



## Research

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## Evolutionary biology

# Flow cytometric sexing of spider sperm reveals an equal sperm production ratio in a female-biased species

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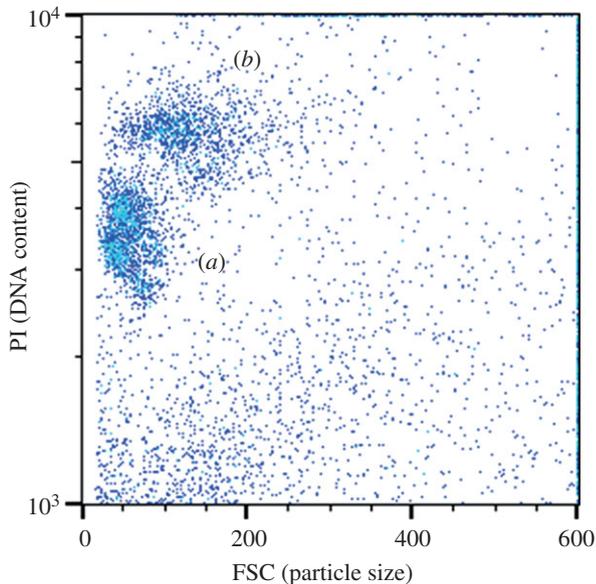
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Producing equal amounts of male and female offspring has long been considered an evolutionarily stable strategy. Nevertheless, exceptions to this general rule (i.e. male and female biases) are documented in many taxa, making sex allocation an important domain in current evolutionary biology research. Pinpointing the underlying mechanism of sex ratio bias is challenging owing to the multitude of potential sex ratio-biasing factors. In the dwarf spider, *Oedothorax gibbosus*, infection with the bacterial endosymbiont *Wolbachia* results in a female bias. However, pedigree analysis reveals that other factors influence sex ratio variation. In this paper, we investigate whether this additional variation can be explained by the unequal production of male- and female-determining sperm cells during sperm production. Using flow cytometry, we show that males produce equal amounts of male- and female-determining sperm cells; thus bias in sperm production does not contribute to the sex ratio bias observed in this species. This demonstrates that other factors such as parental genes suppressing endosymbiont effects and cryptic female choice might play a role in sex allocation in this species.

## 1. Introduction

Frequency-dependent selection acting in a sex-biased population will increase the fitness of the minority sex, and a sex ratio of 50 : 50 is thus expected to be the evolutionarily stable outcome [1,2]. Biased sex ratios therefore have intrigued biologists for many decades, leading to the field of sex allocation theory as an important part of evolutionary thinking [1]. Sex ratio biases are known in a wide range of animal taxa (i.e. arthropods, squamata, birds and mammals) with a variety of sex-determining systems, including male heterogamety [1]. Although such biases are easily observed in laboratory and field populations, determining their mechanistic basis is far more challenging due to the multitude of potential underlying causes [1]. These causes may act at different stages during ontogeny [3], and the bias may manifest itself at, among others, the level of sperm production [4], relative survival of the sexes, and at the phenotypic level (as a consequence of, for example, feminization) [5]. Moreover, determining sex ratios prior to the adult stage is often hampered by the absence of sex-specific morphological traits [6]. It is therefore essential to identify the correct stage at which distorting mechanisms are acting to get a clear insight in the underlying cause.

