first 1 ml of ejaculate was discarded to help avoid seawater, urine and faeces contamination and only samples with >70% motile cells (analysed visually) and in the range of 340–415 mOsm kg^{-1} described for this species by Butts *et al.* (2010) were used in the experiment to assure the use of good quality samples.

SPLIT-EJACULATE EXPERIMENTAL SETUP

Semen from five northern population males was analysed individually in the presence of ovarian fluid from five northern (native) and five southern (foreign) population females in a full factorial design. Each ovarian fluid sample was diluted to two concentrations (25 and 5%) in seawater to determine effects of sperm-egg spatial proximity or time post-spawning on gamete mixing. Semen activated only in the presence of seawater was used as a control.

SPERM MOTILITY ANALYSES

Per cent motile cells ($\%C_{mot}$) and sperm swimming characteristics were analysed as previously described by Beirão *et al.* (2014*a*). Briefly, semen samples were prediluted in a non-activating solution (two thirds fresh water and one third seawater) (Rouxel *et al.*, 2008) and kept at 9° C. One µl of prediluted semen was added to 15 µl of activation solution [31·7 salinity seawater plus 5 or 25% ovarian fluid, with 1% (w/v) bovine serum albumin] on a microscope slide maintained at 9° C. Sample analyses were replicated three times and averaged for statistical analyses. Sperm motility variables were determined 10 s after activation using the open computer-assisted sperm analysis (CASA) software developed by Wilson-Leedy & Ingermann (2007) and modified by Purchase & Earle (2012). Input parameters are available in Table SI (Supporting Information). In addition to $\%C_{mot}$, the following sperm swimming variables obtained with the CASA plugin were analysed: V_{CL} (curvilinear speed, speed according to the actual path; µm s^{-1}), V_{SL} (straight line speed, speed according to the straight path, *i.e.* displacement; µm s^{-1}), V_{AP} (speed according to the smoothed path; µm s^{-1}), W_{OB} (wobble; % calculated from $V_{AP}\ V_{CL}{}^{-1}$) and L_{IN} (linearity; % calculated from $V_{SL}\ V_{AP}{}^{-1}$).

OVARIAN FLUID COMPOSITION ANALYSES

Upon observing effects on sperm motility, the ovarian fluid differences that might constitute a proximate mechanism were investigated. The composition of the frozen ovarian fluid was analysed for all the obtained samples ($n = 10$ and $n = 6$, northern and southern populations). Osmolality was analysed with a model 3320 Osmometer (Advanced Instruments; www.aicompanies.com) and pH with a multi-parameter meter (Accumet XL50; www.fishersci.com). Total protein concentration was measured with the DC Protein Assay (Bio-Rad; www.bio-rad.com) that is based on the Lowry assay. The assay absorbance was measured at 750 nm in a plate reader (Biotek, Powerwave XS; www.biotek.com) and bovine albumin was used as the standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine the proportion of proteins present based on their molecular masses. Pre-diluted ovarian fluid (five or 10 times depending on the initial protein concentration) was mixed with Laemmli buffer (1:3) and loaded into the wells, together with a protein ladder [BioLabs, pre-stained protein marker, broad range (7–175 kDa); www.neb.com]. Gel (10 cm × 8 cm) electrophoresis was conducted for 85 min at 100 V in a vertical electrophoresis system (CBS Scientific, MGV-402; www.cbsscientific.com). After electrophoresis, gels were stained with 0·25% Coomassie blue for 2 h. The molecular masses of the protein bands were estimated by interpolation based on the known molecular masses of the protein ladder. Ovarian fluid ionic composition (Na^+, K^+ and Ca^{2+}) was measured by flame photometry (Jenway, model PFP7; www.jenway.com), using propane as fuel. Each sample was measured three times and a calibration curve was run between every 10 measures.

