Paternal inheritance of the primary sex ratio in a copepod

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Abstract

Uniparentally inherited genetic elements are under strong selection to manipulate sex determination in their host and shift the host sex ratio towards the transmitting sex. For any sex-ratio trait, lineage analysis and quantitative genetics are important tools for characterizing the mode of inheritance (biparental vs. maternal vs. paternal) thereby narrowing the field of possible sex-determining mechanisms (e.g. polygenic, sex chromosomes with meiotic drive, cytoplasmic microorganisms). The primary sex ratio of the harpacticoid copepod, *Tigriopus californicus* is often male-biased and is highly variable among full sib families. We found that this extra-binomial variation for the primary sex ratio is paternally but not maternally transmitted in *T. californicus*. Paternal transmission of the primary sex ratio has been well documented in the haplo–diploid hymenoptera but is relatively rare in diplo–diploid organisms. If the sex-ratio trait is paternally transmitted in other closely related harpacticoid copepods it would explain why male biased primary sex ratios are so common in this group.

Introduction

In sexually reproducing organisms, biparentalism is the cornerstone of Fisher's sex-ratio principle (Bull & Charnov, 1988). From the perspective of an autosomal gene, males and females are equally efficient means of getting to the next generation and the optimal sex ratio is therefore 1 : 1 (Fisher, 1930). In contrast, genetic elements that are predominantly transmitted through either males or females are under strong selection to skew the primary sex ratio towards the transmitting sex (Lewis, 1941; Howard, 1942; Hamilton, 1967). Theory suggests that these asymmetrically inherited sex-ratio distorters can drive their host population to extinction (Hamilton, 1967; Cosmides & Tooby, 1981; Taylor, 1990) and populations with extremely biased sex ratios have been observed (Juchault et al., 1993; Jiggins et al., 2000). Their 'selfish nature' is a striking illustration that not all genes operate in the organism's best interests (Dawkins, 1976).

Sex-ratio distorters include sex chromosomes with meiotic drive (Jaenike, 2001), maternally inherited

microorganisms (O'Neil *et al.*, 1997; Werren, 1997; Stouthamer *et al.*, 1999; Weeks *et al.*, 2002) and supernumerary 'B' chromosomes (Werren & Stouthamer, 2003). Some populations of fruitflies have an X chromosome with meiotic drive that causes males (the heterogametic sex) to produce predominantly X-bearing sperm resulting in a female-biased sex-ratio (Varandas *et al.*, 1997; Carvalho *et al.*, 1998). In several species of isopod, a cytoplasmically (maternally) inherited bacterium, *Wolbachia*, feminizes genetic males into phenotypic females (Rigaud, 1997). In two species of parasitoid wasp, *Nasonia vitripennis* and *Trichogramma kaykai*, a small, paternally transmitted B chromosome converts diploid female embryos into haploid male embryos by destroying the other paternal chromosomes (Werren & Stouthamer, 2003). In each of the above examples, an asymmetric inheritance pattern selects for genetic elements that skew the primary sex ratio towards the transmitting sex.

Traditional lineage analysis of family sex ratios is often the first step in establishing the presence of a uniparentally inherited sex-ratio distorter (Hurst, 1993). For example, the maternal inheritance of a feminizing genetic element in the isopod, *Armadillidium vulgare*, had been characterized for several decades (Legrand *et al.*, 1987) prior to its molecular identification as the

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bacterium *Wolbachia* (Rousset *et al.*, 1992). Likwise, the paternal sex-ratio (PSR) factor of *N. vitripennis* was accidentally discovered during a selection experiment (Werren *et al.*, 1981) and its mechanism was subsequently elucidated (Werren & Stouthamer, 2003). While lineage experiments cannot pinpoint the mechanism per se, the inheritance pattern (i.e. maternal vs. paternal) can narrow the field of potential candidates. For example, a paternally transmitted sex-ratio trait would eliminate *Wolbachia* (or other maternally inherited microorganisms) and would suggest the presence of a male heterogametic system with Y-drive.

Over the last 5 years, we have studied the sex-ratio trait in the harpacticoid copepod, *Tigriopus californicus*. We have repeatedly found that this splash-pool copepod has a large among family component in the primary sex ratio; i.e. full sibs covary in their tendency to develop into a male (Voordouw & Anholt, 2002b). Experiments investigating environmental sex determination (ESD) suggest that temperature (Voordouw & Anholt, 2002a) and density (unpublished data) do not play a significant role in structuring the sex-ratio variance in *T. californicus*. The sex-determining mechanism for *T. californicus* remains unknown. Karyotypes have found no heteromorphic sex chromosomes (Ar-Rushdi, 1963) and several authors have claimed a polygenic sex-determining mechanism (Ar-Rushdi, 1958; Belser, 1959; Egloff, 1966; Voordouw & Anholt, 2002b).

We reported a parent-offspring correlation in the sex ratio between mothers and daughters but this experiment had poor survivorship in both generations so that we cannot rule out the presence of sex-specific mortality (Voordouw & Anholt, 2002b). In addition, the mother-offspring regression is potentially confounded by maternal effects which is not a problem for the father-offspring regression (Falconer, 1989). The recent demonstration

that the primary sex ratio is paternally inherited in the European fairy shrimp, *Branchipus schaefferi* (Beladjal *et al.*, 2002), motivated us to investigate the paternal contribution to the *sex-ratio* trait in *T. californicus*. Like *B. schaefferi*, we found that the sex-ratio trait was paternally transmitted in *T. californicus*, although the mechanism that generates this inheritance pattern is currently unknown.

