Human semen quality and the secondary sex ratio

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The aim of this study was to evaluate the association between semen quality and the secondary sex ratio (SSR), defined as the ratio of male to female live births. Our study cohort comprised 227 male partners who were enrolled prior to conception in Michigan and Texas between 2005 and 2009, and prospectively followed through delivery of a singleton birth. The male partners provided a baseline and a follow-up semen sample a month apart. Semen analysis was conducted to assess 27 parameters including five general characteristics, six sperm head measures, 14 morphology measures, and two sperm chromatin stability assay measures. Modified Poisson regression models with a robust error variance were used to estimate the relative risk (RR) and 95% confidence interval (95% CI) of a male birth for each semen parameter, after adjusting for potential confounders. Of the 27 semen parameters, only the percentage of bicephalic sperm was significantly associated with the SSR (2nd vs 1st quartile, RR, 0.65, 95% CI, 0.45–0.95, P = 0.03; 4th vs 1st quartile, RR, 0.61, 95% CI, 0.38–1.00, P < 0.05 before rounding to two decimal places), suggestive of a higher percentage of bicephalic sperm being associated with an excess of female births. Given the exploratory design of the present study, this preconception cohort study suggests no clear signal that human semen quality is associated with offspring sex determination.

Keywords: fertility; prospective studies; reproduction; semen analysis; sex ratio; sperm

INTRODUCTION

Semen analysis is a key component in population-based studies of male reproductive health, providing information on the functional status of the male reproductive system including the testes and accessory sex glands. In clinical practice, semen analysis is one of the initial tests performed to evaluate male fecundity or the biologic capacity for reproduction, despite its inherent limitations in classifying men by fertility potential. With its fifth edition of the laboratory manual for the examination and processing of human semen, the World Health Organization (WHO) provided reference distributions of semen parameters derived from over 4500 fertile men in 14 countries with a retrospectively reported or prospectively measured time-to-pregnancy (TTP) of ≤12 months. In this edition of the WHO manual for semen analysis, the fifth centile values were newly proposed as the lower cutoff limits for normality, serving as the source of much controversy. The lower reference limits of sperm counts have been decreasing in recent decades, in parallel with longstanding debates on a global decline in human semen quality with equivocal findings and no emerging consensus. Along with other reported adverse trends in male reproductive health including increasing rates of testicular cancer and genitourinary malformations, the testicular dysgenesis syndrome has been proposed as a conceptual paradigm for assessing the effect of environmental and genetic influences on male fecundity.

The secondary sex ratio (SSR) is the ratio of male to female singleton live births, whereas the primary sex ratio is the ratio of male to female conceptions. Given that there is no available biomarker for conceptions at the population level, the SSR has been used as a potential population health and fertility indicator. Motivation for measurement and analysis of the SSR has arisen from multiple hypotheses across social biology, environmental, medical and behavioral science, demography, and epidemiology. Despite debates on its meaningfulness, the SSR has been used as a simple and noninvasive way to monitor population health and fertility, with its strengths being easy to measure, frequently recorded, and rarely subject to recall bias. The expected range of the SSR is from 1.05 to 1.07 in the United States and worldwide, indicative of a slight excess of male births. Recently, a decline in the SSR or the proportion of male births has been reported in the United States, Canada, Japan, and some Northern and Western European countries. The SSR has reportedly been varied by endogenous and exogenous factors, such as parental ages, birth order, race/ethnicity, length of the follicular phase, time of conception within the menstrual cycle, coital rate, endocrine and immunological effects, stress, smoking, anthropometric parameters, and other environmental factors. One of the prevailing hypotheses that may explain a possible decline in male reproductive health is exposure to endocrine-disrupting chemicals. Several studies on the association between endocrine-disrupting chemicals and the SSR have demonstrated possible roles of paternal rather than maternal exposure to select chemicals in offspring sex determination, underscoring the need for investigation into the effect of paternal factors including male fertility on the SSR.