STATISTICAL ANALYSES

Statistics were conducted using R 2.15.1 (R Development Core Team; www.r-project.org). For all analyses, $P < 0{\cdot}05$ was considered significant and assumptions of parametric tests were checked through the analyses of model residuals. Differences between the treatments for the sperm motility variables [$\%C_{mot}$ and the swimming variables (V_{CL}, V_{SL}, V_{AP}, W_{OB} and L_{IN})] were analysed with a repeated-measures MANOVA, with both ovarian fluid origin and dilution considered as fixed effects and the male identification (ID) as a repeated and random factor. Different female ovarian fluid donors provided the replication. The $\%C_{mot}$ and the swimming variables of each male were standardized to his control (seawater sperm motility variables characteristics). *Post hoc* ANOVAs were first analysed as saturated models, and then reanalysed with non-significant interactions removed. In no instance was the three-way interaction between ovarian fluid origin, dilution and male ID significant. Because there was a significant interaction between male ID and ovarian fluid origin in both [$\%C_{mot}$ and V_{CL}], the main effects were not interpreted, and each male was analysed individually with a two-way ANOVA. Results are graphically represented as means ± S.E.

Differences between the control (sperm activated only with seawater, standardized as 0 for each male in each case) and each of the four treatment combinations (two ovarian fluid origins × two concentrations) were analysed with four one-sample *t*-tests ($n = 25$). The standardized values of the sperm motility variables of the five males × five ovarian fluids were used as replicates.

Population differences in ovarian fluid composition were analysed by MANOVA and *post hoc* ANOVAs were conducted on each variable individually. Linear correlations between the different ovarian fluid variables (pH, osmolality, ions, total protein and protein bands) and the sperm motility variables (adjusted to the control) were evaluated by Pearson correlations using the R package ltm (Rizopoulos, 2006). To obtain the motility values for the correlation analyses, the motility variables ($\%C_{mot}$ and sperm swimming characteristics) in the two dilutions were averaged and then the values for the five males were averaged ($n = 10$ ovarian fluid samples and one sperm datum for each). As ovarian fluid Ca^{2+} was significantly correlated with female size (M and L_T), four ANCOVA models were run (one for each sperm motility variable significantly correlated with Ca^{2+}; $\%C_{mot}$, V_{SL}, V_{AP} and W_{OB}) where L_T and calcium were considered as covariates and ovarian fluid origin as the categorical variable. Only L_T was used, and not M, because they were significantly correlated (Pearson correlation $r = 0{\cdot}939$, $n = 16$, $P < 0{\cdot}001$). Numeric results are expressed as means ± S.E.

RESULTS

The null hypothesis that sperm motility variables are unaffected by the female's population was rejected, as the MANOVA revealed a significant interaction between male and ovarian fluid origin (Pillai's = 0·611, $F_{24,348} = 2{\cdot}615$, $P < 0{\cdot}001$). With *post hoc* ANOVAs, four of the six motility variables showed consistency across the males (no interaction). In three variables (V_{SL}, V_{AP} and W_{OB}), swimming performance was higher in native *v*. foreign ovarian fluid ($F_{1,93} \geq 30{\cdot}875$, $P < 0{\cdot}001$); there was no effect of origin on L_{IN} ($F_{1,93} = 1{\cdot}872$, $P > 0{\cdot}05$) (Fig. 2). There were significant differences among males in V_{SL} ($F_{4,93} = 14{\cdot}650$, $P < 0{\cdot}001$), V_{AP} ($F_{4,93} = 18{\cdot}130$, $P < 0{\cdot}001$) and W_{OB} ($F_{4,93} = 28{\cdot}859$, $P < 0{\cdot}001$) independent of ovarian fluid, but not for L_{IN} ($F_{4,93} = 2{\cdot}147$, $P > 0{\cdot}05$). On the other hand, L_{IN} was the only variable affected significantly by ovarian fluid dilution ($F_{1,93} = 13{\cdot}166$, $P < 0{\cdot}001$), with lower values at the higher concentration.

There was a significant interaction between male ID and ovarian fluid origin for $\%C_{mot}$ ($F_{4,93} = 8{\cdot}578$, $P < 0{\cdot}001$) and V_{CL} ($F_{4,93} = 2{\cdot}622$, $P < 0{\cdot}05$), indicating variable responses to ovarian fluids across different males in these two variables (Figs 2 and 3). For $\%C_{mot}$ (Fig. 3), the only significant difference of native (higher) *v*. foreign ovarian fluid involved male 5 ($F_{1,16} = 36{\cdot}313$, $P < 0{\cdot}001$). On the other hand, ovarian

