SEMEN QUALITY IN FAROESE MEN – A CROSS-SECTIONAL POPULATION-BASED STUDY OF 482 MEN

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ABSTRACT

Objectives: To determine semen quality and reproductive hormone levels in young Faroese men.

Design: Descriptive cross-sectional study of Faroese men compared with Danish men.

Setting: Faroese one-center study.

Participants: 484 men born from 1981 to 1987 and investigated from 2007 to 2010.

Outcome measures: Sperm concentration, semen volume, total sperm count, sperm motility, sperm morphology and reproductive hormones levels.

Results: Sperm concentrations for the Faroese men were lower than for the Danish (crude median 40 mill/mL vs. 48, p<0.0005). Semen volume was higher, and thus total sperm counts did not differ (159 vs. 151 mill, p=0.2). Motility and morphology did not differ between Faroese and Danes. Inhibin B/FSH ratio for the Faroese men were lower than for Danes (64 vs. 76, p=0.001). Similarly, lower total testosterone/LH ratio (4.6 vs. 6.0, p<0.0005) and lower calculated free-testosterone/LH ratio (94 vs. 134, p<0.0005) were detected for Faroese men.

Conclusions: Semen quality among Faroese men is at the same low level as reported for Danish men, and the reproductive hormone levels furthermore indicated a lower Leydig cell capacity for testosterone production. The influence of environmental exposure and genetic factors on the semen quality has to be studied further.

ARTICLE SUMMARY

Article focus

Semen quality studies have not been conducted in the Faroe Islands before.

Because the Faroese population differs from other populations regarding e.g. high exposure to persistent organic pollutants (POPs) and genetic diseases, the semen quality of Faroese men from the general population would be expected to be low.

Key messages

The semen quality among Faroese men is at the same low level as reported in Denmark.

The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts and provides independent evidence of poorer testicular function in the Faroese men than in the Danes.

Similarly, the Leydig cell capacity for testosterone production was also lower for Faroese men than for the Danes.

Strengths and limitations of the study

Prospective study of testicular function among young men from the general population unselected with regard to fertility.

Standardized inclusion and investigation procedures.

Clinical examination for one subgroup of Faroese men, but not for the other.

INTRODUCTION

In 1992 Carlsen [1] and co-workers published a combined analysis of results from 61 papers published between 1939 and 1991 and showed a significant decline in sperm counts over a 50 years period. A detailed reanalysis of the results found that the conclusion was supported by the underlying studies [2, 3]. Following the 1992 publication, many analysed retrospectively their historical data for temporal trends, some finding a decline and others not. Realising that the trend analyses indicated that semen quality could have reached a low level where it might affect fecundity, several prospectively designed cross sectional semen quality studies were initiated to determine the current quality in men from general populations. These studies did not only reveal geographical differences [4, 5] but confirmed the general presence of low semen quality in men from all investigated countries when the results were interpreted according to available publications focused on associations between semen quality and fertility chances [6-9].

The causes of decreased semen quality are not clear, but it is feasible that many cases may have been caused by exposure to environmental factors in utero, during adolescence or in adulthood [10], however, most likely acting on a background of different genetic susceptibility.

The Faroese population differs from other populations in many respects. The Faroese population has high exposure to persistent organic pollutants (POPs) derived from traditional marine food, which includes blubber from the pilot whale. At the same time, there are several genetic diseases reported with very high frequency in the Faroese Islands [11]. Thus, our hypothesis was that semen quality of Faroese men from the general population would be quite low.

MATERIALS AND METHODS

To test our hypothesis we investigated semen quality of young Faroese men from the general population in 2007-2010. For comparison we used a recently published similar group of Danish men examined 2006-2010 [12] and the current World Health Organization (WHO) reference levels [13].

Study population: men from the general Faroese population

The entire study population consisted of 484 men, originating from two separate studies. The first study group included 241 randomly selected young men examined between February 2007 and February 2009 (F1). The second group comprised of 243 men examined between November 2009 and November 2010 (F2). A detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1. Within 3 months prior to participation, 132 men (27.3%) had used medication, mainly antibiotics, painkillers, or asthma/allergy medicine.

Table 1: Physical appearance and self-reported information of Faroese and Danish men. Results shown as medians (5-95th percentile) or percentages.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=243)	P value*	Faroese men (F1+F2) 2007-10 (N=484)	Danish men (D) 2006-10 (N=1,274)	P value*
Physical appearance						
Age (years)	25.3 (24.2-26.6)	23.0 (22.0-24.0)	<0.0005	24.0 (22.0-26.2)	19.0 (18.4-21.8)	<0.0005
Height (cm)	180.0 (169.9-189.9)	180.0 (169.8-193.0)	0.1	180.0 (170.0-191.0)	181.6 (171.1-193.0)	<0.0005
Weight (kg)	80.3 (64.2-106.2)	78.00 (62.9-105.1)	0.1	80.0 (63.0-105.8)	74.1 (60.1-96.0)	<0.0005
BMI (kg/m2)	25.0 (20.6-32.3)	24.3 (20.4-32.1)	0.02	24.6 (20.5-32.1)	22.4 (18.7-28.6)	<0.0005
Testes size (mL)**	21.5 (15.3-25.0)	NA		NA	23 (14-29)	
Lifestyle						
Alcohol per week (units)	5(0-25)	6 (0-27)	<0.0005	6 (0-26)	12 (0-42)	<0.0005
Current Smokers	44.5	41.6	0.5	43.2	45.4	0.4
Mother smoked in pregnancy	28.9	37.2	0.06	34.2	29.1	0.4
Taken medication***	22.4	31.0	0.1	27.3	15.1	<0.0005
Been treated for§						
Cryptorchidism†	3.3	7.4	0.05	5.4	5.0	<0.0005
Been diagnosed as having§§						
Cryptorchidism reported	8.8	11.6	0.3	10.2	6.9	0.03
Hypospadias	0.4	0.8	0.06	0.6	0.3	0.007

Sexual transmitted disease††	9.1	10.8	0.6	9.7	6.2	0.008
Phimosis	10.1	11.7	0.4	10.9	4.6	<0.0005
Varicocele	0.8	0.8	1.0	0.8	0.4	0.7
Have§§§						
Ever caused a pregnancy**	25.9	NA		NA	6.8	-
Fatherhood	21.3	7.5	<0.0005	14.9	NA	-
Experienced fertility problems‡	4.6	1.7	0.07	3.1	0.2	<0.0005

*Mann Whitney test

**Testes size was not measured in Faroese men born 1986-87 and the question "Have you ever caused a pregnancy" was not asked in this group

*** Taken any medication 3 months prior to participation in the study

† Hormonal, surgical or combination

†† Chlamydia, gonorrhoea, warts or herpes

‡Ever had regular intercourse without use of contraception for at least 6 months (Faroese men) or 1 year (Danish men) without partner became pregnant

§ "Have you ever been treated for…" §§ "Has a doctor ever diagnosed you as having....." §§§ Have you ever…"

NA: not available

Faroese men, examined 2007-09 (sub-group F1)

Invitation letters to participate in the study were sent to 1,100 men, consecutively listed in the Faroese population register as born between January 1981 and December 1984, followed by a phone call to arrange the examination details. A total of 34 men had emigrated, and 43 letters were returned as undeliverable. Thus, 1,023 were invited. Of these, 490 could not be reached and 292 declined to participate. Hence, the final F1 group was comprised of 241 men (24% of all invited).

Faroese men, examined 2009-10 (sub-group F2)

This group consisted of a cohort generated from consecutive births at the three Faroese hospitals during 1986-1987 (N=509 males) as described elsewhere [14-16]. Detailed information on their physical health and potential environmental factors were collected at the time of birth and during the course of follow-up at ages 7, 14 and 22 years. The 421 men who had participated in the 22 years follow-up were invited to participate in the

semen quality study. This was the sole selection criteria. All men received a letter of invitation and subsequently a phone call to arrange the examination details. Among the 421 invited men, 243 accepted to participate, but 3 did not succeed in delivering a semen sample. Hence, the final F2 group comprised 240 men (57 % of all invited).

Questionnaire

On the day of attendance, the men returned a questionnaire they had received in advance. The questionnaire was based on that used for young Danish men [12, 17] and included information on previous or current diseases, including any known history of fertility potential, and some lifestyle factors like smoking and drinking habits.

Semen samples

Semen samples were produced by masturbation in a room close to the semen laboratory. The period of abstinence was recorded. The abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. The semen sample was analysed according to the World Health Organization 1999 guidelines [18]. Semen volume was estimated by weighing the collection tube with the semen sample and subtracting the weight of the empty pre-weighed tube, assuming that 1 mL semen=1 g. For sperm motility assessment, 10 µL of well-mixed semen was placed on a clean glass slide kept at 37 °C and covered with a 22x22 mm coverslip. The preparation was placed on the heated stage of a microscope at 37 °C and immediately examined at x400 magnification. The sperm were classified as progressive motile, local motile or immotile.

For the assessment of the sperm concentration, the samples were diluted in distilled water. The sperm concentration was subsequently assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). However, a second delivery of Bürker-Türk chambers were 0.05 mm in depth rather than the standard 0.1 mm we had ordered. Because of that 59 samples from F2 were analysed without knowing whether 0.05 mm or 0.1 mm deep counting chambers were used. As the semen samples had been stored in our bio-bank, the 59 samples were measured again with the 0.1 mm deep chamber to indicate if the original concentration assessment was correct (assuming that the 0.1 mm counting chamber was used originally) or should be doubled (assuming that the 0.05 mm counting chamber was used originally). Based on these

re-analyses, the obtained original sperm concentrations were doubled for 30 samples. To ascertain that the concentration could be replicated in thawed samples, samples known to be counted in 0.1 mm deep chamber were counted again. As anticipated, the results were replicated and shown to be accurate. From each semen sample a smear for morphology evaluation was made, Papanicolaou stained and finally assessed according to "strict criteria" at Department of Growth and Reproduction (GR) at the National Hospital (Rigshospitalet, RH) in Denmark.

The Faroese semen analyses were performed by three technicians, who participated in a quality control testing course at Rigshospitalet (RH) and spent two weeks there to ensure comparable results. Furthermore, every three months, 5 blinded samples were sent from RH, and sperm concentration results were compared with the results from their technicians. Throughout the study period, the variation was less than 10% compared with the Danish technicians. Therefore, the methodological differences between the Faroese and the Danes should be considered very limited.

Reproductive hormones

At the examination day a venous blood sample was drawn from each participant and centrifuged. Serum was subsequently separated and kept frozen until they were analysed for reproductive hormones at GR at RH. Levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone-binding globulin (SHBG) were determined using a time-resolved fluoroimmunoassay (Delfia, Wallac, Turku, Finland). Testosterone (T) was determined using a COAT-A-COUNT solid phase radioimmunoassay (RIA) (Siemens Medical Solutions, Malvern, PA, USA) and estradiol by radioimmunoassay **(**RIA) (Pantex, Santa Monica, CA, USA) [19]. Samples were analysed for inhibin B in 2009 using kit material from Oxford Bioinnovation or in 2010 with kit material from DSL Beckman, USA. The free testosterone index (FT) was calculated from total testosterone and SHBG using a fixed albumin value according to Vermeulen et al. [20].

Physical examination

Participants in the F1 sub-group underwent a physical examination performed by one of two examiners at the day of the semen sample delivery. Body weight and height were measured. The Tanner stage of pubic hair and genital development were recorded. Any abnormalities in the testis and penis, the possible presence of varicocele, hydrocele, hypospadias and testis tumour, the location of testis in scrotum and their consistency and that

of epididymis were recorded. Testicular volumes were determined by use of a Prader orchidometer. Participants in the F2 study did not undergo a physical andrological examination.

Comparison population: men from the general Danish population

In Denmark, all young men, except those suffering from severe chronic diseases (<15%), are required to attend a compulsory medical examination before they are considered for military service [5]. Men are called upon at the age of 18-19 years, but some postpone this examination until completion of their education. Men attending the compulsory examination are invited to participate in a semen quality study, using same basic study design including a physical examination as the Faroese, irrespective of whether they are declared with for military service or not. For comparison with results from the Faroese studies, we utilized results for Danish men examined 2006-10, which has recently been published [12]. The detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1.

Comparison population: WHO reference group of fertile men

The Faroese sperm count distribution was compared with the distribution for fertile men reported as the WHO reference group [13].

Statistics

The crude means, medians, standard deviations, 5-95 percentiles and frequencies were used for basic descriptions of obtained results. The main outcome variables were semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and serum level of the reproductive hormones. Differences in semen quality variables and reproductive hormone levels between groups were tested by linear regression adjusted for significant confounders. Semen volume, sperm concentration, and total sperm count were best normalised by a cubic root transformation before regression analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa were logit-transformed. Percentages of morphologically normal spermatozoa were close to normally distributed and entered the model

untransformed. Reproductive hormone levels were natural logarithmic transformed. Correlations between reproductive hormone levels were assessed with Spearman Correlation. Between-group differences for categorical variables were tested with non-parametric tests (Mann Whitney). Total sperm count distribution differences between the Faroese men and the WHO reference distribution was tested by chi square analyses.

For F2, the abstinence time in hours showed a significant positive association to sperm concentration, semen volume and total sperm count. The effect was most pronounced for the period below 96 hours and less above (for sperm concentration: β-value=0.011, p=0.01 and β-value=0.006, p=0.18 respectively). The abstinence time was not significantly associated with sperm concentration in F1 (β-value=0.0001, p=0.98, and β-value=0.0003, p=0.5 respectively) or total sperm count. Thus, abstinence time was entered as piecewise linear functions (linier splines); i.e. one straight line for abstinence below 96 and another straight line for abstinence above 96. The duration from ejaculation to assessment was included as confounder for sperm motility.

The following factors were evaluated as possible confounders for semen parameters and found to have no influence: age, body mass index (BMI) (as continuous variable or categorized as <18.49; 18.5-25; >25), smoking (yes/no) and season of year (spring (March-May), summer (June-August), autumn (September-November) and winter (December-February)).

The same factors were evaluated as possible confounders for the reproductive hormones. Significant associations were observed for BMI and age but not for smoking. The effect of season was not systematic and thus not included as a confounder. Hour of day of blood sampling was included as a confounder for reproductive hormones, although only significantly associated with estradiol, T and FT. p-values below 5% were considered statistically significant. Analyses were performed using PASW GradPack 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In Table 1 physical appearance, lifestyle factors, reproductive health and self-reported information on fertility are displayed for the two sub-groups of Faroese men, the combined Faroese group and the Danish comparison group. The three groups differed in age with the F1 being oldest and the Danish youngest. Similarly the F1 group had the highest BMI and the Danish the lowest. Alcohol intake was lower among Faroese men, and more had caused a pregnancy/fathered a child.

Table 2 summarizes the semen variables. Sperm concentrations for the Faroese men were lower than for the Danish men. However, semen volume was higher, and thus total sperm counts did not differ. Similarly, overall motility and morphology did not differ between Faroese and Danes, but between the two Faroese subgroups with higher values for the F1-subgroup. Figure 1A shows the distributions of total sperm counts of all the investigated Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). According to the figure more Faroese men had lower sperm counts than the reference group ($p<0.0001$).

Table 2: Semen parameters in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=243)	P value	Faroese men (F1+F2) 2007-10 (N=484)	Danish men (D) 2006-10 (N=1,274)	P value
Sperm concentration (mill/mL)						
Mean (SD)	54 (57)	60 (60.0)		57 (58)	62 (55)	
Median (5-95 percentiles)	39 (1.6-157)	41 (2.2-182)	0.5*	40 (1.9-174)	48 (3-169)	□0.0005*
Semen volume (mL)						
Mean (SD)	4.2 (1.7)	4.0 (1.7)		4.1 (1.7)	3.6 (3.1)	
Median (5-95 percentiles)	4.0 (1.9-7.1)	3.9 (1.5-7.2)	0.02*	3.9 (1.8-7.1)	3.3 (1.3-6.3)	□0.0005*
Total sperm count (mill)						
Mean (SD)	215 (205)	216 (206)		215 (206)	206 (258)	
Median (5-95 percentiles)	156 (3-586)	159 (9-638)	0.7*	159 (6-591)	151 (13-559)	0.2*
Normal morphology (%)						
Mean (SD)	7.5 (4.3)	6.3 (4.5)		6.9 (4.4)	7.5 (4.9)	
Median (5-95 percentiles)	7.5 (1.0-15.0)	5.0 (0.5–15.0)	0.007†	6.5 (0.9-15.0)	7.0 (0.5-16.0)	0.1†
Motile sperm (%)						
Mean (SD)	70 (22)	58 (13)		64 (19)	65 (16)	
Median (5-95 percentiles)	74 (19-95)	59 (35-74)	□0.0005‡	64 (24-90)	68 (33-85)	0.01‡
Period of abstinence (h)						
Mean (SD)	81 (38)	86 (35)		83 (37)	77 (96)	
Median (5-95 percentiles)	83 (14-167)	83 (36-168)	0.1†	83 (25-168)	63 (37-134)	□0.0005†

*Regression analysis adjusting for duration of abstinence.
†Mann Whitney test
‡Regression analysis adjusting for delay from time of ejaculation to assessment of motility.

From the entire group of 484 men, 316 (65%) were without any prior knowledge of andrological diseases or conditions that might be associated with risk of impaired testicular function (cryptorchidism, testicular torsion, orchitis, epididymitis, varicocele, hydrocele, prostatitis, hypospadias, phimosis, inguinal hernia, cystitis, sexually transmitted diseases, diabetes, thyroid diseases, experienced fertility problems). In this subgroup 244 men (50% of the entire group) had an ejaculation abstinence period of more than 48 hours. These 244 men also had lower total sperm counts than men from the WHO reference group (Figure 1B, lower panel, p=0.0013).

The percentages of morphologically normal spermatozoa were inversely associated with year of birth, both for the entire group of Faroese men (Figure 2A, trend p=0.002), and for the subgroup of 244 men (Figure 2B, trend p=0.018). Morphology assessments were done in four different time periods, but the results did not differ according to these periods. Similar trends were seen when the estimates according to year of birth were adjusted for age, however, with broader confidence intervals of the estimates. No other semen variables were associated with birth year and age at time of investigation.

Approximately 15% of the participants reported to have fathered a child. The confounder adjusted estimates indicated higher sperm concentration, total sperm count, motility and morphologically normal spermatozoa in these men in comparison to non-fathers, all non-significant except for motility (p<0.0005). Semen volume was non-significantly lower in the fathers (p=0.6). When the distribution of total sperm counts for these fathers were compared with the WHO reference group no difference between the groups could be shown (p=0.1).

Only the men in the F1-group had a physical examination performed. Among these 95% had a normal mean testicular volume (i.e. above 15 mL), but apparently slightly lower than the Danish men (Table 1). A total of 2% did not have both testicles in scrotum, 8% had soft testicles and 4% did not have adult pubic hair distribution (i.e. Tanner stage 5 or 6). A varicocele was detected in 15% (5.8% grade 1, 7.5% grade 2 and 1.7% grade 3). In regression analyses all semen variable estimates were higher in men without any varicocele, but all non-significant (all p>0.2). Hydrocele was detected in 5%, and tended to be associated with a lower percentage of morphologically normal spermatozoa (p=0.05), but not with other semen variables (all p>0.6).

The men's tobacco smoking was non-significantly associated with lower semen volume, sperm concentration and total sperm counts (all p>0.1) and higher percentage of normal forms and motile spermatozoa (p=0.8 and 0.2, respectively). Maternal smoking during pregnancy was significantly associated with lower total sperm count (29% reduction, 95% confidence interval 8;45%, p=0.02), and non-significantly with semen volume (9% reduction, 95% confidence interval -16%;2%, p=0.05) and sperm concentration (22% reduction, 95% confidence interval -40;6%, p=0.06). Associations to motility and morphology were highly non-significant (p=0.5 and 0.8, respectively). BMI above 25 was not associated with adverse effects on any semen variable.

Table 3 summarizes the reproductive hormone levels for the two sub-groups of Faroese men, the combined group and the Danish comparison group. Inhibin B tended to be slightly lower in the Faroese group F1 than in the F2 group, thus showing the same tendency as sperm concentration and total sperm count. FSH showed opposite directions resulting in lower inhibin B/FSH ratio in the F1 group. In the combined Faroese group both inhibin B and FSH individually were higher than in the Danish group, whereas the inhibin B/FSH ratio was lower. In 2010 the laboratory began to use a different kit material for the serum inhibin B analyses. Statistical analyses showed, however, that the inhibin B/FSH results did not differ according to this.