Previous research focusing on the association between human semen quality and the SSR is sparse, especially for population-based cohorts. A study of 15 218 Danish men who sought infertility evaluation between 1963 and 1993 observed no significant association of the SSR with semen characteristics. This study has several potential limitations, including the requirement of a semen sample and a semen analysis as part of the infertility evaluation, and the association may be underestimated due to potential residual confounding. The present study evaluated the association between semen quality measured a month apart and the proportion of male births from populations in the United States.
SSR with sperm concentration (0–20 x 10⁶ ml⁻¹ vs ≥20 x 10⁶ ml⁻¹), sperm motility (poor vs good), and the percentage of morphologically abnormal spermatozoa (75%–100% vs 0%–75%). A study of 46 men undergoing assisted reproductive technologies (ARTs) showed that men with male offspring had slower sperm curvilinear (mean ± standard error of the mean [s.e.m.], 44.2 ± 1.8 µm s⁻¹ vs 49.9 ± 2.7 µm s⁻¹) and average path velocities (mean ± s.e.m., 32.4 ± 1.2 µm s⁻¹ vs 36.3 ± 1.7 µm s⁻¹) in seminal plasma than did men with female offspring. A follow-up expanded study of 187 men corroborated earlier findings on curvilinear and average path velocities in seminal plasma and offspring sex. Although not directly tested for semen quality, the effect of male fertility on the SSR has been evaluated in relation to TTP. Resulting in equivocal findings. The SSR has been also compared by fertility status or infertility etiology such as male or female factor infertility, without evidence supporting an association between infertility and the SSR. In other respects, given that the Y chromosome is the sex-determining chromosome in men, the sperm Y:X chromosome ratio has been assessed relative to semen quality to capture the paternal role in offspring sex determination. In an infertile cohort of 185 men undergoing a semen fluorescence in situ hybridization (FISH) test from 2003 to 2010 in the United States, poor semen quality, which was reflected by semen volume, sperm concentration, and total motile sperm count, significantly decreased the odds of having a Y chromosome-bearing sperm. With increasing speculation that semen quality may affect the SSR, the present study aimed to evaluate a spectrum of semen parameters quantified in men participating in a population-based prospective cohort in relation to the SSR.

MATERIALS AND METHODS

Study population

Our study cohort comprised male partners who participated in the Longitudinal Investigation of Fertility and the Environment (LIFE) Study, provided that they had a singleton birth during the follow-up period. The LIFE study recruited 501 couples discontinuing contraception and attempting pregnancy from 16 counties in Michigan and Texas between 2005 and 2009. Couples were followed until pregnant or through 12 months of trying to conceive and through delivery for those becoming pregnant. Of the 501 couples, 237 couples (47.3%) had a live birth during the follow-up period, including two with multiple births. Of the 235 male partners with a singleton birth, our analysis was restricted to 227 male partners (96.6%) who provided a baseline semen sample. The eligibility criteria for participation were as follows: (a) men in a committed relationship; (b) 18 years of age; (c) no sterilization procedures or physician-diagnosed infertility; and (d) men able to communicate in English or Spanish.

Data collection

Upon enrollment, a pregnancy test was administered to ensure the absence of a preexisting pregnancy. All study participants completed baseline interviews, which were conducted separately with each partner of the couple in the couples’ home. Research assistants obtained information on sociodemographic and lifestyle factors and medical and reproductive histories from all male partners, followed by the completion of standardized anthropometric assessments to ascertain height (in centimeters) and weight (in kilograms). Blood was collected for the quantification of serum cotinine using liquid chromatography-isotope dilution tandem mass spectrometry. Serum cotinine concentrations were reported in nanograms per milliliter and used to differentiate active smokers (≥40.35 ng ml⁻¹) from non- or passive smokers (<40.35 ng ml⁻¹). Couples who had a live birth during the follow-up period were asked to report information on date of birth, sex of the infant, birth size, and delivery mode after delivery. This study was performed in adherence with the guidelines of the Declaration of Helsinki and approved by the Institutional Review Boards at all collaborating institutions. All study participants provided written informed consent before any data collection.

Semen collection and analysis

Male partners were asked to provide a baseline and a follow-up semen sample approximately 1 month apart, irrespective of pregnancy status. Of the 227 male partners with a first semen sample, 200 male partners (88.1%) provided a second semen sample. Semen samples were collected at home via masturbation without the use of any lubricant after 2 days of suggested abstinence. A thermometer was attached to the glass collection jar for monitoring temperature in light of our reliance on next day analysis. In addition, men were instructed to cut one end of a specially prepared glass straw filled with hyaluronic acid (VitroTubes #3520; VitroCom, Mountain Lakes, NJ, USA) and place it into the semen to assess a global marker of motility. This step was taken in light of at home collection, a method for population-based research. The duration of abstinence, time and date of collection, and other information regarding collection including any loss or spillage were recorded on labels. Semen samples were returned using insulated shipping containers (Hamilton Thorne Biosciences, Beverly, MA, USA) and freezer packs to foster the preservation of sperm integrity. Semen samples were shipped overnight to the study’s andrology laboratory for semen analysis.