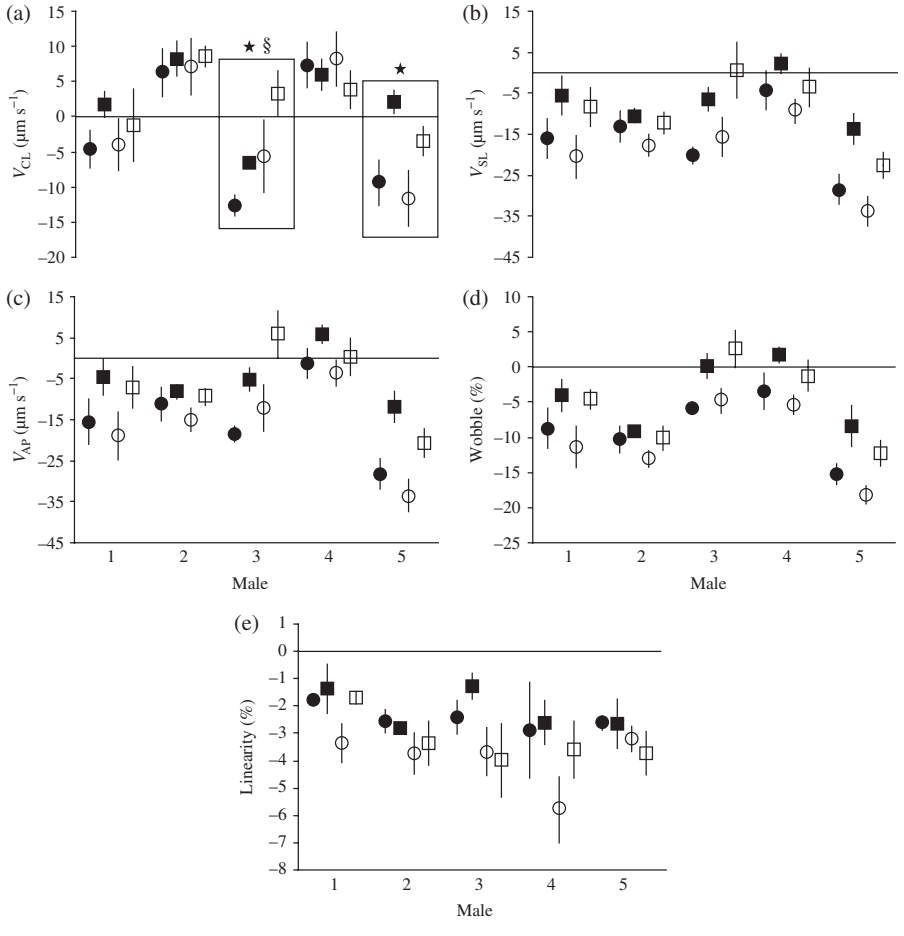

FIG. 2. Mean ± S.E. sperm swimming variables (a) curvilinear speed (V_{CL}), (b) straight line speed (V_{SL}), (c) speed according to the smoothed path (V_{AP}), (d) wobble and (e) linearity 10 s after motility activation for the five northern *Gadus morhua* population males standardized to their values in seawater (as indicated by the line at zero): sperm activated in native (northern; ■,□) and foreign (southern; ●,○) ovarian fluid diluted in seawater by 5% (■,●) and 25% (□,○). Ovarian fluid differences for specific males are only shown for V_{CL} because this is the only variable for which there was a significant interaction in the ANOVA model between male and ovarian fluid origin. Differences in sperm swimming of each male in northern *v.* southern ovarian fluid as detected by two-way ANOVA ($P < 0{\cdot}05$) are indicated by ★ and between 5 and 25% dilution by §. The mean values for the five males were V_{CL}: northern and 5% = 2·29; northern and 25% = 2·19; southern and 5% = −2·62; southern and 25% = −1·19; V_{SL}: northern and 5% = −6·80; northern and 25% = −9·25; southern and 5% = −16·39; southern and 25% = −19·40; V_{AP}: northern and 5% = −4·84; northern and 25% = −6·25; southern and 5% = −14·97; southern and 25% = −16·60; wobble: northern and 5% = −3·94%; northern and 25% = −5·12%; southern and 5% = −8·71%; southern and 25% = −10·58%; linearity: northern and 5% = −2·14%; northern and 25% = −3·27%; southern and 5% = −2·45%; southern and 25% = −3·95%.

