

Y:X Sperm Ratio in Boron-Exposed Men

WENDIE A. ROBBINS,* FUSHENG WEI,† DAVID A. ELASHOFF,‡ GUOPING WU,† LIN XUN,§ AND JUAN JIA‡

*From the *Center for Occupational and Environmental Health, University of California, Los Angeles, California; the †China National Environmental Monitoring Station, Yuhui Nanlu Chaoyang District, Beijing, China; the ‡Department of Biostatistics, School of Public Health; and the §School of Nursing, University of California, Los Angeles, California.*

ABSTRACT: Several epidemiologic investigations have shown shifts in sex ratios at birth toward females in populations with relatively high boron exposure. To investigate the paternal origin of these shifts, we assayed sperm Y:X ratio in men exposed to a range of environmental and workplace boron. Participants included 63 workers in boron industry: 39 men living in an area of high environmental boron but not employed in boron industry, and 44 controls living in an area of low environmental boron. Total daily boron exposure was calculated as the sum of boron in 24-hour duplicate food and fluid intakes plus personal air sampling for workplace inhalable dust. Internal dose was measured in blood, urine, and semen. Sperm were analyzed by fluorescence in situ hybridization for Y- versus X-bearing cells. Potential confounders were identified using a questionnaire. Total exposure was correlated

with internal dose (Pearson correlation for total exposure and boron in blood = 0.63, $P < .0001$; semen = 0.80, $P < .001$; and urine = 0.79, $P < .0001$). Linear regression of logged boron in biologic fluids on Y:X ratio was significant for blood $P = .02$, semen $P = .0003$, and urine $P = .005$. Additionally, when subjects were categorized by exposure group, decreased Y:X sperm ratio was found for boron workers compared with men in a high boron environment and controls ($P < .0001$). Exogenous environmental or workplace boron exposures were associated with decreases in Y- versus X-bearing sperm. This may explain earlier findings from us and others showing changes in offspring sex ratios at birth for men exposed to boron.

Key words: Sperm, spermatogenesis, sex ratio at birth.

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Boron (B^{10} , B^{11}) is distributed throughout the earth as borates in soils, rocks, and waters. Elemental boron is found at, on average, ~10 ppm in the earth's crust; however, some geographic regions are deficient, and a few have concentrations high enough to be of economic interest (Woods, 1994; Howe, 1998). Borates have many industrial and medical applications, for example, in insulation materials, fiberglass, fire retardants, textiles, borosilicate glass, adhesives, detergents/soaps/bleaches, cosmetics, pesticides, soldering and welding fluxes, and neutron capture cancer therapy. Boron is an essential plant element and is found in fertilizers (Richold, 1998). It is likely an essential human nutrient as well (Park et al, 2004), and can be found in some vitamin supplements. Demand for this important element is increasing, and,

subsequently, more workers may be exposed during mining and processing of the ore, and more communities may be exposed to boron-containing industrial wastes. Evidence for safe versus adverse workplace or environmental exposures for humans is limited, particularly for reproductive outcomes. As a result, boric acid was identified as one of 4 top-priority chemicals in need of field study in humans, according to a consensus workshop convened to review and prioritize National Toxicology Program reproductive toxicants (Moorman et al, 2000).

To investigate the effect of a range of boron exposures on reproductive health, we conducted a study in a geographic region in northeast China with extreme variation in environmental boron. This region contains areas rich in borate magnesite ore that support lucrative boron mining and processing plants, as well as geographic areas with very little boron in ground water or soil. Because animal toxicology studies have shown male reproductive system sensitivity at lower doses relative to female or developmental effects (Fail et al, 1991; Ku et al, 1993; Chapin and Ku, 1994; Price et al, 1998), the objective of our study was to evaluate male reproductive outcomes in relationship to total boron exposure that occurred through work, food, and water/liquid consumption. A group of boron workers ($n = 936$) and a comparison group from an area of low environmental

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Correspondence to: Dr Wendie A. Robbins, Room 5-254 Factor Building, Mailcode 956919, UCLA, Los Angeles, CA 90095-6919 (e-mail: wrobbins@sonnet.ucla.edu).

