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Running Title:
Gender Bias after Assisted Conception

Main Title:
Effects of Assisted Reproductive Technologies (ART) on Human Sex Ratio at Birth

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Capsule

We analyse the gender of over 100,000 UK babies born after assisted reproduction treatment and we find that the type of ART treatment used can affect the sex ratio.

Abstract

Objective: To investigate the effect of assisted reproductive technology (ART) treatments on the sex ratio of babies born.

Design: Direct effects of assisted conception through retrospective data analysis on the progeny sex ratio of treated women in the UK.

Setting: The study uses the anonymised register of the Human Fertilisation and Embryology Authority (HFEA).

Patients: 106,066 babies of known gender born to 76,994 treated mothers and 85,511 treatment cycles between 2000 and 2010 in the UK.

Interventions: Intra-Uterine Insemination (IUI), *In Vitro* Fertilization (IVF) or Intra-Cytoplasmic Sperm Injection (ICSI)

Main Outcome Measures: Sex ratio of babies born.

Results: IUI, IVF and ICSI lead to different sex ratios, highest after (proportion male = mean 0.521 ± C.I. 0.0056) and lowest under ICSI cleaving stage embryo transfer (0.493 ± 0.0031). We also find that, for both ISCI and IVF, transferring embryos at a later stage (blastocyst) results in around 5% more males than after early cleavage stage embryo transfer.

Conclusions: As the cumulative number of IVF babies born is increasing significantly in Britain and elsewhere, more research is needed into the causes of gender bias after ART and into the public health impact of such gender bias of offspring born observed on the rest of the population.

Introduction

The prevalence of infertility worldwide and in European countries is estimated to affect around one in seven couples (1). The number of babies born from assisted conception, assisted reproductive technologies (ART), is increasing rapidly: their numbers have quadrupled in the last twenty years, and, to date, approximately 5 million babies worldwide have been born following ART (2, 3). In the UK, the prevalence of infertility is still higher with one in six couples reported to experience infertility problems (4), and ART births constitute 2-2.5% of all births in the country (5, 6). Despite these numbers, the impact of ART treatments on the general human population is poorly understood.

There are three commonly used methods of ART. Intra-Uterine Insemination (IUI) is generally the first line of infertility treatment (1) before proceeding to more invasive procedures such as *In Vitro* Fertilization (IVF) and Intra-Cytoplasmic Sperm Injection (ICSI) (7). IUI requires a catheter to deposit an appropriate number of washed and resuspended sperm directly into the uterus, thereafter the spermatozoa swim through fallopian tubes towards the ovulated egg (or eggs if ovarian stimulation drugs are used). During IVF or ICSI, cumulus oocyte complexes are aspirated form the ovaries after an ovarian stimulation regimen. During IVF, oocytes are incubated with a known concentration of motile spermatozoa. In contrast, during ICSI, the operator selects a single spermatozoon for direct injection into an egg that has been stripped of its cumulus cells using the hyaluronidase enzyme. IVF or ICSI embryos can then be cultured up to 6 days *in vitro* and transferred back to the patient based on their number and morphology. Not only do these three ART methods differ technically, they may differentially affect the sex ratio at birth (8-11), with a general tendency for more males being produced

compared to among naturally born offspring. The sex ratio at conception (primary sex ratio), defined according to the numbers of oocytes fertilized by X- or Y-bearing spermatozoa, is difficult to assess (12) and is thus usually unknown. In contrast, the secondary sex ratio, SSR, which may be defined as the proportion of live-born males out of all live births (13), is straightforward to assess and it is the SSR that most population censuses report in public databases (14-16). At reproductive age, sex ratio bias has the potential to generate substantial public health concerns (8, 12, 17), leading, for instance, to increased socially disruptive behaviour, transmission of sexually transmitted diseases and mental health problems (18-21).

