

## Genetic variation in the primary sex ratio in populations of the intertidal copepod, *Tigriopus californicus*, is widespread on Vancouver Island

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### ABSTRACT

**Hypothesis:** Genetic variation for the primary sex ratio is widespread in a copepod with polygenic sex determination. Cytoplasmic sex ratio distorters (e.g. *Wolbachia* and microsporidians) influence the primary sex ratio in this copepod.

**Organism:** The intertidal copepod, *Tigriopus californicus*; six populations from Vancouver Island, British Columbia.

**Study site:** Quantitative genetics experiment in the laboratory. PCR and antibiotics experiment to test for the presence of cytoplasmic sex ratio distorters.

**Results:** Genetic variation for the primary sex ratio was found in five of the six populations surveyed. The primary sex ratio was paternally transmitted. There was no evidence that *Wolbachia* or microsporidians influenced the primary sex ratio of *T. californicus*.

**Keywords:** copepod, cytoplasmic feminizers, cytoplasmic sex ratio distorters, heritability, microsporidia, polygenic sex determination, primary sex ratio, Rifampin, *Tigriopus californicus*, *Wolbachia*.

### INTRODUCTION

Adaptive sex ratio theory in sexually reproducing animals explains how individuals should allocate their limited resources to the production of male or female offspring. Fisher (1930) pointed out that the best strategy is to invest equally in both because, on average, sons and daughters each supply half of the genes to the next generation. If the costs of producing sons and daughters are the same, Fisher (1930) predicted that at equilibrium, the sex ratio in the population would be 1 : 1. Two important points to understand about Fisher's sex ratio theory are that (1) it makes predictions about the primary sex ratio, defined here as the

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proportion of males at the time of sex determination, and (2) it refers to a population-wide equilibrium and not an individual strategy (Williams, 1979). Kolman (1960) observed that such an equilibrium may be reached if half of the individuals in the population produce only sons and the other half only daughters, if all individuals produce an equal ratio of sons and daughters, or any combination of these two extremes. Hence in large populations at equilibrium, natural selection has no effect on the variance of individual sex ratio strategies (Kolman, 1960).

Finite population size connects Fisher's population equilibrium argument to the fitness of individual sex ratio strategies (Williams, 1979; Charnov, 1982). In finite populations, random fluctuations in the population sex ratio exert strong selection against son and daughter specialists. This happens because such specialists always lose more (in terms of fitness) when their sex is common than they stand to gain when their sex is in short supply (Verner, 1965; Williams, 1979; Charnov, 1982). In general, strong stabilizing selection for balanced sex ratios is expected to reduce genetic variation in individual sex ratio strategies and increase canalization of the sex-determining mechanism (Williams, 1979). Populations with genetic variation for the primary sex ratio are therefore expected to be rare.

The variance in the primary sex ratio depends on the sex-determining mechanism. Sex-determining mechanisms include sex chromosome, haplo-diploid, environmental, and polygenic mechanisms of sex determination. In male heterogamety (XY males, XX females) and female heterogamety (ZZ males, ZW females), sex is determined by sex chromosomes. In the haplo-diploid Hymenoptera, sex is determined by ploidy; males are haploid, females are diploid. In organisms with environmental sex determination, sex is determined some time after conception by an environmental factor (Bull, 1983). In organisms with polygenic sex determination, sex is determined by a number of genes that are located on different chromosomes (Bull, 1983). In sex chromosome systems, the independent assortment of sex chromosomes ensures that the expected sex ratio is 1:1 at conception (Williams, 1979). The expected variance in such systems follows the binomial distribution (Krackow *et al.*, 2002). Hence, Mendelian segregation of sex chromosomes and its corollary, the binomial distribution of the primary sex ratio, provide the null hypothesis to test whether a population contains extra-binomial variation for the primary sex ratio (Krackow *et al.*, 2002).

The causes of extra-binomial variation in the primary sex ratio include: (1) meiotic drive of sex chromosomes, (2) environmental variation in systems with environmental sex determination, (3) genetic variation in systems with polygenic sex determination, and (4) cytoplasmic feminizers. Meiotic drive of sex chromosomes is when one sex chromosome is present in more than half of the functional gametes at the expense of the other sex chromosome (Jaenike, 1996). Systems with environmental sex determination generate extra-binomial variation for the primary sex ratio if clutches of offspring encounter different environmental conditions during development. Most examples of genetic variation for the primary sex ratio come from organisms with environmental sex determination (Conover and Heins, 1987; Naylor *et al.*, 1988; Watt and Adams, 1994) and the Hymenoptera (Parker and Orzack, 1985; Orzack and Gladstone, 1994). Cytoplasmic feminizers are maternally inherited microorganisms, such as the  $\alpha$ -Proteobacteria *Wolbachia*, that manipulate the sex determination of their hosts to increase their transmission to the next generation (Legrand *et al.*, 1987; Rigaud, 1997). *Wolbachia* also induces parthenogenesis, male-killing, and cytoplasmic incompatibility in its arthropod hosts (Stouthamer *et al.*, 1999), but only cytoplasmic feminizers can cause extra-binomial variation in the primary sex ratio. Examples of cytoplasmic feminizers and their arthropod hosts include *Wolbachia* in isopods (Legrand *et al.*, 1987; Bouchon *et al.*, 1998) and

lepidopterans (Kageyama *et al.*, 1998; Hiroki *et al.*, 2002), microsporidians in amphipods (Terry *et al.*, 1998, 1999; Dunn *et al.*, 1993), and a bacterium belonging to the Cytophaga–Flexibacter–Bacteroid group that feminizes a haploid species of mite (Weeks *et al.*, 2001).