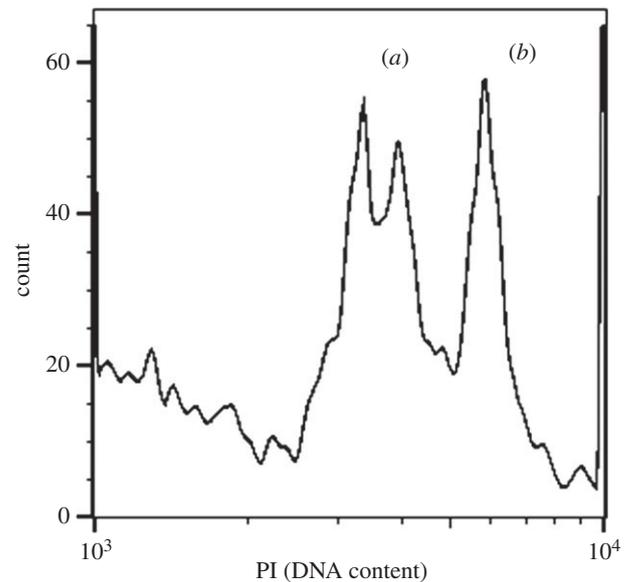
The dwarf spider *Oedothorax gibbosus* is infected by multiple maternally inherited endosymbiont bacteria, of which *Wolbachia* causes a female bias. Using a pedigree analysis based on multiple generations of laboratory rearing, we verified that the largest part of the sex ratio variation in *O. gibbosus* is indeed inherited



**Figure 1.** Dot plot of intensity (corresponding to DNA content) of propidium iodide-stained nuclei and FSC of the nucleus (corresponding to particle size), isolated from a pedipalp of *O. gibbosus* male. Cloud (a) corresponds to sperm nuclei and cloud (b) to somatic nuclei. (Online version in colour.)

from mother to daughter, consistent with maternal inheritance of the endosymbionts [7]. However, significant additional variation was detected that was not inherited maternally, suggesting that *Wolbachia* infection is not the sole factor that determines sex ratio variation [7]. One possible such factor that shows a male inheritance pattern is the production of a biased proportion of male- and female-determining sperm cells (see below). This can be caused by sex chromosome meiotic drive: the unequal transmission of sex chromosomes by heterogametic individuals [4] during meiosis [8]. The driving sex chromosome will be overrepresented in the gametes, and this leads to the biased production of the sex corresponding to this sex chromosome. In the majority of species (excluding birds), males are the heterogametic sex; therefore, the bias will occur during sperm cell production. *Oedothorax gibbosus* follows the most common X1X20 sex determination system in spiders [9], in which males are characterized by one set of sex chromosomes X1X2, while in females two sets are present, X1X2X1X2 (J. Král & D. Vanacker 2002, unpublished results). Hence, males produce two types of sperm cells: a male-determining type without sex chromosomes (0-sperm) and a female-determining type with one set of sex chromosomes (X1X2-sperm). Estimates of the proportion of both types can be obtained through cytological techniques enabling the visualization of the sex chromosomes [10]. Unfortunately, such cytological techniques are very time-consuming and yield a small subsample of sperm cells. By contrast, flow cytometry allows rapid examination of thousands of sperm cells based on DNA content [11]. Flow cytometry has been used in arthropod studies to determine brood sex ratio [12], sperm number [13] and genome size [14], and is often used in mammals for sex preselection of embryos [11]. Here, we expand the use of this technique to sexing sperm in an arthropod species.

In this paper, we show that (i) flow cytometry is an accurate method to determine the proportion of sperm types in arthropods and (ii) that male *O. gibbosus* produce an equal proportion of male- and female-determining sperm cells,



**Figure 2.** Flow cytometric DNA histogram of PI-stained nuclei of a pedipalp of a male *O. gibbosus*. The two overlapping peaks (a) consist of haploid 0 (left, PI value: 3338)- and X1X2-sperm nuclei (right, PI value: 3960). The third peak (b) represents somatic diploid nuclei (PI value: 5777).

hence suggesting that other factors are influencing sex ratio bias in this spider species.

