B-chromosomes and male-biased sex ratio with paternal inheritance in the fairy shrimp *Branchipus schaefferi* (Crustacea, Anostraca)

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This study reports on male-biased sex ratios in west Mediterranean populations of the freshwater anostracan *Branchipus schaefferi* (Crustacea, Anostraca, Branchipodidae), in contrast to populations elsewhere. Crossing experiments over several generations indicate a clear paternal inheritance of the trait, possibly with a dosage effect. Various mechanisms

which may underlie this phenomenon are discussed, the most plausible being the presence of one or more supernumerary ('B') chromosomes – as evidenced by karyological observations – interfering with sex determination and probably having an accumulation mechanism in male individuals. *Heredity* (2002) **88**, 356–360. DOI: 10.1038/sj/hdy/6800061

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Introduction

Many animal species include populations composed of unequal numbers of both sexes, a phenomenon known as sex ratio distortion (SRD). SRD may often be under the control of the mother herself, but in many instances sex ratio distorting elements are involved. Sex ratio distorters are a class of selfish genetic elements (Doolittle and Sapienza, 1980; Orgel and Crick, 1980) enhancing their own spread by favouring the transmitting sex. In invertebrates, facultative SRD may result from extra-chromosomal influences, especially in female-biased populations infected with maternally inherited cytoplasmic microorganisms (O'Neill *et al.*, 1997, and references cited therein). The latter may feminize or kill embryonic males, enhance host fertility, or induce cytoplasmic incompatibility, male sterility, thelytokous (= mother-to-daughter) parthenogenesis or hybrid breakdown. However, in the case of paternally inherited distortion towards the male sex, nuclear transmission of the distorting factor(s) is much more realistic for the mere reason that sperm cell cytoplasm, including extranuclear genes, is largely discarded during spermiogenesis.

Nuclearly transmitted segregation distorters are often associated with B-chromosomes (= Bs, or 'supernumeraries') (Jones and Rees, 1982). Seventy to 80% of all B-chromosomes gain a higher than Mendelian transmission through preferential segregation into functional gametes at meiosis or by directed non-disjunction during mitosis (Beukeboom, 1994). Accumulation may take place mainly in males (Nur, 1962, 1963; Patton, 1977; Camacho *et al.*, 1980) or in females (Hewitt, 1973; Lucov and Nur,

1973). When such selfish DNA is transmitted more efficiently or exclusively by males, it may result in the production of male-biased offspring sex ratios.

An extreme example of paternally inherited SRD towards the male sex has been found in *Nasonia vitripennis* (Hymenoptera: Pteromalidae) populations carrying a B-chromosome called paternal sex ratio (PSR) (Werren *et al.*, 1987; Nur *et al.*, 1988). It occurs in males only and if present, it destroys the paternal chromosome set upon fertilization, rendering the egg haploid with one supernumerary ($n + \text{PSR}$). Due to the haplodiploid sex determination system, the initially diploid (female) embryos will thus develop into PSR-carrying males. Quite obviously, PSR will only spread if more than 50% of the eggs are fertilized (Werren, 1991), a condition observed from maternal sex ratio (MSR) (Skinner, 1982) populations which harbour a cytoplasmic factor causing females to mass-fertilize almost 100% of their eggs. Driving the host's sex ratio toward 100% males would be deleterious for PSR in the long term as females become rare (Hamilton, 1967), so counteracting mechanisms against the driving elements often coexist.

A B-chromosome inheritance pattern resembling that of *N. vitripennis* has been found in the flatworm *Polycelis nigra* (Turbellaria, Tricladida) (Beukeboom *et al.*, 1996). In contrast, a sex ratio effect is lacking due to the animal being hermaphroditic with gynogenetic parthenogenesis; cross-insemination occurs, enabling biparental transmission of the B, whereas the paternally inherited standard chromosomes are always expelled from the fertilized egg.