Materials and methods

General overview

The lineage experiment consists of three generations: the F_1, the F_2 and F_3 generation (Fig. 1). There are seven different types of relatives and all of them are defined in relation to the F_3 generation. The F_1 generation consists of the paternal grandfathers, the paternal grandmothers, the maternal grandfathers and the maternal grandmothers. The F_2 generation consists of the fathers and mothers and the F_3 generation consists of offspring.

Primary sex ratio of a family

The protocol for determining the primary sex ratio of a family is as follows. We removed the egg sac from a gravid female and allowed it to hatch in a glass spot plate. Shortly after hatching, we haphazardly sampled 24 nauplii from the spot plate. We reared these nauplii to sexual maturity (about 2 weeks under laboratory conditions); at which time it is easy to differentiate between males and females.

Rearing protocol

Each nauplius was reared in its own well in a 24-well tissue culture plate. Each well was stocked with 2.5 mL
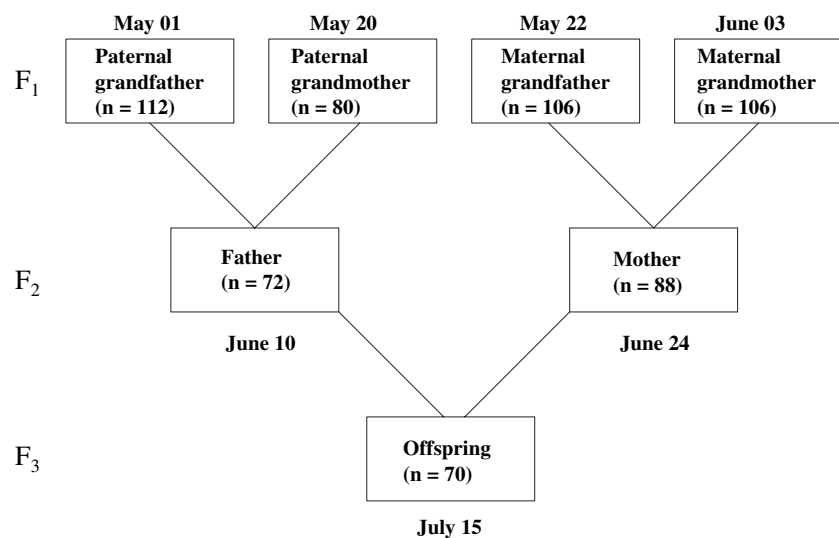
Fig. 1 Experimental design of the lineage experiment showing the three generations (F1, F2 and F3), the seven relatives (paternal grandfathers, paternal grandmothers, maternal grandfathers, maternal grandmothers, fathers, mothers and offspring), the approximate dates on which each relative was born and the sample sizes (*n*).

of filtered sea water, $\sim4.0 \times 10^6$ cells of *Isochrysis galbaena* and ~0.2 mg of Tetramin flakes. Tissue culture plates were stored on top of moist paper towels in covered plastic containers to prevent evaporation. Containers were stored in incubators set at a constant temperature of 20 °C with a 12 h day/night cycle.

Field collection

Like many other crustaceans, *T. californicus* exhibits precopulatory mate-guarding. Under natural mating conditions, adult males typically clasp sexually immature females (copepodite stages I and II) and guard them until they reach sexual maturity (copepodite stage VI) at which time mating occurs (Burton, 1985). To accommodate this precopulatory mate-guarding behaviour, the field collection and rearing of the four grandparents were staggered in time.

We collected ~100 gravid females from the field on each of four sampling dates (1 May, 20 May, 22 May and 3 June 2003) and used the egg sacs from these field-captured females to create the paternal grandfathers ($n = 112$), the paternal grandmothers ($n = 80$), the maternal grandfathers ($n = 106$) and the maternal grandmothers ($n = 106$), respectively (Fig. 1). The 19-day delay (1 May–20 May) between the paternal grandfathers and grandmothers and the 12-day delay (22 May–3 June) between the maternal grandfathers and grandmothers ensured that the sires reached adulthood well before the dams. Likewise, the 14-day delay (20 May–3 June) between the paternal and maternal grandmothers ensured that the F_2 fathers reached adulthood prior to the F_2 mothers.

Mating protocol

The paternal F_1 cross refers to the mating of the paternal grandparents, the maternal F_1 cross refers to the mating of the maternal grandparents, and the F_2 cross refers to the mating of the fathers and mothers (Fig. 1). For all three crosses (paternal F_1, maternal F_1 and F_2), we used a random number generator to pair the families from which the sires and dams were subsequently selected (random pairing).

F_1 mating protocol

For each randomly assigned pair of sire and dam families, we used a mating arena to introduce three haphazardly selected adult males (brothers A, B and C) from the sire family to 12 full-sib copepodites of unknown sex phenotype from the dam family. The mating arena consisted of a single well in a 6-well tissue culture plate stocked with 5 mL of filtered sea water and 5–10 million cells of *I. galbaena*. Once a sexually mature male from the sire family (i.e. a future grandfather) had clasped a

female copepodite from the dam family (i.e. a future grandmother), we removed that couple from the mating arena. We moved the remaining, unclasped copepodites from the mating arena to a 24-well tissue culture plate and reared them to sexual maturity to estimate the dam family sex ratio. The F_1 mating protocol simulated natural courtship in that all female dams were guarded by the sires prior to mating. However, this protocol was very time consuming and so we changed it for the F_2 cross.

F_2 mating protocol

In the F_2 cross, we reared all sire and dam families to sexual maturity prior to mating them. For each randomly assigned pair of sire and dam families, we haphazardly selected two or three adult males from the sire family and introduced them to two or three haphazardly selected adult females from the dam family. In this mating protocol there was no need for a mating arena.