Here we analyse the UK national clinical data for SSR of ART children born between 2006 and 2010 as published by the Human Fertilisation and Embryology Authority (HFEA) which regulates licensing and use of human gametes and embryos across the UK (22). Our main aim is to establish whether the SSR of children born in the UK is affected by the ART method used.

Materials and Methods

Sample data

The Human Fertilisation and Embryology Authority (HFEA) anonymised register was accessed for data published between 2000 and 2010. As the register is anonymised by the HFEA and released as a public document, no ethical approval was needed. The offspring born after 85,511 successful treatment cycles to mothers from across the United Kingdom, with a complete dataset on maternal age, ART method used (IUI, IVF or ICSI), number of eggs collected, number of embryos transferred, the day of embryo transfer

until live birth, were included in this study for analysis: 65,438 of the cycles produced single offspring, 19,595 produced twins, 474 produced triplets and 4 had quadruplets, giving 106,066 babies in total, each of known gender.

Our interest was in evaluating potential influences on the proportion of offspring that were male, i.e. the birth (secondary) sex ratio. The variables considered in this research were: ART procedure (IVF, ICSI or IUI, carried out in 46.00, 46.60 and 7.40% of cycles respectively), the mother's age (range: 18-50 years overall with 55% of mothers aged <35 years and 80% <38 years), the numbers of previous IUI and IVF/ICSI cycles, whether or not gonadotropin stimulation was used and the year that treatment was carried out. For IVF and ICSI, were also evaluated effects of the day of embryo transfer (day 1-3 for early (cleaving) stage and day 4-7 for late (blastocyst) stage) and the number or embryos transferred.

Statistical analyses

The sex ratio of offspring produced from each successful treatment cycle was used as the response variable in statistical analyses with potential influences entered as discrete or continuous explanatory variables within generalized linear models, specifically logistic analyses (using the Genstat statistical package, version 15.1, VSN International, Hemel Hempstead, UK). A single analysis on the effects of the ART procedure with all variables was not possible because embryology data do not apply to the IUI procedure.

We used backward elimination procedures and aggregation of factor levels to obtain the parsimonious 'minimal adequate model' by model simplification (8, 23-25). We report the

percentage of deviance explained (%Dev) as an approximate analogue of r^2. The assumption of quasi-binomially distributed errors (based on empirically estimated scale parameters) was adopted to reduce the probability of Type I errors occurring due to over-dispersion (23, 24). Because multiple successful cycles from the same mother (i.e. those mothers who successfully received further treatment in order to have subsequent children) were initially treated as independent observations, which can promote Type I errors, and because the anonymised nature of HFEA anonymised register prevented the entry of maternal identity as a random factor in a Generalized Linear Mixed Model (26), we repeated the analysis using the sub-set of data on only the first successful treatment cycle (n=76,994). As this generated the same conclusions as the full data analysis, we formally report results from the larger set of data.

The relative risk (RR, sex ratio after treatment/population sex ratio, REF NO: Sheskin DJ (2004) Handbook of parametric and nonparametric statistical procedures. 3rd ed. Boca Raton: Chapman & Hall /CRC) that each treatment group generated SSRs different to that of the general population was then calculated, from the full set of data, and reported with 95% confidence intervals (CI). All tests were two sided and P<0.05 was considered significant.

Results

There were significant sex ratio differences among ART treatment types but none of the other variables influenced the gender of babies born, nor were there significant interactions between any of the explanatory variables (Table 1). The sex ratios of offspring born to IUI, IVF and ICSI are shown separately on Figure 1 but sex ratios from

IVF and IUI did not differ significantly (aggregation of factor levels (23) : $F_{1,85508}$=2.57, P=0.109). Data from ICSI could not be aggregated with either IUI ($F_{1,85508}$=4.05, P=0.044) or IVF ($F_{1,85508}$=54.53, P<0.0001: the overall result is thus due to significantly lower sex ratios (fewer males) being produced under ICSI than under the other treatment methods.