fluid dilution had no significant effect on $\%C_{mot}$ for any of the males. A significantly higher V_{CL} in the presence of native *v.* foreign ovarian fluid was detected for males 3 ($F_{1,16} = 5{\cdot}890$, $P < 0{\cdot}05$) and 5 ($F_{1,16} = 11{\cdot}400$, $P < 0{\cdot}01$) (Fig. 2), and the trend was higher in four of the five males. For male 3, the sperm activated in 25% ovarian fluid

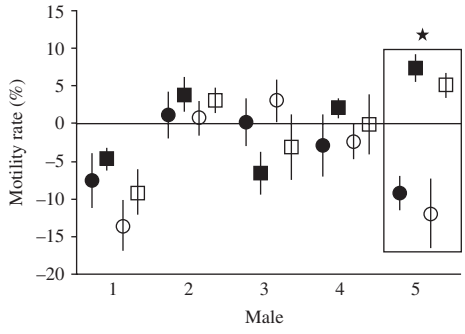

FIG. 3. Mean ± S.E. percentage of motile sperm 10 s after motility activation for the five northern *Gadus morhua* population males standardized to their motility in seawater (as indicated by the line at zero): sperm activated in native (northern; ■,□) and foreign (southern; ●,○) ovarian fluid diluted in seawater by 5% (■,●) and 25% (□,○). Significant differences in motility rate for the sperm of each male in northern *v*. southern ovarian fluid are indicated (★). The mean values for the five males were: northern and 5% = 0·38%; northern and 25% = −0·83%; southern and 5% = −3·69%; southern and 25% = −4·81%.

had higher V_{CL} than those activated in 5% ($F_{1,16} = 7{\cdot}393$, $P < 0{\cdot}05$) (Fig. 2), but concentration had no effect in the other fish.

Unexpectedly, ovarian fluid did not increase the per cent of sperm that were motile or their swimming speeds above the seawater control (Figs 2 and 3). The $\%C_{mot}$ was similar in the control (sperm activated only in seawater) and native ovarian fluid ($t_{24} \geq -0{\cdot}514$, $P > 0{\cdot}05$) but lower in that of foreign females ($t_{24} \leq -2{\cdot}354$, $P < 0{\cdot}05$). The swimming variables were always lower for sperm activated in ovarian fluid compared with the control, regardless of female origin ($t_{24} \leq -3{\cdot}723$, $P \leq 0{\cdot}001$ for V_{SL}, $t_{24} \leq -2{\cdot}439$, $P < 0{\cdot}05$ for V_{AP}, $t_{24} \leq -3{\cdot}321$, $P < 0{\cdot}01$ for W_{OB} and $t_{24} \leq -6{\cdot}590$, $P < 0{\cdot}001$ for L_{IN}), except for V_{CL} whose values were similar to the control (t_{24} between 1·773 and −2·284, $P > 0{\cdot}05$).

To determine the proximate cause of the population differences, the biochemical composition of the ovarian fluids was analysed (Table I). The MANOVA detected significant differences between the two ovarian fluid origins (Pillai's = 0·946, $F_{9,6} = 11{\cdot}745$, $P < 0{\cdot}01$). *Post hoc* *t*-tests revealed that both ovarian fluid pH ($F_{1,14} = 8{\cdot}115$, $P < 0{\cdot}05$) and [Ca^{2+}] ($F_{1,14} = 78{\cdot}814$, $P < 0{\cdot}001$) were significantly different between the two populations, with the southern population (foreign; pH = 7·67 ± 0·11 and 3·11 ± 0·08 mmol l^{-1} Ca^{2+}) having higher values for both variables than the northern population (native; pH = 7·27 ± 0·08 and 2·28 ± 0·05 mmol l^{-1} Ca^{2+}). Several protein bands could be distinguished in the SDS-PAGE gels with molecular masses of 175, 111·2, 80, 60·1, 58, 49·8, 46, 40·9, 31·5, 30, 25 and 23 kDa. Nonetheless, only three protein bands (80, 58 and 23 kDa) were present in all samples, which account for *c.* 90% of the protein amount detected, but none of them present significant differences between the two populations.