Next day semen analysis was conducted using established laboratory protocols inclusive of an ongoing quality assurance and quality control procedures. The distance traveled by the vanguard sperm (millimeters) in the sperm migration straw was measured with a microscope upon removal from the jar. The IVOS system (Hamilton Thorne Biosciences, Beverly, MA, USA) with the IDENT stain was used to determine sperm concentration. Semen smears were prepared for the evaluation of sperm morphology and morphology. Sperm morphometric analysis was conducted using the IVOS METRIX system, and sperm morphology was assessed using both the traditional criteria with differential classification and the strict criteria. Sperm viability was assessed by the hypo-osmotic swelling (HOS) test. For the sperm chromatin stability assay (SCSA) analysis, an aliquot of semen was diluted in sodium chloride-Tris-EDTA buffer with glycerol and kept frozen at -70°C until analysis. The analysis was conducted by SCSA Diagnostics (Brookings, SD, USA) using a Coulter Epics Elite Flow Cytometer. We assessed a total of 27 parameters, including five general characteristics, six sperm head measures, 14 morphology measures, and two SCSA measures. Although eight computer-aided sperm analysis (CASA) motility parameters (i.e., average path velocity, straight line velocity, curvilinear velocity, amplitude head displacement, beat cross frequency, straightness, linearity, and percent motility) were assessed using the HTM-IVOS CASA system, these parameters were not included in the current analysis, given that 24-h semen quality analysis is not ideal for time-sensitive endpoints. Analysis of the second sample was restricted to semen volume, sperm concentration, total sperm count, hypo-osmotic swelling, and sperm head measures to affirm any azoospermia in the first sample.

Statistical analysis

In the descriptive phase of analysis, distributions were summarized as means ± standard deviations for continuous variables and categorically for other variables. Differences in baseline characteristics of male partners by infant sex were assessed using the nonparametric Wilcoxon test for continuous variables and Fisher’s exact test for categorical
variables. We calculated means, standard deviations, and the 5th and 95th percentiles for semen parameters by infant sex and assessed any significant differences in semen parameters using the nonparametric Wilcoxon test. Differences in semen parameters between the first and second semen samples were also evaluated using the nonparametric Wilcoxon test. Proportion tests were used to assess differences in the SSRs by semen parameters (≤ median vs > median; the median values were calculated using the first semen samples).

In the analytic phase, modified Poisson regression models with a robust error variance were used to estimate the relative risk (RR) and 95% confidence interval (95% CI) of a male birth for each semen parameter.6 Fixed and mixed effects models were used for the analysis of semen parameters with one and two measurements, respectively. We modeled each semen parameter both as a continuous variable and as a categorical variable. Specifically, continuous values were rescaled by dividing the original values by 10 (sperm concentration and total sperm count) or 10 (the remaining 25 endpoints) to aid in the interpretation of results in terms of clinical significance, allowing for the estimation of risk per 10 unit increase in semen parameters. Additionally, each semen parameter was categorized into quartiles for analysis. Separate models were run for each semen parameter, consistent with our aim to fully explore the multiple facets of semen quality relative to infant sex. We adjusted a priori for factors thought to be related to semen quality and the SSR, consistent with the definition of confounding, based upon our review of the literature: paternal age (years), body mass index (BMI, kg m²), and smoking (non- or passive smoker/active smoker). Significance was initially set at \( P < 0.05 \), given the exploratory design of this study. In addition, considering the number of semen parameters examined \((n = 27)\), we subsequently assessed the significance at the 0.002 level, the association observed for the percentage of bicephalic sperm being associated with an excess of female births. None of the characteristics differed significantly by infant sex.

RESULTS

Of the 227 singleton live births, 110 (48.5%) were males and 117 (51.5%) were females. The overall SSR was 0.94, indicative of a female excess. The mean (±s.d.) age of the male partners was 31.3 ± 4.5 years. More than half of the male partners had previously fathered a pregnancy upon enrollment. Non-Hispanic white and college-educated male partners comprised the majority of the study participants. Active smokers comprised 11.7% of the study participants. The mean (±s.d.) BMI of the male partners was 29.3 ± 5.4 kg m². None of the baseline characteristics differed significantly by infant sex (Table 1).

The distributions of semen parameters for the male partners by infant sex are presented in Table 2. Of note, there were no statistically significant differences in semen parameters between the first and the second semen samples. In general, the distributions of gross semen parameters such as volume, sperm concentration, total sperm count, and % normal sperm morphology (the strict criteria) were comparable to normative values reported by WHO.6 The sperm migration distance measured using the first semen samples was significantly different between fathers of male infants (9.8 ± 6.8 mm) and fathers of female infants (11.6 ± 6.7 mm; \( P < 0.05 \) before rounding to two decimal places). For the second semen samples, a lower percentage of hypo-osmotic swelling (i.e., viability) was observed for fathers of female infants (65.3% ± 9.6%) versus male infants (68.2% ± 10.1%; \( P = 0.02 \)). No other semen parameters differed significantly by infant sex (Table 2). The differences in the SSRs by semen parameters are presented in Table 3. Statistically significant differences in the SSRs were observed for select dichotomous semen parameters, such as straw distance (≤ median, 0.57; > median, 0.37; \( P = 0.01 \)), sperm head length (≤ median, 0.46; > median, 0.56; \( P < 0.05 \) before rounding to two decimal places), and sperm head perimeter (≤ median, 0.44; > median, 0.55; \( P = 0.03 \)) (Table 3).