Table 3: Reproductive hormones levels in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	**Faroese men (F2) 2009-10 (N=243)**	**P value***	**Faroese men (F1+F2) 2007-10 (N=484)**	**Danish men (D) 2006-10 (N=1,274)**	**P value***
FSH (IU/L)						
Mean (SD)	4.1 (4.7)	3.4 (1.9)		3.8 (3.6)	2.8 (1.8)	
Median (5-95 percentile)	3.1 (1.3-8.5)	2.9 (1.1-7.0)	0.04	3.1 (1.2-7.7)	2.5 (1.0-6.0)	□0.0005
Inhibin B (pg/mL)						
Mean (SD)	196 (79)	220 (84)		208 (82)	189 (70)	
Median (5-95 percentile)	192 (76-333)	210 (99-382)	0.06	202 (85-346)	180 (90-318)	□0.0005
Inhibin B /FSH						
Mean	80 (72)	101 (107)		91 (92)	100 (89)	
Median (5-95 percentile)	57 (11-203)	70 (17-289)	0.03	64 (13-259)	76 (17-272)	0.001

LH (IU/L)						
Mean (SD)	4.8 (2.4)	4.8 (1.8)		4.8 (2.1)	3.5 (1.5)	
Median (5-95 percentile)	4.5 (2.1-8.5)	4.6 (2.1-7.9)	0.9	4.5 (2.1-7.9)	3.3 (1.6-6.3)	□0.0005
Testosterone (nmol/L)						
Mean (SD)	21 (7)	23(7)		22 (7)	20 (6)	
Median (5-95 percentile)	20 (11-32)	22 (14-36)	0.02	21 (12-33)	19 (12-31)	□0.0005
Testosterone/LH						
Mean	5.0 (2.3)	5.9 (7.2)		5.5 (5.3)	6.5 (2.8)	
Median (5-95 percentile)	4.6 (1.9-9.1)	4.6 (2.7-11.6)	0.1	4.6 (2.2-9.4)	6.0 (3.0-12.0)	□0.0005
Free Testosterone						
Mean	409 (134)	462 (140)		439 (134)	454 (142)	
Median (5-95 percentile)	397 (211-633)	442 (262-707)	0.001	424 (236-686)	432 (269-701)	0.2
Free testosteron/LH						
Mean	99 (44)	125 (175)		112 (128)	149 (71)	
Median (5-95 percentile)	92 (39-186)	96 (49-212)	0.04	94 (47-201)	134 (65-273)	□0.0005
Estradiol (nmol/L)						
Mean (SD)	79 (22)	96 (23)		88 (24)	80 (24)	
Median (5-95 percentile)	78 (46-113)	94 (62-140)	□0.0005	86 (54-131)	78 (48-125)	□0.0005
Testosterone/Estradiol						
Mean	268 (83)	243 (69)		255 (78)	258 (70)	
Median (5-95 percentile)	262 (140-409)	236 (146-375)	□0.0005	243 (145-394)	249 (175-383)	0.1
Free Testorsterone/Estradiol						
Mean	5332 (1567)	4955 (1187)		5143 (1400)	5869 (1768)	
Median (5-95 percentile)	5218 (3230-7768)	4876 (3165-7201)	0.001	5026 (3182-7538)	5636 (3519-8956)	□0.0005
SHBG (nmol/L)						
Mean (SD)	38 (15)	37 (15)		38 (15)	30 (12)	
Median (5-95 percentile)	36 (17-62)	35 (19-68)	0.7	36 (18-65)	29 (14-51)	□0.0005
Time of bloodsampling						

Mean (SD)	11:20 (2:12)	10:00 (1:30)		10:10 (2:00)	10:0 (0:15)	
Median (5-95 percentile)	10:12 (9:00-16:00)	9:10 (8:06-12.48)	□0.0005†	10:12 (8:30-15:20)	10:0 (9:05-11:20)	0.008†

*Regression analysis adjusting for hour of day of blood sampling
†Mann Whitney test

Total testosterone (T) only differed slightly between the two Faroese groups and between the combined group and the Danes, although statistically significant. SHBG in contrast were considerably higher for the Faroese men, and with no difference between the F1 and F2 men. Frequency analyses of the SHBG values divided into groups (0-10, 10-20 etc.) showed that the higher SHBG values for Faroese men were not caused by higher concentration in a specific subgroup but an overall shift to higher levels. Approximately 80% of the Faroese men had a SHBG concentration below 50 nmol/L while 80 % of the Danish men had a concentration below 40 nmol/L. The FT differed between the two Faroese groups which combined, however, had non-significantly lower FT than the Danes. Estradiol was higher in the F2 group than in the F1, thus leading to higher levels in the combined group of Faroese men than in the Danish. The ratios T/LH, FT/LH, and FT/estradiol were all lower in the Faroese men than in the Danish whereas the estimated lower total T/estradiol was non-significant.

SHBG and LH were positively correlated with T (P <0.0005), whereas SHBG and LH did not correlate (P=0.1). FT was positively correlated with LH and T (P <0.0005), and negatively but not significantly correlated with SHBG (P=0.07)

BMI was negatively associated with T, FT, SHBG, T/LH, T/estradiol, FT/estradiol and inhibin B (p<0.0005-0.045), and also negatively but non-significant with LH (p=0.8). Faroese smokers tended to have higher T than non-smokers (p=0.6, adjusted for BMI effect), whereas the effect on the Danish men were highly significant (p<0.0005). In the Faroese groups LH, SHBG, estradiol, FT/LH, FT/estradiol and T/estradiol tended to be higher in smokers, although all non-significantly. For the Danes similar trends were detected; the effect on SHBG and LH being non-significant, whereas the remaining were significant with p-values <0.0005-0.05. Further maternal smoking during pregnancy for the Faroese men tended to be associated with lower LH and FT (p=0.04 and 0.02) whereas the effect on the remaining hormone levels were all highly non-significant (p=0.1-0.9). However, the difference (i.e. significant levels in table 3) between Faroese and Danish hormone levels did not change

when including BMI or age as cofactors into the regression analyses indicating that these factors cannot explain the difference in reproductive hormones between Faroese and Danes.

DISCUSSION

This is the first study on testicular function conducted in the Faroe Islands. These young Faroese men had a lower sperm concentration but similar total sperm count compared to Danish men. Recent data have shown sperm counts to be low in young men from several European countries, however, slightly higher than among the Danes [5, 21, 22]. Thus, the semen quality of Danish men seems to be particularly low, and now we have shown the Faroese men to have a similar low level. The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts, and provides independent evidence of poorer testicular function in the Faroese men than in the Danes, although the medians were at a level where the association between sperm counts and inhibin B is weakened [23]. When evaluating the results against the WHO reference population Faroese men also had low sperm counts. The lower T/LH and FT/LH ratios point towards a lower Leydig cell capacity among Faroese men compared to Danes. Thus, the total testicular function among Faroese men may be at the same or lower level than Danes.

The Faroese and the Danish men were not exactly the same age. Semen quality has been reported to decline with increasing age [24-27]. However, the Faroese studies and the comparative Danish studies were conducted at the same time periods and the slight difference in age between groups is not likely to have influenced the semen parameters nor is the actual age of the participants that still can be regarded as young. Semen quality does on average not change between 19 and 23 years of age, and immaturity seems unlikely to explain the poor semen quality both among the Faroese and Danish men [28]. Thus, age difference is not likely to explain the difference in semen quality.

The Faroese men were investigated during a relatively short time period and also represented narrow birth cohorts. Despite of this, we actually detected an inverse association between percentage of morphologically normal spermatozoa and year of birth, a trend that was robust even when controlled for potential confounders. In a cohort like ours the effect of birth year may be difficult to separate from the effect of age. However, when we modelled age as explanatory factor and controlled for birth year, the trend effect of age was not apparent.

The Faroese studies were investigated according to comparable protocols and assessment of sperm concentration was controlled by the same quality control as the Danish study. The Faroese technicians were trained at RH in Denmark and the analyses used in both laboratories were identical, thus reducing the interobserver variation. Additionally, assessment of sperm morphologies was performed randomly by a single person. Frequency of motile sperm, however, is difficult to compare reliably between different groups. The motility assessment is highly subjective and controlling for quality control within this parameter is problematic. Previously, the inter-laboratory variance motility has shown to be of significant importance [29]. Therefore, we hesitate to draw major conclusion regarding sperm motility.

The recording of abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. This latter procedure is believed to be much more accurate, as the men were given time to reflect and were not expected to give an immediate verbal response. This assumption is supported by the fact that the anticipated relationship between abstinence time and sperm concentration only was clearly seen in F2 group.

As explained in the methods section some of the semen samples were analysed with incorrect counting chambers. However, we corrected for this potential testing error. In addition, the statistical analyses performed with or without the 59 samples did not change the overall results, supporting the assumption that the corrections made were valid.

A major advantage of the study was that the participants were not selected on fertility status. According to most of the Danish men, the main incentive for participation was the financial compensation [5]. The F2 men were part of an existing birth cohort study whereas F1 men were randomly recruited. Men with suspected fertility problems may be overrepresented. However, even in the subgroup of men with abstinence period of more than 48 hours and without any prior knowledge of andrological diseases and without know fertility the same low sperm counts were detected.

The finding of low semen quality and lower Leydig cell function in Faroese men cannot be explained by effects of confounders. Both high BMI and maternal smoking have previously been associated with reduced testicular function. In our cohort, the effects of these two usually robust confounders/explanatory factors were not obvious, neither on the reproductive hormones. However, our cohorts may be too small to detect the effect of these two factors. Alternatively, other unidentified factors may have a major influence masking the effect of BMI and maternal smoking on the semen parameters. The reason for the low testicular function of the Faroese young men is unknown but this population is highly exposed to POPs

derived solely from traditional marine food, which includes blubber from the pilot whale. Studies have shown associations between high PCB levels and low semen quality, and since PCBs and p,p'-DDE have the potential to interfere with sex hormone functions, it is plausible to assume that these compounds can affect the function of these organs [30]. There are some reports on the effect of POPs on male reproduction in humans, mainly indicating weak negative effects on sperm motility [30-32]. For the two Faroese sub-groups, we found that the percentage of motile cells was significantly lower compared with Danish men, indicating that increased exposure to endocrine disruptors can be one explanation.

Serum SHBG levels for the Faroese men were strikingly higher than in the Danes. SHBG is a sensitive marker for thyroid function. However, none of the study subjects were under treatment for hyperthyroidism. We did not measure thyroid hormones, but the men did not have any obvious clinical symptoms of hyperthyroidism. If a high thyroid level in general should explain the SHBG level, a significantly elevated LH and testosterone in men with low BMI would have been expected, which was not the case. Therefore, there is no indication that the high SHBG values are due to hyperthyroidism. Furthermore, alcohol consumption could not explain the high levels. One plausible explanation to the high SHBG levels could be the high PCB exposure mentioned above. A recent publication from the Faroe Islands found that SHBG increased at higher PCB exposure, both prenatally and currently. PCBs are known to affect a variety of liver functions, and it could be speculated that PCB-induced hepatic SHBG synthesis could play a possible role, although this possibility remains to be substantiated [33]. We cannot elucidate further if PCB in our current study group can explain the SHBG levels.

It is known that the Faroese population differs genetically from other populations in many respects. There are several genetic diseases reported with very high frequency on the Faroese Islands [11, 34]. However different genetic composition in populations may contribute to the explanation of differences in semen quality and would be worth further exploration.

In conclusion, we found that the semen quality among Faroese men is at the same low level as reported among Danish men. This low quality was corroborated by the reproductive hormone levels in Faroese men. The influence of environmental exposures and genetic factors on the semen quality has to be studied further.

CONTRIBUTORS:

Substantial contribution to conception and design: PG, PW, NJ, TKJ.

Acquisition of data: JH, MSP, PW.

Interpretation of data: All authors.

Drafting the article: JH, MSP, NJ.

Revising the article critically for important intellectual content: all authors.

Final approval of the version to be published: all authors.

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COMPETING INTERESTS. None

ETHICS APPROVAL. The local Science Ethical Committee for the Faroe Islands and the Institutional Review Board at Harvard School of Public Health have approved the study protocol, and all participants had given their informed consent.

DATA SHARING STATEMENT

There are no additional data available.

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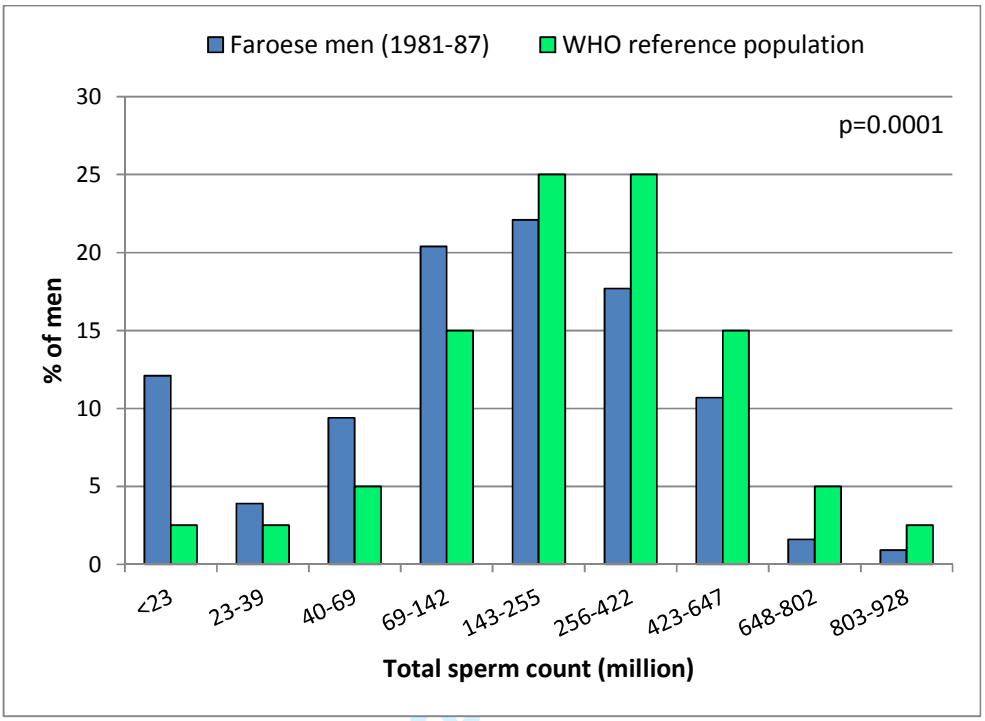


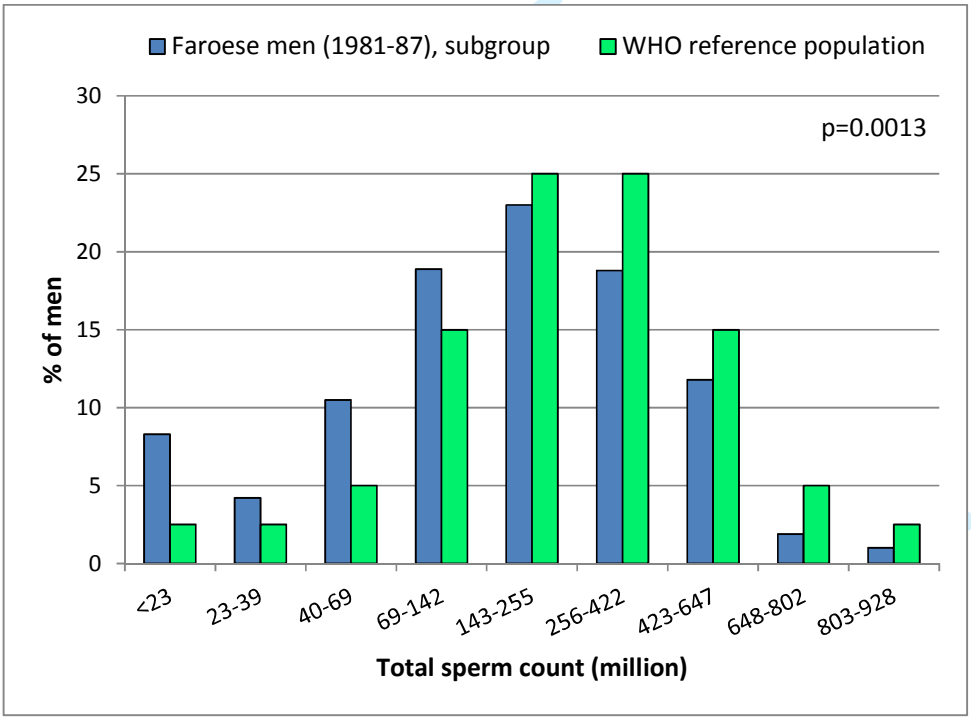
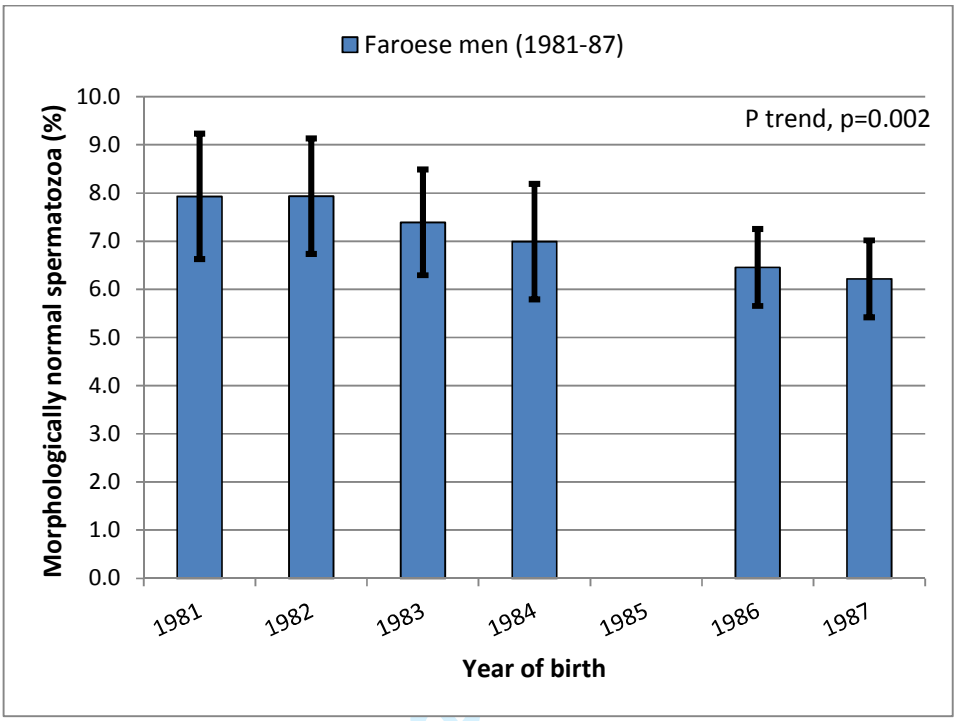


Figure 1. Distribution of total sperm count in Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). In (A) all Faroese men are included. In (B) only men having an ejaculation abstinence period above 48 hours and being "without diseases" (see text for further explanation) are included.



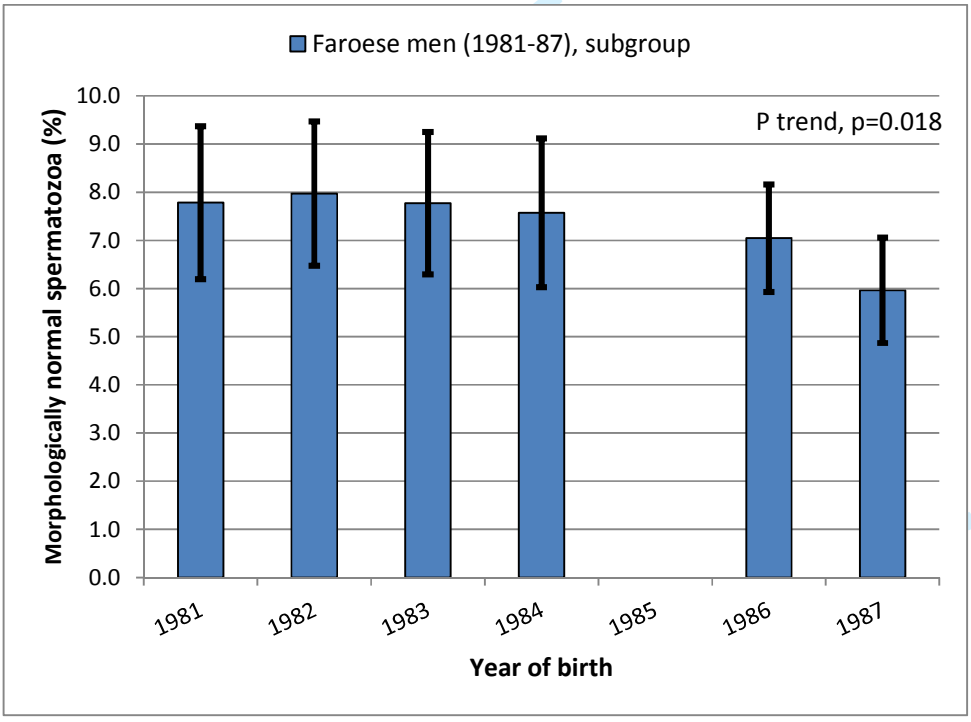


Figure 2. Percentage of morphologically normal spermatozoa according to year of birth. Whiskers show the 95% confidence interval of the estimated mean. In (A) all Faroese men are included. In (B) only men having an ejaculation abstinence period above 48 hours and being "without diseases" (see text for further explanation) are included.

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*	Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses

Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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SEMEN QUALITY AND REPRODUCTIVE HORMONES IN FAROESE MEN – A CROSS-SECTIONAL POPULATION-BASED STUDY OF 481 MEN

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SEMEN QUALITY AND REPRODUCTIVE HORMONES IN FAROESE MEN – A CROSS-SECTIONAL POPULATION-BASED STUDY OF 481 MEN

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Keywords: **Semen quality, reproductive hormones, Faroe Islands**

Word count: 4373

ABSTRACT

Objectives: To determine semen quality and reproductive hormone levels in young Faroese men.

Design: Descriptive cross-sectional study of Faroese men compared with Danish men.

Setting: Faroese one-center study.

Participants: 481 men born from 1981 to 1987 and investigated from 2007 to 2010.

Outcome measures: Sperm concentration, semen volume, total sperm count, sperm motility, sperm morphology and reproductive hormones levels.

Results: Sperm concentrations for the Faroese men were lower than for the Danish (crude median 40 mill/mL vs. 48, p<0.0005). Semen volume was higher, and thus total sperm counts did not differ (159 vs. 151 mill, p=0.2). Motility and morphology did not differ between Faroese and Danes. Inhibin B/FSH ratio for the Faroese men were lower than for Danes (64 vs. 76, p=0.001). Similarly, lower total testosterone/LH ratio (4.6 vs. 6.0, p<0.0005) and lower calculated free-testosterone/LH ratio (94 vs. 134, p<0.0005) were detected for Faroese men.