Total protein in the ovarian fluid of the different females was negatively correlated with V_{CL}, and Ca^{2+} concentration was negatively correlated with several sperm swimming variables [W_{OB} and the speed variables (V_{AP} and V_{SL})] and $\%C_{mot}$ (Table II). L_{IN}, on the other hand, did not present any correlation with the analysed ovarian fluid variables. Furthermore, the only ovarian fluid variable correlated with female size (*M* and L_T) was calcium (Table III). Sizes of the fish from the two populations overlapped

TABLE I. pH, osmolality, ion concentration (Na^+, K^+ and Ca^{2+}), total protein concentration and the proportion of the main protein bands for northern and southern *Gadus morhua* population ovarian fluids. Values are expressed as mean ± S.E. of the six southern and 10 northern female ovarian fluids from each group. Significant differences ($P < 0{\cdot}05$) are indicated in bold

Variable		Population	
		Southern	Northern
pH		**7·67 ± 0·11**	**7·27 ± 0·08**
Osmolality ($mOsm\,kg^{-1}$)		324·8 ± 9·2	343·0 ± 5·1
Ions ($mmol\,l^{-1}$)	Na^+	194·0 ± 9·2	194·6 ± 7·1
	K^+	9·39 ± 1·56	9·73 ± 1·45
	Ca^{2+}	**3·11 ± 0·08**	**2·28 ± 0·05**
Total protein ($mg\,100\,ml^{-1}$)		768·9 ± 155·1	703·6 ± 75·7
Protein bands (kDa)	80	0·676 ± 0·064	0·686 ± 0·026
	58	0·057 ± 0·015	0·045 ± 0·004
	23	0·110 ± 0·010	0·112 ± 0·010

but the northern population was on average larger (Fig. 4). The ANCOVA models for the sperm motility variables correlated with Ca^{2+} indicated that this ion concentration affects V_{SL}, W_{OB} and $\%C_{mot}$ ($F_{1,6} \geq 6{\cdot}203$, $P < 0{\cdot}05$).

DISCUSSION

Different *G. morhua* populations have been shown to vary in reproductive traits when reared under common conditions [spawn timing (Otterå *et al.*, 2006) and thermal plasticity of sperm swimming (Beirão *et al.*, 2014*a*)]. This study revealed that filtering effects of ovarian fluid on sperm performance are more pronounced for foreign *v*. native females, which might be explained by the observed differences in ovarian

TABLE II. Correlation coefficients (r) between the ovarian fluid variables and the percentage of motile sperm ($\%C_{mot}$) and the sperm swimming variables [curvilinear speed (V_{CL}), straight line speed (V_{SL}), speed according to the smoothed path (V_{AP}), wobble (W_{OB}) and linearity (L_{IN})] in *Gadus morhua*. Significant correlations ($P < 0{\cdot}05$) are indicated in bold

		$\%C_{mot}$	V_{CL} ($\mu m\,s^{-1}$)	V_{SL} ($\mu m\,s^{-1}$)	V_{AP} ($\mu m\,s^{-1}$)	W_{OB} (%)	L_{IN} (%)
pH		−0·45	−0·36	−0·56	−0·52	−0·55	−0·51
Osmolality		0·44	−0·17	0·07	0·06	0·18	0·13
Na^+		−0·26	−0·26	−0·28	−0·28	−0·26	−0·14
K^+		0·41	0·01	0·13	0·12	0·17	0·05
Ca^{2+}		**−0·64**	−0·47	**−0·69**	**−0·68**	**−0·73**	−0·42
Total protein		−0·28	**−0·72**	−0·58	−0·62	−0·52	−0·04
Protein bands	80	−0·08	−0·10	−0·58	−0·28	−0·22	0·24
	58	−0·21	0·08	0·47	0·22	0·19	−0·10
	23	0·38	0·35	0·20	0·32	0·37	0·48

TABLE III. Correlation coefficients (r) between the ovarian fluid variables measured and the mass (M) and total length (L_T) of the *Gadus morhua* females. Significant correlations ($P < 0{\cdot}05$) are indicated in bold

		M	L_T
pH		−0·40	−0·57
Osmolality		0·35	0·40
Na^+		0·23	0·21
K^+		−0·08	−0·08
Ca^{2+}		**−0·65**	**−0·78**
Total protein		−0·26	−0·19
Protein bands	80	−0·06	−0·04
	58	−0·23	−0·27
	23	−0·16	−0·20

fluid composition. This mechanism can contribute to reproductive isolation among populations.