IVF and ICSI embryos were transferred between 1 and 7 days after oocyte aspiration and fertilisation, with the number of embryos transferred varying between 1 and 4. Sex ratios were uninfluenced by the number of embryos transferred but the ratio of male births was higher when embryo transfer occurred at later stages of development and, as above, under IVF compared to ICSI (Figure 2).

The relative risk of each ART technique and stage of embryo transfer was compared to the SSR of the UK general population in 2011 (Figure 1) as published by the Office of National Statistics (6). The SSR of babies produced from all ART techniques combined was significantly lower from that of the general population (RR= 0.9889, C.I. 0.9827 to 0.9952, P= 0.0005). Examining only IVF and ICSI showed that each generated significant risks of altering the SSR of offspring (IVF: RR= 1.0158, C.I. 1.0070 to 1.0246, P= 0.0004; ICSI: RR= 0.9616, C.I. 0.9529 to 0.9705, P<0.0001), while IUI was not associated with significant risk of altered SSR (P= 0.493).

Discussion

We have analysed for the first time the effects of assisted conception on the gender of the children born in the UK after treatment. We have found that the type of ART treatment

carried out has been affecting the sex ratios of babies. Our results are similar to those from a previous population-based study on IVF and ICSI in the population of New Zealand (8), but we have analysed a significantly larger sample size, and additionally evaluated effects of IUI. IUI and IVF lead to male biased sex ratios while ISCI leads to female bias overall.

The SSR of babies born following IVF (52.05%) and ICSI (49.28%) did differ significantly from that of the general population of England and Wales, 51.27% (6). The apparent lack of risk of SSR alteration under IUI may reflect a genuine lack of effect of it could be due to the relatively small sample size recorded for IUI. Overall, these results are qualitatively consistent with previous observations of lower SSR after ICSI and higher SSR following IVF (8, 27-31).

For both ICSI and IVF, transferring embryos at a later stage of development (days 4-7) result in sex ratios up to 6% higher than after early transfer (days 1-3); this also accords with prior reports (8, 10, 30, 32). It is also notable that single embryo transfer at the blastocyst stage results in significantly higher SSR (RR= 1.056, C.I. 1.0129 to 1.1009, P= 0.0103), when (selective or elective) single embryo transfer is becoming the preferred treatment for all patients as it avoids complications associated with multiple pregnancies which presents a significant public health concern (33). The use of extended culture conditions during IVF and ICSI may favour selection of more male blastocysts for transfer as it is thought male embryos have greater pre-implantation developmental rates (8-11, 34). Evidence increasingly suggests that female and male embryos respond differently to *in vitro* culture conditions, due to the second X-chromosome present in females but not

males retaining activity to the morula stage (35). The genes controlling glucose uptake and metabolism (glucose-6-phosphate dehydrogenase, G6PD) and anti-oxidants (hypoxanthine phosphoribosyl transferase, HPRT) are located on the X-chromosome, and thus female embryos have been reported to have higher glucose uptake and detoxification of oxygen radicals (36, 37). These radicals also have a growth stimulant effect (38, 39), and are not only involved in mechanisms of cellular damage. Potentially, the double dose of enzyme activity can explain the delayed development of female embryos (35) perhaps resulting in male embryos being more able to withstand stressful *in vitro* conditions. However, not all studies have observed sexually differential development rates or associated effects on SSR (40-42).