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boron ($n = 251$) were interviewed. An interesting finding based on these interviews (Chang et al, 2006) was that boron workers reported a lower percentage of male offspring at birth (52.45%) compared with men in an area of low environmental boron (54.35%) and China as a nation (58%). A shift in sex ratios at birth toward females for boron workers has been reported previously (Whorton et al, 1994). Thus, a subset of men was evaluated using biologic markers for X and Y chromosomes in ejaculated sperm cells to determine whether there might be an association between boron exposure and sex chromosomes in sperm.

Materials and Methods

This research was reviewed and approved by both the University of California Institutional Review Board for the protection of human research subjects and the China National Environmental Monitoring Station Human Subjects Review Board. All study participants gave their informed consent to participate in the research.

Study Subjects

Study subjects resided in Kuandian City, Liaoning Province, PR China. Men comprised 3 groups: 63 employed in 5 different boron mines or processing plants; 39 who did not work in the boron industry but lived in the same area as the boron mines or processing plants where high levels of environmental borates were present (these men are referred to as the *community comparison group*); and 44 who did not work in the boron industry and lived in an area of low levels of environmental borates (these men are referred to as the *control group*). Participation rates approached 95% and did not vary significantly across the 3 exposure groups. Retention rates were 85% across 3 months of follow-up with repeated sampling and did not vary significantly by exposure group.

Reproductive History and Offspring Health

Interviews were conducted using a 51-item questionnaire guide that included domains of work, diet, lifestyle, general health, and reproductive and offspring health as described by Chang et al (2006). Specific reproductive data was collected related to numbers and sex of offspring, pregnancy loss, such as stillbirths, spontaneous, or elected pregnancy terminations, and care or treatment for reproductive health issues. Selected characteristics of the 3 groups are shown in Table 1.

Boron Exposure Assessment

Borates dissociate in the body at physiologic pH to boric acid $[B(OH)_3]$ and borate $[B(OH)_4^-]$ and have similar effects when dose is calculated as elemental boron (Culver et al, 1994); therefore, exposure was calculated in terms of elemental boron (B^{10}) for this study. A composite of total daily exposure was generated by collecting 24-hour duplicate food portions and 24-hour duplicate fluid intakes plus full work shift breathing

zone air samples using Institute of Medicine (IOM) lapel filter cassettes and personal air monitoring pumps (PCXR series; SKC Inc, Eighty Four, Penn). Boron was measured in blood, urine, and semen to estimate internal dose. Samples were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) for semen, blood, and urine and by inductively coupled plasma-atomic emission spectrometry (ICP-AES) for dust, food, and fluid intake samples. A total of 10% field blanks (with a minimum of 2) for each day of sampling were collected and analyzed. Boron analyses were conducted at the National Research Center of Geo-analysis, Chinese Academy of Geological Sciences, Beijing, China.