Conclusions: Semen quality among Faroese men is at the same low level as reported for Danish men, and the reproductive hormone levels furthermore indicated a lower Leydig cell capacity for testosterone production. The influence of environmental exposure and genetic factors on the semen quality has to be studied further.

ARTICLE SUMMARY

Article focus

Semen quality studies have not been conducted in the Faroe Islands before.

Because the Faroese population differs from other populations regarding e.g. high exposure to persistent organic pollutants (POPs) and genetic diseases, the semen quality of Faroese men from the general population would be expected to be low.

Key messages

The semen quality among Faroese men is at the same low level as reported in Denmark.

The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts and provides independent evidence of poorer testicular function in the Faroese men than in the Danes.

Similarly, the Leydig cell capacity for testosterone production was also lower for Faroese men than for the Danes.

Strengths and limitations of the study

Prospectively designed, cross-sectional study of testicular function among young men from the general population unselected with regard to fertility.

Standardized inclusion and investigation procedures.

Clinical examination for one subgroup of Faroese men, but not for the other.

INTRODUCTION

In 1992 Carlsen [1] and co-workers published a combined analysis of results from 61 papers published between 1939 and 1991 and showed a significant decline in sperm counts over a 50 years period. A detailed reanalysis of the results found that the conclusion was supported by the underlying studies [2, 3]. Following the 1992 publication, many analysed retrospectively their historical data for temporal trends, some finding a decline and others not. Realising that the trend analyses indicated that semen quality could have reached a low level where it might affect fecundity, several prospectively designed cross sectional semen quality studies were initiated to determine the current quality in men from general populations. These studies did not only reveal geographical differences [4, 5] but confirmed the general presence of low semen quality in men from all investigated countries when the results were interpreted according to available publications focused on associations between semen quality and fertility chances [6-9].

The causes of decreased semen quality are not clear, but it is feasible that many cases may have been caused by exposure to environmental factors in utero, during adolescence or in adulthood [10], however, most likely acting on a background of different genetic susceptibility.

The Faroese population differs from other populations in many respects. The Faroese population has high exposure to persistent organic pollutants (POPs) derived from traditional marine food, which includes blubber from the pilot whale. At the same time, there are several genetic diseases reported with very high frequency in the Faroese Islands [11]. Thus, our hypothesis was that semen quality of Faroese men from the general population would be quite low.

MATERIALS AND METHODS

To test our hypothesis we investigated semen quality of young Faroese men from the general population in 2007-2010. For comparison we used a recently published similar group of Danish men examined 2006-2010 [12] and the current World Health Organization (WHO) reference levels [13].

Study population: men from the general Faroese population

The entire study population consisted of 484 men, originating from two separate studies. The first study group included 241 randomly selected young men examined between February 2007 and February 2009 (F1). The second group comprised of 243 men examined between November 2009 and November 2010 (F2). A detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1. Within 3 months prior to participation, 132 men (27.4%) had used medication, mainly antibiotics, painkillers, or asthma/allergy medicine.

Table 1: Physical appearance and self-reported information of Faroese and Danish men. Results shown as medians (5-95th percentile) or percentages.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value*	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value *
Physical appearance						
Age (years)	25.3 (24.2-26.7)	23.0 (22.0-24.0)	<0.0005	24.0 (22.0-26.2)	19.0 (18.4-21.8)	<0.0005
Height (cm)	180.0 (169.9-189.9)	180.0 (170.0-193.0)	0.1	180.0 (170.0-191.0)	181.6 (171.1-193.0)	<0.0005
Weight (kg)	80.3 (64.2-106.2)	78.00 (62.9-105.1)	0.2	80.0 (63.1-105.9)	74.1 (60.1-96.0)	<0.0005
BMI (kg/m2)	25.0 (20.6-32.3)	24.3 (20.4-32.1)	0.03	24.6 (20.5-32.1)	22.4 (18.7-28.6)	<0.0005
Testes size (mL)**	21.5 (15.3-25.0)	NA		NA	23 (14-29)	
Lifestyle						
Alcohol per week (units)	5(0-25)	6 (0-26)	0.08	5 (0-25)	12 (0-42)	<0.0005
Current Smokers	44.0	41.3	0.5	42.6	45.4	0.4
Mother smoked in pregnancy	22.4.***	37.5	0.05	33.6	29.1	0.4
Taken medication§	23.7	31.3	0.07	27.4	15.1	<0.0005
Been treated for§§						
Cryptorchidism†	3.3	7.5	0.04	5.4	5.0	0.8
Been diagnosed as having§§						
Cryptorchidism reported	8.7	12.1	0.2	10.4	6.9	0.02
Hypospadias	0.4	0.8	0.6	0.6	0.3	0.4
Sexual transmitted disease††	9.1	10.8	0.5	10.0	6.2	0.007
Phimosis	10.0	11.7	0.5	10.8	4.6	<0.0005
Varicocele	0.8	0.8	1.0	0.8	0.4	0.6
Have§§§						
Ever caused a pregnancy**	25.7	NA		NA	6.8	-
Fatherhood	21.2	6.3	<0.0005	13.7	NA	-
Experienced fertility problems‡	4.6	1.7	0.07	3.1	0.2	<0.0005

*Mann Whitney test

**Mean of two testes. Testes size was not measured in Faroese men born 1986-87 and the question "Have you ever caused a pregnancy" was not asked in this group

*** Information only available for 189 mothers

§ Taken any medication 3 months prior to participation in the study

† Hormonal, surgical

or combination

†† Chlamydia, gonorrhoea, warts or herpes

‡Ever had regular intercourse without use of contraception for at least 6 months (Faroese men) or 1 year (Danish men) without partner became pregnant

§§ "Have you ever been treated for…" §§ "Has a doctor ever diagnosed you as having....." §§§ Have you ever…"

NA: not available

Faroese men, examined 2007-09 (sub-group F1)

Invitation letters to participate in the study were sent to 1,100 men, consecutively listed in the Faroese population register as born between January 1981 and December 1984, followed by a phone call to arrange the examination details. A total of 34 men had emigrated, and 43 letters were returned as undeliverable. Thus, 1,023 were invited. Of these, 490 could not be reached and 292 declined to participate. Hence, the final F1 group was comprised of 241 men (24% of all invited).

Faroese men, examined 2009-10 (sub-group F2)

This group consisted of a cohort generated from consecutive births at the three Faroese hospitals during 1986-1987 (N=509 males) as described elsewhere [14-16]. Detailed information on their physical health and potential environmental factors were collected at the time of birth and during the course of follow-up at ages 7, 14 and 22 years. The 421 men who had participated in the 22 years follow-up were invited to participate in the semen quality study. This was the sole selection criterion. All men received a letter of invitation and subsequently a phone call to arrange the examination details. Among the 421 invited men, 243 accepted to participate, but 3 did not succeed in delivering a semen sample. Hence, the final F2 group comprised 240 men (57 % of all invited).

Questionnaire

On the day of attendance, the men returned a questionnaire they had received in advance. The questionnaire was based on that used for young Danish men [12, 17] and included information on previous or current diseases, including any known history of fertility potential, and some lifestyle factors like smoking and drinking habits.

Semen samples

Semen samples were produced by masturbation in a room close to the semen laboratory. The period of abstinence was recorded. The abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. The semen sample was analysed according to the World Health Organization 1999 guidelines [18]. Semen

volume was estimated by weighing the collection tube with the semen sample and subtracting the weight of the empty pre-weighed tube, assuming that 1 mL semen=1 g. For sperm motility assessment, 10 µL of well-mixed semen was placed on a clean glass slide kept at 37 °C and covered with a 22x22 mm coverslip. The preparation was placed on the heated stage of a microscope at 37 °C and immediately examined at x400 magnification. The sperm were classified as progressive motile (WHO class AB motility), locally motile (WHO class C motility) or immotile (WHO class D motility).

For the assessment of the sperm concentration, the samples were diluted in distilled water. The sperm concentration was subsequently assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). However, a second delivery of Bürker-Türk chambers were 0.05 mm in depth rather than the standard 0.1 mm we had ordered. Because of that 59 samples from F2 were analysed without knowing whether 0.05 mm or 0.1 mm deep counting chambers were used. As the semen samples had been stored in our bio-bank, the 59 samples were measured again with the 0.1 mm deep chamber to indicate if the original concentration assessment was correct (assuming that the 0.1 mm counting chamber was used originally) or should be doubled (assuming that the 0.05 mm counting chamber was used originally). Based on these re-analyses, the obtained original sperm concentrations were doubled for 23 samples (see Supplementary Materials for details). To ascertain that the concentration could be replicated in thawed samples, samples known to be counted in 0.1 mm deep chamber were counted again. As anticipated, the results were replicated and shown to be accurate. From each semen sample a smear for morphology evaluation was made, Papanicolaou stained and finally assessed according to “strict criteria” at Department of Growth and Reproduction (GR) at the National Hospital (Rigshospitalet, RH) in Denmark.

The Faroese semen analyses were performed by three technicians, who participated in a quality control testing course at Rigshospitalet (RH) and spent two weeks there to ensure comparable results. Furthermore, every three months, 5 blinded samples were sent from RH, and sperm concentration results were compared with the results from their technicians. Throughout the study period, the variation was less than 10% compared with the Danish technicians. Therefore, the methodological differences between the Faroese and the Danes should be considered very limited.

Reproductive hormones

On the examination day, a venous blood sample was drawn from each participant and centrifuged (3000 g, 10 minutes). Serum was subsequently separated and kept frozen until it was analysed for reproductive hormones at GR at RH. Levels of follicle stimulating hormone (FSH), luteinizing

hormone (LH), and sex hormone-binding globulin (SHBG) were determined using a time-resolved fluoroimmunoassay (Delfia, Wallac, Turku, Finland). Testosterone (T) was determined using a COAT-A-COUNT solid phase radioimmunoassay (RIA) (Siemens Medical Solutions, Malvern, PA, USA) and estradiol by radioimmunoassay (RIA) (Pantex, Santa Monica, CA, USA) [19]. Inhibin B was analyzed by a double antibody enzyme-immunometric assay using a monoclonal antibody raised against the inhibin βB-subunit in combination with labeled anti-body raised against the α-subunit. Samples were analysed for inhibin B in 2009 using kit material from Oxford Bio-Innovation or in 2010 with kit material from DSL Beckman, USA. For all hormone assessments internal standard samples were analysed and showed no need to correct for potential inter-assay variation, including no need for correction between the kit materials for inhibin B from the two suppliers. The free testosterone index (FT) was calculated from total testosterone and SHBG using a fixed albumin value according to Vermeulen et al. [20].

Physical examination

Participants in the F1 sub-group underwent a physical examination performed by one of two examiners at the day of the semen sample delivery. Body weight and height were measured. The Tanner stage of pubic hair and genital development were recorded. Any abnormalities in the testis and penis, the possible presence of varicocele, hydrocele, hypospadias and testis tumour, the location of testis in scrotum and their consistency and that of epididymis were recorded. Testicular volumes were determined by use of a Prader orchidometer, and reported as the mean of two testes (Table 1). Participants in the F2 study did not undergo a physical andrological examination.

Comparison population: men from the general Danish population

In Denmark, all young men, except those suffering from severe chronic diseases (<15%), are required to attend a compulsory medical examination before they are considered for military service [5]. Men are called upon at the age of 18-19 years, but some postpone this examination until completion of their education. Men attending the compulsory examination are invited to participate in a semen quality study, using same basic study design including a physical examination as the Faroese, irrespective of whether they are declared fit for military service or not. For comparison with results from the Faroese studies, we utilized results for Danish men examined 2006-10, which has recently been published [12]. The detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1.

Comparison population: WHO reference group of fertile men

The Faroese sperm count distribution was compared with the distribution for fertile men reported as the WHO reference group [13].

Statistics

The crude means, medians, standard deviations, 5-95 percentiles and frequencies were used for basic descriptions of obtained results. The main outcome variables were semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and serum level of the reproductive hormones. Differences in semen quality variables and reproductive hormone levels between groups were tested by linear regression adjusted for significant confounders. Semen volume, sperm concentration, and total sperm count were best normalised by a cubic root transformation before regression analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa (ie WHO class AB+C) were logit-transformed. Percentages of morphologically normal spermatozoa were close to normally distributed and entered the model untransformed. Reproductive hormone levels were natural logarithmic transformed. Correlations between reproductive hormone levels were assessed with Spearman Correlation. Between-group differences for categorical variables were tested with non-parametric tests (Mann Whitney). Total sperm count distribution differences between the Faroese men and the WHO reference distribution was tested by chi square analyses. Association between year of birth and semen parameters were tested by linear regression.

For F2, the abstinence time in hours showed a significant positive association to sperm concentration, semen volume and total sperm count. The effect was most pronounced for the period below 96 hours and less above (for sperm concentration: β-value=0.011, p=0.01 and β-value=0.006, p=0.18 respectively). The abstinence time was not significantly associated with sperm concentration in F1 (β-value=0.0001, p=0.98, and β-value=0.0003, p=0.5 respectively) or total sperm count. Thus, abstinence time was entered as piecewise linear functions (linier splines); i.e. one straight line for abstinence below 96 and another straight line for abstinence above 96 for semen volume, sperm concentration and total sperm counts. The duration from ejaculation to assessment was included as a confounder for sperm motility. No confounders were detected to affect morphology.

The following factors were evaluated as possible confounders for semen parameters and found to have no influence ($p > 0.05$): age, body mass index (BMI) (as continuous variable or categorized as <18.49; 18.5-25; >25), smoking (yes/no) and season of year (spring (March-May), summer (June-August), autumn (September-November) and winter (December-February)).

The same factors were evaluated as possible confounders for the reproductive hormones. Significant associations were observed for BMI and age but not for smoking. The effect of season was not systematic and thus not included as a confounder. Hour of day of blood sampling was included as a confounder for reproductive hormones, although only significantly associated with estradiol, T and FT. p-values below 5% were considered statistically significant. Analyses were performed using PASW GradPack 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In Table 1 physical appearance, lifestyle factors, reproductive health and self-reported information on fertility are displayed for the two sub-groups of Faroese men, the combined Faroese group and the Danish comparison group. All men had semen variables assessed. However, morphology scoring was missing for 50 Faroese men and 20 Danes, which is reported in Table 2. Also, assessment of testis size was only performed on sub-group F1, which is stated in Table 1. Semen volume was not recorded for one Faroese man and motility measure was done for one man.

The three groups differed in age with the F1 being oldest and the Danish youngest. Similarly the F1 group had the highest BMI and the Danish the lowest. Alcohol intake was lower among Faroese men, and more had caused a pregnancy/fathered a child.

Semen variables

Table 2 summarizes the semen variables. Sperm concentrations for the Faroese men were lower than for the Danish men. However, semen volume was higher, and thus total sperm counts did not differ. Similarly, overall motility and morphology did not differ between Faroese and Danes, but between the two Faroese subgroups with higher values for the F1-subgroup. Figure 1A shows the distributions of total sperm counts of all the investigated Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). According to the figure more Faroese men had lower sperm counts than the reference group ($p<0.0001$).

Table 2: Semen parameters in Faroese and Danish men.

	Faroese men (F1)	Faroese men (F2)	P value	Faroese men (F1+F2)	Danish men (D)	P value
	2007-09 (N=241)	2009-10 (N=240)		2007-10 (N=481)	2006-10 (N=1,274)	

Sperm concentration (mill/mL)						
Mean (SD)	54 (56)	60 (60)		57 (58)	62 (55)	
Median (5-95 percentiles)	38 (1.6-156)	41 (2.2-182)	0.5*	40 (1.9-174)	48 (4.0-169)	□0.0005*
Semen volume (mL)						
Mean (SD)	4.2 (1.7)	4.0 (1.7)		4.1 (1.7)	3.6 (3.1)	
Median (5-95 percentiles)	4.0 (1.9-7.1)	3.9 (1.5-7.2)	0.02*	3.9 (1.8-7.1)	3.3 (1.3-6.3)	□0.0005*
Total sperm count (mill)						
Mean (SD)	214 (205)	215 (206)		215 (205)	206 (258)	
Median (5-95 percentiles)	154 (3-586)	159 (9-638)	0.8*	159 (6-591)	151 (13-559)	0.2*
Normal morphology (%)[§§]**						
Mean (SD)	7.5 (4.3)	6.3 (4.5)		6.9 (4.4)	7.5 (4.9)	
Median (5-95 percentiles)	7.5 (1.0-15.0)	5.0 (0.5–15.0)	□0.0005**	6.5 (0.9-15.0)	7.0 (0.5-16.0)	0.2†
Motile sperm (%)[#]						
Mean (SD)	70 (22)	58 (13)		64 (19)	65 (16)	
Median (5-95 percentiles)	74 (19-95)	59 (35-74)	□0.0005‡	64 (24-90)	68 (33-85)	0.02‡
Period of abstinence (h)						
Mean (SD)	80 (38)	86 (35)		83 (37)	77 (96)	
Median (5-95 percentiles)	83 (14-167)	83 (35-168)	0.1†	83 (25-168)	63 (37-134)	□0.0005†

*Regression analysis adjusting for duration of abstinence.
†Mann Whitney test
‡Regression analysis adjusting for delay from time of ejaculation to assessment of motility.
** Regression analysis without any adjustment
[§§] Morphology was missing for 50 Faroese men (F1+F2) and 20 Danish men.
[#]Motile sperm is the sum of progressively motile (WHO class AB) and local motile (WHO class C).

From the entire group of 481 men, 313 (65%) were without any prior knowledge of andrological diseases or conditions that might be associated with risk of impaired testicular function (cryptorchidism, testicular torsion, orchitis, epididymitis, varicocele, hydrocele, prostatitis, hypospadias, phimosis, inguinal hernia, cystitis, sexually transmitted diseases, diabetes, thyroid

diseases, experienced fertility problems). In this subgroup 244 men (50% of the entire group) had an ejaculation abstinence period of more than 48 hours. These 244 men also had lower total sperm counts than men from the WHO reference group (Figure 1B, lower panel, p=0.0013).

The percentages of morphologically normal spermatozoa were inversely associated with year of birth, both for the entire group of Faroese men (Figure 2A, trend p=0.002), and for the subgroup of 244 men (Figure 2B, trend p=0.018). Morphology assessments were done in four different time periods, but the results did not differ according to these periods. Similar trends were seen when the estimates according to year of birth were adjusted for age, however, with broader confidence intervals of the estimates. No other semen variables were associated with birth year and age at time of investigation.

Approximately 14% of the participants reported to have fathered a child. The confounder adjusted estimates indicated higher sperm concentration, total sperm count, motility and morphologically normal spermatozoa in these men in comparison to non-fathers, all non-significant except for motility (p<0.0005). Semen volume was non-significantly lower in the father group (p=0.7). When the distribution of total sperm counts for these fathers were compared with the WHO reference group no difference between the groups could be shown (p=0.1).

Physical examination

Only the men in the F1-group had a physical examination performed. Among these 95% had a normal mean testicular volume (i.e. above 15 mL), but apparently slightly lower than the Danish men (Table 1). A total of 2% did not have both testicles in scrotum, 8% had soft testicles and 4% did not have adult pubic hair distribution (i.e. Tanner stage 5 or 6). A varicocele was detected in 15% (5.8% grade 1, 7.5% grade 2 and 1.7% grade 3) of the F1 cohort. In regression analyses all semen variable estimates were higher in men without any varicocele, but all non-significant (all p>0.2). Hydrocele was detected in 5%, and tended to be associated with a lower percentage of morphologically normal spermatozoa (p=0.05), but not with other semen variables (all p>0.6).

Smoking and BMI

The men's tobacco smoking was non-significantly associated with lower semen volume, sperm concentration and total sperm counts (all p>0.1) and higher percentage of normal forms and motile spermatozoa (p=0.8 and 0.2, respectively). Maternal smoking during pregnancy was significantly associated with lower total sperm count (29% reduction, 95% confidence interval 8;45%, p=0.02),

and non-significantly with semen volume (9% reduction, 95% confidence interval -16%;2%, p=0.05) and sperm concentration (22% reduction, 95% confidence interval -40;6%, p=0.06). Associations between mother's smoking motility and morphology were highly non-significant (p=0.5 and 0.8, respectively). BMI above 25 was not associated with adverse effects on any semen variable.

Reproductive hormones

Table 3 summarizes the reproductive hormone levels for the two sub-groups of Faroese men, the combined group and the Danish comparison group. Inhibin B tended to be slightly lower in the Faroese group F1 than in the F2 group, thus showing the same tendency as sperm concentration and total sperm count. FSH showed opposite directions resulting in lower inhibin B/FSH ratio in the F1 group. In the combined Faroese group both inhibin B and FSH individually were higher than in the Danish group, whereas the inhibin B/FSH ratio was lower. In 2010, the laboratory began to use a different kit material for the serum inhibin B analyses. However, that the inhibin B/FSH results did not differ according to this as described in the methods section.