One of the features common to all three techniques is the procedure used to separate motile spermatozoa with normal morphology from seminal plasma and other cells in the ejaculate (43, 44). A Cochrane systematic review of the two most common sperm preparation techniques has concluded that there is no difference in pregnancy rates after using swim-up or density gradient preparation (43). However, density gradient, which is the predominant method in the cohort of patients in our study, can lead to enrichment of Y-bearing spermatozoa (45-47), which could contribute towards increased sex ratios under IVF and IUI. IVF and IUI are closer to natural conception as the sperm compete for fertilization, whereas in ICSI the fertilizing spermatozoon is selected by the embryologist. A low SSR after ICSI has been attributed to male-factor infertility, since ICSI is the technique of choice in cases of semen quality deficit (30) and Y-bearing spermatozoa in infertile men may bear morphological changes which lead to selection bias under this technique (48). However, Luke *et al*. (2009) suggested iatrogenic mechanisms by which

ICSI biases sex ratio towards females, because decreased SSR was observed even in the study group with non-male factor infertility (31). Irrespective of the mechanisms involved, this and prior studies collectively indicate that infertility forms part of the panoply of medical conditions (e.g. (49)) that directly influence human sex ratios.

Conclusion

Irrespective of the cause leading to the deviation of SSR of babies born to ART procedures, our data and those of others, indicate a trend to produce more males, mainly after IVF and also after blastocyst-stage transfer in IVF and ICSI, and single embryo transfer. At the present time, ART births constitute a small proportion of all births (2-3%) and therefore it is not likely that a gender bias within that proportion will be a cause for concern. However, the impact of deviation in SSR should be considered for the future if the proportion of ART births in Human populations continues to rise, and this is particularly so given the trends for using more single embryo transfers and for transferring at the blastocyst-stage.

Author's Role

W.E.M. conceived the project, analysed the data, interpreted the results, and wrote the manuscript. M.N.M. prepared and discussed the data. B.K.C. discussed reproductive physiology aspects. I.C.W.H. analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

Details of Ethics

The data used in this study is from the patient anonymised patient register published by the HFEA and therefore no ethical approval was needed.

Conflict of Interest

The authors declare no competing interest.

References

1.   NICE. Fertility: assessment and treatment for people with fertility problems. RCOG Press, UK 2004.

2.   Sandin S, Nygren K-G, Iliadou A, Hultman CM, Reichenberg A. Autism and mental retardation among offspring born after in vitro fertilization. JAMA : the journal of the American Medical Association 2013;310:75-84.

3.   ESHRE. ART fact sheet. In: Embryology ESoHRa, ed. Vol. 2013, 2009.

4.   Kamel RM. Management of the infertile couple: an evidence-based protocol. Reprod Biol Endocrinol 2010;8:21.

5.   HFEA. Fertility treatment in 2011: trends and figures. In: Authority HFaE, ed. Vol. 2013, 2011.

6.   ONS. Birth Summary Tables - England and Wales, 2011 (Final). In. Vol. 2013: Office for National Statistics, 2011.

7.   Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K *et al.* The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009. Human reproduction (Oxford, England) 2009;24:2683-7.

8.   Dean JH, Chapman MG, Sullivan EA. The effect on human sex ratio at birth by assisted reproductive technology (ART) procedures--an assessment of babies born following single embryo transfers, Australia and New Zealand, 2002-2006. BJOG 2010;117:1628-34.

9.   Luna M, Duke M, Copperman A, Grunfeld L, Sandler B, Barritt J. Blastocyst embryo transfer is associated with a sex-ratio imbalance in favor of male offspring. Fertil Steril 2007;87:519-23.

10.   Menezo YJ, Chouteau J, Torello J, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. Fertil Steril 1999;72:221-4.

11.   Tarin JJ, Bernabeu R, Baviera A, Bonada M, Cano A. Sex selection may be inadvertently performed in in-vitro fertilization-embryo transfer programmes. Hum Reprod 1995;10:2992-8.

12.   Pyeritz RE. Sex: what we make of it. JAMA 1998;279:269-79.

13.   Zarutskie PW, Muller CH, Magone M, Soules MR. The clinical relevance of sex selection techniques. Fertil Steril 1989;52:891-905.

14.   de Mouzon J. ICMART. Preliminary IVF world results,. ESHRE, Barcelona 2008.