Biological differences between the F_1 and the F_2 cross

In the paternal and maternal F_1 crosses, the males engaged in precopulatory mate-guarding for several days prior to mating. In contrast, for the F_2 crosses, there was no precopulatory mate-guarding and mating occurred immediately following introduction. Furthermore, in the F_1 mating arenas, the males had to search for and select a female copepodite while competing with their two brothers. No such sexual selection occurred in the F_2 crosses.

Sample size considerations and nonindependence of crosses

Over the course of the experiment, the sample size inevitably shrank because some crosses failed to produce offspring. To combat this problem, we often kept two or three replicate couples (A, B and C) for a given cross (e.g. sire family 35* dam family 201). For the replicate couples 35A*201A and 35B*201B, the sires (35A and 35B) are full sib brothers (from family 35), the dams (201A and 201B) are full sib sisters (from family 201) and their offspring will be double first cousins. The genetic coefficient of relatedness among double first cousins is 1/4.

In some cases, we used related individuals to increase the sample size in the next generation. The disadvantage of this approach is that it increases the genetic relatedness among individuals over successive generations. Of the 79 F_2 crosses, four involved fathers who were full sib brothers ($r = 1/2$) and five involved fathers who were double first cousins ($r = 1/4$). The genetic coefficient of relatedness among F_3 families whose fathers were full

sibs or double first cousins is 1/8 and 1/16, respectively. In the results we account for this genetic covariance by aggregating the sex phenotype data across paternal grandfathers. Hence the average number of offspring per family is often greater than 24 individuals.

For the paternal F1 cross, 72 of the 80 couples (112 paternal grandfathers*80 paternal grandmothers) produced F2 offspring (the fathers). For the maternal cross, 88 of the 106 couples (106 maternal grandfathers*106 maternal grandmothers) produced F2 offspring (the mothers). For the F2 cross (after aggregating the four and five F2 crosses whose fathers were full sibs and double first cousins, respectively), 70 of the 72 couples (72 fathers*88 mothers) produced F3 offspring (Fig. 1).

Statistical methods

Larval mortality correction

As we have done previously (Voordouw & Anholt, 2002a,b), we used the larval mortality correction to account for sex-biased mortality. For each plate of full sibs, unidentified individuals were assigned to the less common sex for that plate. The larval-mortality correction tends to reduce the variation in the primary sex ratio and is therefore statistically conservative. Throughout this paper we will refer to both the uncorrected (raw) and the larval-mortality corrected data.

Mean and variance in the proportion of males

For each relative, we used binomial randomization tests to determine whether the observed mean and variance in the proportion of males was significantly different from the Mendelian expectation (i.e. independent assortment of sex chromosomes). For each parent – offspring pair, we used a bootstrap procedure to determine the statistical significance of any differences in the sex-ratio mean and variance between parents and offspring.

To compare the distribution of the proportion of males among relatives, we only included those 70 families that actually produced F3 offspring. Excluded families include those families that were not selected in the mating protocol and those families that failed to produce either sons or daughters due to low survivorship and/or extreme sex ratios. Excluding the latter type of family (low survivorship and/or extreme sex ratios) will tend to reduce the variance and/or the skew in the population sex ratio (i.e. if sex-determination and/or larval-mortality is sex-biased). Our estimates of the sex-ratio mean and sex-ratio variance for each population of relatives should therefore be conservative.

Paternal inheritance of the primary sex ratio

To determine whether fathers have a stronger effect than mothers on the primary sex ratio of their offspring (or *vice versa*), we used multiple regression to compare the partial regression coefficients of the father – offspring and the mother – offspring regressions for all three crosses (F1 paternal cross, F1 maternal cross, F2 cross) separately and combined.

To compare this study to Voordouw & Anholt (2002b)), we treated sex as a quantitative threshold trait (Bulmer & Bull, 1982) and estimated the heritability of sex tendency following the method by Roff (1986,1997) with one important exception. For an individual from the ith family, Roff (1986,1997) calculates the phenotypic value of that individual (on the underlying scale) as the ordinate on the standardized normal curve that corresponds to p_i, the proportion of males in that family. This calculation is wrong because families with 10% or 90% males both have the same probability density (0.175). We used the cumulative standardized normal curve instead so that the above two families have a cumulative probability density of 0.035 and 0.965, respectively.

To estimate the heritability of sex tendency (h^2), we doubled the slope (b) and standard error from each of the six parent – offspring regressions: (1) paternal grandfather – F2 offspring, (2) paternal grandmother – F2 offspring, (3) maternal grandfather – F2 offspring, (4) maternal grandmother F2 offspring, (5) F2 father – F3 offspring, (6) F2 mother – F3 offspring (for the three midparent – offspring regressions, $b = h^2$). Again, we only included those 70 families that actually produced F3 offspring.

Results

Survivorship, male-biased mortality and the larval mortality correction

For the seven types of relatives across three generations we reared 18 094 individuals from 608 different families, sexed 15 754 adults (average survivorship = 87.1%) of which 8179 individuals were males (proportion of males = 0.519). For the seven types of relatives, survivorship ranged between 78.5 and 97.9% (Table 1). Survivorship was lowest for the F3 offspring (78.5%) and the maternal grandmothers (81.0%). For the maternal grandmothers, the correlation between survivorship and the raw proportion of males was positive ($r = 0.485$) and highly statistically significant ($P < 0.001$) suggesting that mortality was male-biased in this relative. The correlation between the raw and the larval-mortality corrected proportion of males was lowest for the maternal grandmothers ($r = 0.744$) and this is expected under low survivorship and/or sex-biased mortality. For all other relatives, there was little or no evidence that mortality was sex-biased and the correlation between the raw and corrected proportion of males was high (Table 1). Hence, we are confident that our results are not biased by sex-linked lethal alleles and that they reflect the segregation of genetic factors that affect the primary sex ratio.