In the present study, we investigate variation in the primary sex ratio in the harpacticoid copepod, *Tigriopus californicus* (Baker). *Tigriopus californicus* inhabits splash pools on the Pacific coast and its range extends from Alaska to Baja, California (Haderlie *et al.*, 1980). Splash pools vary in volume from 0.5 to 50 litres and experience substantial annual variation in temperature (5–35°C), salinity (0–100 ppt), and other abiotic factors (Egloff, 1966; Dybdahl, 1995). Pools contain thousands of individuals in the summer; population density in the winter is much lower but reproduction takes place all year round (Burton, 1985; Powlik, 1998). Females mate once, store the sperm, and produce up to a dozen eggs sacs over their lifetime with 40–80 offspring per egg sac (Haderlie *et al.*, 1980). Hatchlings go through six naupliar stages and five copepodite stages before reaching sexual maturity, at which point it is easy to distinguish males from females by their large geniculate antennae. Males use these antennae to clasp virgin female copepodites and guard them until they reach sexual maturity, at which time they mate.

Fifty years ago, Belser (1959) claimed that *T. californicus* was the first example of polygenic sex determination known to science after Ar-Rushdi (1958) selected populations for both male-biased (99.5% male) and female-biased (20.0%) primary sex ratios. Cytological work on its 24 chromosomes has found no heteromorphic sex chromosomes in *T. californicus* or its congeners *T. brevicornis* and *T. japonicus* (Ar-Rushdi, 1963). Microsatellite analysis of the *T. californicus* genome has not identified any sex-linked loci (Harrison and Edmands, 2006). The sex-determining mechanism of *T. californicus* remains unknown. A variety of environmental factors have small effects on the primary sex ratio of *T. californicus*, including temperature (Egloff, 1966; Voordouw and Anholt, 2002a), salinity (Egloff, 1966), UV-B irradiation (Chalker-Scott, 1995), and hydrostatic pressure (Vacquier, 1962; Vacquier and Belser, 1965) but not larval density (Voordouw *et al.*, 2005b). Studies using genotype  $\times$  environment experimental designs have found that the variation in the primary sex ratio among full-sib families is typically much larger than the effect of the environmental factor (Voordouw and Anholt, 2002a; Voordouw *et al.*, 2005b). More importantly, environmental sex determination cannot account for extra-binomial variation in the primary sex ratio in laboratory experiments where environmental factors (temperature, salinity, light regime, density, food levels, etc.) are strictly controlled (Voordouw and Anholt, 2002b; Voordouw *et al.*, 2005a).

We have previously documented extra-binomial variation in the primary sex ratio in two populations of *T. californicus* on Vancouver Island, British Columbia, Canada: one from Arbutus Cove (Voordouw and Anholt, 2002b) and the other from East Sooke Park (Voordouw *et al.*, 2005a). The purpose of the present study was to determine whether extra-binomial genetic variation for the primary sex ratio exists in other populations of *T. californicus* on Vancouver Island. In addition, we wished to determine whether cytoplasmic feminizers influence the primary sex ratio of *T. californicus*. Cytoplasmic feminizers are an untested but plausible explanation of extra-binomial variation in the primary sex ratio of *T. californicus* because they have been reported in a number of other crustacean taxa, including 22 species of isopod (Bouchon *et al.*, 1998) and several species of amphipod (Terry *et al.*, 2004). In isopods, the causal agent is the bacterium *Wolbachia* (Rousset *et al.*, 1992), whereas amphipods are feminized by several microsporidia (Terry *et al.*, 2004). After finding extra-binomial variation in the primary sex ratio in six populations of *T. californicus* distributed across South West Vancouver Island, we assayed these populations for vertically

transmitted *Wolbachia* and microsporidia using standard PCR protocols (and a DAPI staining technique for microsporidia). Recent studies have shown that *Cardinium*, a newly discovered bacterial symbiont from the *Bacteroidetes*, causes feminization as well as parthenogenesis and cytoplasmic incompatibility in a variety of arthropod hosts (Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001; Hunter *et al.*, 2003). We therefore exposed adult *T. californicus* to an antibiotic treatment, which has been used in a number of studies to cure invertebrate hosts of vertically transmitted bacteria such as *Wolbachia* and *Cardinium* and restore normal reproduction (Stouthamer *et al.*, 1990; Weeks *et al.*, 2001; Zchori-Fein *et al.*, 2001; Hunter *et al.*, 2003).

## MATERIALS AND METHODS

### Geographic distribution of the sex-ratio trait in *T. californicus*

In the summer of 2003, we sampled gravid females from six different geographic locations on Vancouver Island, British Columbia: Arbutus Cove (AC), Victoria (48°28'36"N, 123°18'00"W), Cattle Point (CP), Victoria (48°26'16"N, 123°17'29"W), East Sooke Park (ES), Sooke (48°19'32"N, 123°37'49"W), Ross Bay Cemetery (RB), Victoria (48°24'34"N, 123°20'12"W), Crystal Cove (CC), Tofino (49°07'46"N, 125°54'08"W), and Botanical Beach (BB), Port Renfrew (48°31'33"N, 124°26'36"W). *Tigriopus californicus* often swims in the water column and we captured copepods by simply scooping an empty yoghurt container into the splash pool.