15.   James WH. The human sex ratio. Part 1: A review of the literature. Hum Biol 1987;59:721-52.

16.   Visaria PM. Sex ratio at birth in territories with a relatively complete registration. Eugen Q 1967;14:132-42.

17.   Hesketh T, Xing ZW. Abnormal sex ratios in human populations: causes and consequences. Proc Natl Acad Sci U S A 2006;103:13271-5.

18.   Bhattacharya PC. Gender inequality and the sex ratio in three emerging economies. Prog Dev Stud 2013;13:117-33.

19.   Zhou C, Wang XL, Zheng WJ, Zhou XD, Li L, Hesketh T. The High Sex Ratio in China: What Do the Chinese Think? Journal of Biosocial Science 2012;44:121-5.

20.   Zhou X, Yan Z, Therese H. Depression and aggression in never-married men in China: a growing problem. Social psychiatry and psychiatric epidemiology 2012.

21.   Zhou XD, Wang XL, Li L, Hesketh T. The very high sex ratio in rural China: Impact on the psychosocial wellbeing of unmarried men. Soc Sci Med 2011;73:1422-7.

22.   HFEA. Long-term data - birth rates 2009.

23.   Crawley MJ. GLIM for Ecologists. Oxford: Blackwell Scientific Publications, 1993.

24.   Wilson K, Hardy ICW. Statistical analysis of sex ratios: an introduction. In: Hardy ICW, ed. Sex Ratios: Concepts and Research Methods. Cambridge: Cambridge University Press, 2002:48-92.

25.   Warton DI, Hui FK. The arcsine is asinine: the analysis of proportions in ecology. Ecology 2011;92:3-10.

26.   Krackow S, Tkadlec E. Analysis of brood sex ratios: implications of offspring clustering. Behavioral Ecology and Sociobiology 2001;50:293-301.

27.   Ghazzawi IM, Sarraf M, Al Hasani S. Children born after ICSI: are we altering sex ratio? International Journal of Gynaecology and Obstetrics 2000;70:B66.

28.   Ericson A, Kallen B. Congenital malformations in infants born after IVF: a population-based study. Hum Reprod 2001;16:504-9.

29.   Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P *et al.* Neonatal data on a cohort of 2889 infants born after ICSI (1991-1999) and of 2995 infants born after IVF (1983-1999). Hum Reprod 2002;17:671-94.

30.   Hentemann MA, Briskemyr S, Bertheussen K. Blastocyst transfer and gender: IVF versus ICSI. J Assist Reprod Genet 2009.

31.   Luke B, Brown MB, Grainger DA, Baker VL, Ginsburg E, Stern JE. The sex ratio of singleton offspring in assisted-conception pregnancies. Fertil Steril 2009;92:1579-85.

32.   Chang HJ, Lee JR, Jee BC, Suh CS, Kim SH. Impact of blastocyst transfer on offspring sex ratio and the monozygotic twinning rate: a systematic review and meta-analysis. Fertil Steril 2009;91:2381-90.

33.   HFEA. Multiple births and single embryo transfer review. In, 2013.

34.   Ray PF, Conaghan J, Winston RM, Handyside AH. Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization. J Reprod Fertil 1995;104:165-71.

35.   Gutierrez-Adan A, Oter M, Martinez-Madrid B, Pintado B, De La Fuente J. Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage. Mol Reprod Dev 2000;55:146-51.

36.   Tiffin GJ, Rieger D, Betteridge KJ, Yadav BR, King WA. Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. J Reprod Fertil 1991;93:125-32.

37.   Peippo J, Bredbacka P. Sex-related growth rate differences in mouse preimplantation embryos in vivo and in vitro. Mol Reprod Dev 1995;40:56-61.

38.   Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. Reproduction, fertility, and development 1992;4:547-57.

39.   Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro. J Reprod Fertil 1992;95:585-95.