Table 1: Baseline characteristics of male partners with a singleton birth by infant sex (2005–2009)

| Characteristic | Male (n=110), n (%) | Female (n=117), n (%) |
|---------------|-------------------|----------------------|
| Previously fathered a pregnancy | No | 44 (43.6)| 48 (42.9)|
| | Yes | 57 (56.4)| 64 (57.1)|
| Research site | Michigan | 21 (19.1)| 26 (22.2)|
| | Texas | 89 (80.9)| 91 (77.8)|
| Annual income ($) | <70 000 | 23 (21.3)| 36 (31.0)|
| | ≥70 000 | 85 (78.7)| 80 (69.0)|
| Education | ≤High school graduate/GED | 2 (1.8)| 4 (3.4)|
| | Some college/technical school | 31 (28.4)| 23 (19.8)|
| | College graduate or higher | 76 (69.7)| 89 (76.7)|
| Race/ethnicity | Non-Hispanic white | 89 (81.7)| 103 (88.0)|
| | Non-Hispanic black | 3 (2.8)| 2 (1.7)|
| | Hispanic | 11 (10.1)| 8 (6.8)|
| | Other | 6 (5.5)| 4 (3.4)|
| Serum cotinine (ng ml⁻¹) | <40.35 (non- or passive smoker) | 96 (89.7)| 100 (87.0)|
| | ≥40.35 (active smoker) | 11 (10.3)| 15 (13.0)|
| Age (year), mean (±s.d.) | 32.0 (±5.0)| 30.7 (±3.9)|
| Body mass index (kg m⁻²), mean (±s.d.) | 29.7 (±5.7)| 28.9 (±5.1)|
| First semen sample age (h), mean (±s.d.) | 27.6 (±8.5)| 27.1 (±3.8)|
| Second semen sample age (h), mean (±s.d.) | 28.2 (±10.2)| 28.4 (±8.8)|
| First semen sample abstinence time (day), mean (±s.d.) | 4.2 (±6.5)| 4.0 (±3.2)|
| Second semen sample abstinence time (day), mean (±s.d.) | 4.8 (±9.1)| 3.9 (±2.8)|

Note – Of the 227 male partners with a first semen sample, 200 (88.1%) provided a second semen sample. None of the characteristics differed significantly by infant sex. GED: general educational development; s.d.: standard deviation.

DISCUSSION

In our comprehensive analysis of preconception semen quality parameters and the SSR, we found no clear signal that human semen...
quality is associated with the SSR. In fact, our exploratory analysis identified only one semen parameter being associated with alterations in the SSR, when analyzing semen parameters categorically and controlling for potential confounders. Specifically, a higher percentage