Table 1. Mean survivorship (expressed as percent) ± standard error, the correlation (*r*) between the raw proportion of males and survivorship and the correlation between the raw and larval-mortality corrected proportion of males for all seven types of relatives. Also shown are the sample size (*n*) and the statistical significance (*P*) of the two correlations. Statistically significant correlations are outlined in boldface type.

Relative	*n*	Survivorship ± SE	Raw sex ratio vs. survivorship *r*	*P*	Corrected vs. raw sex ratio *r*	*P*
Paternal grandfather	112	85.3 ± 1.43	0.175	0.066	**0.884**	<0.001
Paternal grandmother	80	96.4 ± 0.88	0.023	0.843	**0.978**	<0.001
Maternal grandfather	106	97.9 ± 0.36	**0.200**	0.040	**0.991**	<0.001
Maternal grandmother	106	81.0 ± 2.16	**0.485**	<0.001	**0.744**	<0.001
F_2 father	72	89.8 ± 2.19	−0.065	0.590	**0.948**	<0.001
F_2 mother	88	90.8 ± 0.99	0.157	0.144	**0.963**	<0.001
F_3 offspring	70	78.5 ± 1.92	−0.081	0.508	**0.899**	<0.001

Mean proportion of males within generations

For the seven relatives, the mean proportion of males for the raw data was generally similar to that of the larval-mortality corrected data (Table 2). The only exception was the maternal grandmothers where – due to a combination of low survivorship and male-biased mortality (Table 1) – the larval mortality correction increased the proportion of males from 0.33 to 0.42. In either case, the mean proportion of males for the maternal grandmothers was significantly female-biased whereas it was significantly male-biased for all other grandparents. The sex ratio was not significantly different from 0.5 for the fathers, female-biased for the mothers and male-biased in the F_3 offspring (Table 2). The observation that the primary sex ratio was significantly male-biased for four of the seven relatives is consistent with the idea of a paternally transmitted sex-ratio factor that has drive relative to the rest of the genome.

Mean proportion of males among generations

Multiple independent two-sample *t*-tests found that the balanced sex ratio in the F_2 fathers was not significantly different from the male-biased sex ratio in either the paternal grandfathers or the paternal grandmothers (Table 3). In contrast, the mildly female-biased sex ratio in the F_2 mothers was significantly different from both the highly male-biased sex ratio of the maternal grandfathers and the highly female-biased sex ratio of the maternal grandmothers (Table 3). Finally the proportion of males in the F_3 offspring was significantly more male-biased than either that of their F_2 mothers or their F_2 fathers (Table 3). Hence, the bias in the primary sex ratio decreased from the F_1 to the F_2 generation and increased slightly from the F_2 to the F_3 generation (Fig. 2). From our random mating protocol, we expect the primary sex ratio to fluctuate at random around the mean of 0.500. With only two observations it is impossible for us to determine whether the fluctuations are random or directional.

Table 2. The mean proportion of males (*P*.male) ± SE and the observed variance in the proportion of males (Obs. Var.) for all seven types of relatives. We only included those families that actually produced F3 offspring (*n* = 70 for each relative). We conducted randomization tests to determine if (1) a relative's mean proportion of males was significantly different from 0.500 (H_0: *P*.male = 0.50) and (2) whether the observed variance (Obs. Var) in the proportion of males was significantly greater than the expected variance (Exp. Var) under Mendelian segregation of sex chromosomes (H_0: Obs. Var. ≤ Exp. Var.). The ratio is the observed variance in the proportion of males divided by the expected variance. For each type of relative, the top and bottom row show the raw and larval mortality-corrected (LMC) data, respectively (as indicated in the 'Data' column). Means and variances that are signifcantly different from the Mendelian expectation are shown in boldface type.

Relative	*n*	Data	H_0: *P*.male = 0.50		H_0: Obs. Var. ≤ Exp. Var.			
			P.male ± SE	*P*	Obs. Var.	Exp. Var.	Ratio	*P*
Paternal grandfather	70	Raw	**0.55 ± 0.020**	<0.001	**0.028**	0.011	2.5	<0.001
		LMC	**0.55 ± 0.017**	<0.001	**0.020**	0.010	1.9	<0.001
Paternal grandmother	70	Raw	**0.56 ± 0.019**	<0.001	**0.025**	0.011	2.3	<0.001
		LMC	**0.55 ± 0.018**	<0.001	**0.022**	0.010	2.1	<0.001
Maternal grandfather	70	Raw	**0.60 ± 0.025**	<0.001	**0.042**	0.010	4.2	<0.001
		LMC	**0.59 ± 0.024**	<0.001	**0.039**	0.010	3.9	<0.001
Maternal grandmother	70	Raw	**0.33 ± 0.021**	<0.001	**0.032**	0.013	2.5	<0.001
		LMC	**0.42 ± 0.015**	<0.001	**0.015**	0.009	1.6	<0.001
F2 father	70	Raw	0.51 ± 0.024	0.497	**0.039**	0.012	3.2	<0.001
		LMC	0.50 ± 0.021	0.799	**0.030**	0.010	3.0	<0.001
F2 mother	70	Raw	**0.46 ± 0.024**	<0.001	**0.041**	0.010	4.2	<0.001
		LMC	**0.47 ± 0.021**	0.016	**0.030**	0.009	3.4	<0.001
F3 offspring	70	Raw	**0.58 ± 0.021**	<0.001	**0.030**	0.008	3.8	<0.001
		LMC	**0.54 ± 0.014**	<0.001	**0.015**	0.006	2.5	<0.001