In the laboratory, we removed egg sacs from gravid females ( $n = 112, 40, 47, 34, 92,$  and  $68$  for AC, CP, ES, RB, CC, and BB, respectively) and selected 24 live and vigorous nauplii (i.e. large size and exhibit fast escape response) from each egg sac (i.e. a full sib family). To test for full-sib correlations in the primary sex ratio (Roff, 1997), we reared nauplii from the same full-sib family in two separate 24-well tissue culture plates (plates 1 and 2 with 12 nauplii per plate). Within each plate, nauplii were reared individually in wells stocked with 2.5 ml of filtered seawater, approximately 4 million cells of *Isochrysis galbaena*, and ~0.2 mg of Tetramin flakes. Plates 1 and 2 were stored in separate incubators set at a constant temperature of 20°C with a 12-h day/night cycle. We assayed the sex phenotype after individuals had reached sexual maturity (~3 weeks post-hatching).

### Pre-hatching sex-biased mortality protocol check

We were concerned that our selection of the 24 nauplii for each family might inadvertently bias our estimates of the primary sex ratio (i.e. if live, vigorous nauplii are more likely to belong to one sex than the other). In addition, a number of vertically transmitted microorganisms are known to specifically kill male embryos in insects (Hurst and Majerus, 1993), which would also bias our selection. To test whether there was a selection bias, we conducted a side experiment where we chose 24 nauplii using our selection criteria (see previous paragraph) and reared them (see previous paragraph) for a sample of 65 families obtained from Arbutus Cove. We also counted all unhatched eggs and reared all those nauplii that were not chosen by our criteria. The average fecundity of the 65 females was  $44.7 \pm 1.71$  eggs per egg sac and approximately 94% of all eggs reached sexual maturity. A paired-sample *t*-test found that the mean proportion of males in our selected sample ( $0.54 \pm 0.025$ ) was almost identical to that in the unselected sample ( $0.55 \pm 0.024$ ;  $t = -0.342$ , d.f. = 55,  $P = 0.734$ ), demonstrating that selection bias was not a problem in this experiment. Furthermore, there

was a strong correlation in the proportion of males between the selected sample and the egg sac as a whole ( $r = 0.919$ ,  $n = 65$ ,  $P < 0.0001$ ), indicating that the former provides a good estimate of the latter.

### ***Wolbachia* and microsporidia detection**

We tested for feminizing *Wolbachia* and microsporidia in five populations of *Tigriopus californicus*: Arbutus Cove South (ACS), Arbutus Cove North (ACN), Cattle Point (CP), Ross Bay Cemetery (RB), and East Sooke Park (ES). For each of these five populations we isolated DNA from 12 separate samples, each sample containing five egg-sac carrying females that were ground up together for DNA extraction. Hence, there were a total of 60 independent PCR tests in the experiment and a total of 300 female copepods. We used the mitochondrial primer (679<sup>f</sup> – 1241<sup>r</sup>) to determine if our DNA extraction was successful (Bouchon *et al.*, 1998). To detect *Wolbachia*, we used the 16S rDNA primer [99f – 994r (O'Neil *et al.*, 1992)], and *Wolbachia*-infected *Drosophila simulans* females as a positive control (a strain from the Seychelles kindly provided by J.F. Ferveur). To detect microsporidia, we used the SSU rRNA microsporidian-specific primer [V1f – 0530r (Terry *et al.*, 2004)], and used microsporidia-infected *Gammarus roeseli* females as a positive control (Haine *et al.*, 2004). Since a number of pathogenic, not vertically transmitted microsporidia can infect several aquatic organisms including crustaceans (e.g. Freeman and Sommerville, 2003; Stentiford *et al.*, 2007), we had to ensure the detected parasites were vertically transmitted, which is a prerequisite for them to be potential sex-ratio distorters (Terry *et al.*, 2004). We therefore tried to visualize microsporidia in *T. californicus* eggs using DAPI staining and fluorescence microscopy [following the technique of Dunn *et al.* (1993) and Haine *et al.* (2004)] for the two populations (ES and RB) that tested positive for microsporidian primers. For each of the RB and ES populations, we observed the eggs from ten females and for a positive control we DAPI stained the microsporidian-infected embryos of *Gammarus roeseli*.

### **Antibiotics experiment to cure *T. californicus* from bacterial feminizers**

To determine whether bacteria other than *Wolbachia* influence the sex-ratio trait of *T. californicus*, we treated the parental generation with the antibiotic Rifampin™ (obtained from Fisher) and checked the sex ratio of the offspring resulting from all four possible mating combinations: (i) both parents treated with Rifampin™, (ii) only the father treated, (iii) only the mother treated, (iv) neither parent treated. Rifampin™ inhibits prokaryotic DNA-dependent RNA polymerase and we chose it because it has been used to cure a number of arthropod hosts from *Wolbachia* (Stouthamer *et al.*, 1990; Noda *et al.*, 2001; Fenollar *et al.*, 2003; Gotoh *et al.*, 2005) and *Cardinium* (Hunter *et al.*, 2003). A series of pilot experiments determined that two consecutive exposures to 1% Rifampin™ solution (e.g. 100 mg of Rifampin™ in 10 ml of Isochrysis solution), with each exposure lasting 3 h and spaced 24 h apart, was the treatment that maximized survivorship (~75%). Exposures to higher Rifampin™ concentrations (1.5%, 2.0%, 2.5%) or of longer duration (6 h, 24 h, 48 h) had unacceptably low survivorship.