### Table 2: Distributions of semen parameters by infant sex, 2005–2009 (n=227)

| Semen parameters                                  | Mean (s.d.) | Centiles (5th, 95th) | Mean (s.d.) | Centiles (5th, 95th) |
|---------------------------------------------------|-------------|----------------------|-------------|----------------------|
|                                                    | Male        |                      | Female      |                      |
| General semen characteristics                      |             |                      |             |                      |
| Volume (ml)                                        | 3.4 (±1.7)  | 1.1, 6.8             | 3.6 (±1.7)  | 1.0, 6.8             |
| 1<sup>st</sup> sample                             | 3.5 (±1.6)  | 1.3, 6.5             | 3.4 (±1.5)  | 1.1, 5.7             |
| 2<sup>nd</sup> sample                             |             |                      |             |                      |
| Sperm concentration (×10<sup>6</sup> ml<sup>−1</sup>) |             |                      |             |                      |
| 1<sup>st</sup> sample                             | 77.7 (±55.6) | 17.6, 187.8          | 75.8 (±51.7) | 15.0, 190.7          |
| 2<sup>nd</sup> sample                             | 85.2 (±56.8) | 19.7, 210.5          | 73.8 (±56.0) | 15.3, 186.5          |
| Total sperm count (×10<sup>9</sup>/ejaculate)      |             |                      |             |                      |
| 1<sup>st</sup> sample                             | 249.8 (±203.8) | 44.3, 714.4         | 244.4 (±159.9) | 51.4, 558.4          |
| 2<sup>nd</sup> sample                             | 278.3 (±216.0) | 63.1, 737.7         | 240.2 (±186.3) | 36.3, 680.9          |
| Hypo-osmotic swelling (%)                         |             |                      |             |                      |
| 1<sup>st</sup> sample                             | 68.6 (±9.8)  | 51.1, 82.0           | 68.7 (±8.2)  | 55.0, 80.9           |
| 2<sup>nd</sup> sample                             | 68.2 (±10.1)<sup>a</sup> | 51.9, 81.9        | 65.3 (±9.6)<sup>a</sup> | 48.8, 79.8           |
| Straw (mm distance sperm traveled)                |             |                      |             |                      |
| 1<sup>st</sup> sample                             | 9.8 (±6.8)<sup>b</sup> | 3.0, 24.9         | 11.6 (±6.7)<sup>de</sup> | 3.4, 26.1           |
| Sperm head measurement                            |             |                      |             |                      |
| Length (µm)                                       | 4.9 (±0.3)  | 4.5, 5.3             | 4.9 (±0.3)  | 4.5, 5.3             |
| 1<sup>st</sup> sample                             | 4.9 (±0.3)  | 4.4, 5.4             | 4.9 (±0.3)  | 4.5, 5.4             |
| 2<sup>nd</sup> sample                             |             |                      |             |                      |
| Area (µm)                                         | 12.3 (±0.9) | 10.6, 13.8           | 12.2 (±0.8) | 10.6, 13.7           |
| 1<sup>st</sup> sample                             | 12.3 (±0.8) | 11.1, 13.8           | 12.2 (±0.9) | 11.0, 14.0           |
| 2<sup>nd</sup> sample                             |             |                      |             |                      |
| Width (µm)                                        | 3.2 (±0.2)  | 2.9, 3.5             | 3.2 (±0.2)  | 2.9, 3.4             |
| 1<sup>st</sup> sample                             | 3.2 (±0.2)  | 3.0, 3.4             | 3.2 (±0.2)  | 2.9, 3.5             |
| 2<sup>nd</sup> sample                             |             |                      |             |                      |
| Perimeter (µm)                                    | 13.3 (±0.5) | 12.3, 14.0           | 13.2 (±0.5) | 12.4, 13.9           |
| 1<sup>st</sup> sample                             | 13.3 (±0.5) | 12.6, 14.2           | 13.2 (±0.5) | 12.5, 14.2           |
| 2<sup>nd</sup> sample                             |             |                      |             |                      |
| Elongation factor (%)                             | 66.0 (±5.1) | 58.0, 74.4           | 65.9 (±5.3) | 56.9, 74.4           |
| 1<sup>st</sup> sample                             | 65.8 (±5.1) | 58.1, 75.1           | 66.3 (±5.4) | 57.9, 74.8           |
| 2<sup>nd</sup> sample                             |             |                      |             |                      |
| Acrosome area of head (%)                         | 26.1 (±5.7) | 17.0, 36.0           | 25.7 (±4.5) | 17.7, 33.4           |
| 1<sup>st</sup> sample                             | 26.6 (±4.8) | 19.2, 34.2           | 25.8 (±3.8) | 19.7, 31.7           |
| Morphology (1<sup>st</sup> sample) (%)            |             |                      |             |                      |
| Strict criteria                                   | 21.2 (±9.3) | 6.0, 37.0            | 21.7 (±10.1) | 7.0, 39.5            |
| WHO normal                                        | 32.0 (±11.2) | 14.0, 51.5          | 31.7 (±12.6) | 12.0, 53.5          |
| Amorphous                                         | 30.0 (±10.2) | 14.5, 46.5          | 29.4 (±10.9) | 13.0, 49.5          |
| Round                                             | 1.0 (±1.1)  | 0.0, 3.0             | 1.2 (±1.5)  | 0.0, 3.5             |
| Pyriform                                          | 6.3 (±6.0)  | 0.5, 18.0            | 5.9 (±5.5)  | 0.5, 19.5            |
| Bicephalic                                        | 0.9 (±1.3)  | 0.0, 3.0             | 1.3 (±2.0)  | 0.0, 5.5             |
| Tapered                                           | 2.5 (±2.3)  | 0.0, 6.0             | 3.0 (±3.0)  | 0.0, 9.5             |
| Megalo head                                       | 2.4 (±2.0)  | 0.5, 6.0             | 2.3 (±1.8)  | 0.0, 6.0             |
| Micro head                                        | 1.4 (±1.2)  | 0.0, 3.0             | 1.5 (±1.5)  | 0.0, 4.0             |
| Neck and midpiece abnormalities                   | 25.4 (±8.8) | 14.0, 40.5           | 25.7 (±10.1) | 12.0, 44.0           |
| Coiled tail                                       | 23.0 (±11.0) | 9.5, 46.0           | 23.5 (±10.6) | 10.0, 44.0           |
| Other tail abnormalities                          | 5.0 (±3.0)  | 1.0, 10.5            | 5.5 (±6.1)  | 1.5, 12.0            |
| Cytoplasmic droplet                               | 10.1 (±5.5) | 3.0, 18.5            | 9.9 (±5.3)  | 3.0, 20.0            |
| Immature germ cell count                          | 4.3 (±4.7)  | 0.0, 13.0            | 4.8 (±5.4)  | 0.0, 17.0            |
| SCSA (1<sup>st</sup> sample) (%)                  |             |                      |             |                      |
| DNA fragmentation index                           | 15.2 (±13.1) | 4.5, 43.5           | 13.3 (±6.9) | 4.6, 27.3            |
| High DNA stainability                             | 6.6 (±4.6)  | 1.9, 17.2            | 7.1 (±5.0)  | 2.2, 19.9            |