Table 3: Reproductive hormones levels in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value*	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value*
FSH (IU/L)						
Mean (SD)	4.1 (4.7)	3.4 (1.9)		3.8 (3.6)	2.8 (1.8)	
Median (5-95 percentile)	3.1 (1.3-8.5)	2.9 (1.1-7.0)	0.05	3.1 (1.2-7.7)	2.5 (1.0-6.0)	□0.0005
Inhibin B (pg/mL)						
Mean (SD)	196 (79)	220 (84)		208 (82)	189 (70)	
Median (5-95 percentile)	192 (76-333)	210 (99-382)	0.04	202 (85-346)	180 (90-318)	0.05
Inhibin B /FSH						
Mean	80 (72)	101 (107)		91 (92)	100 (89)	
Median (5-95 percentile)	57 (11-203)	70 (17-289)	0.02	64 (13-259)	76 (17-272)	□0.0005
LH (IU/L)						
Mean (SD)	4.8 (2.4)	4.8 (1.8)		4.8 (2.1)	3.5 (1.5)	
Median (5-95 percentile)	4.5 (2.1-8.5)	4.6 (2.1-7.9)	1.0	4.5 (2.1-7.9)	3.3 (1.6-6.3)	□0.0005
Testosterone (nmol/L)						
Mean (SD)	21 (7)	23(7)		22 (7)	20 (6)	
Median (5-95	20 (11-32)	22 (14-36)	0.006	21 (12-33)	19 (12-31)	□0.00

percentile)						05
Testosterone/LH						
Mean	5.0 (2.3)	5.9 (7.2)		5.5 (5.3)	6.5 (2.8)	
Median (5-95 percentile)	4.6 (1.9-9.1)	4.6 (2.7-11.6)	0.09	4.6 (2.2-9.4)	6.0 (3.0-12.0)	□0.00 05
Free Testosterone (pmol/L)						
Mean	409 (134)	462 (140)		439 (134)	454 (142)	
Median (5-95 percentile)	397 (211-633)	442 (262-707)	□0.000 5	424 (236-686)	432 (269-701)	0.1
Free testosteron/LH						
Mean	99 (44)	125 (175)		112 (128)	149 (71)	
Median (5-95 percentile)	92 (39-186)	96 (49-212)	0.03	94 (47-201)	134 (65-273)	□0.00 05
Estradiol (nmol/L)						
Mean (SD)	79 (22)	96 (23)		88 (24)	80 (24)	
Median (5-95 percentile)	78 (46-113)	94 (62-140)	□0.000 5	86 (54-131)	78 (48-125)	□0.00 05
Testosterone/Estradiol						
Mean	268 (83)	243 (69)		255 (78)	258 (70)	
Median (5-95 percentile)	262 (140-409)	236 (146-375)	□0.000 5	243 (145-394)	249 (175-383)	0.1
Free Testorsterone/Estradiol						
Mean	5332 (1567)	4955 (1187)		5143 (1400)	5869 (1768)	
Median (5-95 percentile)	5218 (3230-7768)	4876 (3165-7201)	0.002	5026 (3182-7538)	5636 (3519-8956)	□0.00 05
SHBG (nmol/L)						
Mean (SD)	38 (15)	37 (15)		38 (15)	30 (12)	
Median (5-95 percentile)	36 (17-62)	35 (19-68)	0.7	36 (18-65)	29 (14-51)	□0.00 05
Time of bloodsampling						
Mean (SD)	11:56 (2:11)	9:57 (1:29)		10:27 (1:56)	10:04 (0:42)	
Median (5-95 percentile)	10:15 (8:40-15:40)	9:37 (8:02-12.45)	□0.000 5†	10:10 (8:30-15:00)	10:00 (9:05-11:20)	0.6†

*Regression analysis adjusting for hour of day of blood sampling
†Mann Whitney test

Total testosterone (T) only differed slightly between the two Faroese groups and between the combined group and the Danes, although statistically significant. SHBG in contrast wasconsiderably higher for the Faroese men and with no difference between the F1 and F2 men. Frequency analyses of the SHBG values divided into groups (0-10, 10-20 etc.) showed that the higher SHBG values for Faroese men were not caused by higher concentration in a specific

subgroup but an overall shift to higher levels. Approximately 80% of the Faroese men had a SHBG concentration below 50 nmol/L while 80 % of the Danish men had a concentration below 40 nmol/L. The FT differed between the two Faroese groups which combined, however, had non-significantly lower FT than the Danes. Estradiol was higher in the F2 group than in the F1, thus leading to higher levels in the combined group of Faroese men than in the Danish. The ratios T/LH, FT/LH, and FT/estradiol were all lower in the Faroese men than in the Danish whereas the estimated lower total T/estradiol was non-significant.

SHBG and LH were positively correlated with T (P <0.0005), whereas SHBG and LH did not correlate (P=0.1). FT was positively correlated with LH and T (P <0.0005), and negatively but not significantly correlated with SHBG (P=0.06)

BMI was negatively associated with T, FT, SHBG, T/LH, T/estradiol, FT/estradiol and inhibin B (p<0.0005-0.042), and also negatively but non-significant with LH (p=0.3). Faroese smokers tended to have higher T than non-smokers (p=0.7, adjusted for BMI effect), whereas the effect on the Danish men were highly significant (p<0.0005). In the Faroese groups LH, SHBG, estradiol, FT/LH, FT/estradiol and T/estradiol tended to be higher in smokers, although all non-significantly. For the Danes similar trends were detected; the effect on SHBG and LH being non-significant, whereas the remaining were significant with p-values <0.0005-0.05. Further maternal smoking during pregnancy for the Faroese men tended to be associated with lower LH and FT (p=0.04 and 0.02) whereas the effect on the remaining hormone levels were all highly non-significant (p=0.1-0.9). However, the difference (i.e. significant levels in table 3) between Faroese and Danish hormone levels did not change when including BMI or age as cofactors into the regression analyses indicating that these factors cannot explain the difference in reproductive hormones between Faroese and Danes.

DISCUSSION

This is the first study on testicular function conducted in the Faroe Islands. These young Faroese men had a lower sperm concentration but similar total sperm count compared to Danish men. Recent data have shown sperm counts to be low in young men from several European countries, however, slightly higher than among the Danes [5, 21, 22]. Thus, the semen quality of Danish men seems to be particularly low, and now we have shown the Faroese men to have a similar low level. The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts, and provides independent evidence of poorer testicular function in the Faroese men than in the Danes, although the medians were at a level where the association between sperm counts and inhibin B is weakened [23]. When evaluating the results against the WHO reference population, Faroese men

also had low sperm counts. The lower T/LH and FT/LH ratios point towards a lower Leydig cell capacity among Faroese men compared to Danes. Thus, the total testicular function among Faroese men may be at the same or lower level than Danes.

A greater proportion of men from the Faroese study populations had previously caused a pregnancy, which also would be expected since these men in average were almost 6 years older than the Danes. Furthermore, Faroese people are in general younger when parenting for the first time (25.5 years in 2011) [24], and the fertility rate in the Faroe Islands is the highest in European countries [25] (with 2.3 children per women in 2011 [24]. Traditionally, the family culture in the Faroe Islands is to have large families with many children; however, during the last 40 years the fertility rate in the Faroe Islands has decreased form 3.4 children per women in 1970 to 2.8 children per women in 1990 to the current fertility rate of 2.3 children per women in 2011 [24]. Thus, the high birth rate most likely reflects socio-economic factors rather than male fecundity. Despite of this more Faroese men from the study populations had experienced fertility problems than the Danish men. This is in agreement with the lower sperm concentration found in the Faroese men, but it should be taken into account that the Faroese men were asked about fertility problems on the basis of 6 months unprotected intercourse without achieving a pregnancy and for the Danes it was based on 1 year. Therefore, the Danish men were more likely to have obtained a successful fertilization having tried for twice as long. Furthermore, fewer Danish men may be aware of a fecundity problem because they were younger and had not yet tried to conceive.

The Faroese and the Danish men were not exactly the same age. Semen quality has been reported to decline with increasing age [26-29]. However, the Faroese studies and the comparative Danish studies were conducted at the same time periods and the difference in age between groups is not likely to have influenced the semen parameters nor is the actual age of the participants that still can be regarded as young. Semen quality does on average not change between 19 and 23 years of age, and immaturity seems unlikely to explain the poor semen quality both among the Faroese and Danish men [30]. Thus, age difference is not likely to explain the difference in semen quality.

The Faroese men were investigated during a relatively short time period and also represented narrow birth cohorts. Despite of this, we actually detected an inverse association between percentage of morphologically normal spermatozoa and year of birth, a trend that was robust even when controlled for potential confounders. In a cohort like ours, the effect of birth year may be difficult to separate from the effect of age. However, when we modelled age as explanatory factor and controlled for birth year, the trend effect of age was not apparent. Thus, our results suggest that younger men produced ejaculates with lower percentages of normal spermatozoa. This

could be caused by certain unhealthy life-style factors among the younger cohorts, but not the older cohorts. Our currently existing data does not support that explanation, however, but the information we have could also be insufficient to exclude this suggestion. Alternatively, the birth cohort effect could reflect an increasing exposure during fetal life leading to impaired testicular development which in adulthood would be reflected by reduced semen quality in line with the suggested Testicular Dysgenesis Syndrome hypothesis [31-32].The Faroese studies were investigated according to almost similar protocols and assessment of sperm concentration was controlled by the same quality control as the Danish study. The Faroese technicians were trained at RH in Denmark and the analyses used in both laboratories were identical, thus reducing the interobserver variation. Additionally, assessment of sperm morphologies was performed randomly by a single person. Frequency of motile sperm, however, is difficult to compare reliably between different groups. The motility assessment is highly subjective and controlling for quality control within this parameter is problematic. Previously, the inter-laboratory variance of motility has shown to be of significant importance [33]. Therefore, we hesitate to draw major conclusion regarding sperm motility.

The recording of abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. This latter procedure is believed to be much more accurate, as the men were given time to reflect and were not expected to give an immediate verbal response. This assumption is supported by the fact that the anticipated relationship between abstinence time and sperm concentration only was clearly seen in F2 group. Assuming the reported abstinence time actually was longer among the Faroese men these men still produced similar numbers of sperm as the Danish men, suggesting that the sperm production rate is lower in the Faroese population compared to Danes. This was, however, only confirmed for sperm concentration but not total sperm count when abstinence period was accounted for in the statistical model.

As explained in the methods section some of the semen samples were analysed with incorrect counting chambers. However, we corrected for this potential testing error. In addition, the statistical analyses performed with or without the 59 samples did not change the overall results, supporting the assumption that the corrections made were valid. A major advantage of the study was that the participants were not selected on fertility status. According to most of the Danish men, the main incentive for participation was the financial compensation [5]. The F2 men were part of an existing birth cohort study whereas F1 men were randomly recruited. Thus, we cannot exclude that men with suspicion of infertility may have been more interested in participating in the study. However, to control for this we defined a subgroup of men with abstinence period of more than 48

hours and without any prior knowledge of andrological diseases and without know fertility, and still the same low sperm counts were detected. This problem with counting chambers, and the differences in semen quality and population characteristics between the two Faroese sub-population exemplifies one of the main problems when comparing semen characteristics globally and over time, namely that standardised methods have to be applied and sufficiently large study populations investigated.

The finding of low semen quality and lower Leydig cell function in Faroese men cannot be explained by effects of confounders. Both high BMI and maternal smoking have previously been associated with reduced testicular function in some studies but not all [34-40].In our cohort, the effects of these two usually quite robust confounders/explanatory factors were not obvious, neither on the reproductive hormones. However, our cohorts may be too small to detect the effect of these two factors. Alternatively, other unidentified factors may have a major influence masking the effect of BMI and maternal smoking on the semen parameters. The reason for the low testicular function of the Faroese young men is unknown but this population is highly exposed to POPs derived solely from traditional marine food, which includes blubber from the pilot whale. Studies have shown associations between high polychlorinated biphenhyls (PCB) levels and low semen quality, and since PCBs and p,p'-DDE have the potential to interfere with sex hormone functions, it is plausible to assume that these compounds can affect the function of these organs [41]. There are some reports on the effect of POPs on male reproduction in humans, mainly indicating weak negative effects on sperm motility [41-43]. For the two Faroese sub-groups, we found that the percentage of motile cells was significantly lower compared with Danish men, indicating that increased exposure to endocrine disruptors can be one explanation.

Serum SHBG levels for the Faroese men were strikingly higher than in the Danes. SHBG is a sensitive marker for thyroid function. However, none of the study subjects were under treatment for hyperthyroidism. We did not measure thyroid hormones, but the men did not have any obvious clinical symptoms of hyperthyroidism. If a high thyroid level in general should explain the SHBG level, a significantly elevated LH and testosterone in men with low BMI would have been expected, which was not the case. Therefore, there is no indication that the high SHBG values are due to hyperthyroidism. Furthermore, alcohol consumption could not explain the high levels. One plausible explanation to the high SHBG levels could be the high PCB exposure mentioned above. A recent publication from the Faroe Islands found that SHBG increased at higher PCB exposure, both prenatally and currently. PCBs are known to affect a variety of liver functions, and it could be speculated that PCB-induced hepatic SHBG synthesis could play a possible role, although this

possibility remains to be substantiated [44]. We cannot elucidate further if PCB in our current study group can explain the SHBG levels.

It is known that the Faroese population differs genetically from other populations in many respects. There are several genetic diseases reported with very high frequency on the Faroese Islands [11, 45]. However different genetic composition in populations may contribute to the explanation of differences in semen quality and would be worth further exploration.

In conclusion, we found that the semen quality among Faroese men is at the same low level as reported among Danish men. This low quality was corroborated by the reproductive hormone levels in Faroese men. The influence of environmental exposures and genetic factors on the semen quality has to be studied further.

CONTRIBUTORS:

Substantial contribution to conception and design: PG, PW, NJ, TKJ.

Acquisition of data: JH, MSP, PW.

Interpretation of data: All authors.

Drafting the article: JH, MSP, NJ.

Revising the article critically for important intellectual content: all authors.

Final approval of the version to be published: all authors.

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COMPETING INTERESTS. None

ETHICS APPROVAL. The local Science Ethical Committee for the Faroe Islands and the Institutional Review Board at Harvard School of Public Health have approved the study protocol,

and all participants had given their informed consent.

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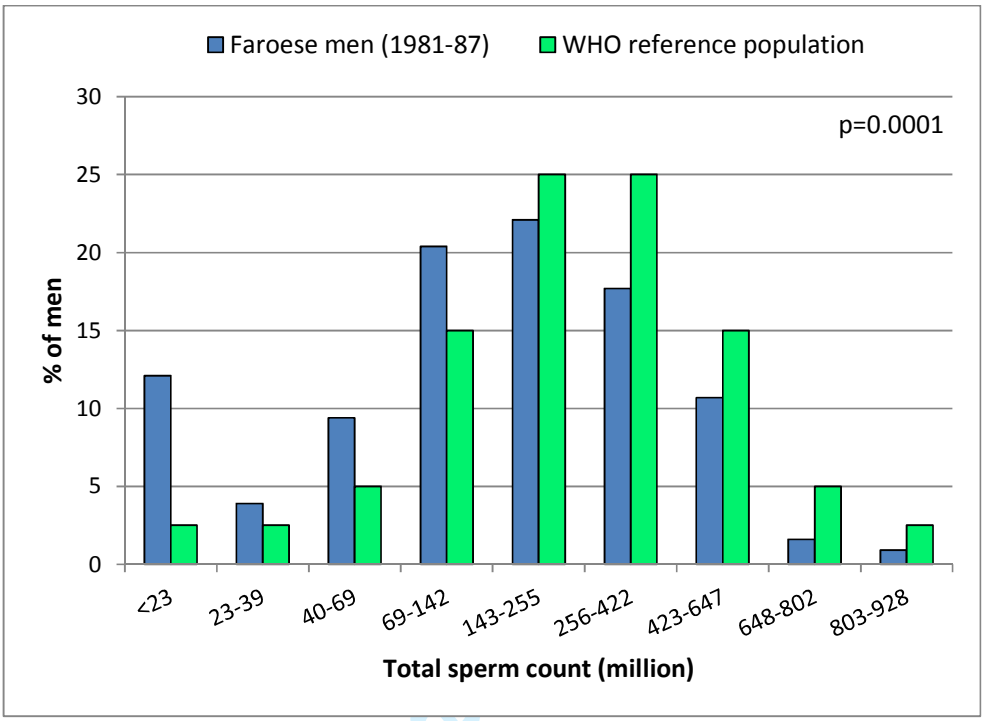


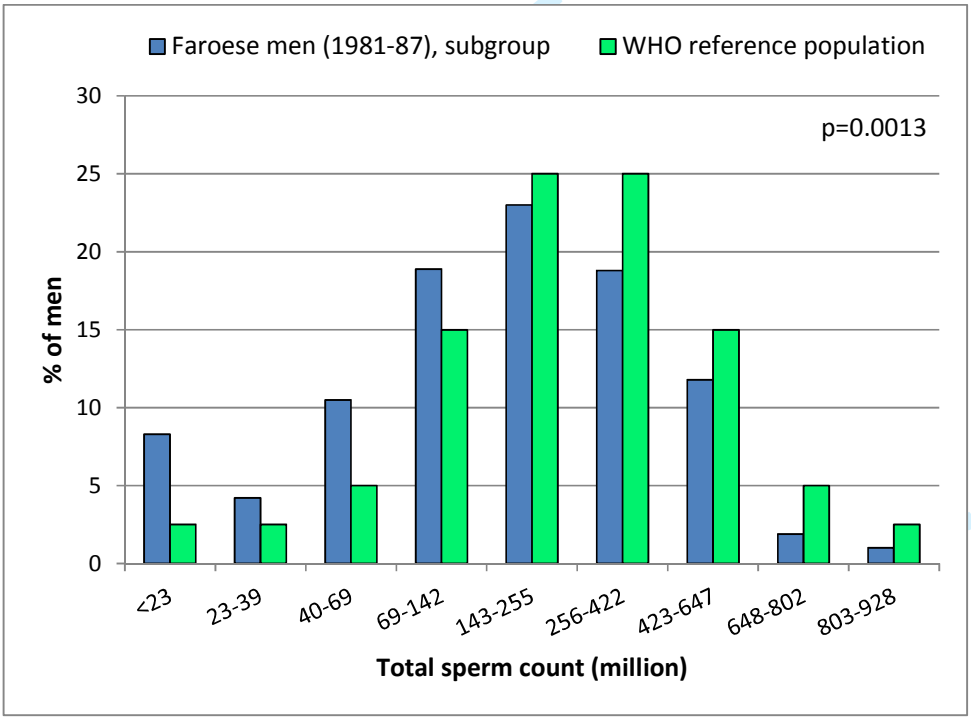
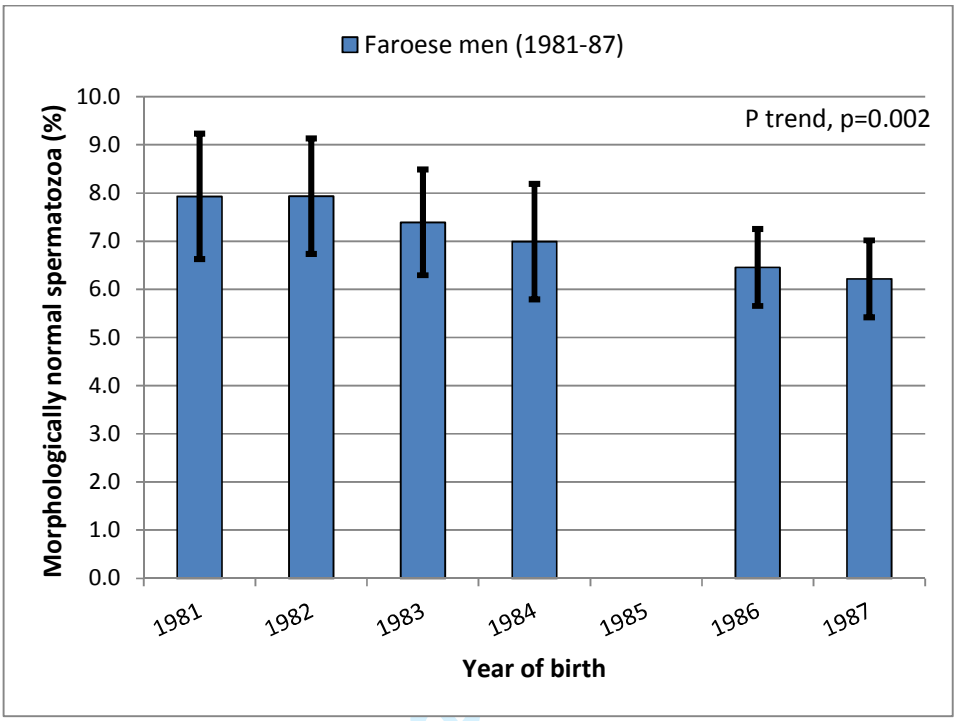


Figure 1. Distribution of total sperm count in Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). In (A) all Faroese men are included. In (B) only men having an ejaculation abstinence period above 48 hours and being "without diseases" (see text for further explanation) are included.