Semen Y:X ratios

Sperm cells were assayed for Y- or X-bearing cells using sperm fluorescence in situ hybridization (FISH) adapted from Robbins et al (1995). Briefly, direct labeled enumerator probes to the alpha satellite region for X (Xp11.1-q11.1) labeled with fluorophore SpectrumGreen and Y (Yp11.1-q11.1) labeled with SpectrumOrange were obtained from Vysis Inc (Downers Grove, Ill) as part of the Aneuvysion DNA Probe Kit. For an internal control on hybridization efficiency, chromosome 18 (18p11.1-q11.1) labeled with Spectrum Aqua (Vysis Inc) was used. In situ hybridization was performed on 7 μ L semen dried onto microscope slides and decondensed with 10 mM dithiothreitol (DTT; Sigma, St Louis, MO) in 0.1 M Tris buffer, pH 7.6, on ice for 20 minutes, followed by 4 mM lithium diiodosalicylate (LIS; Sigma) in 0.1 M Tris buffer, pH 7.6, at room temperature for 28 minutes. Slides were air dried and then denatured at 75°C for 3 minutes in 70% formamide, 2 \times SSC (0.15 M sodium chloride, 0.015 M sodium citrate), pH 7.0, followed by ethanol incubations of 70%, 85%, and 100% for 2.5 minutes each. Predenatured probe (10 μ L) was added to each slide, then covered by a 22 \times 22 mm cover slip and hybridized overnight at 37°C. Slides then were washed in 50% formamide, 2 \times SSC at 45°C, for 10 minutes followed by a 10-minute wash in 2 \times SSC at 45°C, a 10-minute wash in 1 \times SSC at 45°C, and a 10-minute wash in 1 \times SSC at room temperature. Air-dried slides were counterstained with a 9 μ L mixture of DAPI 125 ng/mL in mounting media (Vysis), covered by a 22 \times 22 mm cover slip, and stored at 4°C until scoring. Sperm nuclei were observed via fluorescence microscopy using a Zeiss Axiophot microscope with DAPI/FITC/Texas Red triple band pass filter set-up (61002; Carl Zeiss, Inc, Thornwood, NY) for simultaneous visualization of DAPI and the Spectrum orange, green, and aqua fluorochromes (Chroma Technology Corp, Brattleboro, Vt). A single band pass emission filter for green (41001: exciter 480/40, emitter 535/50), red (41004: exciter 560/55, emitter 645/75), or aqua (30-150092: exciter 420/30, emitter 460/40) was used to confirm discrimination of green, orange, or aqua signals during scoring. Slides were analyzed using strict scoring criteria to discriminate normal from abnormal fluorescence phenotypes as described by Ong et al (2002). Sperm were scored only if they were morphologically preserved with no clumping or overlapping and if they had a well-defined outline and tail. A total of 5000 cells were scored per sample, and a single scorer analyzed all samples. Hybridization efficiency was greater than

Table 1. Selected characteristics of the study population of 146 men*

	Boron Worker (n = 63)	Community Comparison (n = 39)	Control (n = 44)	<i>P</i> < .05
Age, y, mean ± SD	31.2 ± 4.4	30.0 ± 6.1	31.3 ± 5.4	
Education, n (%)				
Lower than high school	45 (71.4%)	30 (76.9%)	32 (72.7%)	
High school or higher	18 (28.6%)	9 (23.1%)	12 (27.3%)	
Alcohol (% yes)	30 (47.6%)	22 (56.4%)	23 (52.3%)	
Smoke (% yes)	44 (69.8%)	22 (56.4%)	30 (68.2%)	
Pesticide exposure (% yes)	13 (20.6%)	5 (12.8%)	14 (31.8%)	
Spontaneous abortion (% yes)	4 (6.4%)	2 (5.1%)	3 (6.8%)	
Elective pregnancy termination (% yes)	31 (49.2%)	11 (28.2%)	22 (50.0%)	
More boys than girls at birth (% yes)	30 (57.7%)	11 (42.3%)	23 (76.7%)	.03

* *P* values are based on ANOVA comparing the 3 exposure groups.

99%. Slides were coded so the scorer did not know whether the subject was from the boron worker group or a comparison group. Prior to beginning the scoring, the study group slides were mixed so that slides were scored in random order.

Statistical Analyses

All variables were plotted, and those that were highly skewed (eg, the biologic boron measures) were log transformed to normalize the data prior to analysis. χ^2 tests and 1-way ANOVA were used to evaluate relationships between the reproductive outcome variables (eg, elective abortion, spontaneous abortion, gender of offspring), demographic, lifestyle, and environmental variables (eg, age, education, smoking, diet). To examine differences with respect to the sex of offspring at birth, we compared the proportion of subjects with more male than female offspring at birth across the groups (thus, men with equal numbers of male and female offspring at birth were not included in this comparison). Those variables that were significantly different among the 3 groups were compared between pairs of groups with the *t* test with Tukey multiple comparison correction. To investigate possible correlates of the Y:X ratio, univariate linear regression models were constructed with the Y:X ratio as the outcome and the following variables as predictors: conventional semen parameters (total concentration, total motile cells, morphology), days of abstinence prior to semen collection, boron in biologic fluids, total daily boron exposure, diet, years of marriage, medications, chronic medical conditions, exposures to other known reproductive toxicants, or history of reproductive problems. A multiple linear regression model for Y:X ratio was constructed to determine whether the effect of group remained significant after controlling for potential confounders that might affect semen quality. The final model included terms for age, smoking, alcohol, education, and pesticide exposure. All statistical analyses were carried out in SAS (version 9.1.3; SAS Institute Inc, Cary, NC) and R (www.r-project.org).