Table 3. Comparing the sex-ratio mean and the sex-ratio variance between parents and offspring. We only included those families that actually produced F3 offspring ($n = 70$ for each parent–offspring pair). For each of the six parent–offspring pairs, we compared the sex-ratio mean between parents and offspring using an independent two-sample t-test (t_{138}, P_1) and a bootstrap test (P_2). We compared the sex-ratio variance between parents and offspring using an F-test ($F_{69,69}$, P_3) and a bootstrap test (P_4). For each type of relative, the top and bottom row show the raw and larval mortality-corrected (LMC) data, respectively (as indicated in the 'Data' column). Statistically significant t-tests and F-tests are shown in boldface type.

| | | | H_0: $\mu_{parent} = \mu_{offspring}$ | | | H_0: $\sigma^2_{parent} = \sigma^2_{offspring}$ | | |
| | | | t-test | | | F-test | | |
Parent	Offspring	Data	t_{138}	P_1	P_2	$F_{69,69}$	P_3	P_4
Paternal grandfathers	F₂ fathers	Raw	1.364	0.175	0.175	1.406	0.159	0.103
		LMC	1.671	0.097	0.097	1.513	0.088	0.098
Paternal grandmothers	F₂ fathers	Raw	1.646	0.102	0.101	1.577	0.061	0.039
		LMC	1.826	0.070	0.070	1.380	0.184	0.198
Maternal grandfathers	F₂ mothers	Raw	**3.970**	<0.001	<0.001	0.957	0.857	0.809
		LMC	**3.888**	<0.001	<0.001	0.769	0.278	0.194
Maternal grandmothers	F₂ mothers	Raw	**−4.158**	<0.001	<0.001	1.282	0.305	0.265
		LMC	**−2.252**	0.026	0.029	**1.961**	0.006	0.016
F₂ fathers	F₃ offspring	Raw	**−2.330**	0.021	0.024	0.754	0.244	0.171
		LMC	−1.621	0.107	0.109	**0.490**	0.004	0.010
F₂ mothers	F₃ offspring	Raw	**−3.850**	<0.001	<0.001	0.728	0.191	0.109
		LMC	**−2.828**	0.006	0.005	**0.487**	0.003	0.008

Variance in the proportion of males within generations

For the raw data, the observed variance in the proportion of males was, on average, 3.2 times larger than the Mendelian expectation (Table 2; range: 2.3–4.2). For the larval-mortality corrected proportion of males, the average ratio of the observed to the expected variance was 2.6 (Table 2: range:1.6–3.9). Regardless of whether the data were corrected for larval mortality, the observed variance in the primary sex ratio was always significantly greater than the binomial expectation (Table 2). The observation that the variance in the primary sex ratio is conserved across generations is consistent with our random mating protocol (i.e. random mating does not exert any directional selection on the primary sex ratio).

Variance in the proportion of males among generations

For the uncorrected data, multiple F-tests found that the change in sex-ratio variance between parents and offspring was not statistically significant for any of the six parent–offspring pairs (Table 3). For the larval-mortality corrected data, the sex-ratio variance in F₂ mothers is almost twice as great as that of the maternal grandmothers. Conversely, the variance in F₃ offspring is half as great as that of their F₂ fathers and mothers (Table 3). In both cases, this change in variance between generations appears to be the result of differences in survivorship (Table 1; the maternal grandmothers and the F₃ offspring both had low survivorship, compared to their F₂ offspring and F₂ parents, respectively). Hence, there do

not appear to be any meaningful changes in sex-ratio variance between generations suggesting, again, that it was conserved in this experiment.

Paternal inheritance of the primary sex ratio

For the paternal F₁ cross (larval mortality corrected data), the partial regression coefficient for the paternal grandfathers ($b = 0.41 \pm 0.143$) is highly statistically significant ($t = 2.842$, $P = 0.006$) and is much larger than the partial regression coefficient for the paternal grandmothers ($b = -0.01 \pm 0.137$) which is not statistically significant ($t = -0.063$, $P = 0.950$). For the maternal F₁ cross (larval mortality corrected data), the highly significant partial regression coefficient for the maternal grandfathers ($b = 0.34 \pm 0.100$, $t = 3.379$, $P = 0.001$) is almost eight times larger than the nonsignificant partial regression coefficient for the maternal grandmothers ($b = 0.04 \pm 0.159$, $t = 0.272$, $P = 0.787$). For the maternal F₂ cross (larval mortality corrected data), the partial regression coefficient for the fathers ($b = 0.08 \pm 0.088$) is not statistically significant ($t = 0.922$, $P = 0.360$) but still larger than the nonsignificant partial regression coefficient for the mothers ($b = -0.02 \pm 0.088$, $t = -0.207$, $P = 0.837$). When all three crosses are combined (larval mortality corrected data), the highly significant partial regression coefficient for the father ($b = 0.22 \pm 0.062$, $t = 3.463$, $P = 0.001$) was almost eight times larger than that of the mother ($b = 0.03 \pm 0.068$, $t = 0.410$, $P = 0.682$). Hence fathers appear to have a stronger effect than mothers on the offspring sex ratio.