We discovered paternally inherited SRD towards the male sex in North African and Spanish populations of the diploid fairy shrimp *Branchipus schaefferi* (Crustacea, Anostraca, Branchipodidae), as opposed to the normal 1:1 sex ratio seen elsewhere in the Mediterranean region. This paper reports on crossing experiments and cyto-

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logical observations suggesting the SRD may be induced by one or more supernumerary chromosomes in males.

Materials and methods

Most of the experimental work was carried out on one or more of the following three stock populations in culture in our laboratory: (i) Algeria (Boughzoul, Medea), (ii) Spain (Pueblo de Alcolea, Castellón), and (iii) Austria (Neustadt, Wien),

According to Bianchi Bullini *et al* (1968), *B. visnyai* has a diploid genome with $2n = 20$ chromosomes. Since *B. visnyai* is synonymous to *B. schaefferi* (Beladjal and Mertens, 1999; Zarattini *et al*, 2001), $2n = 20$ ($n = 10$) chromosomes are expected to make up the *B. schaefferi* nucleus. Therefore, 10 males and 10 females from each *B. schaefferi* population were fixed (5 min) in a freshly made 1:1 mixture of glacial acetic acid and absolute ethanol, wherein the gonads were dissected. Upon rinsing (3 min) in distilled water, the gonads were transferred to microscope slides and a drop of the DNA-specific fluorescent dye DAPI (4',6-diamidino-2-phenylindole) (1 µg/ml) was added. The preparation was gently squeezed with a cover slip, sealed and viewed under a light microscope (Leitz Laborlux S) equipped with epifluorescence optics in 360 nm UV. This protocol was also adopted to karyotype different Algerian F1 descendants, the latter either displaying SRD or not. In this way, a correlation could be made between numbers of B-chromosomes and the extent of SRD.

Progeny sex ratios were studied as follows. Starting from resting eggs (cysts) from Algeria, Spain and Austria, laboratory populations were initiated. Upon hatching, nauplii to be used in later crossing experiments were transferred to individual 50-ml containers filled with aerated tap water enriched with unicellular algae (*Scenedesmus* sp.) as food source (a few drops of a suspension approximately 5×10^5 cells/ml). At sexual maturity they were pair-mated in plastic bottles (20 replicas for F1 crosses) containing 450-ml aerated tap water and algae. The temperature was kept at a constant 25°C ($\pm 1^\circ\text{C}$). Culture vessels were cleaned and food added daily; one-third of the medium was replaced weekly. Cyst production was checked every day and if cysts were present they were harvested with 100-µm sieves, dried at 35°C and counted to determine the clutch size. A few days later the cysts were artificially hatched in distilled water and virgin adults could be sexed by the end of their first week on the basis of external sexual characters (females have an ovisac and males possess typically-shaped second antennae). Sex ratios were calculated as % males.

SRD was assayed by binomial tests based on critical values for proportions by Rohlf and Sokal (1995). Further statistics were carried out with Statistica for Windows (Kernel release 5.5, Statsoft Inc, 2000). The Spearman test was used to address the question of differential female mortality as a hypothetical explanation for male-biased sex ratios. Other important variables such as fecundity, cyst hatchability (or fertility) and longevity were also tested (using the Mann-Whitney U-test) in order to determine their relation to the SRD trait.

Finally, *B. schaefferi* samples collected from five countries (Algeria, Morocco, Spain, Jordan and Turkey) were checked for sex ratios in the field.

Results

Chromosome counts of the Algerian SRD *B. schaefferi* sperm cells clearly showed different haploid conditions: 10 to 13 chromosomes (Figure 1), in contrast to the usual 10. Bianchi Bullini *et al* (1968) have mentioned that in a *B. visnyai* (a synonym of *B. schaefferi*) telophase sometimes one or two neatly isolated chromosomes could be seen. These were probably the same supernumeraries as shown in Figure 1. Interestingly, females had no more than 10 chromosomes per haploid set; the same holds true for both sexes from Spain and Austria.