40.   Kausche A, Jones GM, Trounson AO, Figueiredo F, MacLachlan V, Lolatgis N. Sex ratio and birth weights of infants born as a result of blastocyst transfers compared with early cleavage stage embryo transfers. Fertil Steril 2001;76:688-93.

41.   Milki AA, Jun SH, Hinckley MD, Westphal LW, Giudice LC, Behr B. Comparison of the sex ratio with blastocyst transfer and cleavage stage transfer. J Assist Reprod Genet 2003;20:323-6.

42.   Csokmay JM, Hill MJ, Cioppettini FV, Miller KA, Scott RT, Jr., Frattarelli JL. Live birth sex ratios are not influenced by blastocyst-stage embryo transfer. Fertil Steril 2009;92:913-7.

43.   Boomsma CM, Heineman MJ, Cohlen BJ, Farquhar C. Semen preparation techniques for intrauterine insemination. Cochrane Database Syst Rev 2004:CD004507.

44.   WHO. WHO laboratory manual for the Examination and processing of human semen. 5th ed. Switzerland: World Health Organization, 2010.

45.   Ericsson RJ, Langevin CN, Nishino M. Isolation of fractions rich in human Y sperm. Nature 1973;246:421-4.

46.   Claassens OE, Oosthuizen CJ, Brusnicky J, Franken DR, Kruger TF. Fluorescent in situ hybridization evaluation of human Y-bearing spermatozoa separated by albumin density gradients. Fertil Steril 1995;63:417-8.
47.   Dineen T, Nolan A, Harrington J, Greer A, Kennedy R, Houghton JA. Fluorescence in situ hybridization studies on the sex chromosome constitution of human sperm. Arch Androl 1997;39:217-22.
48.   Setti AS, Figueira RC, Braga DP, Iaconelli A, Jr., Borges E, Jr. Gender incidence of intracytoplasmic morphologically selected sperm injection-derived embryos: a prospective randomized study. Reprod Biomed Online 2012;24:420-3.
49.   James WH. The relevance of the epidemiology of human sex ratios at birth to some medical problems. Paediatric and perinatal epidemiology 2012;26:181-9.

Table 1. Factors affecting sex ratios of offspring born following IUI, IVF or ICSI.

Results are from logistic Analysis of Covariance

Source	d.f.	Deviance	Mean Deviance	F	P
ART Treatment type (IUI, IVF or ICSI)	2	76.468	38.234	27.33	**<0.001**
Mother's age	5	8.348	1.670	1.19	0.309
Number of previous IUI cycles	1	0.603	0.603	0.43	0.511
Number of previous IVF/ICSI cycles	1	0.538	0.538	0.38	0.535
Gonadotropin Stimulation	1	0.046	0.046	0.03	0.856
Year of Treatment	1	1.698	1.698	1.21	0.270
ART Treatment type × Mother's age	10	12.208	1.221	0.87	0.558
ART Treatment type × Previous IUI	2	1.287	0.643	0.46	0.631
ART Treatment type × Previous IVF/ICSI	2	0.396	0.198	0.14	0.868
ART Treatment type × Stimulation	2	1.780	0.890	0.64	0.529
ART Treatment type × Year	2	1.237	0.618	0.44	0.643
Mother's age × Previous IUI	5	1.679	0.336	0.24	0.945
Mother's age × Previous IVF/ICSI	5	1.385	0.277	0.20	0.963
Mother's age × Stimulation	5	8.221	1.644	1.18	0.318
Mother's age × Year	5	2.696	0.539	0.39	0.859
Previous IUI × Previous IVF/ICSI	1	0.351	0.351	0.25	0.616
Previous IUI × Stimulation	1	0.509	0.509	0.36	0.546
Previous IUI × Year	1	4.480	4.480	3.20	0.074
Previous IVF/ICSI × Stimulation	1	0.014	0.014	0.01	0.919
Previous IVF/ICSI × Year	1	0.314	0.314	0.22	0.636
Stimulation × Year	1	0.000	0.000	0.00	0.991
Residual	85455	119534.663	1.399		
Total	85510	119658.920	1.399		

Figure legends

Figure 1. Sex ratio at birth following IUI, IVF and ICSI. Confidence Intervals at 95% are shown around each mean. The dashed horizontal line shows the overall sex ratio of babies born following all treatments (0.507±0.02). The dotted horizontal line shows the England and Wales population sex ratio (0.513, (6)).