Results

Interview data collected from the 146 participants indicated that the 3 comparison groups did not differ

on general health status or on specific reproductive outcomes, including elective pregnancy termination, spontaneous abortion, stillbirths, and birth defects in offspring. However, when men were compared as to whether they had more male than female offspring at birth, the 3 groups differed (*P* = .03), with 57.7% of the boron workers reporting more boys than girls compared with 76.7% for men from an area of low environmental boron and 42.3% for men living in a high-environmental boron area. Comparisons of selected characteristics are shown in Table 1.

Boron in biologic fluids of the 146 men varied across the 3 groups (ANOVA *P* < .0001), with boron workers being higher on all measures (blood, semen, urine) than men sampled from either the community with high environmental boron (*t* test *P* < .0001) or controls from the low-environmental boron area (*t* test *P* < .0001). Nonboron workers from the community with high environmental boron also had more boron in their biologic fluids than men in the low-environmental boron area (*t* tests: blood, *P* < .03; semen, *P* = .06; urine, *P* = .02). Total boron exposure was estimated for a convenience sample of men who collected 24-hour food/fluid intakes and workplace inhalable dust monitoring. Boron workers had the highest total exposure and differed from both comparison groups (*t* test *P* < .0001). Total exposure for nonboron workers living in the community with high environmental boron was nearly twice that of men in the control group, but this difference did not reach statistical significance (*t* test *P* < .06). Biologic measures of internal dose were correlated with total boron exposure (Pearson correlation for total exposure and boron in blood = 0.63, *P* < .0001; semen = 0.80, *P* < .001; and urine = 0.79, *P* < .0001). These relationships are shown in Table 2.

The 3 groups differed in ratio of Y- to X-bearing sperm in their ejaculates, as shown in Table 2 (ANOVA *P* < .0001). Among the 146 men, boron workers had

Table 2. Boron exposure and Y:X ratio in sperm

	Boron Worker	High Boron Community Comparison	Low Boron Control	P
Biologic measures of dose, n	63	39	44	
Blood boron, ppb, mean ± SD	515.4 ± 805.7	109.11 ± 111.2	45.5 ± 22.5	<.0001
Semen boron, ppb, mean ± SD	806.0 ± 612.6	297.3 ± 273.0	203.9 ± 105.7	<.0001
Postshift urine boron, mg/L, mean ± SD*	17.3 ± 32.0	6.67 ± 19.1	1.98 ± 0.8	<.0001
Total daily boron exposure, n	15	15	15	
Dust, food, and fluid intake, mg, mean ± SD	41.2 ± 37.4	4.3 ± 3.1	2.3 ± 3.0	<.001
Sperm FISH for Y:X ratio				
Sperm cells (5000 scored per subject)	n = 63	n = 39	n = 44	
Y:X ratio, mean ± SD	0.93 ± 0.03	0.96 ± 0.04	0.99 ± 0.03	<.0001

* Urine is corrected for creatinine clearance; P values are based on ANOVA comparing the 3 exposure groups.

a lower ratio of Y-bearing to X-bearing sperm compared with men in both the high-environmental boron area (t test $P < .0001$) and low-environmental boron area (t test $P < .0001$). Of note, the community comparison group living in an area with high environmental boron also had significantly lower sperm Y:X ratios than the low-environmental boron control group (t test $P < .0001$). Linear regression analysis adjusting for age, smoking, alcohol, education, and pesticide exposure found a significant relationship between Y:X ratio and the boron worker group ($P < .0001$) and community comparison group ($P = .0001$) when using the control group from the area of low environmental boron as baseline, as shown in Table 3. Linear regression models of logged boron in biologic fluids on Y:X ratio were all statistically significant (blood $P = .02$, semen $P = .0003$, urine $P = .005$). A separate within-group regression between Y:X ratio and the biologic boron measures found no statistically significant within group correlations.