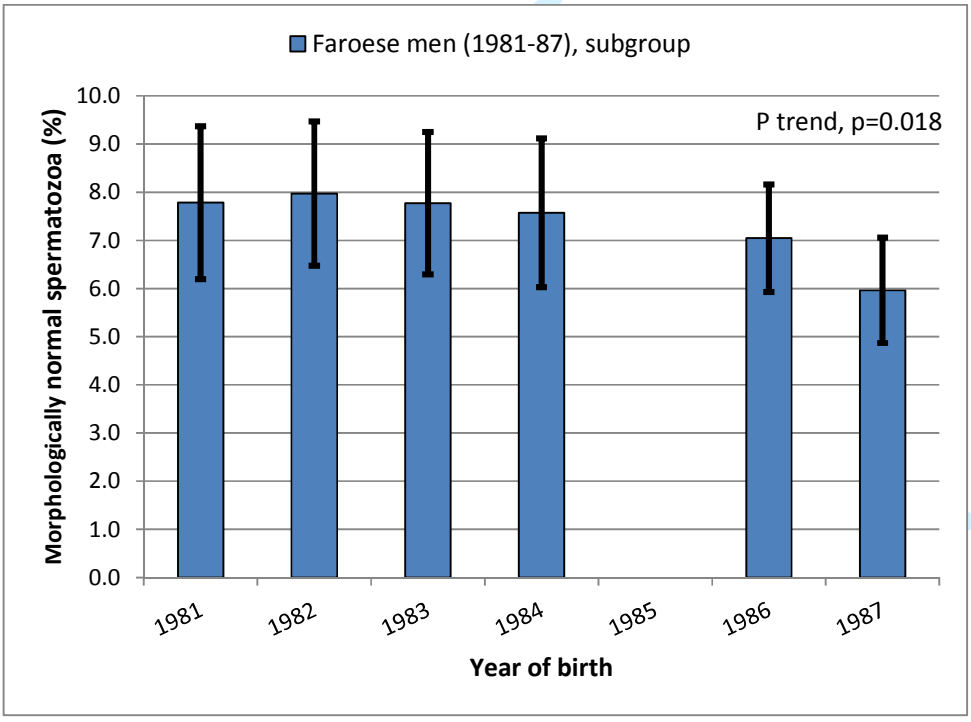


Figure 2. Percentage of morphologically normal spermatozoa according to year of birth. Whiskers show the 95% confidence interval of the estimated mean. In (A) all Faroese men are included. In (B) only men having an ejaculation abstinence period above 48 hours and being "without diseases" (see text for further explanation) are included.

Supplementary Information

In our study of young Faroese men, the sperm concentration was assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) as described in the main text. As stated there, a second delivery of Bürker-Türk chambers were 0.05 mm in depth rather than the standard 0.1 mm and therefore, 59 samples from F2 were analyzed without knowing whether 0.05 mm or 0.1 mm deep counting chambers were used. As the semen samples had been stored in our bio-bank, the 59 samples were measured again with the 0.1 mm deep chamber to indicate if the original concentration assessment was correct (assuming that the 0.1 mm counting chamber was used originally) or should be doubled (assuming that the 0.05 mm counting chamber was used originally). This was done manually by investigating the agreement between the values of the two measures. Based on these re-analyses, the obtained original sperm concentrations were doubled for 23 samples (table 1):

Table 1

ID number	Original count (mill/ml)	Recount (mill/ml)	Factor	Final concentration (mill/ml)
1	31,05	75,19	x2	62,10
2	63,28	61,33	x1	63,28
3	56,64	96,09	x2	113,28
4	44,14	76,95	x2	88,28
5	35,94	31,41	x1	35,94
6	57,42	66,02	x1	57,42
7	62,50	58,98	x1	62,50
8	37,73	36,17	x1	37,73
9	29,06	27,11	x1	29,06
10	37,50	77,93	x2	75,00
11	86,72	86,52	x1	86,72
12	76,17	78,52	x1	76,17
13	23,59	22,97	x1	23,59
14	109,76	104,3	x1	109,76
15	19,33	40,82	x2	38,66
16	27,34	28,13	x1	27,34
17	42,18	46,09	x1	42,18
18	37,89	31,25	x1	37,89
19	23,44	20,31	x1	23,44
20	75,39	63,67	x1	75,39
21	43,75	92,19	x2	87,50
22	29,69	29,69	x1	29,69
23	28,30	27,42	x1	28,30
24	112,50	88,67	x1	112,50
25	126,18	95,31	x1	126,18
26	20,90	41,02	x2	41,80
27	20,31	18,95	x1	20,31
28	40,43	46,88	x1	40,43
29	48,05	90,23	x2	96,10
30	44,53	102,34	x2	89,06

31	23,05	44,53	x2	46,10
32	44,53	55,07	x1	44,53
33	49,80	41,79	x1	49,80
34	30,86	53,52	x2	61,72
35	28,05	31,56	x1	28,05
36	80,47	220,3	x2	160,94
37	27,93	47,85	x2	55,86
38	28,91	56,45	x2	57,82
39	2,69	2,46	x1	2,69
40	54,49	66,01	x1	54,49
41	24,02	47,46	x2	48,04
42	22,46	39,45	x2	44,92
43	91,02	180,07	x2	182,04
44	13,13	37,89	x2	26,26
45	3,18	8,28	x2	6,36
46	14,84	14,38	x1	14,84
47	9,09	6,33	x1	9,09
48	6,57	9,22	x1	6,57
49	1,96	1,73	x1	1,96
50	15,31	35,23	x2	30,62
51	3,22	0,85	x1	3,22
52	5,27	3,95	x1	5,27
53	5,63	10,47	x2	11,26
54	10,55	22,42	x2	21,10
55	5,16	5,39	x1	5,16
56	17,34	17,27	x1	17,34
57	4,18	8,16	x1	4,18
58	5,30	4,35	x1	5,30
59	19,30	41,02	x2	38,60

To ascertain that the concentration could be replicated in thawed samples, 15 thawed samples known to be counted in 0.1 mm deep chamber were counted again. As anticipated, the results were replicated and shown to be accurate (Table 2):

Table 2:

Sample	Original count (mill/ml)	Recount (mill/ml)	Factor
1	224,4	203,9	1,10
2	8,55	7,03	1,22
3	20,74	20,31	1,02
4	21,64	20,39	1,06
5	33,7	31,4	1,07
6	62,9	60,5	1,04
7	51,4	56,8	0,91
8	35,9	46,7	0,77
9	55,5	60,2	0,92

10	37,89	27,89	1,36
11	5,39	4,2	1,28
12	8,52	1,84	4,63
13	1,78	1,21	1,47
14	154,3	149,22	1,03
15	13,91	11,44	1,22

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*	Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses

Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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SEMEN QUALITY AND REPRODUCTIVE HORMONES IN FAROESE MEN – A CROSS-SECTIONAL POPULATION-BASED STUDY OF 481 MEN

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SEMEN QUALITY AND REPRODUCTIVE HORMONES IN FAROESE MEN – A CROSS-SECTIONAL POPULATION-BASED STUDY OF 481 MEN

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ABSTRACT

Objectives: To determine semen quality and reproductive hormone levels in young Faroese men.

Design: Descriptive cross-sectional study of Faroese men compared with Danish men.

Setting: Faroese one-center study.

Participants: 481 men born from 1981 to 1987 and investigated from 2007 to 2010.

Outcome measures: Sperm concentration, semen volume, total sperm count, sperm motility, sperm morphology and reproductive hormones levels.

Results: Sperm concentrations for the Faroese men were lower than for the Danish (crude median 40 mill/mL vs. 48, p<0.0005). Semen volume was higher, and thus total sperm counts did not differ (159 vs. 151 mill, p=0.2). Motility and morphology did not differ between Faroese and Danes. Inhibin B/FSH ratio for the Faroese men were lower than for Danes (64 vs. 76, p=0.001). Similarly, lower total testosterone/LH ratio (4.6 vs. 6.0, p<0.0005) and lower calculated free-testosterone/LH ratio (94 vs. 134, p<0.0005) were detected for Faroese men.

Conclusions: Semen quality among Faroese men is at the same low level as reported for Danish men, and the reproductive hormone levels furthermore indicated a lower Leydig cell capacity for testosterone production. The influence of environmental exposure and genetic factors on the semen quality has to be studied further.

ARTICLE SUMMARY

Article focus

Semen quality studies have not been conducted in the Faroe Islands before.

Because the Faroese population differs from other populations regarding e.g. high exposure to persistent organic pollutants (POPs) and genetic diseases, the semen quality of Faroese men from the general population would be expected to be low.

Key messages

The semen quality among Faroese men is at the same low level as reported in Denmark.

The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts and provides independent evidence of poorer testicular function in the Faroese men than in the Danes.

Similarly, the Leydig cell capacity for testosterone production was also lower for Faroese men than for the Danes.

Strengths and limitations of the study

Prospectively designed, cross-sectional study of testicular function among young men from the general population unselected with regard to fertility.

Standardized inclusion and investigation procedures.

Clinical examination for one subgroup of Faroese men, but not for the other.

INTRODUCTION

In 1992 Carlsen [1] and co-workers published a combined analysis of results from 61 papers published between 1939 and 1991 and showed a significant decline in sperm counts over a 50 years period. A detailed reanalysis of the results found that the conclusion was supported by the underlying studies [2, 3]. Following the 1992 publication, many analysed retrospectively their historical data for temporal trends, some finding a decline and others not. Realising that the trend analyses indicated that semen quality could have reached a low level where it might affect fecundity, several prospectively designed cross sectional semen quality studies were initiated to determine the current quality in men from general populations. These studies did not only reveal geographical differences [4, 5] but confirmed the general presence of low semen quality in men from all investigated countries when the results were interpreted according to available publications focused on associations between semen quality and fertility chances [6-9].

The causes of decreased semen quality are not clear, but it is feasible that many cases may have been caused by exposure to environmental factors in utero, during adolescence or in adulthood [10], however, most likely acting on a background of different genetic susceptibility.

The Faroese population differs from other populations in many respects. The Faroese population has high exposure to persistent organic pollutants (POPs) derived from traditional marine food, which includes blubber from the pilot whale. At the same time, there are several genetic diseases reported with very high frequency in the Faroese Islands [11]. Thus, our hypothesis was that semen quality of Faroese men from the general population would be quite low.

MATERIALS AND METHODS

To test our hypothesis we investigated semen quality of young Faroese men from the general population in 2007-2010. For comparison we used a recently published similar group of Danish men examined 2006-2010 [12] and the current World Health Organization (WHO) reference levels [13].

Study population: men from the general Faroese population

The entire study population consisted of 484 men, originating from two separate studies. The first study group included 241 randomly selected young men examined between February 2007 and February 2009 (F1). The second group comprised of 243 men examined between November 2009 and November 2010 (F2). A detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1. Within 3 months prior to participation, 132 men (27.4%) had used medication, mainly antibiotics, painkillers, or asthma/allergy medicine.

Table 1: Physical appearance and self-reported information of Faroese and Danish men. Results shown as medians (5-95th percentile) or percentages.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value*	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value*
Physical appearance						
Age (years)	25.3 (24.2-26.7)	23.0 (22.0-24.0)	<0.0005	24.0 (22.0-26.2)	19.0 (18.4-21.8)	<0.0005
Height (cm)	180.0 (169.9-189.9)	180.0 (170.0-193.0)	0.1	180.0 (170.0-191.0)	181.6 (171.1-193.0)	<0.0005
Weight (kg)	80.3 (64.2-106.2)	78.00 (62.9-105.1)	0.2	80.0 (63.1-105.9)	74.1 (60.1-96.0)	<0.0005
BMI (kg/m2)	25.0 (20.6-32.3)	24.3 (20.4-32.1)	0.03	24.6 (20.5-32.1)	22.4 (18.7-28.6)	<0.0005
Testes size (mL)**	21.5 (15.3-25.0)	NA		NA	23 (14-29)	
Lifestyle						
Alcohol per week (units)	5(0-25)	6 (0-26)	0.08	5 (0-25)	12 (0-42)	<0.0005
Current Smokers	44.0	41.3	0.5	42.6	45.4	0.4
Mother smoked in pregnancy	22.4.***	37.5	0.05	33.6	29.1	0.4
Taken medication§	23.7	31.3	0.07	27.4	15.1	<0.0005
Been treated for§§						
Cryptorchidism†	3.3	7.5	0.04	5.4	5.0	0.8
Been diagnosed as having§§						
Cryptorchidism reported	8.7	12.1	0.2	10.4	6.9	0.02
Hypospadias	0.4	0.8	0.6	0.6	0.3	0.4

Sexual transmitted disease††	9.1	10.8	0.5	10.0	6.2	0.007
Phimosis	10.0	11.7	0.5	10.8	4.6	<0.0005
Varicocele	0.8	0.8	1.0	0.8	0.4	0.6
Have[§§§]						
Ever caused a pregnancy**	25.7	NA		NA	6.8	-
Fatherhood	21.2	6.3	<0.0005	13.7	NA	-
Experienced fertility problems‡	4.6	1.7	0.07	3.1	0.2	<0.0005

*Mann Whitney test

**Mean of two testes. Testes size was not measured in Faroese men born 1986-87 and the question "Have you ever caused a pregnancy" was not asked in this group

*** Information only available for 189 mothers

§ Taken any medication 3 months prior to participation in the study

† Hormonal, surgical or combination

†† Chlamydia, gonorrhoea, warts or herpes

‡Ever had regular intercourse without use of contraception for at least 6 months (Faroese men) or 1 year (Danish men) without partner became pregnant

§§ "Have you ever been treated for…" §§ "Has a doctor ever diagnosed you as having....." §§§ Have you ever…"

NA: not available

Faroese men, examined 2007-09 (sub-group F1)

Invitation letters to participate in the study were sent to 1,100 men, consecutively listed in the Faroese population register as born between January 1981 and December 1984, followed by a phone call to arrange the examination details. A total of 34 men had emigrated, and 43 letters were returned as undeliverable. Thus, 1,023 were invited. Of these, 490 could not be reached and 292 declined to participate. Hence, the final F1 group was comprised of 241 men (24% of all invited).

Faroese men, examined 2009-10 (sub-group F2)

This group consisted of a cohort generated from consecutive births at the three Faroese hospitals during 1986-1987 (N=509 males) as described elsewhere [14-16]. Detailed information on their physical health and potential environmental factors were collected at the time of birth and during the course of follow-up at ages 7, 14 and 22 years. The 421 men who had participated in the 22 years follow-up were invited to participate in the

semen quality study. This was the sole selection criterion. All men received a letter of invitation and subsequently a phone call to arrange the examination details. Among the 421 invited men, 243 accepted to participate, but 3 did not succeed in delivering a semen sample. Hence, the final F2 group comprised 240 men (57 % of all invited).

Questionnaire

On the day of attendance, the men returned a questionnaire they had received in advance. The questionnaire was based on that used for young Danish men [12, 17] and included information on previous or current diseases, including any known history of fertility potential, and some lifestyle factors like smoking and drinking habits.

Semen samples

Semen samples were produced by masturbation in a room close to the semen laboratory. The period of abstinence was recorded. The abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. The semen sample was analysed according to the World Health Organization 1999 guidelines [18]. Semen volume was estimated by weighing the collection tube with the semen sample and subtracting the weight of the empty pre-weighed tube, assuming that 1 mL semen=1 g. For sperm motility assessment, 10 µL of well-mixed semen was placed on a clean glass slide kept at 37 °C and covered with a 22x22 mm coverslip. The preparation was placed on the heated stage of a microscope at 37 °C and immediately examined at x400 magnification. The sperm were classified as progressive motile (WHO class AB motility), locally motile (WHO class C motility) or immotile (WHO class D motility).

For the assessment of the sperm concentration, the samples were diluted in distilled water. The sperm concentration was subsequently assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). However, a second delivery of Bürker-Türk chambers were 0.05 mm in depth rather than the standard 0.1 mm we had ordered. Because of that 59 samples from F2 were analysed without knowing whether 0.05 mm or 0.1 mm deep counting chambers were used. As the semen samples had been stored in our bio-bank, the 59 samples were measured again with the 0.1 mm deep chamber to indicate if the original concentration assessment was correct (assuming that the 0.1

mm counting chamber was used originally) or should be doubled (assuming that the 0.05 mm counting chamber was used originally). Based on these re-analyses, the obtained original sperm concentrations were doubled for 23 samples (see Supplementary Materials for details). To ascertain that the concentration could be replicated in thawed samples, samples known to be counted in 0.1 mm deep chamber were counted again. As anticipated, the results were replicated and shown to be accurate. From each semen sample a smear for morphology evaluation was made, Papanicolaou stained and finally assessed according to "strict criteria" at Department of Growth and Reproduction (GR) at the National Hospital (Rigshospitalet, RH) in Denmark.

The Faroese semen analyses were performed by three technicians, who participated in a quality control testing course at Rigshospitalet (RH) and spent two weeks there to ensure comparable results. Furthermore, every three months, 5 blinded samples were sent from RH, and sperm concentration results were compared with the results from their technicians. Throughout the study period, the variation was less than 10% compared with the Danish technicians. Therefore, the methodological differences between the Faroese and the Danes should be considered very limited.

Reproductive hormones

On the examination day, a venous blood sample was drawn from each participant and centrifuged (3000 g, 10 minutes). Serum was subsequently separated and kept frozen until it was analysed for reproductive hormones at GR at RH. Levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone-binding globulin (SHBG) were determined using a time-resolved fluoroimmunoassay (Delfia, Wallac, Turku, Finland). Testosterone (T) was determined using a COAT-A-COUNT solid phase radioimmunoassay (RIA) (Siemens Medical Solutions, Malvern, PA, USA) and estradiol by radioimmunoassay (RIA) (Pantex, Santa Monica, CA, USA) [19]. Inhibin B was analyzed by a double antibody enzyme-immunometric assay using a monoclonal antibody raised against the inhibin βB-subunit in combination with labeled anti-body raised against the α-subunit. Samples were analysed for inhibin B in 2009 using kit material from Oxford Bio-Innovation or in 2010 with kit material from DSL Beckman, USA. For all hormone assessments internal standard samples were analysed and showed no need to correct for potential inter-assay variation, including no need for correction between the kit materials for inhibin B from the two suppliers. The free testosterone index (FT) was calculated from total testosterone and SHBG using a fixed albumin value according to Vermeulen et al. [20].

Physical examination

Participants in the F1 sub-group underwent a physical examination performed by one of two examiners at the day of the semen sample delivery. Body weight and height were measured. The Tanner stage of pubic hair and genital development were recorded. Any abnormalities in the testis and penis, the possible presence of varicocele, hydrocele, hypospadias and testis tumour, the location of testis in scrotum and their consistency and that of epididymis were recorded. Testicular volumes were determined by use of a Prader orchidometer, and reported as the mean of two testes (Table 1). Participants in the F2 study did not undergo a physical andrological examination.

Comparison population: men from the general Danish population

In Denmark, all young men, except those suffering from severe chronic diseases (<15%), are required to attend a compulsory medical examination before they are considered for military service [5]. Men are called upon at the age of 18-19 years, but some postpone this examination until completion of their education. Men attending the compulsory examination are invited to participate in a semen quality study, using same basic study design including a physical examination as the Faroese, irrespective of whether they are declared fit for military service or not. For comparison with results from the Faroese studies, we utilized results for Danish men examined 2006-10, which has recently been published [12]. The detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1.

Comparison population: WHO reference group of fertile men

The Faroese sperm count distribution was compared with the distribution for fertile men reported as the WHO reference group [13].

Statistics

The crude means, medians, standard deviations, 5-95 percentiles and frequencies were used for basic descriptions of obtained results. The main outcome variables were semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and serum level of the reproductive hormones. Differences in semen quality variables and reproductive hormone levels between

groups were tested by linear regression adjusted for significant confounders. Semen volume, sperm concentration, and total sperm count were best normalised by a cubic root transformation before regression analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa (ie WHO class AB+C) were logit-transformed. Percentages of morphologically normal spermatozoa were close to normally distributed and entered the model untransformed. Reproductive hormone levels were natural logarithmic transformed. Correlations between reproductive hormone levels were assessed with Spearman Correlation. Between-group differences for categorical variables were tested with non-parametric tests (Mann Whitney). Total sperm count distribution differences between the Faroese men and the WHO reference distribution was tested by chi square analyses. Association between year of birth and semen parameters were tested by linear regression.

For F2, the abstinence time in hours showed a significant positive association to sperm concentration, semen volume and total sperm count. The effect was most pronounced for the period below 96 hours and less above (for sperm concentration: β-value=0.011, p=0.01 and β-value=0.006, p=0.18 respectively). The abstinence time was not significantly associated with sperm concentration in F1 (β-value=0.0001, p=0.98, and β-value=0.0003, p=0.5 respectively) or total sperm count. Thus, abstinence time was entered as piecewise linear functions (linier splines); i.e. one straight line for abstinence below 96 and another straight line for abstinence above 96 for semen volume, sperm concentration and total sperm counts. The duration from ejaculation to assessment was included as a confounder for sperm motility. No confounders were detected to affect morphology.

The following factors were evaluated as possible confounders for semen parameters and found to have no influence (p >0.05): age, body mass index (BMI) (as continuous variable or categorized as <18.49; 18.5-25; >25), smoking (yes/no) and season of year (spring (March-May), summer (June-August), autumn (September-November) and winter (December-February)).

The same factors were evaluated as possible confounders for the reproductive hormones. Significant associations were observed for BMI and age but not for smoking. The effect of season was not systematic and thus not included as a confounder. Hour of day of blood sampling was included as a confounder for reproductive hormones, although only significantly associated with estradiol, T and FT. p-values below 5% were considered statistically significant. Analyses were performed using PASW GradPack 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In Table 1 physical appearance, lifestyle factors, reproductive health and self-reported information on fertility are displayed for the two sub-groups of Faroese men, the combined Faroese group and the Danish comparison group. All men had semen variables assessed. However, morphology scoring was missing for 50 Faroese men and 20 Danes, which is reported in Table 2. Also, assessment of testis size was only performed on sub-group F1, which is stated in Table 1. Semen volume was not recorded for one Faroese man and motility measure was missing for one man.

The three groups differed in age with the F1 being oldest and the Danish youngest. Similarly the F1 group had the highest BMI and the Danish the lowest. Alcohol intake was lower among Faroese men, and more had caused a pregnancy/fathered a child.

Semen variables

Table 2 summarizes the semen variables. Sperm concentrations for the Faroese men were lower than for the Danish men. However, semen volume was higher, and thus total sperm counts did not differ. Similarly, overall motility and morphology did not differ between Faroese and Danes, but between the two Faroese subgroups with higher values for the F1-subgroup. Figure 1A shows the distributions of total sperm counts of all the investigated Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). According to the figure more Faroese men had lower sperm counts than the reference group ($p<0.0001$).