Only the Algerian shrimps showed a biased sex ratio of about 2:1 (mean 63.1% males). Since the experimental conditions were identical throughout, sex ratio differences could not be ascribed to environmental influences on sex determination. In order to clarify these findings, experimental crosses were set up. Out of 20 parental couples per population, 12 of the Algerian, eight of the Austrian and seven of the Spanish were useful for F1 sex ratio counts (Table 1). The remainder produced an insufficient number of cysts. The Algerian F1 sex ratio was approximately 2:1 (pooled 63.7% overall, ranging from 40.2% to 99.3% – see Table 1 for details). None of the Austrian or Spanish populations produced male-biased offspring and the sex ratio was very close to 1:1 (51.0% in the Austrian, 49.3% in the Spanish).

Crosses were extended over several generations. Since the Algerian 9F1 (Table 1) generated a male-biased progeny with an acceptable number of females, it was selected for the following reciprocal crosses (performed in twentyfold) with the non-biased 6F1 (see Table 1) to determine the hereditary pattern of the bias: σ 9F1 $\times$ φ 9F1; σ 9F1 $\times$ φ 6F1; σ 6F1 $\times$ φ 9F1. As a control for non-bias, we randomly chose σ 7F1 $\times$ φ 7F1, but σ 6F1 $\times$ φ 6F1 was also included (as depicted in Figure 2). The results (Table 2) indicate that the high male ratio is strictly paternally inherited, more specifically through some fathers rather than through all males. SRD may range from a mild male preponderance to for example over 90% males in some of the 9F1 inbreeding crosses and from crossing σ 9F1 $\times$ φ 6F1 crosses. The offspring of the

Figure 1 DAPI fluorescence micrograph of chromosomes from Algerian *Branchipus schaefferi* sperm cells; left, $n = 11$ in the haploid set, whereas $n = 12$ in the nucleus on the right. Arrowheads point to the latter's two unattached supernumeraries: one very small, the other approximately as large as the smallest A chromosome (= regular, pertaining to the standard *B. schaefferi* genome).

Table 1 F1 progeny sex ratios^a of inbred crosses stemming from cyst populations from three different locations

F1 result ^b	Origin of parental population		
	Algeria	Austria	Spain
1F1	40.2 (368) ^c	46.3 (229)	46.0 (172)
2F1	43.7 (261)	46.4 (252)	47.8 (251)
3F1	48.5 (136)	50.4 (137)	48.7 (115)
4F1	50.3 (457)	50.7 (67)	49.2 (236)
5F1	50.5 (295)	50.8 (354)	49.5 (303)
6F1	53.4 (221)	53.0 (434)	50.2 (309)
7F1	55.8 (120)	53.4 (487)	50.7 (517)
8F1	58.9 (207) ^d	55.7 (79)	
9F1	74.1 (224) ^c		
10F1	77.5 (435) ^c		
11F1	90.0 (440) ^c		
12F1	99.3 (292) ^c		
Pooled Mean	63.7 (3456) ^c	51.0 (2039)	49.3 (1903)
	61.9	50.8	48.9

^aExpressed as % males; total numbers of offspring are given between parentheses; figures in bold face indicate male-biased SR.

^bRanked arbitrarily in ascending order of % males.

^cSRD significant at 0.01 level.

^dSRD significant at 0.02 level.

^eSRD towards female sex significant at 0.01 level.

reciprocal cross ♂ 6F1 × ♀ 9F1 had a sex ratio comparable to that of the parental 6F1. Thus a father from a clutch with a high percentage of males has a reasonable chance of producing skewed offspring, whereas a mother from the same clutch has not. Similar results were obtained when the crosses were repeated (surveyed in Figure 2).

Although the karyotyping of Algerian F1 males was preliminary, there are very sound indications that the SRD is indeed tightly associated with the presence of one or more B-chromosomes. For example, the 2F1 progeny was not distorted and the single karyotyped male had the standard haploid chromosome number of $n = 10$. A 5F1 male, also belonging to a clutch showing no SRD, had $n = 10$ in some cells, but $n = 10 + 1$ in others. A male from the mildly distorted 9F1 had $n = 10 + 1$ chromosomes per haploid cell, whereas $n = 10 + 3$ in the two investigated males from the heavily distorted 12F1.