In the summer of 2006, we collected gravid females from two sites: Gordon Head (GH; 48°29'48"N, 123°18'24"W) and Arbutus Cove (AC; 48°28'36"N, 123°18'00"W). For each site we obtained 40 egg sacs (i.e. 40 full-sib families), reared 20 offspring per family, and determined the family sex ratio using standard methods (Voordouw *et al.*, 2005a). For each family

(when possible), we randomly selected four males and four females (hereafter referred to as fathers and mothers, respectively), half of which were exposed to the Rifampin™ treatment at 30 days after hatching. Within each site, we randomly paired the families of fathers with the families of mothers subject to the constraint that matings between brothers and sisters were not allowed. For the Gordon Head and Arbutus Cove treatment we obtained 26 and 27 such pairings, respectively. For each of these 53 pairings, we conducted all four mating combinations (see previous paragraph) at 35–38 days after hatching for a total of 212 such matings. For each mated mother we reared the offspring from a single egg sac to determine the offspring sex ratio. Altogether, 149 of the 212 matings produced at least one adult offspring and therefore an estimate of the offspring sex ratio.

## Statistical methods

### *Larval mortality correction*

Sex-biased mortality during development can generate variation in the sex ratio among families. To control for this problem, we assigned all unidentified individuals to the less common sex of that family. This larval mortality correction (Voordouw and Anholt, 2002a, 2002b) is statistically conservative as it tends to reduce extra-binomial variation in the primary sex ratio as well as the full-sib genetic correlation in the proportion of males. Hence we refer to both the raw (uncorrected) and the larval-mortality corrected data.

### *Geographic distribution of the sex-ratio trait*

A female's clutch of 24 offspring was defined as female-biased if it contained 7 or fewer males ( $7/24 = 0.292$ ) because this is significantly different from the Fisherian expectation ( $P = 0.032$ ). Conversely, a female's clutch of 24 offspring was defined as male-biased if it contained 17 or more males ( $17/24 = 0.708$ ,  $P = 0.032$ ). To determine whether the mean primary sex ratio was significantly biased (towards either males or females) for the six populations, we wrote a randomization protocol (similar to a one-sample *t*-test) in R version 2.7.0 (R Development Core Team, 2007). To test whether the observed variance in the primary sex ratio was greater than expected under Mendelian segregation of sex chromosomes, we wrote another randomization protocol (similar to an *F*-test) in R. For each of the six populations, we estimated the correlation in the proportion of males between plates 1 and 2. This correlation estimates the full sibling genetic correlation in the primary sex ratio (Via, 1984). Finally, we tested whether the mean proportion of males was significantly different among populations using a simple one-way analysis of variance (ANOVA).

### *Wolbachia and microsporidia PCR*

Across the five populations, we tested a total of 60 samples (each sample contained DNA from five gravid females) for the presence of *Wolbachia* or microsporidia DNA. The probability that at least one of these samples will test positive for parasite DNA (i.e. the power of the experiment) is given by the binomial equation

$$P = 1 - (1 - \rho)^n, \quad (1)$$

where  $n$  is the number of samples ( $n = 60$ ) and  $\rho$  is the true prevalence of the parasite across the five populations (i.e. the proportion of female copepods with parasite loads above the detection limit of the assay). Rearranging equation (1) we can calculate, for a given level of

type II error ( $\beta = 1 - P$ ), the minimum true parasite prevalence that our experiment was able to detect:

$$\rho = 1 - (\beta)^{1/n}. \quad (2)$$

For most power analyses,  $\beta = 0.20$  is considered an acceptable level of type II error (i.e. the power of the experiment is 80%).

#### *Antibiotics experiment to cure *T. californicus* from bacterial feminizers*

We used the generalized linear model (glm) function in R to model offspring sex ratio as a function of three fixed factors – Rifampin<sup>TM</sup> treatment in the male, Rifampin<sup>TM</sup> treatment in the female, and site – and two covariates: the paternal and maternal sex ratios, which are the sex ratios of the families from which the fathers and mothers were sampled, respectively. We tested the significance of all the interaction terms by sequentially removing them from the model. The minimal adequate model was the one with the main effects and all statistically significant interaction terms. The residual deviance was approximately five times the residual degrees of freedom, so we used the quasi-binomial distribution to model the error distribution and the *F*-test to assess the statistical significance of terms. The quasi-binomial distribution models the inflated error term directly and is nearly as efficient as the binomial when this distribution applies. The approach is generally preferred over using an *ad hoc* variance inflation factor (McCullagh and Nelder, 1989).

#### *Antibiotics experiment power analysis*

We conducted a retrospective power analysis for the antibiotics experiment. We assumed that mothers contain maternally transmitted bacteria that feminize their offspring and that exposing mothers to the Rifampin<sup>TM</sup> treatment cures them from these bacteria and increases their production of sons. For each of the 149 pairings that produced offspring (i.e. across both the GH and AC sites), we transformed the offspring proportion of males ( $p$ ) to a logit value ( $x$ ) using the relationship,  $x = \ln(p/(1-p))$ . We added a small treatment effect ( $\delta$ ) to the logit values of the offspring from Rifampin<sup>TM</sup>-cured mothers. The logit values were then back-transformed to the expected probability ( $p_e$ ) that individual offspring develop as males using  $p_e = \exp(y)/(1 + \exp(y))$ . We used the rbinom() function in R to generate observed proportions of males ( $p_o$ ) using the  $p_e$  probabilities and the number of offspring obtained from each of the 149 pairings (thereby incorporating binomial variance into the data). We used the glm function in R on the  $p_o$  data to test for a significant difference between Rifampin<sup>TM</sup>-treated and control mothers. We used ten different values of  $\delta$  that generated a range in power from 0 to 1. For each value of  $\delta$ , we ran 1000 randomizations and power was the proportion of times where the  $p$ -value was statistically significant ( $P < 0.05$ ).