<sup>a</sup> P<0.05. Note – Of the 227 male partners with a first semen sample, 200 (88.1%) provided a second semen sample. Sperm morphology was assessed using both the traditional criteria with differential classification and the strict criteria. s.d.: standard deviation; WHO: World Health Organization; SCSA: sperm chromatin stability assay.
Table 3: Differences in the SSRs by semen parameters, 2005–2009 (n=227)

| General semen characteristics | Median ≤ median | Median > median |
|-------------------------------|---------------|---------------|
| Volume (ml)                   | 3.3           | 0.51          | 0.48          |
| Sperm concentration (×10^6 ml⁻¹) | 65.5         | 0.48          | 0.51          |
| Total sperm count (×10⁶ ejaculate) | 200.8       | 0.49          | 0.50          |
| Hypo-osmotic swelling (%)     | 69.0          | 0.45          | 0.54          |
| Straw (mm distance sperm traveled) | 8.9          | 0.57⁺         | 0.37⁺         |

| Sperm head measurement | Length (µm) | Area (µm) | Width (µm) | Perimeter (µm) | Elongation factor (%) | Acrosome area of head (%) |
|------------------------|------------|-----------|------------|---------------|-----------------------|---------------------------|
|                        | 4.9        | 12.3      | 3.2        | 13.2          | 65.8                  | 25.7                      |
|                        | 0.46⁺      | 0.47      | 0.49       | 0.44⁺         | 0.51                  | 0.48                      |
|                        | 0.56⁺      | 0.52      | 0.50       | 0.56⁺         | 0.51                  | 0.51                      |

| Morphology (%) | Strict criteria | WHO normal | Amorphous | Round | Pyriform | Bicephalic | Tapered | Megalo head | Micro head | Neck and midpiece abnormalities | Coiled tail | Other tail abnormalities | Cytoplasmic droplet | Immature germ cell count | SCSA (%) |
|----------------|----------------|------------|-----------|-------|----------|------------|---------|-------------|-----------|-------------------------------|-------------|--------------------------|---------------------|----------------------|----------|
|                | 21.0           | 31.5       | 28.5      | 0.5   | 4.0      | 0.5        | 2.0     | 2.0         | 1.5       | 24.0                           | 21.0        | 4.5                       | 9.0                  | 3.0                  | 11.8     |
|                | 0.52           | 0.49       | 0.46      | 0.52  | 0.45     | 0.50       | 0.50    | 0.48        | 0.49      | 0.50                           | 0.53        | 0.46                      | 0.47                 | 0.48                 | 5.3      |
|                | 0.44           | 0.47       | 0.50      | 0.43  | 0.51     | 0.46       | 0.46    | 0.47        | 0.46      | 0.46                           | 0.43        | 0.51                      | 0.47                 | 0.47                 | 0.49     |
|                | 0.44⁺          | 0.47       | 0.50      | 0.43  | 0.51     | 0.46       | 0.46    | 0.47        | 0.46      | 0.46                           | 0.53        | 0.51                      | 0.47                 | 0.47                 | 0.49     |

| SCSA (%) | DNA fragmentation index | High DNA stainability |
|----------|-------------------------|-----------------------|
|          | 11.8                    | 5.3                   |
|          | 0.48                    | 0.49                  |
|          | 0.48                    | 0.53                  |

Table 4: Semen parameters and the relative risks of a male birth, 2005–2009 (n=227)

| General semen characteristics | Volume | Sperm concentration (×10^6 ml⁻¹) | Total sperm count (×10⁶ ejaculate) | Hypo-osmotic swelling (%) | Straw (mm distance sperm traveled) |
|-------------------------------|--------|-------------------------------|----------------------------------|--------------------------|----------------------------------|
| Unadjusted RR (95% CI)        |        | Unadjusted RR (95% CI)        | Unadjusted RR (95% CI)        | Unadjusted RR (95% CI)        | Unadjusted RR (95% CI)        |
|                               | 1.00   | 1.00                          | 1.00                             | 1.00                      | 1.00                             |
| Adjusted RR (95% CI)          |        | Unadjusted RR (95% CI)        | Unadjusted RR (95% CI)        | Unadjusted RR (95% CI)        | Unadjusted RR (95% CI)        |
|                               | 1.00   | 1.00                          | 1.00                             | 1.00                      | 1.00                             |

Note: Of the 227 male partners with a first semen sample, 200 (88.1%) provided a second semen sample. Sperm morphology was assessed using both the traditional criteria with differential classification and the strict criteria. The first semen samples were used to calculate the median value of each semen parameter.WHO: World Health Organization; SCSA: sperm chromatin stability assay; SSR: secondary sex ratio.