A general improvement in sperm motility variables in the presence of ovarian fluid has been documented for several fish species (Elofsson *et al.*, 2006; Rosengrave *et al.*, 2008; Diogo *et al.*, 2010), including *G. morhua* (Litvak & Trippel, 1998). Similar to results testing wild and farmed *G. morhua* ovarian fluid (Beirão *et al.*, 2014*b*), there was no such enhancement over seawater control in this study. In fact, ovarian fluid appeared to act more as a filter that selects for some males' sperm rather than others. Whether the differences from previous work were because of the methodology applied [sperm motility measured at 10 s in this study *v.* 30 s and a less automatized technique in Litvak & Trippel (1998)], the individual's life history [farmed individuals in this study *v*. wild individuals adapted to captivity in Litvak & Trippel (1998)], ovarian fluid storage [ovarian fluid previously frozen at −80° C in this study that could alter some physical properties *v*. freshly collected ovarian fluid in Litvak & Trippel (1998)] or the ovarian fluid concentration tested [in Litvak & Trippel (1998), the ovarian fluid

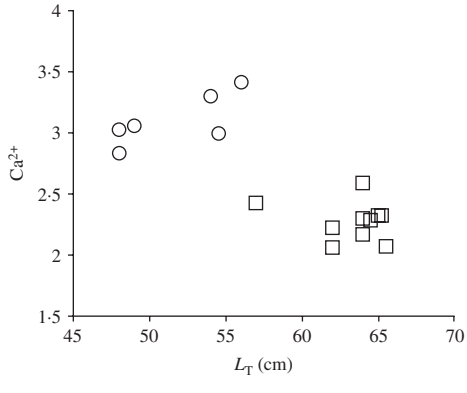

FIG. 4. Relationships between the ovarian fluid calcium concentration and female total length (L_T) for the northern (□) and southern (○) *Gadus morhua* population.

concentration is not specified] are not clear. In any case, some sperm motility variables (V_{SL}, V_{AP} and W_{OB}) were higher for all males in the presence of ovarian fluid from native (northern) than from foreign females (southern), while others ($\%C_{mot}$ and V_{CL}) were higher just for specific males. These differences in sperm motility variables are probably sufficient to affect fertilization rates in relation to female origin, particularly during sperm competition, as V_{CL} (Skjaeraasen *et al.*, 2009) and sperm progressiveness (closely related with the W_{OB} variable considered as an index of the forward movement efficiency) (Rudolfsen *et al.*, 2008) have been shown to be positively correlated with fertilization success in *G. morhua*.

Ca^{2+} was the only ovarian fluid variable analysed that would appear responsible for the differential effects of the two sources of females on sperm motility variables. Ca^{2+} was correlated with some of the sperm motility variables and was different between the northern and southern females. In other species, such as *G. aculeatus* and various salmonids, improvement in sperm motility variables is related to the saline composition of ovarian fluid (Elofsson *et al.*, 2006; Hatef *et al.*, 2009; Rosengrave *et al.*, 2009). Nonetheless, only in Chinook salmon *Oncorhynchus tshawytscha* (Walbaum 1792), a negative effect of ovarian fluid Ca^{2+} on sperm motility variables has been observed (Rosengrave *et al.*, 2009), similar to the results obtained in this study. In marine teleosts, the general concept is that extracellular ions are not responsible for the motility initiation of sperm, which is controlled by the increased osmolality due to contact with seawater that causes the intracellular release of Ca^{2+} (Morita *et al.*, 2003; Alavi & Cosson, 2006; Zilli *et al.*, 2008). This together with pH triggers a number of signalling molecules and cytoskeletal elements that lead to the activation of the motile machinery (Inaba, 2008). In some marine teleosts, extracellular Ca^{2+} has been described as being required for motility initiation (Cherr *et al.*, 2008) and even crucial for the spermatozoa entry into the micropyle (Yanagimachi *et al.*, 2013). In other cases, it may negatively affect sperm swimming speed (Detweiler & Thomas, 1998; Zilli *et al.*, 2008) and flagellar symmetry (Cosson *et al.*, 1989; Boitano & Omoto, 1992; Böhmer *et al.*, 2005), and increase turning rate (Detweiler & Thomas, 1998) at high concentrations. Similarly, in this experiment, higher Ca^{2+} concentrations in the ovarian fluids of females from the southern population appear to negatively affect sperm speed and wobble (reflective of increased turning rate), compared with that of northern population females, but not linearity. The importance of these different Ca^{2+} concentrations should be considered with caution because the values of Ca^{2+} in seawater are normally around 10 mmol l^{-1}; thus, in the tested ovarian fluid concentrations (25 and 5% diluted in seawater), the different ovarian fluid Ca^{2+} values could be masked by the seawater Ca^{2+} values. Obviously, other unmeasured variables in the ovarian fluid can affect sperm motility, such as Mg^{2+} (Rosengrave *et al.*, 2009) or specific proteins. In the case of the ovarian fluid proteins, Johnson *et al.* (2014) observed that its composition was variable among females, while Lahnsteiner (2002) found that molecular masses between 20 and 60 kDa improve fertilization rates in brown trout *Salmo trutta* L. 1758. In this study, albeit there was a negative effect of the total protein content on the speed (V_{CL}), there were no correlations between the different protein bands and the sperm motility variables.