The estimates of the heritability of sex tendency were calculated from parent–offspring regressions (i.e. not
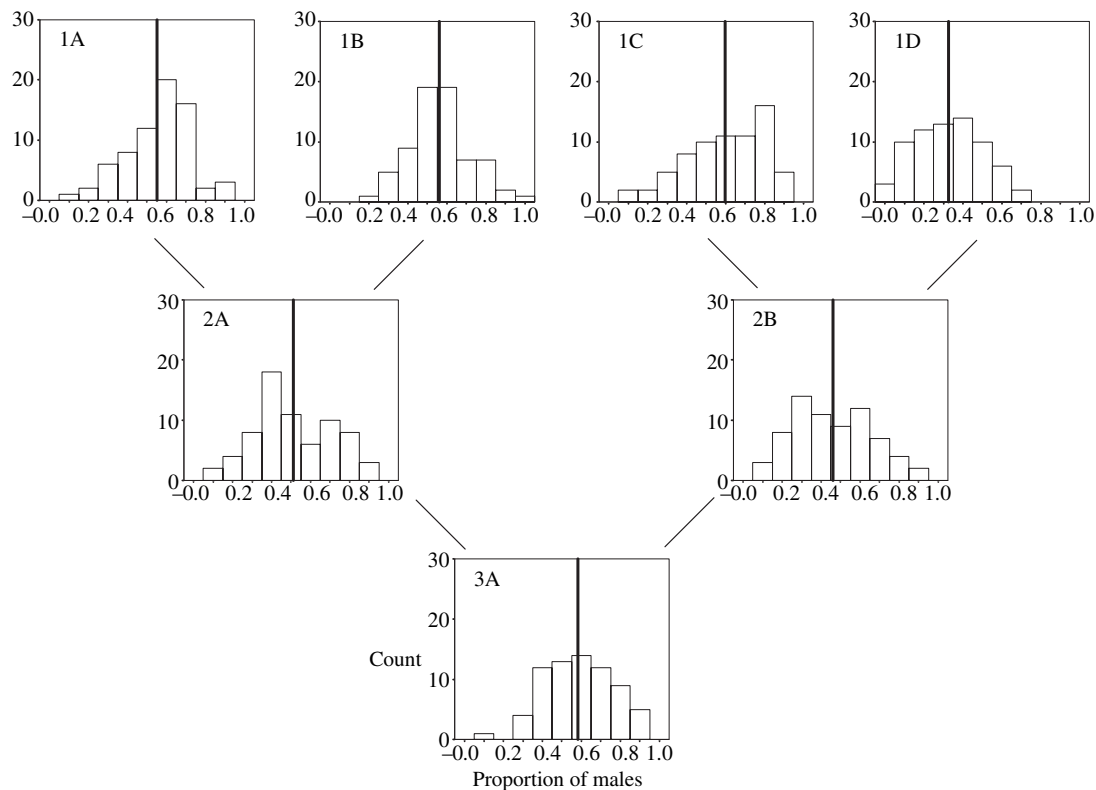
Fig. 2 Distribution of the raw proportion of males for three generations (F_1, F_2 and F_3) and seven relatives (1A: paternal grandfathers; 1B: paternal grandmothers; 1C: maternal grandfathers; 1D: maternal grandmothers; 2A: F_2 fathers; 2B: F_2 mothers; 3A: F3 offspring). We only included those families that actually produced F3 offspring ($n = 70$ for each relative). For each relative, the mean proportion of males (uncorrected for larval mortality) is depicted by the bold-black line.

from the multiple regression involving both parents) and reflect the pattern of transmission of the sex-ratio trait (Fig. 3; Table 4). The heritability estimates are high for the paternal grandfather – F_2 offspring regression and the maternal grandfather – F_2 offspring regression. For the F_2 father – F_3 offspring regression, the heritability of sex tendency is not significantly different from zero, however, if we exclude just two families (F_2 fathers 137 and 151), the heritability from the corrected data is significantly different from zero ($h^2 = 0.36 \pm 0.173$), although the heritability from the raw data is not ($h^2 = 0.07 \pm 0.218$). The F_2 fathers 137 and 151 (marked in black in Fig. 3) were taken from highly female-biased families (two males per 24 adults and three males per 23 adults) but produced highly male-biased families (18 males per 20 adults and 19 males per 22 adults). While we have no *a priori* justification for removing these two families, it is worthwhile to consider their impact on the F_2 father – F_3 offspring heritability estimate. For the mother – offspring regressions, none of the heritability estimates were significantly different from zero (Table 4). The midparent – offspring heritability was statistically significant for both F_1 crosses (due to the large contribution of the grandfathers) but not for the F_2 cross. Hence

the sex-ratio trait in *T. californicus* was clearly paternally inherited in the F_2 generation but not in the F_3 generation.

Discussion

The strength of our results lies in the large sample size (>18 000 individuals), high survivorship (>87%) and our conservative use of the larval mortality correction, which accounts for sex-biased mortality (especially in the maternal grandmothers in Tables 1 and 2). The three major findings of this study are: (1) the mean primary sex ratio is often biased (usually towards males; Table 2) and can change significantly between generations (i.e. maternal grandparents vs. F_2 mothers in Table 3), (2) the observed variance in the primary sex ratio is always larger than the binomial expectation (Table 2) and is similar among generations (Table 3) and (3) this extra-binomial variation in the primary sex ratio appears to be paternally transmitted (at least from F_1 to F_2).