The percentage of males was not significantly correlated with the number of hatched nauplii (Table 3) (Spearman's coefficient of rank correlation, $r_s = -0.007$, $P = 0.98$). Neither was there any significant difference between the SRD (= Algerian) and normal (= either Spanish or Austrian) shrimp populations with respect to fecundity, fertility and longevity (Table 4).

Skew in the field-collected samples was observed in two out of four Algerian populations (Plateau du Fadnoun: 37/42 males, Sidi Makhoulouf: 87/126), one out of

Figure 2 Schematic overview of the exclusive paternal inheritance of SRD observed in *Branchipus schaefferi*, based on further experimental data from the Algerian laboratory stock. × denotes loss of the trait.

Table 2 Algerian F2 progeny sex ratios^a of several crossing types (males written in *italics*)

F2 result ^b	Crossing type			
	9F1 × 9F1	9F1 × 6F1	6F1 × 9F1	7F1 × 7F1
1F1	46.2 (117)	52.5 (162)	45.7 (173)	42.4 (66)
2F2	50.5 (95)	52.9 (206)	46.3 (240)	42.4 (59)
3F2	53.1 (81)	53.3 (212)	46.6 (116)	43.2 (37)
4F2	64.7 (34)	53.8 (145)	47.7 (197)	44.2 (154)
5F2	66.7 (18)	57.4 (190)	48.9 (225)	48.2 (220)
6F2	67.6 (37) ^c	58.3 (72)	51.4 (148)	49.3 (144)
7F2	71.6 (109) ^c	60.6 (66)	52.4 (170)	50.0 (124)
8F2	74.4 (82) ^c	64.3 (28)		50.4 (113)
9F2	75.0 (20) ^c	71.4 (224) ^c		52.1 (117)
10F2	75.0 (32) ^c	71.9 (185) ^c		53.8 (52)
11F2	75.9 (112) ^c	80.8 (177) ^c		55.4 (92)
12F2	77.0 (148) ^c	83.8 (68) ^c		56.8 (118)
13F2	80.0 (15) ^c	94.7 (169) ^c		60.2 (123) ^c
14F2	83.9 (93) ^c			60.3 (146) ^d
15F2	90.4 (73) ^c			61.9 (113) ^d
Pooled Mean	69.1 (1006) ^c	65.5 (1904) ^c	48.3 (1269)	52.0 (1678)
	70.1	65.8	48.4	51.4

^aExpressed as % males; total numbers of offspring are given between parentheses; figures in bold face indicate male-biased SR.

^bRanked arbitrarily in ascending order of % males.

^cSRD significant at 0.01 level.

^dSRD significant at 0.02 level.

^eSRD significant at 0.05 level.

Table 3 Algerian artificial hatching (as described in the text) and male percentage from a given number of cysts

Clutch	No. cysts	No. nauplii	% hatching	% ♂
1	2285	1386	60.66	50.2
2	1520	1136	74.74	58.5
3	1077	922	85.61	99.3
4	1587	1350	85.07	92.5
5	207	197	95.17	51.2
6	291	165	56.70	89.6
7	267	203	76.03	52.3
8	612	441	72.06	54.5
9	590	473	80.17	68.2
10	292	172	58.90	63.8
11	342	227	70.06	62.8
12	488	347	71.11	53.1
13	161	106	65.84	60.6
14	182	120	65.93	71.9
15	238	176	73.95	52.6
Mean	674.73	494.73	72.80	65.41

Table 4 Mean values (± standard deviation) for life parameters other than sex ratio. All groups consisted of 13 female and 13 male parents. The Mann-Whitney U-test pointed out there were no significant differences between the groups

	Fecundity (no. of cysts)	Hatchability of cysts (no.)	Longevity of parents (days)
Algeria (SRD)	1869 (±652)	1153 (±413)	35 (±10)
Spain (no SRD)	1671 (±610)	1052 (±384)	35 (±9)
Austria (no SRD)	2235 (±929)	1348 (±561)	37 (±7)

two Spanish populations (Benicarló: 183/300), and one out of four Moroccan populations (Settat: 67/84), but neither in the Jordanian nor the Turkish stocks, indicating the *B. schaefferi* SRD trait is limited to the west Mediterranean.