## RESULTS

### **Survivorship, sex-biased mortality, and the larval mortality correction**

Survivorship was generally high (range = 85.3–98.8%; Table 1) and the correlation between it and the proportion of males was not statistically significant for any of the six populations (Table 1), suggesting that sex-biased mortality did not occur in this experiment. The correlation between the raw and the larval-mortality corrected proportion of males was close (range = 0.88–1.00) and highly significant (Table 1), indicating that these two estimates

**Table 1.** For each of the six populations, the number of full-sib families ( $N$ ) and the mean survivorship to adulthood (%)  $\pm$  standard error are shown

| Pop. | $N$ | Survivorship $\pm$ S.E. | $r_1$  | $P_1$ | $r_2$ | $P_2$  |
|------|-----|-------------------------|--------|-------|-------|--------|
| AC   | 112 | 85.3 $\pm$ 1.43         | 0.175  | 0.066 | 0.883 | <0.001 |
| CP   | 40  | 91.5 $\pm$ 2.06         | -0.038 | 0.817 | 0.962 | <0.001 |
| ES   | 47  | 87.9 $\pm$ 1.95         | 0.151  | 0.312 | 0.933 | <0.001 |
| RB   | 34  | 94.5 $\pm$ 1.49         | 0.262  | 0.134 | 0.968 | <0.001 |
| CC   | 92  | 96.6 $\pm$ 0.69         | -0.055 | 0.601 | 0.987 | <0.001 |
| BB   | 68  | 98.8 $\pm$ 0.44         | 0.076  | 0.537 | 0.996 | <0.001 |

*Note:* For each family, survivorship is based on 24 offspring. Also shown are the correlation between the raw proportion of males and survivorship ( $r_1$ ), its statistical significance ( $P_1$ ), the correlation between the raw and larval-mortality corrected proportion of males ( $r_2$ ), and its statistical significance ( $P_2$ ).

of the primary sex ratio are virtually interchangeable. In what follows, the results for the raw and larval-mortality corrected data were similar, so for simplicity we only present the raw sex ratio data.

### The primary sex ratio in the geographic survey of *T. californicus* populations

The mean primary sex ratio was generally male-biased (5/6 populations), although the magnitude of the bias was weak and statistically significant in only three populations (Table 2). A one-way ANOVA found no significant difference in the primary sex ratio among the six populations ( $F_{5,387} = 0.82$ ,  $P = 0.534$ ). Across all six populations, 14.8% of the families were strongly female biased (proportion of males  $\leq 0.300$ ), which is almost five times higher than the null expectation of 3.2% ( $\chi^2 = 165.8$ , d.f. = 1,  $P < 0.001$ ). The frequency of female-biased families was highest in Crystal Cove (21.7%) and lowest in Botanical Beach (8.8%; Table 3). Male-biased families (proportion of males  $\geq 0.700$ ) were more common (19.6%). The frequency of male-biased families was highest in East Sooke Park (25.5%) and lowest in Ross Bay (8.8%; Table 3).

The observed variance in the primary sex ratio was, on average, 2.8 times larger and was always significantly greater than the expected variance under Mendelian segregation of sex chromosomes (Table 2). The full sib correlation between plates 1 and 2 was statistically significant for all the populations except Ross Bay (Table 2). Across all six populations, the mean ( $\pm$  standard error) full sib correlation in the proportion of males between plates 1 and 2 was  $0.48 \pm 0.063$ .

### *Wolbachia* and microsporidia PCR

None of the 60 samples from the five *T. californicus* populations and none of the negative controls tested positive for *Wolbachia* DNA, whereas all of the *Drosophila simulans* positive controls tested positive for *Wolbachia*. When the 60 PCR samples from the five populations are considered together, our experiment had sufficient power ( $\geq 80\%$ ) to detect any *Wolbachia* prevalence greater than 3%. Hence we conclude that *T. californicus* is not infected with *Wolbachia*. Microsporidia were PCR-detected in two samples (RB01 and ES06) from two populations (Ross Bay Cemetery and East Sooke Park). Using DAPI staining and



**Table 2.** For each of the six populations, the number of full-sib families ( $N$ ) and the mean sex ratio (proportion of males = p.male)  $\pm$  standard error are shown

| Pop. | $N$ | p.male $\pm$ s.e. | $P_1$ | Obs. Var.<br>( $\times 10^{-2}$ ) | Exp. Var.<br>( $\times 10^{-2}$ ) | Ratio | $P_2$  | $r$   | $P_3$  |
|------|-----|-------------------|-------|-----------------------------------|-----------------------------------|-------|--------|-------|--------|
| AC   | 112 | 0.52 $\pm$ 0.017  | 0.040 | 3.18                              | 1.29                              | 2.5   | <0.001 | 0.318 | <0.001 |
| CP   | 40  | 0.48 $\pm$ 0.029  | 0.199 | 3.28                              | 1.20                              | 2.7   | <0.001 | 0.575 | <0.001 |
| ES   | 47  | 0.55 $\pm$ 0.029  | 0.001 | 3.84                              | 1.25                              | 3.1   | <0.001 | 0.395 | 0.006  |
| RB   | 34  | 0.52 $\pm$ 0.026  | 0.282 | 2.34                              | 1.20                              | 1.9   | <0.001 | 0.312 | 0.072  |
| CC   | 92  | 0.52 $\pm$ 0.023  | 0.160 | 4.89                              | 1.26                              | 3.9   | <0.001 | 0.670 | <0.001 |
| BB   | 68  | 0.54 $\pm$ 0.021  | 0.001 | 3.09                              | 1.10                              | 2.8   | <0.001 | 0.588 | <0.001 |

Note: For each family, the sex ratio is based on 24 offspring. The probability that a population's mean sex ratio was significantly biased from 0.50 is given by  $P_1$ . The probability that the observed sex ratio variance (Obs. Var.) is significantly greater than the expected sex ratio variance (Exp. Var.) is given by  $P_2$ . The ratio is the observed divided by the expected sex ratio variance. For each population, the full-sib correlation in the sex ratio ( $r$ ) and its  $P$ -value ( $P_3$ ) are also shown.