## 2. Material and methods

### (a) Experimental set-up

Subadult *O. gibbosus* (Blackwell, 1841) males were caught by hand from two populations in Belgium (Damvallei (51,057° N; 3.831° E) and Walenbos (50.927° N; 4.863° E)) and reared in the laboratory under standard conditions [7]. Upon reaching adulthood, males transfer a droplet of sperm onto a sperm web, after which loading of the pedipalps occurs. The charged pedipalps, being a modified first pair of legs, subsequently transfer the sperm to the reproductive organ of the female (epigyne) [15]. Reloading of the palp occurs after mating (Bram Vanthournout 2010, personal observation). Thirteen adult males were mated with up to four virgin females. Offspring were reared to adulthood to determine tertiary sex ratio (number of adult males/total number of adult offspring). Probability of difference from an even sex ratio was calculated using a binomial test. After their last mating and reloading of the palps, males were anaesthetized by placing them in a freezer for 1 min. Pedipalps were clipped off, and DNA of the isolated nuclei was stained with propidium iodide (PI) using the protocol described in [12,16]. Since pedipalps contain diploid somatic cells and haploid sperm cells with and without sex chromosomes, it is expected that three populations of nuclei are observed. Pedipalps of seven virgin males were used to determine sperm ratio before mating and thus to verify whether the sperm ratio remains constant over successive ejaculates. DNA content analysis of nuclei was performed on a FACSaria flow cytometer (Argon laser emitting at 488 nm) and the resulting data were processed using FlowJo (TreeStar Inc).

### (b) Statistical analysis

Before analysis, nuclei were selected by visual inspection of a two-dimensional plot depicting PI fluorescence intensity, which reflects the DNA content of each nucleus, and forward scatter (FSC), which is used as a proxy for particle size (figure 1) [17].

Estimates of the proportion of 0- and X1X2-containing sperm,  $p_1$  and  $p_2$ , respectively, were obtained by fitting the observed PI fluorescence intensity to a mixture of two normal distributions

**Table 1.** Proportion of male-determining sperm (0-sperm) in Damvallei (D) and Walenbos (W) males of *O. gibbosus*. Sex ratios represent the proportion of male offspring per female. When males were mated with multiple females, multiple sex ratios are shown. Sex ratios that differ significantly from 0.5 (as calculated by a binomial test) are indicated with an asterisk.