Table 2: Semen parameters in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value
Sperm concentration (mill/mL)						
Mean (SD)	54 (56)	60 (60)		57 (58)	62 (55)	
Median (5-95 percentiles)	38 (1.6-156)	41 (2.2-182)	0.5*	40 (1.9-174)	48 (4.0-169)	□0.0005*
Semen volume (mL)						
Mean (SD)	4.2 (1.7)	4.0 (1.7)		4.1 (1.7)	3.6 (3.1)	
Median (5-95 percentiles)	4.0 (1.9-7.1)	3.9 (1.5-7.2)	0.02*	3.9 (1.8-7.1)	3.3 (1.3-6.3)	□0.0005*
Total sperm count (mill)						

Mean (SD)	214 (205)	215 (206)		215 (205)	206 (258)	
Median (5-95 percentiles)	154 (3-586)	159 (9-638)	0.8*	159 (6-591)	151 (13-559)	0.2*
Normal morphology (%)[§§]**						
Mean (SD)	7.5 (4.3)	6.3 (4.5)		6.9 (4.4)	7.5 (4.9)	
Median (5-95 percentiles)	7.5 (1.0-15.0)	5.0 (0.5–15.0)	□0.0005**	6.5 (0.9-15.0)	7.0 (0.5-16.0)	0.2†
Motile sperm (%)[#]						
Mean (SD)	70 (22)	58 (13)		64 (19)	65 (16)	
Median (5-95 percentiles)	74 (19-95)	59 (35-74)	□0.0005‡	64 (24-90)	68 (33-85)	0.02‡
Period of abstinence (h)						
Mean (SD)	80 (38)	86 (35)		83 (37)	77 (96)	
Median (5-95 percentiles)	83 (14-167)	83 (35-168)	0.1†	83 (25-168)	63 (37-134)	□0.0005†

*Regression analysis adjusting for duration of abstinence.
†Mann Whitney test
‡Regression analysis adjusting for delay from time of ejaculation to assessment of motility.
** Regression analysis without any adjustment
[§§] Morphology was missing for 50 Faroese men (F1+F2) and 20 Danish men.
[#]Motile sperm is the sum of progressively motile (WHO class AB) and local motile (WHO class C).

From the entire group of 481 men, 313 (65%) were without any prior knowledge of andrological diseases or conditions that might be associated with risk of impaired testicular function (cryptorchidism, testicular torsion, orchitis, epididymitis, varicocele, hydrocele, prostatitis, hypospadias, phimosis, inguinal hernia, cystitis, sexually transmitted diseases, diabetes, thyroid diseases, experienced fertility problems). In this subgroup 244 men (50% of the entire group) had an ejaculation abstinence period of more than 48 hours. These 244 men also had lower total sperm counts than men from the WHO reference group (Figure 1B, lower panel, p=0.0013).

The percentages of morphologically normal spermatozoa were inversely associated with year of birth, both for the entire group of Faroese men (Figure 2A, trend p=0.002), and for the subgroup of 244 men (Figure 2B, trend p=0.018). Morphology assessments were done in four different time periods, but the results did not differ according to these periods. Similar trends were seen when the estimates according to year of birth

were adjusted for age, however, with broader confidence intervals of the estimates. No other semen variables were associated with birth year and age at time of investigation.

Approximately 14% of the participants reported to have fathered a child. The confounder adjusted estimates indicated higher sperm concentration, total sperm count, motility and morphologically normal spermatozoa in these men in comparison to non-fathers, all non-significant except for motility ($p<0.0005$). Semen volume was non-significantly lower in the father group ($p=0.7$). When the distribution of total sperm counts for these fathers were compared with the WHO reference group no difference between the groups could be shown ($p=0.1$).

Physical examination

Only the men in the F1-group had a physical examination performed. Among these 95% had a normal mean testicular volume (i.e. above 15 mL), but apparently slightly lower than the Danish men (Table 1). A total of 2% did not have both testicles in scrotum, 8% had soft testicles and 4% did not have adult pubic hair distribution (i.e. Tanner stage 5 or 6). A varicocele was detected in 15% (5.8% grade 1, 7.5% grade 2 and 1.7% grade 3) of the F1 cohort. In regression analyses all semen variable estimates were higher in men without any varicocele, but all non-significant (all $p>0.2$). Hydrocele was detected in 5%, and tended to be associated with a lower percentage of morphologically normal spermatozoa ($p=0.05$), but not with other semen variables (all $p>0.6$).

Smoking and BMI

The men's tobacco smoking was non-significantly associated with lower semen volume, sperm concentration and total sperm counts (all $p>0.1$) and higher percentage of normal forms and motile spermatozoa ($p=0.8$ and 0.2, respectively). Maternal smoking during pregnancy was significantly associated with lower total sperm count (29% reduction, 95% confidence interval 8;45%, $p=0.02$), and non-significantly with semen volume (9% reduction, 95% confidence interval -16%;2%, $p=0.05$) and sperm concentration (22% reduction, 95% confidence interval -40;6%, $p=0.06$). There were no significant associations between maternal smoking, sperm motility, and sperm morphology ($p=0.5$ and 0.8, respectively). BMI above 25 was not associated with adverse effects on any semen variable.

Reproductive hormones

Table 3 summarizes the reproductive hormone levels for the two sub-groups of Faroese men, the combined group and the Danish comparison group. Inhibin B tended to be slightly lower in the Faroese group F1 than in the F2 group, thus showing the same tendency as sperm concentration and total sperm count. FSH showed opposite directions resulting in lower inhibin B/FSH ratio in the F1 group. In the combined Faroese group both inhibin B and FSH individually were higher than in the Danish group, whereas the inhibin B/FSH ratio was lower. In 2010, the laboratory began to use a different kit material for the serum inhibin B analyses. However, that the inhibin B/FSH results did not differ according to this is described in the methods section.

Table 3: Reproductive hormones levels in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value*	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value*
FSH (IU/L)						
Mean (SD)	4.1 (4.7)	3.4 (1.9)		3.8 (3.6)	2.8 (1.8)	
Median (5-95 percentile)	3.1 (1.3-8.5)	2.9 (1.1-7.0)	0.05	3.1 (1.2-7.7)	2.5 (1.0-6.0)	□0.0005
Inhibin B (pg/mL)						
Mean (SD)	196 (79)	220 (84)		208 (82)	189 (70)	
Median (5-95 percentile)	192 (76-333)	210 (99-382)	0.04	202 (85-346)	180 (90-318)	0.05
Inhibin B /FSH						
Mean	80 (72)	101 (107)		91 (92)	100 (89)	
Median (5-95 percentile)	57 (11-203)	70 (17-289)	0.02	64 (13-259)	76 (17-272)	□0.0005
LH (IU/L)						
Mean (SD)	4.8 (2.4)	4.8 (1.8)		4.8 (2.1)	3.5 (1.5)	
Median (5-95 percentile)	4.5 (2.1-8.5)	4.6 (2.1-7.9)	1.0	4.5 (2.1-7.9)	3.3 (1.6-6.3)	□0.0005
Testosterone (nmol/L)						
Mean (SD)	21 (7)	23(7)		22 (7)	20 (6)	
Median (5-95 percentile)	20 (11-32)	22 (14-36)	0.006	21 (12-33)	19 (12-31)	□0.0005

Testosterone/LH						
Mean	5.0 (2.3)	5.9 (7.2)		5.5 (5.3)	6.5 (2.8)	
Median (5-95 percentile)	4.6 (1.9-9.1)	4.6 (2.7-11.6)	0.09	4.6 (2.2-9.4)	6.0 (3.0-12.0)	□0.0005
Free Testosterone (pmol/L)						
Mean	409 (134)	462 (140)		439 (134)	454 (142)	
Median (5-95 percentile)	397 (211-633)	442 (262-707)	□0.0005	424 (236-686)	432 (269-701)	0.1
Free testosteron/LH						
Mean	99 (44)	125 (175)		112 (128)	149 (71)	
Median (5-95 percentile)	92 (39-186)	96 (49-212)	0.03	94 (47-201)	134 (65-273)	□0.0005
Estradiol (nmol/L)						
Mean (SD)	79 (22)	96 (23)		88 (24)	80 (24)	
Median (5-95 percentile)	78 (46-113)	94 (62-140)	□0.0005	86 (54-131)	78 (48-125)	□0.0005
Testosterone/Estradiol						
Mean	268 (83)	243 (69)		255 (78)	258 (70)	
Median (5-95 percentile)	262 (140-409)	236 (146-375)	□0.0005	243 (145-394)	249 (175-383)	0.1
Free Testorsterone/Estradiol						
Mean	5332 (1567)	4955 (1187)		5143 (1400)	5869 (1768)	
Median (5-95 percentile)	5218 (3230-7768)	4876 (3165-7201)	0.002	5026 (3182-7538)	5636 (3519-8956)	□0.0005
SHBG (nmol/L)						
Mean (SD)	38 (15)	37 (15)		38 (15)	30 (12)	
Median (5-95 percentile)	36 (17-62)	35 (19-68)	0.7	36 (18-65)	29 (14-51)	□0.0005
Time of bloodsampling						
Mean (SD)	11:56 (2:11)	9:57 (1:29)		10:27 (1:56)	10:04 (0:42)	
Median (5-95 percentile)	10:15 (8:40-15:40)	9:37 (8:02-12.45)	□0.0005†	10:10 (8:30-15:00)	10:00 (9:05-11:20)	0.6†

*Regression analysis adjusting for hour of day of blood sampling
†Mann Whitney test

Total testosterone (T) only differed slightly between the two Faroese groups and between the combined group and the Danes, although statistically significant. SHBG in contrast was considerably higher for the Faroese men and with no difference between the F1 and F2 men. Frequency analyses of the SHBG values divided into groups (0-10, 10-20 etc.) showed that the higher SHBG values for Faroese men were not caused by higher concentration in a specific subgroup but an overall shift to higher levels. Approximately 80% of the Faroese men had a SHBG concentration below 50 nmol/L while 80 % of the Danish men had a concentration below 40 nmol/L. The FT differed between the two Faroese groups which combined, however, had non-significantly lower FT than the Danes. Estradiol was higher in the F2 group than in the F1, thus leading to higher levels in the combined group of Faroese men than in the Danish. The ratios T/LH, FT/LH, and FT/estradiol were all lower in the Faroese men than in the Danish whereas the estimated lower total T/estradiol was non-significant.

SHBG and LH were positively correlated with T ($P < 0.0005$), whereas SHBG and LH did not correlate ($P=0.1$). FT was positively correlated with LH and T ($P < 0.0005$), and negatively but not significantly correlated with SHBG ($P=0.06$)

BMI was negatively associated with T, FT, SHBG, T/LH, T/estradiol, FT/estradiol and inhibin B ($p<0.0005$-0.042), and also negatively but non-significant with LH ($p=0.3$). Faroese smokers tended to have higher T than non-smokers ($p=0.7$, adjusted for BMI effect), whereas the effect on the Danish men were highly significant ($p<0.0005$). In the Faroese groups LH, SHBG, estradiol, FT/LH, FT/estradiol and T/estradiol tended to be higher in smokers, although all non-significantly. For the Danes similar trends were detected; the effect on SHBG and LH being non-significant, whereas the remaining were significant with p-values <0.0005-0.05. Further maternal smoking during pregnancy for the Faroese men tended to be associated with lower LH and FT ($p=0.04$ and 0.02) whereas the effect on the remaining hormone levels were all highly non-significant ($p=0.1$-0.9). However, the difference (i.e. significant levels in table 3) between Faroese and Danish hormone levels did not change when including BMI or age as cofactors into the regression analyses indicating that these factors cannot explain the difference in reproductive hormones between Faroese and Danes.

DISCUSSION

This is the first study on testicular function conducted in the Faroe Islands. These young Faroese men had a lower sperm concentration but similar total sperm count compared to Danish men. Recent data have shown sperm counts to be low in young men from several European countries,

however, slightly higher than among the Danes [5, 21, 22]. Thus, the semen quality of Danish men seems to be particularly low, and now we have shown the Faroese men to have a similar low level.

The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts, and provides independent evidence of poorer testicular function in the Faroese men than in the Danes, although the medians were at a level where the association between sperm counts and inhibin B is weakened [23]. When evaluating the results against the WHO reference population, Faroese men also had low sperm counts. The lower T/LH and FT/LH ratios point towards a lower Leydig cell capacity among Faroese men compared to Danes. Thus, the total testicular function among Faroese men may be at the same or lower level than Danes.

A greater proportion of men from the Faroese study populations had previously caused a pregnancy, which also would be expected since these men in average were almost 6 years older than the Danes. Furthermore, Faroese people are in general younger when parenting for the first time (25.5 years in 2011) [24], and the fertility rate in the Faroe Islands is the highest in European countries [25] with 2.3 children per woman in 2011 [24]. Traditionally, the family culture in the Faroe Islands is to have large families with many children; however, during the last 40 years the fertility rate in the Faroe Islands has decreased form 3.4 children per woman in 1970 to 2.8 children per woman in 1990 to the current fertility rate of 2.3 children per woman in 2011 [24]. Thus, the high birth rate most likely reflects socio-economic factors rather than male fecundity. Despite of this more Faroese men from the study populations had experienced fertility problems than the Danish men. This is in agreement with the lower sperm concentration found in the Faroese men, but it should be taken into account that the Faroese men were asked about fertility problems on the basis of 6 months unprotected intercourse without achieving a pregnancy and for the Danes it was based on 1 year. Therefore, the Danish men were more likely to have obtained a successful fertilization having tried for twice as long. Furthermore, fewer Danish men may be aware of a fecundity problem because they were younger and had not yet tried to conceive.

The Faroese and the Danish men were not exactly the same age. Semen quality has been reported to decline with increasing age [26-29]. However, the Faroese studies and the comparative Danish studies were conducted at the same time periods and the difference in age between groups is not likely to have influenced the semen parameters nor is the actual age of the participants that still can be regarded as young. Semen quality does on average not change between 19 and 23 years of age, and immaturity seems unlikely to explain the poor semen quality both among the Faroese and Danish men [30]. Thus, age difference is not likely to explain the difference in semen quality.

The Faroese men were investigated during a relatively short time period and also represented narrow birth cohorts. Despite of this, we actually detected an inverse association between percentage of morphologically normal spermatozoa and year of birth, a trend that was robust even when controlled for potential confounders. In a cohort like ours, the effect of birth year may be difficult to separate from the effect of age. However, when we modelled age as explanatory factor and controlled for birth year, the trend effect of age was not apparent. Thus, our results suggest that younger men produced ejaculates with lower percentages of normal spermatozoa. This could be caused by certain unhealthy life-style factors among the younger cohorts, but not the older cohorts. Our currently existing data does not support that explanation, however, but the information we have could also be insufficient to exclude this suggestion. Alternatively, the birth cohort effect could reflect an increasing exposure during fetal life leading to impaired testicular development which in adulthood would be reflected by reduced semen quality in line with the suggested Testicular Dysgenesis Syndrome hypothesis [31-32].The Faroese studies were investigated according to similar protocols and assessment of sperm concentration was controlled by the same quality control as the Danish study. The Faroese technicians were trained at RH in Denmark and the analyses used in both laboratories were identical, thus reducing the interobserver variation. Additionally, assessment of sperm morphologies was performed randomly by a single person. Frequency of motile sperm, however, is difficult to compare reliably between different groups. The motility assessment is highly subjective and controlling for quality control within this parameter is problematic. Previously, the inter-laboratory variance of motility has shown to be of significant importance [33]. Therefore, we hesitate to draw major conclusion regarding sperm motility.

The recording of abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. This latter procedure is believed to be much more accurate, as the men were given time to reflect and were not expected to give an immediate verbal response. This assumption is supported by the fact that the anticipated relationship between abstinence time and sperm concentration only was clearly seen in F2 group. Assuming the reported abstinence time actually was longer among the Faroese men these men still produced similar numbers of sperm as the Danish men, suggesting that the sperm production rate is lower in the Faroese population compared to Danes. This was, however, only confirmed for sperm concentration but not total sperm count when abstinence period was accounted for in the statistical model.

As explained in the methods section some of the semen samples were analysed with incorrect counting chambers. However, we corrected for this potential testing error. In addition, the statistical analyses performed with or without the 59 samples did not change the overall

results, supporting the assumption that the corrections made were valid. A major advantage of the study was that the participants were not selected on fertility status. According to most of the Danish men, the main incentive for participation was the financial compensation [5]. The F2 men were part of an existing birth cohort study whereas F1 men were randomly recruited. Thus, we cannot exclude that men with suspicion of infertility may have been more interested in participating in the study. However, to control for this we defined a subgroup of men with abstinence period of more than 48 hours and without any prior knowledge of andrological diseases and without know fertility, and still the same low sperm counts were detected. This problem with counting chambers, and the differences in semen quality and population characteristics between the two Faroese sub-population exemplifies one of the main problems when comparing semen characteristics globally and over time, namely that standardised methods have to be applied and sufficiently large study populations investigated.

The finding of low semen quality and lower Leydig cell function in Faroese men cannot be explained by effects of confounders. Both high BMI and maternal smoking have previously been associated with reduced testicular function in some studies but not all [34-40].In our cohort, the effects of these two usually quite robust confounders/explanatory factors were not obvious, neither on the reproductive hormones. However, our cohorts may be too small to detect the effect of these two factors. Alternatively, other unidentified factors may have a major influence masking the effect of BMI and maternal smoking on the semen parameters. The reason for the low testicular function of the Faroese young men is unknown but this population is highly exposed to POPs derived solely from traditional marine food, which includes blubber from the pilot whale. Studies have shown associations between high polychlorinated biphenhyls (PCB) levels and low semen quality, and since PCBs and p,p'-DDE have the potential to interfere with sex hormone functions, it is plausible to assume that these compounds can affect the function of these organs [41]. There are some reports on the effect of POPs on male reproduction in humans, mainly indicating weak negative effects on sperm motility [41-43]. For the two Faroese sub-groups, we found that the percentage of motile cells was significantly lower compared with Danish men, indicating that increased exposure to endocrine disruptors can be one explanation.

Serum SHBG levels for the Faroese men were strikingly higher than in the Danes. SHBG is a sensitive marker for thyroid function. However, none of the study subjects were under treatment for hyperthyroidism. We did not measure thyroid hormones, but the men did not have any obvious clinical symptoms of hyperthyroidism. If a high thyroid level in general should explain the SHBG level, a significantly elevated LH and

testosterone in men with low BMI would have been expected, which was not the case. Therefore, there is no indication that the high SHBG values are due to hyperthyroidism. Furthermore, alcohol consumption could not explain the high levels. One plausible explanation to the high SHBG levels could be the high PCB exposure mentioned above. A recent publication from the Faroe Islands found that SHBG increased at higher PCB exposure, both prenatally and currently. PCBs are known to affect a variety of liver functions, and it could be speculated that PCB-induced hepatic SHBG synthesis could play a possible role, although this possibility remains to be substantiated [44]. We cannot elucidate further if PCB in our current study group can explain the SHBG levels.

It is known that the Faroese population differs genetically from other populations in many respects. There are several genetic diseases reported with very high frequency on the Faroese Islands [11, 45]. However different genetic composition in populations may contribute to the explanation of differences in semen quality and would be worth further exploration.

In conclusion, we found that the semen quality among Faroese men is at the same low level as reported among Danish men. This low quality was corroborated by the reproductive hormone levels in Faroese men. The influence of environmental exposures and genetic factors on the semen quality has to be studied further.

CONTRIBUTORS:

Substantial contribution to conception and design: PG, PW, NJ, TKJ.

Acquisition of data: JH, MSP, PW.

Interpretation of data: All authors.

Drafting the article: JH, MSP, NJ.

Revising the article critically for important intellectual content: all authors.

Final approval of the version to be published: all authors.

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ETHICS APPROVAL. The local Science Ethical Committee for the Faroe Islands and the Institutional Review Board at Harvard School of Public Health have approved the study protocol, and all participants had given their informed consent.