**Table 3.** For each of the six populations, the numbers of male-biased (MB; proportion of males  $\geq 0.700$ ) and female-biased (FB; proportion of males  $\leq 0.300$ ) families are shown

| Pop.  | $N$ | MB | f(MB)        | p(MB)  | FB | f(FB)        | p(FB)  |
|-------|-----|----|--------------|--------|----|--------------|--------|
| AC    | 112 | 19 | <b>0.170</b> | <0.001 | 14 | <b>0.125</b> | <0.001 |
| CP    | 40  | 5  | <b>0.125</b> | 0.009  | 7  | <b>0.175</b> | <0.001 |
| ES    | 47  | 12 | <b>0.255</b> | <0.001 | 7  | <b>0.149</b> | 0.001  |
| RB    | 34  | 3  | 0.088        | 0.094  | 4  | <b>0.118</b> | 0.023  |
| CC    | 92  | 23 | <b>0.250</b> | <0.001 | 20 | <b>0.217</b> | <0.001 |
| BB    | 68  | 15 | <b>0.221</b> | <0.001 | 6  | <b>0.088</b> | 0.022  |
| Total | 393 | 77 | <b>0.196</b> | <0.001 | 58 | <b>0.148</b> | <0.001 |

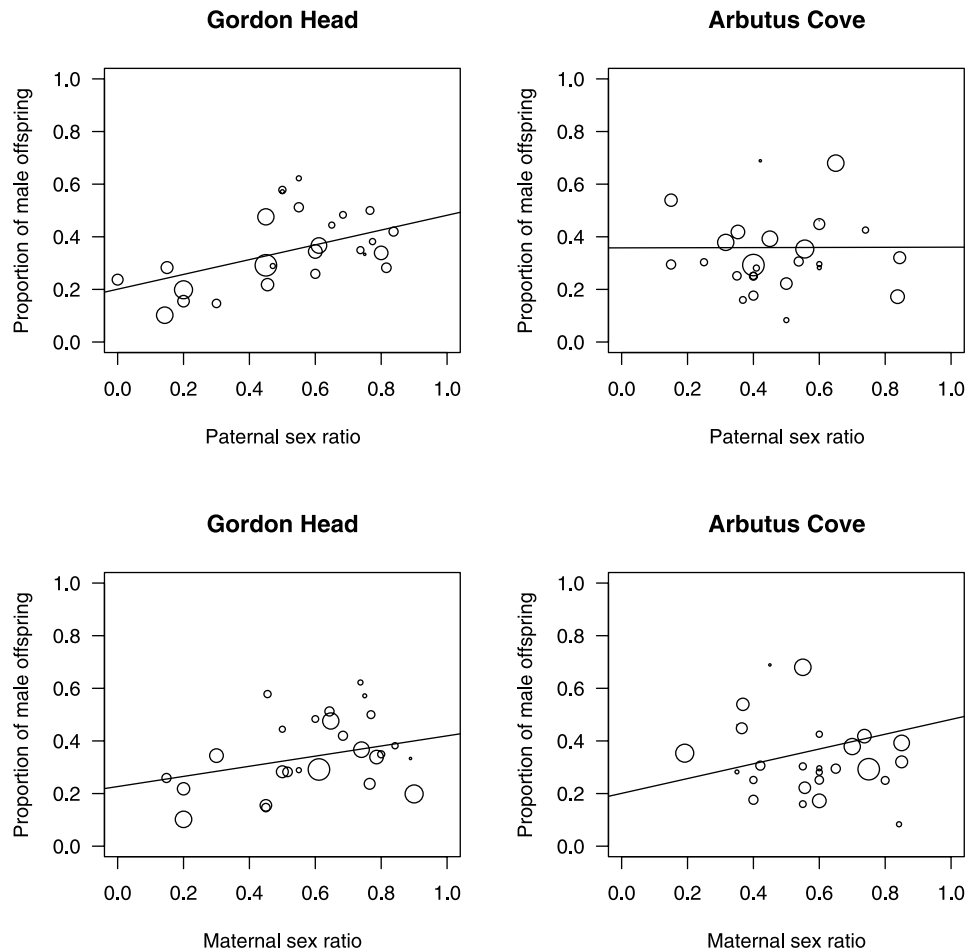
Note: For each population, the number of families ( $N$ ), the frequencies of male-biased (f(MB)) and female-biased (f(FB)) families, and the probabilities of obtaining the observed number of male (p(MB)) and female-biased families (p(FB)) are shown.

fluorescence microscopy, we did not observe microsporidian nuclei in any of the *T. californicus* eggs sampled from either the Ross Bay or East Sooke Park females ( $n = 10$  females for each population).