| male | pedipalp | proportion of 0-sperm<br>(male-determining)<br>[credibility interval] | no. counted<br>sperm nuclei | no. hatched<br>offspring | no. adult<br>males | no. adult<br>offspring | sex<br>ratio |
|------|----------|-----------------------------------------------------------------------|-----------------------------|--------------------------|--------------------|------------------------|--------------|
| D    | 1 and 2  | 0.51 [0.47; 0.54]                                                     | 2286                        | 40                       | 16                 | 33                     | 0.48         |
|      |          |                                                                       |                             | 26                       | 3                  | 12                     | 0.25         |
|      |          |                                                                       |                             | 12                       | 0                  | 9                      | 0**          |
| D    | 1        | 0.51 [0.46; 0.57]                                                     | 1413                        | 49                       | 2                  | 43                     | 0.05**       |
|      |          |                                                                       |                             | 19                       | 2                  | 10                     | 0.2          |
| D    | 1        | 0.53 [0.49; 0.58]                                                     | 1119                        | 57                       | 28                 | 55                     | 0.51         |
|      | 2        | 0.54 [0.50; 0.59]                                                     | 907                         | 21                       | 5                  | 10                     | 0.5          |
| D    | 1        | 0.53 [0.50; 0.56]                                                     | 2877                        | 14                       | 1                  | 14                     | 0.07**       |
|      |          |                                                                       |                             | 15                       | 3                  | 15                     | 0.2*         |
| D    | 1        | 0.50 [0.44; 0.58]                                                     | 1742                        | 37                       | 14                 | 30                     | 0.47         |
|      |          |                                                                       |                             | 55                       | 14                 | 53                     | 0.26**       |
|      |          |                                                                       |                             | 13                       | 1                  | 7                      | 0.14         |
| D    | 1        | 0.50 [0.46; 0.54]                                                     | 1628                        | 29                       | 16                 | 26                     | 0.62         |
|      |          |                                                                       |                             | 17                       | 3                  | 15                     | 0.2*         |
|      |          |                                                                       |                             | 28                       | 8                  | 25                     | 0.32         |
| D    | 1        | 0.51 [0.47; 0.55]                                                     | 1214                        | 44                       | 20                 | 35                     | 0.57         |
|      | 2        | 0.51 [0.48; 0.55]                                                     | 1970                        |                          |                    |                        |              |
| D    | 1        | 0.53 [0.46; 0.59]                                                     | 840                         | 38                       | 12                 | 35                     | 0.34         |
|      | 2        | 0.53 [0.47; 0.58]                                                     | 1213                        |                          |                    |                        |              |
| W    | 1        | 0.53 [0.50; 0.57]                                                     | 1614                        | 14                       | 3                  | 4                      | 0.75         |
|      | 2        | 0.46 [0.41; 0.50]                                                     | 1178                        | 44                       | 20                 | 37                     | 0.54         |
| W    | 1        | 0.53 [0.50; 0.56]                                                     | 2607                        | 92                       | 47                 | 76                     | 0.62*        |
| W    | 1        | 0.52 [0.48; 0.55]                                                     | 1514                        | 45                       | 22                 | 45                     | 0.49         |
|      |          |                                                                       |                             | 44                       | 16                 | 35                     | 0.46         |
|      |          |                                                                       |                             | 43                       | 15                 | 36                     | 0.42         |
|      |          |                                                                       |                             | 55                       | 19                 | 47                     | 0.40         |
| W    | 1        | 0.51 [0.47; 0.54]                                                     | 1422                        | 54                       | 33                 | 54                     | 0.61         |
|      |          |                                                                       |                             | 20                       | 5                  | 13                     | 0.38         |
|      |          |                                                                       |                             | 63                       | 29                 | 60                     | 0.48         |
| W    | 1        | 0.52 [0.49; 0.55]                                                     | 2016                        | 63                       | 22                 | 60                     | 0.37*        |
|      | 2        | 0.52 [0.49; 0.55]                                                     | 2606                        |                          |                    |                        |              |
| W    | 1        | 0.49 [0.42; 0.56]                                                     | 1362                        |                          | virgin             |                        |              |
|      | 2        | 0.48 [0.41; 0.55]                                                     | 1807                        |                          |                    |                        |              |
| W    | 1        | 0.50 [0.46; 0.53]                                                     | 1445                        |                          | virgin             |                        |              |
|      | 2        | 0.47 [0.43; 0.52]                                                     | 1242                        |                          |                    |                        |              |
| W    | 1        | 0.47 [0.42; 0.54]                                                     | 803                         |                          | virgin             |                        |              |
|      | 2        | 0.48 [0.46; 0.50]                                                     | 1741                        |                          |                    |                        |              |
| W    | 1        | 0.49 [0.42; 0.56]                                                     | 1362                        |                          | virgin             |                        |              |
|      | 2        | 0.48 [0.41; 0.55]                                                     | 1807                        |                          |                    |                        |              |
| D    | 1        | 0.49 [0.43; 0.54]                                                     | 1210                        |                          | virgin             |                        |              |
|      | 2        | 0.48 [0.44; 0.52]                                                     | 1101                        |                          |                    |                        |              |
| D    | 1        | 0.52 [0.48; 0.57]                                                     | 1390                        |                          | virgin             |                        |              |
|      | 2        | 0.53 [0.48; 0.58]                                                     | 2038                        |                          |                    |                        |              |
| D    | 1        | 0.54 [0.48; 0.60]                                                     | 1443                        |                          | virgin             |                        |              |

\* $\leq 0.05$ ; \*\* $< 0.01$ .

with unknown means ( $\mu_i$ ), proportion ( $p_i$ ) and a common unknown variance ( $\sigma^2$ ), i.e.

$$X \approx \sum_{i=1}^2 p_i N(\mu_i, \sigma),$$

where  $X$  represents the particle PI fluorescence intensity.