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Supplementary Information

In our study of young Faroese men, the sperm concentration was assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) as described in the main text. As stated there, a second delivery of Bürker-Türk chambers were 0.05 mm in depth rather than the standard 0.1 mm and therefore, 59 samples from F2 were analyzed without knowing whether 0.05 mm or 0.1 mm deep counting chambers were used. As the semen samples had been stored in our bio-bank, the 59 samples were measured again with the 0.1 mm deep chamber to indicate if the original concentration assessment was correct (assuming that the 0.1 mm counting chamber was used originally) or should be doubled (assuming that the 0.05 mm counting chamber was used originally). This was done manually by investigating the agreement between the values of the two measures. Based on these re-analyses, the obtained original sperm concentrations were doubled for 23 samples (table 1):

Table 1

ID number	Original count (mill/ml)	Recount (mill/ml)	Factor	Final concentration (mill/ml)
1	31,05	75,19	x2	62,10
2	63,28	61,33	x1	63,28
3	56,64	96,09	x2	113,28
4	44,14	76,95	x2	88,28
5	35,94	31,41	x1	35,94
6	57,42	66,02	x1	57,42
7	62,50	58,98	x1	62,50
8	37,73	36,17	x1	37,73
9	29,06	27,11	x1	29,06
10	37,50	77,93	x2	75,00
11	86,72	86,52	x1	86,72
12	76,17	78,52	x1	76,17
13	23,59	22,97	x1	23,59
14	109,76	104,3	x1	109,76
15	19,33	40,82	x2	38,66
16	27,34	28,13	x1	27,34
17	42,18	46,09	x1	42,18
18	37,89	31,25	x1	37,89
19	23,44	20,31	x1	23,44
20	75,39	63,67	x1	75,39
21	43,75	92,19	x2	87,50
22	29,69	29,69	x1	29,69
23	28,30	27,42	x1	28,30
24	112,50	88,67	x1	112,50
25	126,18	95,31	x1	126,18
26	20,90	41,02	x2	41,80
27	20,31	18,95	x1	20,31
28	40,43	46,88	x1	40,43
29	48,05	90,23	x2	96,10
30	44,53	102,34	x2	89,06

31	23,05	44,53	x2	46,10
32	44,53	55,07	x1	44,53
33	49,80	41,79	x1	49,80
34	30,86	53,52	x2	61,72
35	28,05	31,56	x1	28,05
36	80,47	220,3	x2	160,94
37	27,93	47,85	x2	55,86
38	28,91	56,45	x2	57,82
39	2,69	2,46	x1	2,69
40	54,49	66,01	x1	54,49
41	24,02	47,46	x2	48,04
42	22,46	39,45	x2	44,92
43	91,02	180,07	x2	182,04
44	13,13	37,89	x2	26,26
45	3,18	8,28	x2	6,36
46	14,84	14,38	x1	14,84
47	9,09	6,33	x1	9,09
48	6,57	9,22	x1	6,57
49	1,96	1,73	x1	1,96
50	15,31	35,23	x2	30,62
51	3,22	0,85	x1	3,22
52	5,27	3,95	x1	5,27
53	5,63	10,47	x2	11,26
54	10,55	22,42	x2	21,10
55	5,16	5,39	x1	5,16
56	17,34	17,27	x1	17,34
57	4,18	8,16	x1	4,18
58	5,30	4,35	x1	5,30
59	19,30	41,02	x2	38,60

To ascertain that the concentration could be replicated in thawed samples, 15 thawed samples known to be counted in 0.1 mm deep chamber were counted again. As anticipated, the results were replicated and shown to be accurate (Table 2):

Table 2:

Sample	Original count (mill/ml)	Recount (mill/ml)	Factor
1	224,4	203,9	1,10
2	8,55	7,03	1,22
3	20,74	20,31	1,02
4	21,64	20,39	1,06
5	33,7	31,4	1,07
6	62,9	60,5	1,04
7	51,4	56,8	0,91
8	35,9	46,7	0,77
9	55,5	60,2	0,92

10	37,89	27,89	1,36
11	5,39	4,2	1,28
12	8,52	1,84	4,63
13	1,78	1,21	1,47
14	154,3	149,22	1,03
15	13,91	11,44	1,22

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
Outcome data	15*	Report numbers of outcome events or summary measures
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses

Discussion		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

SEMEN QUALITY AND REPRODUCTIVE HORMONES IN FAROESE MEN – A CROSS-SECTIONAL POPULATION-BASED STUDY OF 481 MEN

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Keywords: **Semen quality, reproductive hormones, Faroe Islands**

Word count: 4373

ABSTRACT

Objectives: To determine semen quality and reproductive hormone levels in young Faroese men.

Design: Descriptive cross-sectional study of Faroese men compared with Danish men.

Setting: Faroese one-center study.

Participants: 481 men born from 1981 to 1987 and investigated from 2007 to 2010.

Outcome measures: Sperm concentration, semen volume, total sperm count, sperm motility, sperm morphology and reproductive hormones levels.

Results: Sperm concentrations for the Faroese men were lower than for the Danish (crude median 40 mill/mL vs. 48, p<0.0005). Semen volume was higher, and thus total sperm counts did not differ (159 vs. 151 mill, p=0.2). Motility and morphology did not differ between Faroese and Danes. Inhibin B/FSH ratio for the Faroese men were lower than for Danes (64 vs. 76, p=0.001). Similarly, lower total testosterone/LH ratio (4.6 vs. 6.0, p<0.0005) and lower calculated free-testosterone/LH ratio (94 vs. 134, p<0.0005) were detected for Faroese men.

Conclusions: Semen quality among Faroese men is at the same low level as reported for Danish men, and the reproductive hormone levels furthermore indicated a lower Leydig cell capacity for testosterone production. The influence of environmental exposure and genetic factors on the semen quality has to be studied further.

ARTICLE SUMMARY

Article focus

Semen quality studies have not been conducted in the Faroe Islands before.

Because the Faroese population differs from other populations regarding e.g. high exposure to persistent organic pollutants (POPs) and genetic diseases, the semen quality of Faroese men from the general population would be expected to be low.

Key messages

The semen quality among Faroese men is at the same low level as reported in Denmark.

The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts and provides independent evidence of poorer testicular function in the Faroese men than in the Danes.

Similarly, the Leydig cell capacity for testosterone production was also lower for Faroese men than for the Danes.

Strengths and limitations of the study

Prospectively designed, cross-sectional study of testicular function among young men from the general population unselected with regard to fertility.

Standardized inclusion and investigation procedures.

Clinical examination for one subgroup of Faroese men, but not for the other.

INTRODUCTION

In 1992 Carlsen [1] and co-workers published a combined analysis of results from 61 papers published between 1939 and 1991 and showed a significant decline in sperm counts over a 50 years period. A detailed reanalysis of the results found that the conclusion was supported by the underlying studies [2, 3]. Following the 1992 publication, many analysed retrospectively their historical data for temporal trends, some finding a decline and others not. Realising that the trend analyses indicated that semen quality could have reached a low level where it might affect fecundity, several prospectively designed cross sectional semen quality studies were initiated to determine the current quality in men from general populations. These studies did not only reveal geographical differences [4, 5] but confirmed the general presence of low semen quality in men from all investigated countries when the results were interpreted according to available publications focused on associations between semen quality and fertility chances [6-9].

The causes of decreased semen quality are not clear, but it is feasible that many cases may have been caused by exposure to environmental factors in utero, during adolescence or in adulthood [10], however, most likely acting on a background of different genetic susceptibility.

The Faroese population differs from other populations in many respects. The Faroese population has high exposure to persistent organic pollutants (POPs) derived from traditional marine food, which includes blubber from the pilot whale. At the same time, there are several genetic diseases reported with very high frequency in the Faroese Islands [11]. Thus, our hypothesis was that semen quality of Faroese men from the general population would be quite low.

MATERIALS AND METHODS

To test our hypothesis we investigated semen quality of young Faroese men from the general population in 2007-2010. For comparison we used a recently published similar group of Danish men examined 2006-2010 [12] and the current World Health Organization (WHO) reference levels [13].

Study population: men from the general Faroese population

The entire study population consisted of 484 men, originating from two separate studies. The first study group included 241 randomly selected young men examined between February 2007 and February 2009 (F1). The second group comprised of 243 men examined between November 2009 and November 2010 (F2). A detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1. Within 3 months prior to participation, 132 men (27.4%) had used medication, mainly antibiotics, painkillers, or asthma/allergy medicine.

Table 1: Physical appearance and self-reported information of Faroese and Danish men. Results shown as medians (5-95th percentile) or percentages.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value*	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value*
Physical appearance						
Age (years)	25.3 (24.2-26.7)	23.0 (22.0-24.0)	<0.0005	24.0 (22.0-26.2)	19.0 (18.4-21.8)	<0.0005
Height (cm)	180.0 (169.9-189.9)	180.0 (170.0-193.0)	0.1	180.0 (170.0-191.0)	181.6 (171.1-193.0)	<0.0005
Weight (kg)	80.3 (64.2-106.2)	78.00 (62.9-105.1)	0.2	80.0 (63.1-105.9)	74.1 (60.1-96.0)	<0.0005
BMI (kg/m2)	25.0 (20.6-32.3)	24.3 (20.4-32.1)	0.03	24.6 (20.5-32.1)	22.4 (18.7-28.6)	<0.0005
Testes size (mL)**	21.5 (15.3-25.0)	NA		NA	23 (14-29)	
Lifestyle						
Alcohol per week (units)	5(0-25)	6 (0-26)	0.08	5 (0-25)	12 (0-42)	<0.0005
Current Smokers	44.0	41.3	0.5	42.6	45.4	0.4
Mother smoked in pregnancy	22.4.***	37.5	0.05	33.6	29.1	0.4
Taken medication§	23.7	31.3	0.07	27.4	15.1	<0.0005
Been treated for§§						
Cryptorchidism†	3.3	7.5	0.04	5.4	5.0	0.8
Been diagnosed as having§§						
Cryptorchidism reported	8.7	12.1	0.2	10.4	6.9	0.02
Hypospadias	0.4	0.8	0.6	0.6	0.3	0.4

Sexual transmitted disease††	9.1	10.8	0.5	10.0	6.2	0.007
Phimosis	10.0	11.7	0.5	10.8	4.6	<0.0005
Varicocele	0.8	0.8	1.0	0.8	0.4	0.6
Have[§§§]						
Ever caused a pregnancy**	25.7	NA		NA	6.8	-
Fatherhood	21.2	6.3	<0.0005	13.7	NA	-
Experienced fertility problems‡	4.6	1.7	0.07	3.1	0.2	<0.0005

*Mann Whitney test

**Mean of two testes. Testes size was not measured in Faroese men born 1986-87 and the question "Have you ever caused a pregnancy" was not asked in this group

*** Information only available for 189 mothers

§ Taken any medication 3 months prior to participation in the study

† Hormonal, surgical or combination

†† Chlamydia, gonorrhoea, warts or herpes

‡Ever had regular intercourse without use of contraception for at least 6 months (Faroese men) or 1 year (Danish men) without partner became pregnant

§§ "Have you ever been treated for…" §§ "Has a doctor ever diagnosed you as having....." §§§ Have you ever…"

NA: not available

Faroese men, examined 2007-09 (sub-group F1)

Invitation letters to participate in the study were sent to 1,100 men, consecutively listed in the Faroese population register as born between January 1981 and December 1984, followed by a phone call to arrange the examination details. A total of 34 men had emigrated, and 43 letters were returned as undeliverable. Thus, 1,023 were invited. Of these, 490 could not be reached and 292 declined to participate. Hence, the final F1 group was comprised of 241 men (24% of all invited).

Faroese men, examined 2009-10 (sub-group F2)

This group consisted of a cohort generated from consecutive births at the three Faroese hospitals during 1986-1987 (N=509 males) as described elsewhere [14-16]. Detailed information on their physical health and potential environmental factors were collected at the time of birth and during the course of follow-up at ages 7, 14 and 22 years. The 421 men who had participated in the 22 years follow-up were invited to participate in the

semen quality study. This was the sole selection criterion. All men received a letter of invitation and subsequently a phone call to arrange the examination details. Among the 421 invited men, 243 accepted to participate, but 3 did not succeed in delivering a semen sample. Hence, the final F2 group comprised 240 men (57 % of all invited).

Questionnaire

On the day of attendance, the men returned a questionnaire they had received in advance. The questionnaire was based on that used for young Danish men [12, 17] and included information on previous or current diseases, including any known history of fertility potential, and some lifestyle factors like smoking and drinking habits.

Semen samples

Semen samples were produced by masturbation in a room close to the semen laboratory. The period of abstinence was recorded. The abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. The semen sample was analysed according to the World Health Organization 1999 guidelines [18]. Semen volume was estimated by weighing the collection tube with the semen sample and subtracting the weight of the empty pre-weighed tube, assuming that 1 mL semen=1 g. For sperm motility assessment, 10 µL of well-mixed semen was placed on a clean glass slide kept at 37 °C and covered with a 22x22 mm coverslip. The preparation was placed on the heated stage of a microscope at 37 °C and immediately examined at x400 magnification. The sperm were classified as progressive motile (WHO class AB motility), locally motile (WHO class C motility) or immotile (WHO class D motility).

For the assessment of the sperm concentration, the samples were diluted in distilled water. The sperm concentration was subsequently assessed using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). However, a second delivery of Bürker-Türk chambers were 0.05 mm in depth rather than the standard 0.1 mm we had ordered. Because of that 59 samples from F2 were analysed without knowing whether 0.05 mm or 0.1 mm deep counting chambers were used. As the semen samples had been stored in our bio-bank, the 59 samples were measured again with the 0.1 mm deep chamber to indicate if the original concentration assessment was correct (assuming that the 0.1

mm counting chamber was used originally) or should be doubled (assuming that the 0.05 mm counting chamber was used originally). Based on these re-analyses, the obtained original sperm concentrations were doubled for 23 samples (see Supplementary Materials for details). To ascertain that the concentration could be replicated in thawed samples, samples known to be counted in 0.1 mm deep chamber were counted again. As anticipated, the results were replicated and shown to be accurate. From each semen sample a smear for morphology evaluation was made, Papanicolaou stained and finally assessed according to "strict criteria" at Department of Growth and Reproduction (GR) at the National Hospital (Rigshospitalet, RH) in Denmark.

The Faroese semen analyses were performed by three technicians, who participated in a quality control testing course at Rigshospitalet (RH) and spent two weeks there to ensure comparable results. Furthermore, every three months, 5 blinded samples were sent from RH, and sperm concentration results were compared with the results from their technicians. Throughout the study period, the variation was less than 10% compared with the Danish technicians. Therefore, the methodological differences between the Faroese and the Danes should be considered very limited.

Reproductive hormones

On the examination day, a venous blood sample was drawn from each participant and centrifuged (3000 g, 10 minutes). Serum was subsequently separated and kept frozen until it was analysed for reproductive hormones at GR at RH. Levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone-binding globulin (SHBG) were determined using a time-resolved fluoroimmunoassay (Delfia, Wallac, Turku, Finland). Testosterone (T) was determined using a COAT-A-COUNT solid phase radioimmunoassay (RIA) (Siemens Medical Solutions, Malvern, PA, USA) and estradiol by radioimmunoassay (RIA) (Pantex, Santa Monica, CA, USA) [19]. Inhibin B was analyzed by a double antibody enzyme-immunometric assay using a monoclonal antibody raised against the inhibin βB-subunit in combination with labeled anti-body raised against the α-subunit. Samples were analysed for inhibin B in 2009 using kit material from Oxford Bio-Innovation or in 2010 with kit material from DSL Beckman, USA. For all hormone assessments internal standard samples were analysed and showed no need to correct for potential inter-assay variation, including no need for correction between the kit materials for inhibin B from the two suppliers. The free testosterone index (FT) was calculated from total testosterone and SHBG using a fixed albumin value according to Vermeulen et al. [20].

Physical examination

Participants in the F1 sub-group underwent a physical examination performed by one of two examiners at the day of the semen sample delivery. Body weight and height were measured. The Tanner stage of pubic hair and genital development were recorded. Any abnormalities in the testis and penis, the possible presence of varicocele, hydrocele, hypospadias and testis tumour, the location of testis in scrotum and their consistency and that of epididymis were recorded. Testicular volumes were determined by use of a Prader orchidometer, and reported as the mean of two testes (Table 1). Participants in the F2 study did not undergo a physical andrological examination.

Comparison population: men from the general Danish population

In Denmark, all young men, except those suffering from severe chronic diseases (<15%), are required to attend a compulsory medical examination before they are considered for military service [5]. Men are called upon at the age of 18-19 years, but some postpone this examination until completion of their education. Men attending the compulsory examination are invited to participate in a semen quality study, using same basic study design including a physical examination as the Faroese, irrespective of whether they are declared fit for military service or not. For comparison with results from the Faroese studies, we utilized results for Danish men examined 2006-10, which has recently been published [12]. The detailed description of the study population based on questionnaire information and results from the physical examination (see below) is summarized in Table 1.

Comparison population: WHO reference group of fertile men

The Faroese sperm count distribution was compared with the distribution for fertile men reported as the WHO reference group [13].

Statistics

The crude means, medians, standard deviations, 5-95 percentiles and frequencies were used for basic descriptions of obtained results. The main outcome variables were semen volume, sperm concentration, total sperm count, percentage of motile spermatozoa, percentage of morphologically normal spermatozoa and serum level of the reproductive hormones. Differences in semen quality variables and reproductive hormone levels between

groups were tested by linear regression adjusted for significant confounders. Semen volume, sperm concentration, and total sperm count were best normalised by a cubic root transformation before regression analysis to correct for skewed distribution of residuals. The percentages of motile spermatozoa (ie WHO class AB+C) were logit-transformed. Percentages of morphologically normal spermatozoa were close to normally distributed and entered the model untransformed. Reproductive hormone levels were natural logarithmic transformed. Correlations between reproductive hormone levels were assessed with Spearman Correlation. Between-group differences for categorical variables were tested with non-parametric tests (Mann Whitney). Total sperm count distribution differences between the Faroese men and the WHO reference distribution was tested by chi square analyses. Association between year of birth and semen parameters were tested by linear regression.

For F2, the abstinence time in hours showed a significant positive association to sperm concentration, semen volume and total sperm count. The effect was most pronounced for the period below 96 hours and less above (for sperm concentration: β-value=0.011, p=0.01 and β-value=0.006, p=0.18 respectively). The abstinence time was not significantly associated with sperm concentration in F1 (β-value=0.0001, p=0.98, and β-value=0.0003, p=0.5 respectively) or total sperm count. Thus, abstinence time was entered as piecewise linear functions (linier splines); i.e. one straight line for abstinence below 96 and another straight line for abstinence above 96 for semen volume, sperm concentration and total sperm counts. The duration from ejaculation to assessment was included as a confounder for sperm motility. No confounders were detected to affect morphology.

The following factors were evaluated as possible confounders for semen parameters and found to have no influence (p >0.05): age, body mass index (BMI) (as continuous variable or categorized as <18.49; 18.5-25; >25), smoking (yes/no) and season of year (spring (March-May), summer (June-August), autumn (September-November) and winter (December-February)).

The same factors were evaluated as possible confounders for the reproductive hormones. Significant associations were observed for BMI and age but not for smoking. The effect of season was not systematic and thus not included as a confounder. Hour of day of blood sampling was included as a confounder for reproductive hormones, although only significantly associated with estradiol, T and FT. p-values below 5% were considered statistically significant. Analyses were performed using PASW GradPack 19.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

In Table 1 physical appearance, lifestyle factors, reproductive health and self-reported information on fertility are displayed for the two sub-groups of Faroese men, the combined Faroese group and the Danish comparison group. All men had semen variables assessed. However, morphology scoring was missing for 50 Faroese men and 20 Danes, which is reported in Table 2. Also, assessment of testis size was only performed on sub-group F1, which is stated in Table 1. Semen volume was not recorded for one Faroese man and motility measure was ~~done~~missing for one man.

The three groups differed in age with the F1 being oldest and the Danish youngest. Similarly the F1 group had the highest BMI and the Danish the lowest. Alcohol intake was lower among Faroese men, and more had caused a pregnancy/fathered a child.

Semen variables

Table 2 summarizes the semen variables. Sperm concentrations for the Faroese men were lower than for the Danish men. However, semen volume was higher, and thus total sperm counts did not differ. Similarly, overall motility and morphology did not differ between Faroese and Danes, but between the two Faroese subgroups with higher values for the F1-subgroup. Figure 1A shows the distributions of total sperm counts of all the investigated Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). According to the figure more Faroese men had lower sperm counts than the reference group ($p<0.0001$).

Table 2: Semen parameters in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value
Sperm concentration (mill/mL)						
Mean (SD)	54 (56)	60 (60)		57 (58)	62 (55)	
Median (5-95 percentiles)	38 (1.6-156)	41 (2.2-182)	0.5*	40 (1.9-174)	48 (4.0-169)	□0.0005*
Semen volume (mL)						
Mean (SD)	4.2 (1.7)	4.0 (1.7)		4.1 (1.7)	3.6 (3.1)	
Median (5-95 percentiles)	4.0 (1.9-7.1)	3.9 (1.5-7.2)	0.02*	3.9 (1.8-7.1)	3.3 (1.3-6.3)	□0.0005*
Total sperm count (mill)						
Mean (SD)	214 (205)	215 (206)		215 (205)	206 (258)	

Median (5-95 percentiles)	154 (3-586)	159 (9-638)	0.8*	159 (6-591)	151 (13-559)	0.2*
Normal morphology (%)[§§]**						
Mean (SD)	7.5 (4.3)	6.3 (4.5)		6.9 (4.4)	7.5 (4.9)	
Median (5-95 percentiles)	7.5 (1.0-15.0)	5.0 (0.5–15.0)	□0.0005**	6.5 (0.9-15.0)	7.0 (0.5-16.0)	0.2†
Motile sperm (%)[#]						
Mean (SD)	70 (22)	58 (13)		64 (19)	65 (16)	
Median (5-95 percentiles)	74 (19-95)	59 (35-74)	□0.0005‡	64 (24-90)	68 (33-85)	0.02‡
Period of abstinence (h)						
Mean (SD)	80 (38)	86 (35)		83 (37)	77 (96)	
Median (5-95 percentiles)	83 (14-167)	83 (35-168)	0.1†	83 (25-168)	63 (37-134)	□0.0005†

*Regression analysis adjusting for duration of abstinence.
†Mann Whitney test
‡Regression analysis adjusting for delay from time of ejaculation to assessment of motility.
** Regression analysis without any adjustment
[§§] Morphology was missing for 50 Faroese men (F1+F2) and 20 Danish men.
[#]Motile sperm is the sum of progressively motile (WHO class AB) and local motile (WHO class C).

From the entire group of 481 men, 313 (65%) were without any prior knowledge of andrological diseases or conditions that might be associated with risk of impaired testicular function (cryptorchidism, testicular torsion, orchitis, epididymitis, varicocele, hydrocele, prostatitis, hypospadias, phimosis, inguinal hernia, cystitis, sexually transmitted diseases, diabetes, thyroid diseases, experienced fertility problems). In this subgroup 244 men (50% of the entire group) had an ejaculation abstinence period of more than 48 hours. These 244 men also had lower total sperm counts than men from the WHO reference group (Figure 1B, lower panel, p=0.0013).