### Antibiotics experiment to cure *T. californicus* from bacterial feminizers

There was no effect of the antibiotics treatment on whether a mating produced at least one adult offspring or not ( $\chi^2 = 4.79$ , d.f. = 3,  $P = 0.188$ ), but there was a significant effect of site ( $\chi^2 = 15.52$ , d.f. = 1,  $P < 0.001$ ). In Gordon Head, 82.7% (86/104) of the matings produced at least one adult offspring compared with 58.3% (63/108) of the matings in Arbutus Cove. For this subset of 149 matings with an estimate of the offspring sex ratio, the minimal adequate model contained the four main effects – maternal sex ratio, paternal sex ratio, antibiotics treatment, and site – and the site  $\times$  paternal sex ratio interaction. In this model, the antibiotics treatment had no statistically significant effect on the offspring sex ratio ( $F_{3,141} = 1.29$ ,  $P = 0.279$ ). The significant site  $\times$  paternal sex ratio interaction ( $F_{1,138} = 4.05$ ,

$P = 0.046$ ) indicated that the relationship between the sex ratio of the offspring and that of the father was different between Gordon Head and Arbutus Cove. A separate analysis for each site revealed that the paternal sex ratio had a significant effect on the offspring sex ratio in Gordon Head ( $F_{1,81} = 13.21$ ,  $P < 0.001$ ) but not in Arbutus Cove ( $F_{1,58} = 0.02$ ,  $P = 0.886$ ). The relationship between the paternal and offspring sex ratio in Gordon Head remained significant ( $F_{1,24} = 10.19$ ,  $P = 0.004$ ; Figure 1) after combining the offspring for fathers that were sampled from the same full-sib family to avoid pseudo-replication (i.e. the 149 offspring sex ratios in the previous analysis were sampled from 58 different full sib-families). The sex ratio was significantly female-biased in both the Gordon Head (proportion of males = 0.36, 95% CI = 0.31–0.42,  $t = 5.17$ , d.f. = 85,  $P < 0.001$ ) and Arbutus Cove population (proportion of males = 0.36, 95% CI = 0.31–0.41,  $t = 5.81$ , d.f. = 62,  $P < 0.001$ ).



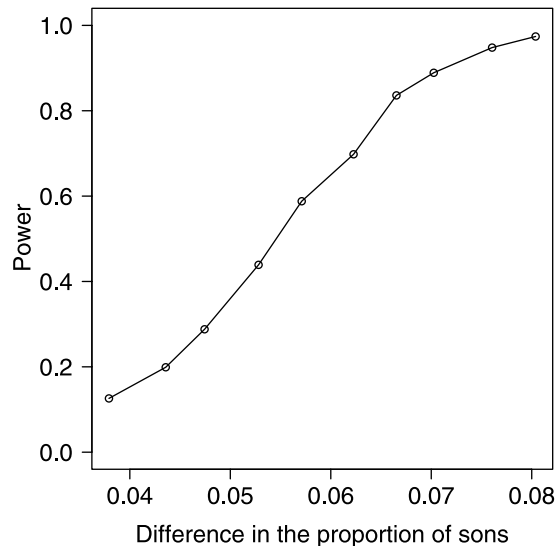
**Fig. 1.** The relationship between the offspring sex ratio and the parental sex ratio in the two sites. The rows show the paternal and maternal sex ratio and the columns show the Gordon Head ( $n = 26$  crosses) and Arbutus Cove ( $n = 27$  crosses) sites. The sizes of the circles are relative to the number of offspring produced by the cross. The lines of best fit from the linear regressions are shown.

### Antibiotics experiment power analysis

The power analysis found that our antibiotics experiment contained more than enough replication to detect all but the smallest effects of the Rifampin™ treatment on the offspring sex ratio (Figure 2). For example, we had >80% chance of detecting a significant effect of the Rifampin™ treatment when the difference in the proportion of sons between the Rifampin™-cured (0.39) and control mothers (0.33) was only 0.06 (Figure 2). In other words, our experiment had the ability to detect changes much smaller than if Rifampin™-cured females produced clutches with equal sex ratios.

### DISCUSSION

Our geographic survey of *T. californicus* populations on Vancouver Island found extra-binomial variation in the primary sex ratio among full-sib families in all six populations and significant genetic correlations in the primary sex ratio in five populations (Table 2). Previous work in our laboratory had primarily focused on a population from Arbutus Cove (Voordouw and Anholt, 2002a, 2002b), but the present results show that genetic variation in the primary sex ratio in populations of *T. californicus* is potentially widespread on Vancouver Island. This result is surprising because *T. californicus* populations go through annual bottlenecks in the winter when their population size is reduced by wave wash (Powlik, 1998). We would expect such drastic variation in population density to eliminate genetic variation for the primary sex ratio and for the sex determination of *T. californicus* to evolve from a polygenic to a two-factor (i.e. a sex chromosome) mechanism (Bull, 1983). We are confident that our results are not biased by sex-biased mortality because the results were the same for the larval-mortality corrected data. Our protocol check shows that the sex ratio of our selected sample of 24 nauplii represents the sex ratio of the whole egg sac.



**Fig. 2.** The probability that our antibiotics experiment detects a statistically significant effect (power) of the Rifampin™ treatment on the proportion of sons increases as the effect size (i.e. the difference in the proportion of sons between the Rifampin™-cured and the control mothers) increases.