Unfortunately, fish from the two populations tested in this study did not have the same spawning season even though they were under common conditions during their entire lives. Therefore, it was not possible to test southern population sperm samples in the

presence of the two ovarian fluid origins. If southern population males' sperm also have better motility variables in the presence of southern population females' ovarian fluid (their native ovarian fluid), taken together this would be an example of a cryptic female choice towards males from the same population, similar to the results observed by Butts *et al.* (2012) in lake trout *Salvelinus namaycush* (Walbaum 1792). This cryptic choice mechanism could contribute to reproductive isolation among populations. The other possibility is that independent of the male population origin (northern or southern), all sperm have better motility variables in the northern females' (lower Ca^{2+}) ovarian fluid.

In other taxa, experimentally increasing ovarian fluid concentration from the same fish increases sperm swimming linearity (Turner & Montgomerie, 2002; Diogo *et al.*, 2010). Contrary to this, in this study, there was a decrease in linearity with increasing ovarian fluid concentration. This decrease appeared unrelated to Ca^{2+} concentration as there was no correlation between the two variables. Other (unquantified) ovarian fluid variables are probably responsible for the decrease in linearity, such as viscosity or undetected proteins. In *C. pallasii*, a mechanism mediated by egg-derived proteins has been described to induce highly circular trajectories (lower linearity) in sperm as they near the egg micropyle as a means of increasing the likelihood of finding and entering the micropylar canal (Cherr *et al.*, 2008). Because ovarian fluid is more viscous than water, it remains close to the eggs after spawning (Rosengrave *et al.*, 2009), in other words, the closer to the egg the higher the ovarian fluid concentration and the lower the linearity. Thus, this study results suggest that a similar mechanism to the one described for *C. pallasii* (Cherr *et al.*, 2008) may exist in *G. morhua*.

In this study, there was variable male response in some sperm motility variables to ovarian fluid origin. In several other species, sperm motility variables varied significantly among males in response to ovarian fluids of individual females (Urbach *et al.*, 2005; Dietrich *et al.*, 2008; Rosengrave *et al.*, 2008), suggesting that it may be a mechanism of cryptic female choice whereby females differentially affect the sperm motility of different males. Evidence of a significant male–female interaction in regards to fertilization has been reported in *G. morhua* (Rakitin *et al.*, 1999). While this study is not specifically set out to evaluate interactions among individual male/female pairings, these results together with those of Rakitin *et al.* (1999) suggest the existence of cryptic female choice mediated through ovarian fluid in *G. morhua*.

Although there is evidence that female *G. morhua* behaviourally select for specific males (Hutchings *et al.*, 1999), more work is clearly needed to better understand how ovarian fluid composition affects sperm motility, including whether there is sperm selection at the population level. In Atlantic salmon *Salmo salar* L. 1758, there is evidence of selection for genetically similar individuals based on the major histocompatibility complex (MHC) (Yeates *et al.*, 2009). There is a dearth of studies, however, on the effect of ions on sperm motility activation in marine teleosts. For example, it would be interesting to test the effects of Ca^{2+} in *G. morhua* sperm motility. In any case, differences in reproductive traits among *G. morhua* populations are in line with other studies (Purchase & Brown, 2000; McIntyre & Hutchings, 2003; Otterå *et al.*, 2006; Harrald *et al.*, 2010), suggesting adaptive responses to the local environments. Broadly, this study contributes to the literature that reflects the variation in phenotypes and genotypes between different *G. morhua* populations and the importance of treating them as independent evolutionary units when establishing resource management programmes.

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Supporting Information

Supporting Information may be found in the online version of this paper:
Table S1. Parameters used to set the ImageJ computer-assisted sperm analysis (CASA) plugin

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