Men with male offspring had slower sperm in seminal plasma, in terms of curvilinear and average path velocities, in comparison with men with female offspring. These findings on curvilinear and average path velocities in seminal plasma and offspring sex were confirmed in an expanded study of 187 men. In contrast, although not directly tested for the SSR, in a study of 500 men attending an andrology laboratory in the United Kingdom evaluating sibling sex composition relative to CASA sperm motility measures, men with female-biased siblings had significantly slower sperm than men from male-biased siblings. Meanwhile, a Danish study of 15 218 men seeking infertility evaluation reported no evidence for an association between sperm motility and the SSR. With regard to sperm morphology, this Danish study also found no evidence for an association between % morphologically abnormal spermatozoa (the...
Table 5: Categorized semen parameters and the adjusted relative risks of a male birth, 2005–2009 (n=227)

| Semen parameters | 1st quartile | 2nd quartile | 3rd quartile | 4th quartile |
|------------------|-------------|-------------|-------------|-------------|
| General semen characteristics |             |             |             |             |
| Volume | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Sperm concentration | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Total sperm count | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Hypo-osmotic swelling | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Straw distance sperm traveled | 1.00 (referent) | 0.95 (0.66–1.36) | 0.73 (0.47–1.13) | 0.64 (0.40–1.02) |
| Sperm head measurement |             |             |             |             |
| Length | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Area | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Width | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Perimeter | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Elongation factor | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.01) |
| Acrosome area of head | 1.00 (referent) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) | 1.00 (1.00–1.00) |
| Morphology |             |             |             |             |
| Strict criteria | 1.00 (referent) | 1.35 (0.93–1.96) | 1.01 (0.65–1.55) | 1.05 (0.69–1.60) |
| WHO normal | 1.00 (referent) | 1.21 (0.83–1.75) | 0.94 (0.60–1.45) | 1.17 (0.80–1.70) |
| Amorphous | 1.00 (referent) | 1.30 (0.87–1.94) | 1.06 (0.69–1.62) | 1.36 (0.91–2.03) |
| Round | 1.00 (referent) | 1.00 (0.70–1.42) | 0.75 (0.51–1.11) | 0.89 (0.59–1.35) |
| Pyriform | 1.00 (referent) | 0.88 (0.57–1.34) | 1.13 (0.79–1.62) | 0.97 (0.66–1.45) |
| Bicephalic | 1.00 (referent) | 0.65 (0.45–0.95) | 0.86 (0.61–1.21) | 0.61 (0.38–1.00) |
| Tapered | 1.00 (referent) | 0.94 (0.65–1.37) | 0.86 (0.57–1.30) | 0.98 (0.69–1.41) |
| Megalo head | 1.00 (referent) | 1.34 (0.94–1.92) | 1.14 (0.74–1.75) | 1.05 (0.68–1.63) |
| Micro head | 1.00 (referent) | 1.22 (0.87–1.71) | 1.26 (0.77–2.05) | 0.86 (0.53–1.39) |
| Neck and midpiece abnormalities | 1.00 (referent) | 1.07 (0.74–1.56) | 0.88 (0.59–1.31) | 0.99 (0.68–1.44) |
| Coiled tail | 1.00 (referent) | 1.02 (0.71–1.47) | 0.69 (0.45–1.05) | 0.90 (0.63–1.30) |
| Other tail abnormalities | 1.00 (referent) | 0.78 (0.52–1.16) | 1.06 (0.73–1.54) | 0.91 (0.64–1.29) |
| Cytoplasmic droplet | 1.00 (referent) | 1.07 (0.73–1.56) | 1.04 (0.69–1.57) | 1.14 (0.77–1.68) |
| Immature germ cell count | 1.00 (referent) | 1.00 (0.69–1.45) | 1.19 (0.83–1.69) | 0.88 (0.57–1.34) |
| SCmA |             |             |             |             |
| DNA fragmentation index | 1.00 (referent) | 0.94 (0.64–1.37) | 1.00 (0.69–1.44) | 0.80 (0.53–1.21) |
| High DNA stallability | 1.00 (referent) | 1.02 (0.70–1.49) | 1.11 (0.78–1.58) | 0.77 (0.51–1.17) |