We do not believe that some as yet undiscovered environmental factor operating during development caused the extra-binomial variance in the primary sex ratio. The offspring from all of the gravid females were reared under identical conditions. Furthermore, an environmental factor operating during development cannot account for the fact that siblings reared in separate plates have a similar probability of differentiating into a male. The full-sib correlation in the primary sex ratio could be caused by a maternal effect that operates before the siblings were separated. However, we have found no evidence that maternal factors such as the duration of the pre-copulatory mate-guarding period experienced by the mother as a virgin copepodite (a cue for the operational sex ratio) (M.J. Voordouw, unpublished data), the duration of egg sac attachment to the mother, maternal age, or clutch parity affect the primary sex ratio in *T. californicus* (A.-M. Madden, unpublished data). We also found extra-binomial variation in the offspring primary sex ratio when mothers were reared under controlled laboratory conditions (Voordouw *et al.*, 2005a). Thus we do not believe that some unidentified maternal effect caused the extra-binomial variance in the primary sex ratio. As claimed by Belser (1959) almost 50 years ago, *T. californicus* has a polygenic sex-determining mechanism and this appears to be true for all of the populations on Vancouver Island sampled in this study.

The primary sex ratio was slightly male-biased in the geographic survey of *T. californicus* (Table 2). We have found this pattern of male bias in most of our studies on *T. californicus* (Voordouw and Anholt, 2002a, 2002b; Voordouw *et al.*, 2005a, 2005b) as well as in other harpacticoid copepods such as *T. japonicus* (Igarashi, 1963) and *Tisbe gracilis* (Battaglia, 1958). In contrast, the sex ratios in the antibiotics experiment were highly female-biased. The antibiotics experiment was conducted 3 years (~40 generations) after the geographic survey, so it was not surprising that the mean population sex ratio in Arbutus Cove was different between 2003 ( $0.52 \pm 0.017$ ) and 2006 ( $0.36 \pm 0.025$ ). We have seen larger changes in the mean population sex ratio between field samples taken less than 2 weeks apart from the same tide pool (Voordouw *et al.*, 2005a) and between consecutive generations reared under identical conditions in the laboratory (M.J. Voordouw, unpublished data). Similarly, variable sex ratios have been observed in populations of *Armadillidium vulgare* where an unstable *Wolbachia*-derived transposable element has integrated into the wood louse genome and replaced the female sex chromosome (Juchault and Mocquard, 1993; Juchault *et al.*, 1993).

Across the five populations of *T. californicus*, we found no *Wolbachia* and a low prevalence of microsporidians (3.3%). We were unable to detect microsporidian nuclei in the embryos of these animals, suggesting that the parasites are not vertically transmitted to offspring. Furthermore, the microsporidia were not abundant enough to account for the observed sex ratio variance. Pathogenic, non-vertically transmitted microsporidia can exist in a number of crustaceans where they generally reach 2–10% prevalence (e.g. Freeman and Sommerville, 2003; Stentiford *et al.*, 2007; Wattier *et al.*, 2007). The antibiotics experiment further confirmed that Rifampin<sup>TM</sup>-sensitive bacteria such as *Wolbachia* and *Cardinium* do not influence sex determination in *T. californicus*.

The primary sex ratio was paternally transmitted in the Gordon Head but not in the Arbutus Cove population. We have previously shown that the primary sex ratio is paternally transmitted in a *T. californicus* population from East Sooke Park (Voordouw *et al.*, 2005a). Paternal transmission or inheritance of the sex ratio has been well characterized in two species of parasitoid wasp (Werren and Stouthamer, 2003). Here, the mechanism is a selfish B-chromosome that kills the paternal genome upon fertilization. This strategy is adaptive because B-chromosomes are more likely to be transmitted during mitosis in haploid males

than during meiosis in diploid females (Werren and Stouthamer, 2003). Paternal inheritance of the sex ratio has also been documented in the European fairy shrimp, *Branchipus schaefferi* (Beladjal *et al.*, 2002), the polychaete worm, *Ophryotrocha labronica* (Premoli *et al.*, 1996), and the woodlouse, *Armadillidium vulgare* (Juchault and Mocquard, 1993). In *B. schaefferi*, the sperm contains a variable number (0–3) of B-chromosomes, which appear to be associated with the paternally transmitted sex ratio trait. 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 *A. vulgare*, the putative transposable element responsible for sex ratio bias could also be paternally inherited (Juchault and Mocquard, 1993).

Cytogenetic studies of meiosis and the karyotypes of copepods and other crustaceans have revealed a number of unusual features, including chromatin diminution and gonometry (Rasch and Wyngaard, 2006; Rasch *et al.*, 2008), endopolyploidy (Rasch and Wyngaard, 2008; Rubenstein *et al.*, 2008), chromosomes that form rings resulting in achiasmatic meiosis (Chinnappa and Victor, 1979), and intraspecific variation in chromosome number (Grishanin *et al.*, 2006; Torrentera and Abreu-Grobois, 2002). Whether these mechanisms play a role in the extra-binomial variation of the sex ratio in *T. californicus* is not known but future work should search for associations between karyotype and sex ratio. In the closely related *T. japonicus*, a mate-recognition protein that is highly expressed in sexually immature females has been sequenced (Ting and Snell, 2003). Recent developments in gene expression profiling in *T. japonicus* and two other species of copepod (Lee *et al.*, 2005; Eichner *et al.*, 2008; Tarrant *et al.*, 2008) will further enhance our understanding of sex-linked gene expression in copepods.

In conclusion, extra-binomial variation in the primary sex ratio in populations of *T. californicus* appears to be widespread across Vancouver Island. We conclude that populations of *T. californicus* on Vancouver Island have a polygenic sex-determining mechanism that generates the observed full-sib correlation. Cytoplasmic feminizers such as *Wolbachia* and microsporidians that manipulate the primary sex ratio in other crustacean taxa do not influence the primary sex ratio of *T. californicus*. The processes that allow *T. californicus* to maintain such high levels of genetic variation for the primary sex ratio remain to be elucidated.

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