Discussion

In this group of 146 men, we found that boron in biologic fluids was significantly correlated with Y:X ratio in sperm ($P = .02$ for blood, 0.0003 for semen, and 0.005 for urine), and this relationship held when men

were categorized into 3 groups: boron workers, men living in a boron-contaminated environment but not working with boron, and men living and working in an area of little environmental boron. The lower ratio of Y- to X-bearing sperm in ejaculates of boron workers (Y:X = 0.93) compared with men not working in the industry living in an area of low environmental boron (Y:X = 0.99, $P < .0001$) was consistent with self-report of fathering comparatively fewer male than female offspring. After excluding families with equal numbers of boys and girls at birth, 57.7% of boron workers reported fathering more boys than girls compared with 76.7% for men in the area of low environmental boron, although direct comparison of these 2 groups did not reach statistical significance ($P < .08$). We used proportions of families with more male than female offspring at birth for this comparison instead of comparing total numbers of male offspring across the groups, because our study population was limited to 146 men with an average of ~1.3 births per man, and it would have required a sample size of ~1700 total births to boron-exposed men and an equal number of total births to boron-unexposed men to verify statistically significant differences between the groups if total numbers of male offspring were used. The finding of reduced Y:X ratios in the present study replicated our pilot data collected the year prior on 60 boron workers and 9 controls ($P < .009$, data not shown). The Y:X sperm ratios reported

Table 3. Linear regression model adjusting for age, lifestyle, education, and pesticide exposure*

Parameter	Estimate	Standard Error	t Value	Pr > t
Intercept	0.995	0.017	58.15	<0.0001
Age	-0.00008	0.0005	-0.15	0.88
Smoke (yes/no)	-0.0025	0.0066	-0.38	0.70
Alcohol (yes/no)	-0.00065	0.0056	-0.12	0.901
Education (less than high school vs high school or above)	-0.0029	0.0062	-0.46	0.65
Pesticide (yes/no)	-0.00004	0.0067	-0.01	0.99
Group: boron worker vs control	-0.056	0.0064	-8.74	<0.0001
Group: community comparison vs control	-0.028	0.0072	-3.91	0.0001

* Model: Y:X ratio = age + smoke + alcohol + education + pesticide + group.