In the present study, the primary sex ratio in two successive field samples dropped from 0.60 ± 0.025 (maternal grandfathers) to 0.33 ± 0.021 (maternal
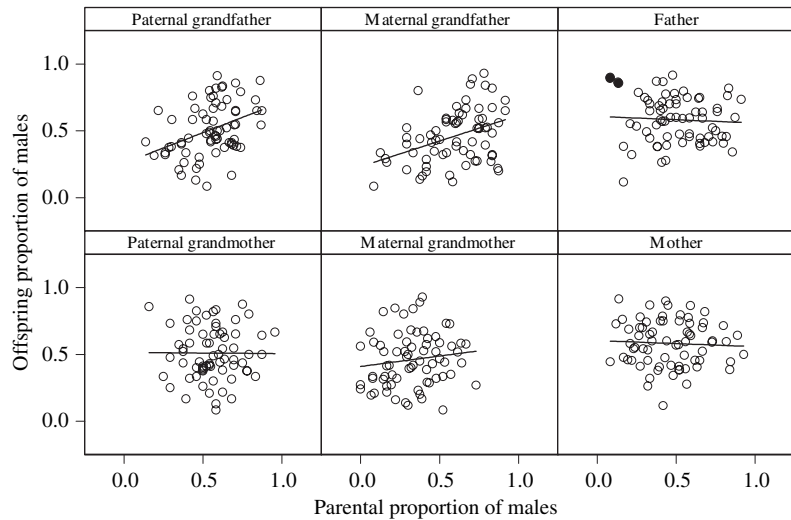
Fig. 3 The relationship in the raw proportion of males for the six parent–offspring pairs (paternal grandfathers: F2 fathers; maternal grandfathers: F2 mothers; F2 fathers: F3 offspring; paternal grandmothers: F2 fathers; maternal grandmothers: F2 mothers; F2 mothers: F3 offspring). We only included those families that actually produced F3 offspring ($n = 70$ for each parent–offspring pair). Shown is the line of best fit from the linear regression of the offspring proportion of males vs. the parental proportion of males.

grandmothers) in less than 2 weeks and changed to 0.46 ± 0.024 in the next generation (F_2 mothers). In the summer of 2002, the mean primary sex ratio in a sample of 47 field-captured females and 167 of their lab-reared daughters changed from 0.59 ± 0.027 to 0.46 ± 0.015 (unpublished data). We have previously suggested that the sex phenotype in adult copepods is influenced by environmental factors operating during naupliar development (Voordouw & Anholt, 2002a). However, the fact that all of these copepods were reared under similar conditions (i.e. 20 °C, 12 h light : 12 h dark cycle, abundant *I. galbaena* food source) argues against this interpretation.

The primary sex ratio is often male-biased in *T. californicus*. In Voordouw & Anholt (2002a), all eight independent estimates of the primary sex ratio (uncorrected for larval mortality) were ≥0.50 (range: 0.50–0.68). In a recent experiment where we manipulated larval density, the mean primary sex ratio in the low (0.71 ± 0.027) and high (0.64 ± 0.030) density treatment was highly male-biased (unpublished data). In the present study, male bias was less obvious but still more common than female bias (Table 2). Igarashi (1963b) reported highly male-biased sex ratios (range: 0.50–0.91) among families in the closely related *T. japonicus*. However, his practice of excluding any family with less than

Table 4. The relationship in the proportion of males between parents and offspring. We only included those families that actually produced F3 offspring ($n = 70$ for each parent–offspring pair). For each of the six parent–offspring regressions, we show the correlation coefficient (r), the F-statistic ($F_{1,68}$), the statistical significance (P) and the heritability of sex tendency and its standard error ($h^2 \pm SE$). For each type of relative, the top and bottom row show the raw and larval mortality-corrected (LMC) data, respectively (as indicated in the 'Data' column). Significant parent–offspring correlations are outlined in boldface type.

Parent	Offspring	n	Data	r	$F_{1,68}$	P	$h^2 \pm SE$
Paternal grandfathers	F_2 fathers	70	Raw	**0.386**	11.902	0.001	0.92 ± 0.260
			LMC	**0.330**	8.308	0.005	0.80 ± 0.272
Paternal grandmothers	F_2 fathers	70	Raw	−0.008	0.005	0.946	-0.01 ± 0.304
			LMC	0.039	0.104	0.748	0.09 ± 0.282
Maternal grandfathers	F_2 mothers	70	Raw	**0.392**	12.338	<0.001	0.78 ± 0.220
			LMC	**0.390**	12.176	<0.001	0.70 ± 0.199
Maternal grandmothers	F_2 mothers	70	Raw	0.138	1.324	0.254	0.32 ± 0.290
			LMC	0.092	0.577	0.450	0.24 ± 0.332
F_2 fathers	F_3 offspring	70	Raw	−0.057	0.221	0.640	-0.08 ± 0.212
			LMC	0.109	0.821	0.368	0.16 ± 0.173
F_2 mothers	F_3 offspring	70	Raw	−0.053	0.191	0.664	-0.07 ± 0.208
			LMC	0.006	0.002	0.962	0.01 ± 0.172
Paternal grandparents	F_2 fathers	70	Raw	**0.266**	5.173	0.026	0.45 ± 0.191
			LMC	**0.240**	4.155	0.045	0.38 ± 0.183
Maternal grandparents	F_2 mothers	70	Raw	**0.384**	11.780	0.001	0.58 ± 0.170
			LMC	**0.355**	9.777	0.003	0.50 ± 0.160
F_2 parents	F_3 offspring	70	Raw	−0.069	0.330	0.568	-0.06 ± 0.133
			LMC	0.072	0.355	0.554	0.06 ± 0.107

perfect survivorship may have left him with an unrepresentative sample, especially considering that female bias and mortality appear to be correlated in this species (Igarashi, 1963a). To date, the male bias in *Tigriopus* and other closely related copepods (Battaglia, 1958; Battaglia & Malesani, 1959) has not been satisfactorily explained.

The absence of maternal transmission in this study was surprising because it contradicts our earlier work (Voordouw & Anholt, 2002b). The mother–offspring heritability of sex tendency in Voordouw & Anholt (2002b) was 1.24 ± 0.400 (recalculated using the cumulative standardized normal curve) and is comparable to the father–offspring heritabilities in Table 4. In Voordouw & Anholt (2002b), the sample size was small (17 mother–offspring pairs), and we could not rule out maternal inheritance of sex-biased mortality (i.e. survivorship was ∼65% in both generations). In the present study, the maternal grandmothers had low survivorship, sex-biased mortality and the highest (although nonsignificant) mother–offspring heritability estimate. This suggests maternal transmission of sex-specific viability differences and highlights the importance of minimizing larval mortality in this study. In addition, the lack of maternal transmission suggests that cytoplasmic sex-ratio distorters such as *Wolbachia* do not play a role in the sex determination of *T. californicus*.