The model was fitted to the data using a Bayesian approach as implemented in WinBUGS v. 1.4 [18]. As prior distributions, we specified a Dirichlet (1,1) distribution for sampling both proportions, a normal distribution with mean 0 and precision (i.e.  $1/\sigma^2$ ) of  $1.0 \times 10^{-6}$  as prior distribution for  $\mu_i$  and a uniform (0,100) distribution for  $\sigma$ . Markov chain Monte Carlo chains were run for 10 000 generations, and the first 2000 generations were discarded as a burn-in period when obtaining the posterior densities. This procedure enables the determination of posterior densities of the produced proportion of each class of sperm cell. From these estimates, we calculated the mean and coefficient of variation for each peak. WinBUGS code to perform the analysis can be found in the electronic supplementary material.

### 3. Results

Based on the dot plot of nuclei isolated from the pedipalp of an adult male, two dense clouds can be distinguished (figure 1). Cloud (*a*) is characterized by a small nucleus size and a PI fluorescence intensity approximately half that of the nuclei forming cloud (*b*). The distribution of the PI fluorescence intensity values in cloud (*a*) shows a bimodal pattern, suggesting that 0-sperm nuclei (left peak) and X1X2-nuclei (right peak) are present, while the double PI fluorescence intensity of cloud (*b*) suggests that these correspond to diploid nuclei in the pedipalp (figure 2). To test whether cloud (*a*) indeed corresponds to sperm nuclei, we also analysed two male legs and revealed that a cloud of nuclei similar to (*b*) is retained, while a cloud similar to (*a*) is absent (electronic supplementary material, figure S1). For all males ( $n = 11$  and  $n = 9$ , for Damvallei and Walenbos, respectively), estimates of the proportion of male-determining sperm were obtained with an average precision of about 5% (table 1). Moreover, a high repeatability was found between the estimates of both pedipalps of one male. As the 95% credibility interval of the proportion of 0-sperm included 0.5 for all investigated males, no evidence was obtained for the production of unequal amounts of X1X2-sperm and 0-sperm nor for any difference in proportion between the two populations nor between the sperm ratios of mated and virgin males.

### 4. Discussion

We investigated whether the unequal production of sperm with or without sex chromosomes can explain the sex ratio variation in a female-biased erigonid spider species. Using flow cytometry, we found no evidence of a biased

production. As the offspring sex ratio of these males was often significantly female-biased (table 1), this is strong evidence that biased sperm production is not contributing to the sex ratio bias in this species.

It is therefore likely that other mechanisms, active during or after sperm transfer, play a role. As the female-biasing effect of the endosymbiont *Wolbachia* is considered to be maladaptive from a host's perspective, spider nuclear genes suppressing the biasing factor or male-biased sex ratios are likely to evolve in the framework of genetic conflict theory [19]. If these are active in only males or in both sexes, they would account for the observed additional sex ratio variation in our previously published pedigree analysis [7]. Alternatively, the additional variation could also be explained by cryptic female choice. This is particularly expected in spiders, as encapsulated sperm is transferred by the male and activation within the female reproductive tract is required for fertilization [20]. In line with this, in another erigonid spider, *Pityohyphantes phrygianus*, female post-mating position influences offspring sex ratio, perhaps as a consequence of differential storage within the spermatheca [21].

These results should ideally be complemented with a screening of the number of live and dead sperm combining live- and dead-cell stains to account for non-functional 0-spermatozoa, as this could contribute to the female bias. This is not detectable with the current methodology, as the protocol requires free nuclei for an accurate DNA content determination [22]. The accuracy of the estimated proportions is highly dependent on the difference in DNA content between the two classes of sperm cells, which is influenced by the ratio of autosomal to sex chromosomes and the difference in DNA content between the sex chromosomes. This difference has to be large enough to allow an irrefutable assignment of nuclei to either class. Although the study species has an X1X20 sex determination system, this technique is not limited to species with sperm cells with and without sex chromosomes as it is often used in mammalian species with an XY sex determination [11].

By introducing a novel flow cytometry method, we show that the female-biased spider *O. gibbosus* produces equal amounts of sperm types and, therefore, that mechanisms in addition to endosymbiont infection are contributing to the sex ratio variation.

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**Data accessibility.** A text file containing the WinBUGS code with an example dataset can be found in the electronic supplementary material. The full dataset can be found in the electronic supplementary material.

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