Figure 2. Sex ratio at birth following the IVF and ICSI at late and early stage embryo transfer. Confidence intervals are shown around each mean. The dotted horizontal line shows the England and Wales population sex ratio (0.513 males, (6)).
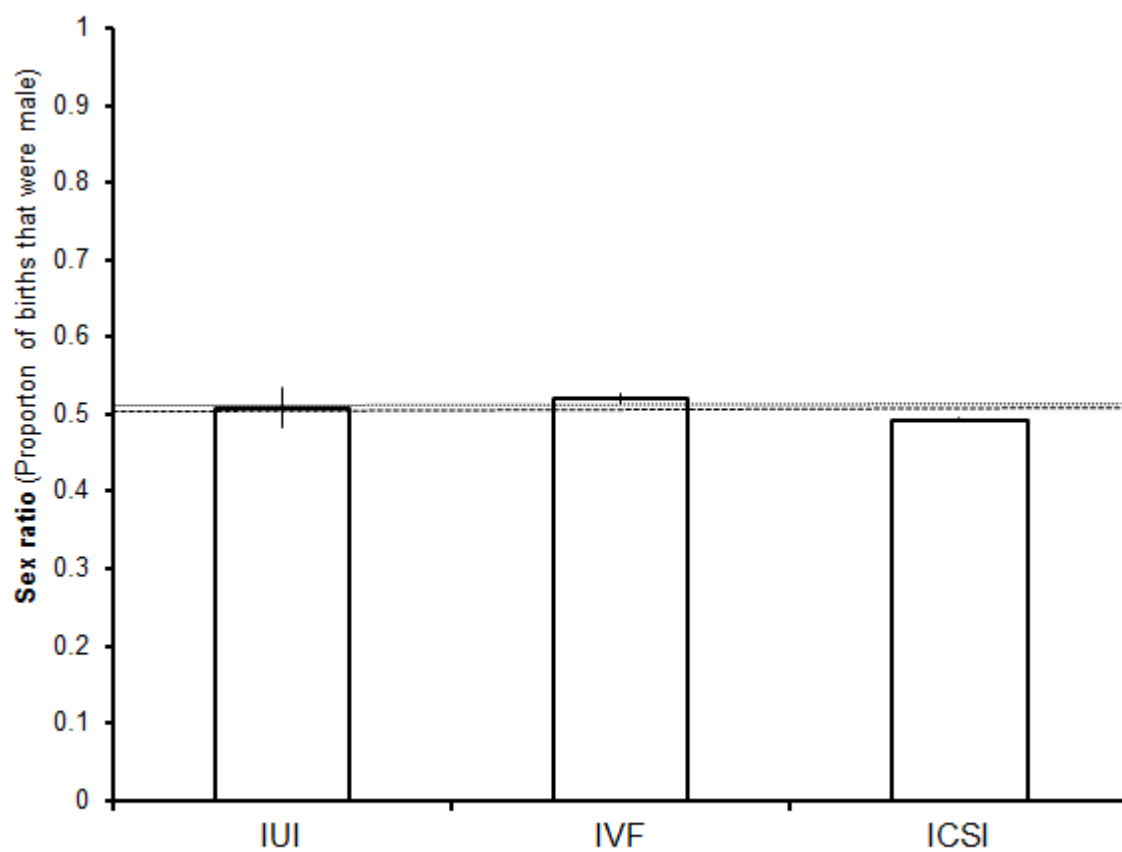
Figure 1.

Figure 2.