Discussion

Several causes may underlie the paternal sex ratio phenomenon seen in *B. schaefferi*: (1) B-chromosome action, (2) differential mortality of females, eg due to a female sex-linked lethal allele, (3) differential female diapausing, and (4) a Y-chromosome with meiotic drive. Let us consider the different options.

B (PSR) transmission and expression were detected in *Nasonia* by single pair crosses (Werren *et al*, 1987) between males bearing PSR and standard females, as we found with *B. schaefferi*. Our results show a continual variation in the offspring sex ratio, suggesting it may be determined by several factors (interaction between different allele pairs), or by a variable dosage of one sex factor. In the latter case, a supernumerary chromosome with an accumulation mechanism through spermatogenesis may be a potential candidate. Indeed, a dosage effect may be linked to a variable number of Bs. Moreover, in at least one well-studied example (the New Zealand frog, *Leiopelma hochstetteri*), sex determination is under control of one single B-chromosome (Green, 1988). However, this does not seem to have an accumulation mechanism and, contrary to *B. schaefferi*, the female is the transmitting sex.

From a parasitologist's viewpoint, the skewed sex ratio in the Algerian *B. schaefferi* population, if B-chromosome directed, should be seen as an equilibrium between parasite accumulation (ie the transmission drive of selfish DNA, in this case B-chromosomes) and the accompanying negative fitness effects on the host population (Harvey, 1989). The equilibrium sex ratio is seemingly established around 2:1 in the laboratory culture but seems to be 4:1 in nature. Decreased fitness resulting from B overload may arise through lower viability and/or fecundity, in addition to an increased developmental time in males (Nur, 1966a, b), lower viability in both sexes (Nur, 1977), and/or sperm dysfunction (Hewitt *et al*, 1987). On the other hand, in B-carrying individuals an increased chiasma formation among autosomes is often observed (John and Hewitt, 1965; Patton, 1977; Camacho *et al*, 1980), perhaps leading to genetic rearrangements resistant to B-chromosome action (Harvey, 1989). Possible effects on fecundity, fertility (or hatchability of eggs) and longevity associated with SRD in *B. schaefferi* tested negative.

Nonetheless, parameters such as hatchability need careful interpretation in our case since over 90% of all anostracan cysts enter diapause after deposition. These will hatch to a variable degree if artificially induced to do so by suspending them in distilled water for a few hours. A total eclosion rate of 70–100% may be reached (Table 3). Considering this, differential female diapause or embryonic lethality can be ruled out as the SRD source in *B. schaefferi*; otherwise progeny sex ratios over 70% (the minimum that ecloses) would be impossible.

The fourth possibility, a Y chromosome with meiotic drive as is known for example from mosquitoes (Hickey and Craig, 1966), cannot be entirely excluded. Notwithstanding this, the dosage effect for which preliminary

indications are apparent from our experiments would then have to be accounted for by a second, variable factor, again suggesting Bs as the driving force.

Figure 2 summarizes the transmission pathways, deduced from crossing experiments as described above spanning five generations, of the unique SRD trait with paternal inheritance of *B. schaefferi*. There is also evidence for the occasional loss of the trait, which could be considered as a natural event counterbalancing SRD and reducing the probability of creating all-male host populations which would soon become extinct.

Further research is now being focused on the dosage effect of the Bs as the most likely force underlying continual variation of SRD in *B. schaefferi*. If confirmed, this could potentially become an important empirical system.

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