In this study, the sex-ratio trait was paternally transmitted for both the paternal and the maternal F_1 cross but not for the F_2 cross (Fig. 3). Differences in the mating protocol between F_1 and F_2 crosses included precopulatory mate-guarding, age of fertilization and parity of the egg sac. In an earlier study, we found no effect of precopulatory mate-guarding on the primary sex ratio (unpublished data) but it is possible that the lack of mate-guarding in the F_2 cross somehow inhibited paternal transmission of the sex-ratio factor (although we have no idea how this would occur). Volkmann-Rocco (1972) found that delayed fertilization caused females to increase the proportion of males in three species of *Tisbe* (a closely related genus of harpacticoid copepods). If this behaviour exists in *T. californicus* females, variance in the age of fertilization could compromise the father–offspring covariance in the primary sex ratio. If there are parity effects on the primary sex ratio (i.e. due to sperm or maternal aging), differences in egg sac parity between the three generations would likewise lower the parent–offspring covariance. For all the grandmothers in the F_1 cross, we used their first egg sac to produce the F_2 generation. In the F_2 cross by contrast, some of the mothers had already produced a sterile egg sac before they were mated and so we used their second egg sac to produce the F_3 generation. Although parental age and clutch parity were more variable in the F_3 than the F_2 generation these variables were, presumably, the most variable in the F_1 generation where they comprised a haphazard sample from the field population. More likely, paternal transmission in the F_2 cross was compromised by poor survivorship in the F_3 generation (Table 1).

Paternal transmission of the sex-ratio is well known from two species of parasitoid wasp (Werren & Stouthamer, 2003); it was first discovered in *N. vitripennis* (Werren *et al.*, 1981) and was subsequently characterized in *T. kaykai* (Stouthamer *et al.*, 2001). In these two species of parasitoid wasp, a supernumerary chromosome (B-chromosome) in the male's sperm destroys the paternal genome (except themselves) shortly after fertilization. In the haplo–diploid Hymenoptera, males develop from unfertilized eggs and females develop from fertilized eggs. The destruction of the paternal genome by the B-chromosome results in a haploid embryo that then develops into a male (Werren & Stouthamer, 2003). Haploid males produce sperm via mitosis whereas diploid females produce eggs via meiosis. B-chromosomes are lost during meiosis but not during mitosis (Werren & Stouthamer, 2003). Hence from the perspective of the B-chromosome, destruction of the paternal genome is adaptive because it creates a mitotic lineage through which it can be transmitted to the next generation.

Paternal inheritance of a sex-ratio trait has also been reported in a polychaete worm, *Ophryotrocha labronica* (Premoli *et al.*, 1996), and in the European fairy shrimp, *B. schaefferi* (Beladjal *et al.*, 2002). Premoli *et al.* (1996) speculated that the sex-determining mechanism in *O. labronica* is a combination of female heterogamety and a polygenic system that modifies the sex of the embryo following fertilization. However, it is not clear to us why such a system would not generate a mother–offspring correlation as the polygenic modifiers are presumably transmitted through the mother as well. Beladjal *et al.* (2002) showed that the sperm of *B. schaefferi* contains 10 autosomes and anywhere from one to three B-chromosomes. They suggested that the paternally transmitted sex-ratio trait in *B. schaefferi* is associated with the presence of B-chromosomes and did not explain how B-chromosomes would create such a pattern of inheritance in a diplo–diploid organism.

Associations between B chromosomes and sex ratios have been found in other organisms (Henderson, 1988; Lopez-Leon *et al.*, 1996; Vicente *et al.*, 1996). In the ladybird beetle, *Exochomus quadripustulatus*, B-chromosome frequency was positively correlated with the adult proportion of females across 14 populations (Henderson, 1988). In the fish, *Astyanax scabripinnis*, B-chromosomes were more common in females than males and populations with a high prevalence of these B-chromosomes had female-biased sex ratios (Vicente *et al.*, 1996). In the grasshopper, *Eyprepocnemis plorans*, high B-chromosome load in field-mated parents was associated with a male-biased sex ratio in the offspring but this sex-ratio distortion was not detected in lab-mated individuals (Lopez-Leon *et al.*, 1996). Again, for all of these examples, it is not clear how B-chromosomes and the observed sex-ratio bias are related.

One possibility is that these B-chromosomes carry a major sex-determining gene so that all individuals

with one or more B-chromosome develop into one sex (e.g. males) and all individuals who lack a B-chromosome develop into the other sex (e.g. females). We further envision a transmission rate that is some function of the B-chromosome load in the father. Fathers with higher B-chromosome loads transmit more of them to their offspring and consequently have more sons. Barring some sort of fitness advantage, it is not clear how this male-determining B-chromosome could invade and replace an existing sex chromosome system (Camacho *et al.*, 2000). However, we believe that the mechanism described above would account for the paternal inheritance pattern documented in this study and the male biased primary sex ratios commonly observed in *T. californicus* and other harpacticoid copepods.

Future research efforts will concentrate on karyotyping *T. californicus* to check for the presence of heteromorphic sex chromosomes (re: Ar-Rushdi, 1963) and to karyotype the sperm to test whether *T. californicus* males contain B-chromosomes (re: Beladjal *et al.*, 2002) and how these B-chromosomes affect sex determination in their crustacean host.

Acknowledgments

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