As derived from the most extensive evaluation of semen quality parameters in relation to the SSR to date, comparison of our findings with prior research focusing on the association between semen quality and the SSR requires caution, in that much of the past research has relied on men seeking infertility evaluation or treatment, and whose semen quality may differ from fertile men. A Danish study assessed only three semen parameters (sperm concentration, motility, and % morphologically abnormal spermatozoa) that were dichotomized for analysis with the SSR, and observed no associations. Also of note is that the results were generated from single semen analysis that had been conducted before or after having one or more children, preventing the authors from being able to speak to semen quality around time of conception. In studies evaluating sperm motility relative to SSR, sperm in seminal plasma rather than ejaculated sperm were assessed in men undergoing ART. Prior research on the association between semen quality and sperm Y: X chromosome ratio may, in part, explain the effect of semen quality on the SSR, given that the SSR is affected by not only sperm Y: X chromosome ratio but also other factors, such as sperm selection within the female reproductive tract and differential implantation and survival rates of embryos. In spite of research efforts to date, the effect of male fertility including semen quality on the SSR remains elusive. There has been conflicting evidence for an association between TTP and the SSR from large European and Australian datasets. In a retrospective cohort of 30 448 women who sought infertility evaluation or treatment in California between 1990 and 1998, no significant difference was found for the SSR in comparison with matched fertile women derived from vital statistics records, irrespective of infertility etiology (male or female factor infertility). In a study of 15 164 singleton births from the Society for Assisted Reproductive Technology Outcomes Reporting System (SART CORS) database for 2005 in the United States, a diagnosis of male factor infertility was not associated with an altered SSR, although the reliability of the male factor infertility diagnosis in SART CORS is uncertain. Some previous studies have suggested that siring male offspring may be linked to male reproductive potential. A study of 185 American men undergoing a semen FISH test showed that there was a positive relationship between the production of Y chromosome-bearing sperm and sperm production, as an indicator of male fertility. A study of 14 red deer stags, a positive relationship between the percentage of morphologically normal spermatozoa and the proportion of male offspring was observed. However, our study did not clearly...
demonstrate such a link between semen parameters and male reproductive potential.

One might also postulate that sperm motility or morphology may serve as a possible predictor of infant sex, in accordance with an existing theory focusing on the difference in sperm swimming velocity or size between Y chromosome-bearing sperm and X chromosome-bearing sperm as a potential influence of human sex selection. Specifically, the “over-ripeness ovopathy” concept hypothesizes that Y chromosome-bearing sperm are more suitable for navigating nonoptimally liquefied cervical mucus accompanied by nonoptimally matured oocytes than are X chromosome-bearing sperm, as Y chromosome-bearing sperm are smaller than X chromosome-bearing sperm in terms of the length, perimeter, and area of sperm head, and the length of sperm neck and tail. As a consequence, the preferential fertilization of nonoptimally matured oocytes by Y chromosome-bearing sperm may contribute to disproportional loss of male embryos and fetuses, resulting in alterations in the SSR. Intrinsic differences in sperm motility, viability, and fertility potential between Y- and X-chromosome bearing sperm have been suggested to alter the SSR on the paternal side, whereas the condition of the reproductive tract, including sex-specific transcriptomic responses of the oviduct, and the penetrability of the oocyte’s zona pellucida have been suggested as sex-biasing mechanisms controlled by the mother. Still, biological mechanisms underlying the impact of a specific sperm morphology parameter on infant sex observed in our study remain unclear.

Our study is strengthened by several unique features, including the most extensive evaluation of semen parameters in relation to the SSR as well as the preconception measurement of semen quality. We also used data from both the first and second semen samples for analysis, while addressing biologic variability of semen parameters and correlations between the two semen samples. However, caution should be exercised when interpreting our results. In our study, 24-h semen quality analysis was used, which is not a conventional clinical or diagnostic evaluation tool. As such, our findings are not directly comparable to those derived from the clinical gold standard. Due to our inability to measure conceptions, we could not evaluate the primary sex ratio and possible disproportional male losses following conception to birth. Given the uncertainty as to factors affecting the SSR, we cannot eliminate residual confounding or model misclassification in the interpretation of our results. In light of our sampling on couples planning pregnancies, our findings may not be generalizable to the general population or among couples with unplanned pregnancy. Finally, given the likely small changes in the SSR that have been reported in the literature, it is conceivable that the present study is underpowered to detect all associations between semen quality and the SSR. For this reason, we refrain from in-depth discussion about the observed nonsignificant associations.

CONCLUSION
In a population-based preconception cohort, we found no clear signal that semen quality was associated with sex selection as measured by the SSR. With the need to identify novel surrogate markers of male reproductive health by applying new technologies, emphasis should be placed on determining possible interrelatedness of potential biomarkers of male fertility and offspring sex determination, such as hormonal profiles, semen quality, sperm Y:X chromosome ratio, and genetic and epigenetic sperm abnormalities. More comprehensive investigation incorporating these factors may help elucidate the underlying biological mechanisms of male fertility and human sex selection.

AUTHOR CONTRIBUTIONS
GMBL supervised cohort recruitment and data collection. All authors designed the study. SK and ZC analyzed the data. All authors interpreted the data. JB and SK drafted the manuscript. ZC, MLE and GMBL provided critical revisions to the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

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