The percentages of morphologically normal spermatozoa were inversely associated with year of birth, both for the entire group of Faroese men (Figure 2A, trend p=0.002), and for the subgroup of 244 men (Figure 2B, trend p=0.018). Morphology assessments were done in four different time periods, but the results did not differ according to these periods. Similar trends were seen when the estimates according to year of birth

were adjusted for age, however, with broader confidence intervals of the estimates. No other semen variables were associated with birth year and age at time of investigation.

Approximately 14% of the participants reported to have fathered a child. The confounder adjusted estimates indicated higher sperm concentration, total sperm count, motility and morphologically normal spermatozoa in these men in comparison to non-fathers, all non-significant except for motility ($p<0.0005$). Semen volume was non-significantly lower in the father group ($p=0.7$). When the distribution of total sperm counts for these fathers were compared with the WHO reference group no difference between the groups could be shown ($p=0.1$).

Physical examination

Only the men in the F1-group had a physical examination performed. Among these 95% had a normal mean testicular volume (i.e. above 15 mL), but apparently slightly lower than the Danish men (Table 1). A total of 2% did not have both testicles in scrotum, 8% had soft testicles and 4% did not have adult pubic hair distribution (i.e. Tanner stage 5 or 6). A varicocele was detected in 15% (5.8% grade 1, 7.5% grade 2 and 1.7% grade 3) of the F1 cohort. In regression analyses all semen variable estimates were higher in men without any varicocele, but all non-significant (all $p>0.2$). Hydrocele was detected in 5%, and tended to be associated with a lower percentage of morphologically normal spermatozoa ($p=0.05$), but not with other semen variables (all $p>0.6$).

Smoking and BMI

The men's tobacco smoking was non-significantly associated with lower semen volume, sperm concentration and total sperm counts (all $p>0.1$) and higher percentage of normal forms and motile spermatozoa ($p=0.8$ and 0.2, respectively). Maternal smoking during pregnancy was significantly associated with lower total sperm count (29% reduction, 95% confidence interval 8;45%, $p=0.02$), and non-significantly with semen volume (9% reduction, 95% confidence interval -16%;2%, $p=0.05$) and sperm concentration (22% reduction, 95% confidence interval -40;6%, $p=0.06$). There were no significant associations between maternal smoking, sperm motility, and sperm morphology ~~Associations between mother's smoking~~

motility and morphology were highly non-significant (p=0.5 and 0.8, respectively). BMI above 25 was not associated with adverse effects on any semen variable.

Reproductive hormones

Table 3 summarizes the reproductive hormone levels for the two sub-groups of Faroese men, the combined group and the Danish comparison group. Inhibin B tended to be slightly lower in the Faroese group F1 than in the F2 group, thus showing the same tendency as sperm concentration and total sperm count. FSH showed opposite directions resulting in lower inhibin B/FSH ratio in the F1 group. In the combined Faroese group both inhibin B and FSH individually were higher than in the Danish group, whereas the inhibin B/FSH ratio was lower. In 2010, the laboratory began to use a different kit material for the serum inhibin B analyses. However, that the inhibin B/FSH results did not differ according to this as is described in the methods section.

Table 3: Reproductive hormones levels in Faroese and Danish men.

	Faroese men (F1) 2007-09 (N=241)	Faroese men (F2) 2009-10 (N=240)	P value*	Faroese men (F1+F2) 2007-10 (N=481)	Danish men (D) 2006-10 (N=1,274)	P value*
FSH (IU/L)						
Mean (SD)	4.1 (4.7)	3.4 (1.9)		3.8 (3.6)	2.8 (1.8)	
Median (5-95 percentile)	3.1 (1.3-8.5)	2.9 (1.1-7.0)	0.05	3.1 (1.2-7.7)	2.5 (1.0-6.0)	□0.0005
Inhibin B (pg/mL)						
Mean (SD)	196 (79)	220 (84)		208 (82)	189 (70)	
Median (5-95 percentile)	192 (76-333)	210 (99-382)	0.04	202 (85-346)	180 (90-318)	0.05
Inhibin B /FSH						
Mean	80 (72)	101 (107)		91 (92)	100 (89)	
Median (5-95 percentile)	57 (11-203)	70 (17-289)	0.02	64 (13-259)	76 (17-272)	□0.0005
LH (IU/L)						

Mean (SD)	4.8 (2.4)	4.8 (1.8)		4.8 (2.1)	3.5 (1.5)	
Median (5-95 percentile)	4.5 (2.1-8.5)	4.6 (2.1-7.9)	1.0	4.5 (2.1-7.9)	3.3 (1.6-6.3)	□0.0005
Testosterone (nmol/L)						
Mean (SD)	21 (7)	23(7)		22 (7)	20 (6)	
Median (5-95 percentile)	20 (11-32)	22 (14-36)	0.006	21 (12-33)	19 (12-31)	□0.0005
Testosterone/LH						
Mean	5.0 (2.3)	5.9 (7.2)		5.5 (5.3)	6.5 (2.8)	
Median (5-95 percentile)	4.6 (1.9-9.1)	4.6 (2.7-11.6)	0.09	4.6 (2.2-9.4)	6.0 (3.0-12.0)	□0.0005
Free Testosterone (pmol/L)						
Mean	409 (134)	462 (140)		439 (134)	454 (142)	
Median (5-95 percentile)	397 (211-633)	442 (262-707)	□0.0005	424 (236-686)	432 (269-701)	0.1
Free testosteron/LH						
Mean	99 (44)	125 (175)		112 (128)	149 (71)	
Median (5-95 percentile)	92 (39-186)	96 (49-212)	0.03	94 (47-201)	134 (65-273)	□0.0005
Estradiol (nmol/L)						
Mean (SD)	79 (22)	96 (23)		88 (24)	80 (24)	
Median (5-95 percentile)	78 (46-113)	94 (62-140)	□0.0005	86 (54-131)	78 (48-125)	□0.0005
Testosterone/Estradiol						
Mean	268 (83)	243 (69)		255 (78)	258 (70)	
Median (5-95 percentile)	262 (140-409)	236 (146-375)	□0.0005	243 (145-394)	249 (175-383)	0.1
Free Testorsterone/Estradiol						
Mean	5332 (1567)	4955 (1187)		5143 (1400)	5869 (1768)	
Median (5-95 percentile)	5218 (3230-7768)	4876 (3165-7201)	0.002	5026 (3182-7538)	5636 (3519-8956)	□0.0005
SHBG (nmol/L)						
Mean (SD)	38 (15)	37 (15)		38 (15)	30 (12)	
Median (5-95 percentile)	36 (17-62)	35 (19-68)	0.7	36 (18-65)	29 (14-51)	□0.0005
Time of bloodsampling						
Mean (SD)	11:56 (2:11)	9:57 (1:29)		10:27 (1:56)	10:04 (0:42)	

Median (5-95 percentile)	10:15 (8:40-15:40)	9:37 (8:02-12.45)	□0.0005†	10:10 (8:30-15:00)	10:00 (9:05-11:20)	0.6†

*Regression analysis adjusting for hour of day of blood sampling
†Mann Whitney test

Total testosterone (T) only differed slightly between the two Faroese groups and between the combined group and the Danes, although statistically significant. SHBG in contrast was considerably higher for the Faroese men and with no difference between the F1 and F2 men. Frequency analyses of the SHBG values divided into groups (0-10, 10-20 etc.) showed that the higher SHBG values for Faroese men were not caused by higher concentration in a specific subgroup but an overall shift to higher levels. Approximately 80% of the Faroese men had a SHBG concentration below 50 nmol/L while 80 % of the Danish men had a concentration below 40 nmol/L. The FT differed between the two Faroese groups which combined, however, had non-significantly lower FT than the Danes. Estradiol was higher in the F2 group than in the F1, thus leading to higher levels in the combined group of Faroese men than in the Danish. The ratios T/LH, FT/LH, and FT/estradiol were all lower in the Faroese men than in the Danish whereas the estimated lower total T/estradiol was non-significant.

SHBG and LH were positively correlated with T (P <0.0005), whereas SHBG and LH did not correlate (P=0.1). FT was positively correlated with LH and T (P <0.0005), and negatively but not significantly correlated with SHBG (P=0.06)

BMI was negatively associated with T, FT, SHBG, T/LH, T/estradiol, FT/estradiol and inhibin B (p<0.0005-0.042), and also negatively but non-significant with LH (p=0.3). Faroese smokers tended to have higher T than non-smokers (p=0.7, adjusted for BMI effect), whereas the effect on the Danish men were highly significant (p<0.0005). In the Faroese groups LH, SHBG, estradiol, FT/LH, FT/estradiol and T/estradiol tended to be higher in smokers, although all non-significantly. For the Danes similar trends were detected; the effect on SHBG and LH being non-significant, whereas the remaining were significant with p-values <0.0005-0.05. Further maternal smoking during pregnancy for the Faroese men tended to be associated with lower LH and FT (p=0.04 and 0.02) whereas the effect on the remaining hormone levels were all highly non-significant (p=0.1-0.9). However, the difference (i.e. significant levels in table 3) between Faroese and Danish hormone levels did not change when including BMI or age as cofactors into the regression analyses indicating that these factors cannot explain the difference in reproductive hormones between Faroese and Danes.

DISCUSSION

This is the first study on testicular function conducted in the Faroe Islands. These young Faroese men had a lower sperm concentration but similar total sperm count compared to Danish men. Recent data have shown sperm counts to be low in young men from several European countries, however, slightly higher than among the Danes [5, 21, 22]. Thus, the semen quality of Danish men seems to be particularly low, and now we have shown the Faroese men to have a similar low level.

The low inhibin B/FSH ratio for the Faroese men corroborates the finding of low sperm counts, and provides independent evidence of poorer testicular function in the Faroese men than in the Danes, although the medians were at a level where the association between sperm counts and inhibin B is weakened [23]. When evaluating the results against the WHO reference population, Faroese men also had low sperm counts. The lower T/LH and FT/LH ratios point towards a lower Leydig cell capacity among Faroese men compared to Danes. Thus, the total testicular function among Faroese men may be at the same or lower level than Danes.

A greater proportion of men from the Faroese study populations had previously caused a pregnancy, which also would be expected since these men in average were almost 6 years older than the Danes. Furthermore, Faroese people are in general younger when parenting for the first time (25.5 years in 2011) [24], and the fertility rate in the Faroe Islands is the highest in European countries [25] ~~(~~with 2.3 children per woma~~e~~n in 2011 [24]. Traditionally, the family culture in the Faroe Islands is to have large families with many children; however, during the last 40 years the fertility rate in the Faroe Islands has decreased form 3.4 children per woma~~e~~n in 1970 to 2.8 children per woma~~e~~n in 1990 to the current fertility rate of 2.3 children per woma~~e~~n in 2011 [24]. Thus, the high birth rate most likely reflects socio-economic factors rather than male fecundity. Despite of this more Faroese men from the study populations had experienced fertility problems than the Danish men. This is in agreement with the lower sperm concentration found in the Faroese men, but it should be taken into account that the Faroese men were asked about fertility problems on the basis of 6 months unprotected intercourse without achieving a pregnancy and for the Danes it was based on 1 year. Therefore, the Danish men were more likely to have obtained a successful fertilization having tried for twice as long. Furthermore, fewer Danish men may be aware of a fecundity problem because they were younger and had not yet tried to conceive.

The Faroese and the Danish men were not exactly the same age. Semen quality has been reported to decline with increasing age [26-29]. However, the Faroese studies and the comparative Danish studies were conducted at the same time periods and the difference in age between groups is not likely to have influenced the semen parameters nor is the actual age of the participants that still can be regarded as young. Semen quality does on average not change between 19 and 23 years of age, and immaturity seems unlikely to explain the poor semen quality both among the Faroese and Danish men [30]. Thus, age difference is not likely to explain the difference in semen quality.

The Faroese men were investigated during a relatively short time period and also represented narrow birth cohorts. Despite of this, we actually detected an inverse association between percentage of morphologically normal spermatozoa and year of birth, a trend that was robust even when controlled for potential confounders. In a cohort like ours, the effect of birth year may be difficult to separate from the effect of age. However, when we modelled age as explanatory factor and controlled for birth year, the trend effect of age was not apparent. Thus, our results suggest that younger men produced ejaculates with lower percentages of normal spermatozoa. This could be caused by certain unhealthy life-style factors among the younger cohorts, but not the older cohorts. Our currently existing data does not support that explanation, however, but the information we have could also be insufficient to exclude this suggestion. Alternatively, the birth cohort effect could reflect an increasing exposure during fetal life leading to impaired testicular development which in adulthood would be reflected by reduced semen quality in line with the suggested Testicular Dysgenesis Syndrome hypothesis [31-32].The Faroese studies were investigated according to ~~almost~~ similar protocols and assessment of sperm concentration was controlled by the same quality control as the Danish study. The Faroese technicians were trained at RH in Denmark and the analyses used in both laboratories were identical, thus reducing the interobserver variation. Additionally, assessment of sperm morphologies was performed randomly by a single person. Frequency of motile sperm, however, is difficult to compare reliably between different groups. The motility assessment is highly subjective and controlling for quality control within this parameter is problematic. Previously, the inter-laboratory variance of motility has shown to be of significant importance [33]. Therefore, we hesitate to draw major conclusion regarding sperm motility.

The recording of abstinence time was obtained differently in the two Faroese sub-groups. In F1, the men were asked directly by the physician about the abstinence time while the F2 men themselves wrote down their abstinence time after they had some time to consider. This latter procedure is believed to be much more accurate, as the men were given time to reflect and were not expected to give an immediate verbal response. This assumption is supported by the fact that the anticipated relationship between abstinence time and sperm concentration only was clearly seen in

F2 group. Assuming the reported abstinence time actually was longer among the Faroese men these men still produced similar numbers of sperm as the Danish men, suggesting that the sperm production rate is lower in the Faroese population compared to Danes. This was, however, only confirmed for sperm concentration but not total sperm count when abstinence period was accounted for in the statistical model.

As explained in the methods section some of the semen samples were analysed with incorrect counting chambers. However, we corrected for this potential testing error. In addition, the statistical analyses performed with or without the 59 samples did not change the overall results, supporting the assumption that the corrections made were valid. A major advantage of the study was that the participants were not selected on fertility status. According to most of the Danish men, the main incentive for participation was the financial compensation [5]. The F2 men were part of an existing birth cohort study whereas F1 men were randomly recruited. Thus, we cannot exclude that men with suspicion of infertility may have been more interested in participating in the study. However, to control for this we defined a subgroup of men with abstinence period of more than 48 hours and without any prior knowledge of andrological diseases and without know fertility, and still the same low sperm counts were detected. This problem with counting chambers, and the differences in semen quality and population characteristics between the two Faroese sub-population exemplifies one of the main problems when comparing semen characteristics globally and over time, namely that standardised methods have to be applied and sufficiently large study populations investigated.

The finding of low semen quality and lower Leydig cell function in Faroese men cannot be explained by effects of confounders. Both high BMI and maternal smoking have previously been associated with reduced testicular function in some studies but not all [34-40].In our cohort, the effects of these two usually quite robust confounders/explanatory factors were not obvious, neither on the reproductive hormones. However, our cohorts may be too small to detect the effect of these two factors. Alternatively, other unidentified factors may have a major influence masking the effect of BMI and maternal smoking on the semen parameters. The reason for the low testicular function of the Faroese young men is unknown but this population is highly exposed to POPs derived solely from traditional marine food, which includes blubber from the pilot whale. Studies have shown associations between high polychlorinated biphenhyls (PCB) levels and low semen quality, and since PCBs and p,p'-DDE have the potential to interfere with sex hormone functions, it is plausible to assume that these compounds can affect the function of these organs [41]. There are some reports on the effect of POPs on male reproduction in humans, mainly indicating weak negative effects on sperm motility [41-43]. For the two

Faroese sub-groups, we found that the percentage of motile cells was significantly lower compared with Danish men, indicating that increased exposure to endocrine disruptors can be one explanation.

Serum SHBG levels for the Faroese men were strikingly higher than in the Danes. SHBG is a sensitive marker for thyroid function. However, none of the study subjects were under treatment for hyperthyroidism. We did not measure thyroid hormones, but the men did not have any obvious clinical symptoms of hyperthyroidism. If a high thyroid level in general should explain the SHBG level, a significantly elevated LH and testosterone in men with low BMI would have been expected, which was not the case. Therefore, there is no indication that the high SHBG values are due to hyperthyroidism. Furthermore, alcohol consumption could not explain the high levels. One plausible explanation to the high SHBG levels could be the high PCB exposure mentioned above. A recent publication from the Faroe Islands found that SHBG increased at higher PCB exposure, both prenatally and currently. PCBs are known to affect a variety of liver functions, and it could be speculated that PCB-induced hepatic SHBG synthesis could play a possible role, although this possibility remains to be substantiated [44]. We cannot elucidate further if PCB in our current study group can explain the SHBG levels.

It is known that the Faroese population differs genetically from other populations in many respects. There are several genetic diseases reported with very high frequency on the Faroese Islands [11, 45]. However different genetic composition in populations may contribute to the explanation of differences in semen quality and would be worth further exploration.

In conclusion, we found that the semen quality among Faroese men is at the same low level as reported among Danish men. This low quality was corroborated by the reproductive hormone levels in Faroese men. The influence of environmental exposures and genetic factors on the semen quality has to be studied further.

CONTRIBUTORS:

Substantial contribution to conception and design: PG, PW, NJ, TKJ.

Acquisition of data: JH, MSP, PW.

Interpretation of data: All authors.

Drafting the article: JH, MSP, NJ.

Revising the article critically for important intellectual content: all authors.

Final approval of the version to be published: all authors.

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ETHICS APPROVAL. The local Science Ethical Committee for the Faroe Islands and the Institutional Review Board at Harvard School of Public Health have approved the study protocol, and all participants had given their informed consent.

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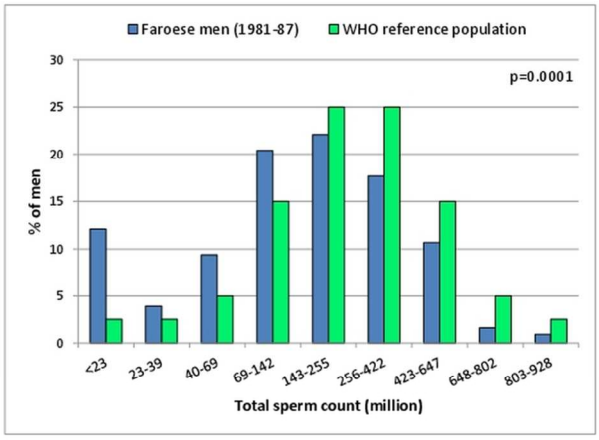

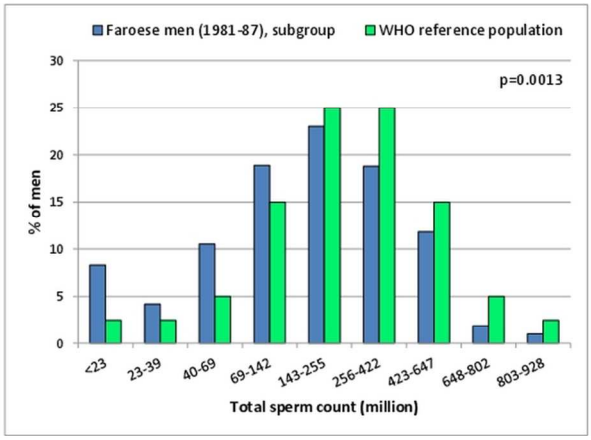

Figure 1. Distribution of total sperm count in Faroese men (blue bars) in categories defined from the reference levels according to centiles as described by WHO (green bars). In (A) all Faroese men are included. In (B) only men having an ejaculation abstinence period above 48 hours and being "without diseases" (see text for further explanation) are included.

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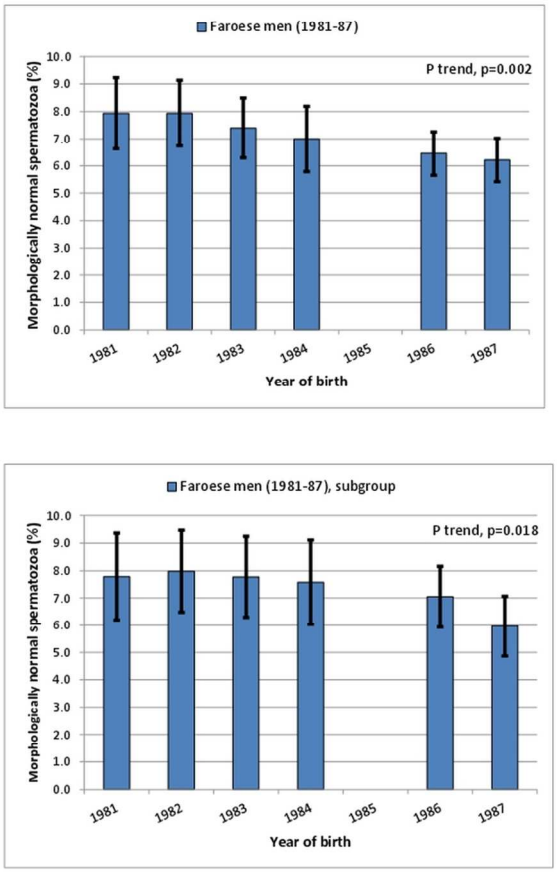

Figure 2. Percentage of morphologically normal spermatozoa according to year of birth. Whiskers show the 95% confidence interval of the estimated mean. In (A) all Faroese men are included. In (B) only men having an ejaculation abstinence period above 48 hours and being "without diseases" (see text for further explanation) are included.

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