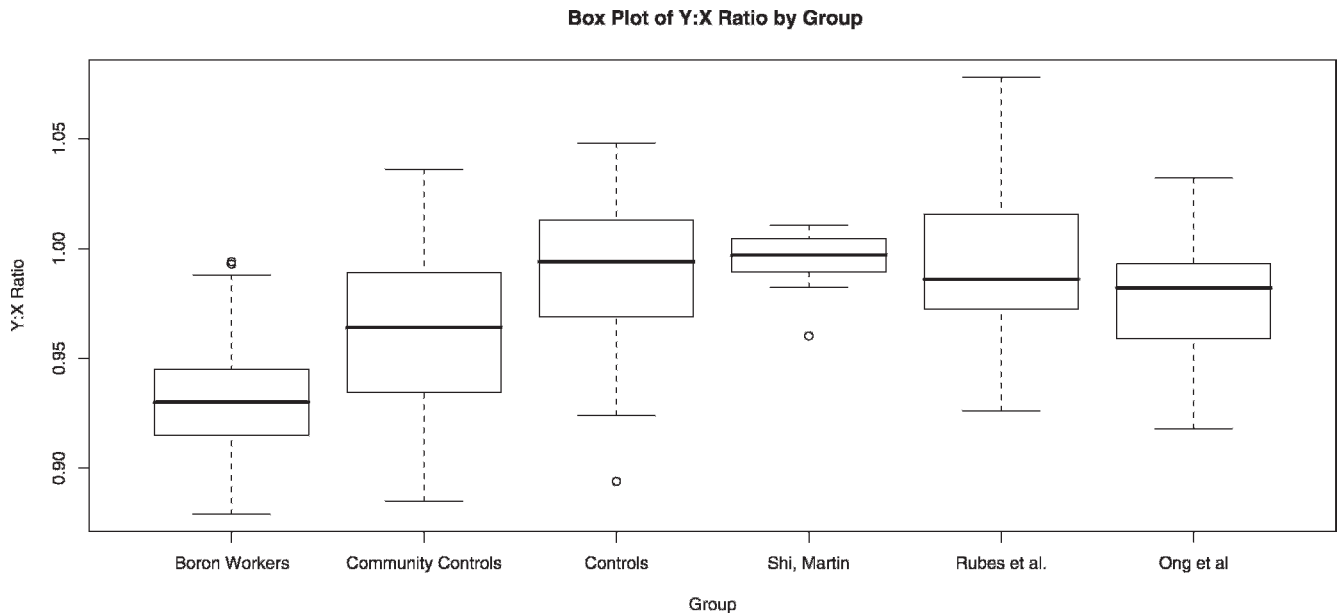


Figure. Comparison of Y:X ratio in boron worker group, community comparison group, and low-boron exposure control group, as well as three groups of healthy men from the published literature (cohort of 10 healthy Chinese [Shi and Martin, 2000]; cohort of 14 healthy Czech [Rubes et al, 2002]; cohort of 20 healthy US men [Ong et al, 2002]). For the box and whisker plots, the box is drawn from the first and third quartiles, with the thick line representing the median. The whiskers above the box are either the maximum value of data or the largest value that is equal to or below the third + 1.5* interquartile range, whichever is smaller. The lower bar is similarly defined. The figure demonstrates that control men from the low environmental boron area in the present study are similar in Y:X ratio to men evaluated in other published research studies. Comparatively, boron workers have the lowest Y:X ratio.

for men from the area of low environmental boron in our study are consistent with those reported for other cohorts of healthy men from around the world (Figure, constructed from data of published studies by Shi and Martin, 2000; Ong et al, 2002; and Rubes et al, 2002, where enough detail is available on Y- and X-bearing sperm in the publication to construct box and whisker plots).

The lower Y:X ratios in sperm of boron-exposed men could not be explained by other variables tested, including conventional semen parameters (total concentration, total motile cells, morphology), additional elements assayed simultaneously in the biologic fluids (Ca, Mg, Pb, Se, Cr, Cu, Sr, Zn), or particulars of diet, years of marriage, medications, chronic medical conditions, exposures to other known reproductive toxicants, history of reproductive problems, elective and spontaneous abortion, or days of abstinence prior to the semen collection. Within each comparison group, correlations between biologic levels of boron and Y:X ratio were not significant; however, previous modeling of creatinine corrected urine boron as a predictor of total exposure (Xiaoru et al, 2006) demonstrated a boron exposure–dose relationship that predicted best for high exposures and not as well for low exposures. This plus the small within-group numbers may explain a lack of within-group statistical significance.

Only a few published epidemiologic studies have examined human male reproductive health related to environmental or workplace borates, and these studies have reported either no adverse effect (Whorton et al, 1994; Sayli, 1998, 2003; Yazbeck et al, 2005) or testicular atrophy and sterility (Tarasenko et al, 1972). It is interesting that epidemiologic investigations that collected data on offspring of men with high exposures to borates all suggest trends in the sex ratio at birth toward females when the ratio is compared across exposure groups (Whorton et al, 1994; Chang et al, 2006) or compared to year-appropriate ratios for the home country of the study (Sayli, 1998, 2003) or the sex ratio 1.05–1.06 generally reported for most countries of the world (Mathews and Hamilton, 2005; Yazbeck et al, 2005). For example, the drinking water study by Yazbeck et al (2005) reported slightly higher female offspring in regions of France with boron in water of ≥ 0.3 mg/L compared with geographic regions with boron in water ranging from 0.00–0.09 mg/L based on 72 993 births and 84 305 births, respectively. This difference (sex ratio 1.05 versus 1.04) was not statistically significant. Of note, Yazbeck et al measured blood boron in a subset of their population using military recruits from various regions, including 36 from the region with comparatively high boron in water. The average blood boron level for these recruits was 27%

lower than the average blood boron for the 68 workers in our boron industry study. None of the above publications reported on Y:X ratios in sperm. In our study, we were able to demonstrate a shift in Y:X ratio across the groups ($P < .0001$) that was consistent with self-report of a shift toward female offspring ($P < .03$). To date, published animal toxicology studies have not reported on boron-related changes in offspring sex ratios.

Shifts in sex ratios at birth toward more females have been reported for extreme exposures to men—for example, the 1979 Yucheng polychlorinated biphenyl (PCB)-contaminated cooking oil poisonings (del Rio Gomez et al, 2002), the 1976 Seveso tetrachlorodibenzo-*p*-dioxin (TCDD) explosion disaster (Mocarelli et al, 2000)—as well as workplace exposures, such as dibromochloropropane (DBCP) exposures (Whorton and Foliart, 1983). In each of these cases, significant changes in conventional semen quality were noted. Markers of Y- and X-bearing sperm were not analyzed, so effects after fertilization cannot be ruled out. Tiido et al (2005) reported a significant association between Y:X ratios in sperm and the persistent organochlorine pollutants CB-153 ($P = .05$) and p,p'-DDE ($P < .001$) in 149 Swedish fishermen. The magnitude of change in Y:X ratio in the Tiido study (1.6% and 0.8%, respectively) did not change observed sex ratios in offspring of the fishermen and are considerably smaller than the 6% change in Y:X ratio found and replicated by us between boron workers and controls.

Delayed release of sperm into the lumen of the seminiferous tubule (retained sperm) and retention of residual cytoplasm in maturing sperm cells (residual bodies) are the first signs of boron toxicity noted in nonhuman test systems (Wang et al, 2005; Namekawa et al, 2006), although Y:X ratios at this juncture have not been studied. Data from our study are epidemiologic (observational); however, they fit with mechanistic models of disruption of normal events during the process of meiotic sex chromosome pairing and inactivation, subsequent segregation of sex chromosomes, as well as postmeiotic gene reactivation patterns conferring selective advantage to X-bearing sperm during spermiation. If fewer Y-bearing sperm remain following errors in meiotic gene inactivation/postmeiotic reactivation patterns that confer selective advantage to X-bearing sperm and loss of Y chromosome sperm, overall lower sperm concentrations in the ejaculate might be expected. For our data, based on the change in the Y:X ratio found, we would expect a 3% decrease in total concentration. A decrease was observed, although it did not reach statistical significance. Given the level of variability in sperm concentration among the study subjects, the statistical power to detect this expected

decrease in concentration was only 6%, indicating that even if there was a change, it would be too small to detect for sperm concentration with the current sample size. Evidence in the ejaculated sperm cells of residual bodies or morphologic size/shape differences were not seen, nor were differential levels of apoptotic sperm carrying X versus Y chromosomes (data not shown), suggesting the shift in Y:X ratio occurred earlier in spermatogenesis, at some point before or close to spermiation.

Lower Y:X ratio in sperm cells of boron workers indicates paternal origin for the shift seen in sex ratios at birth rather than an effect of maternal factors or selective survival of the female fetus. Families in northeast China face strong socio-political and cultural preference for sons. Although we cannot know the sex of pregnancies that were not brought to term in our study group, we did see that boron workers were not different from men in the comparison groups in numbers of reported elective pregnancy terminations, spontaneous pregnancy losses, or stillbirths.

Sex ratio at birth has an impact on critical population dynamics (eg, doubling time), socio-economics, and health factors. The ratio of males to females at birth has been used to assess the effects of environmental and other factors on human reproductive health (Mathews and Hamilton, 2005) but reflects maternal, fetal, and paternal components. Our findings demonstrate both changes in sex ratios at birth and changes in Y- to X-bearing sperm. We present these findings as evidence that exogenous environmental or workplace exposures can result in selective genetic pressure in human males (X- versus Y-bearing sperm) and that this could be expressed as changed sex ratios in offspring. A decline in the number of males born relative to females has been reported in a number of countries over the past few decades (Davis et al, 1998; Mathews and Hamilton, 2005). Social and environmental scientists implicate a range of population stressors for this downward trend, including, most commonly, environmental pollution, increasing parental ages, parental hormones, and race (Mathews and Hamilton, 2005; Bay et al, 2006; Catalano and Bruckner, 2006; James, 2006). Findings confirm the need to be concerned about changing sex ratios at birth for humans that are experienced in some areas around the world, as well as the need to explore the underlying etiology.

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