

HERITABILITY OF SEX TENDENCY IN A HARPACTICOID COPEPOD, *TIGRIOPUS CALIFORNICUS*

MAARTEN J. VOORDOUW¹ AND BRADLEY R. ANHOLT²

Department of Biology, University of Victoria, P.O. Box 3020, Victoria, British Columbia V8W 3N5, Canada

¹E-mail: voordouw@uvic.ca ²E-mail: banholt@uvic.ca

Abstract. Systems with genetic variation for the primary sex ratio are important for testing sex-ratio theory and for understanding how this variation is maintained. Evidence is presented for heritable variation of the primary sex ratio in the harpacticoid copepod *Tigriopus californicus*. Variation in the primary sex ratio among families cannot be accounted for by Mendelian segregation of sex chromosomes. The covariance in sex phenotype between full-sibling clutches and between mothers and offspring suggests that this variation has a polygenic basis. Averaged over four replicates, the full-sibling heritability of sex tendency is 0.13 ± 0.040 ; and the mother-offspring heritability of sex tendency is 0.31 ± 0.216 . Genetic correlations in the sex phenotype across two temperature treatments indicate large genotype-by-temperature interactions. Future experiments need to distinguish between zygotic, parental, or cytoplasmic mechanisms of sex determination in *T. californicus*.

Key words. Genotype-by-environment interactions, heritability of sex ratio, heritability of sex tendency, polygenic sex determination, primary sex ratio, temperature-dependent sex determination, *Tigriopus californicus*.

The demonstration of heritable genetic variation for the primary sex ratio is important from both a theoretical and an experimental perspective. From a theoretical perspective, Fisher's (1930) adaptive explanation of the primary sex ratio is contingent on the supply of genetic variation for the primary sex ratio. From an experimental perspective, organisms with genetic variation for the primary sex ratio are necessary for testing Fisher's theory and for determining the overall importance of frequency-dependent (Fisherian) selection in shaping the evolution of the primary sex ratio (Bull and Charnov 1988). Hence, in sex-ratio theory there is a general need to demonstrate that heritable variation of the primary sex ratio does in fact exist (Bull et al. 1982a).

There are two main types of sex-determining mechanisms: genotypic (GSD) and environmental (ESD) sex determination (Charnov 1982; Bull 1983). GSD includes sex chromosomes and multiple-factor and polygenic mechanisms where sex is predominantly determined by an individual's genotype (Bull 1983). In contrast, under ESD, an individual's sex is predominantly determined in response to an environmental factor (Bull 1983; Adams et al. 1987). Most ESD mechanisms exhibit some genetic effects on sex determination, likewise, many GSD mechanisms may be affected by environmental influences (Bull 1983). Thus, the distinction between these two categories is not always clear.

In sex-ratio theory, it is important to distinguish between the sex-determining mechanism and the genes responsible for generating variation in the primary sex ratio, because these are not necessarily one and the same. In the haplodiploid hymenopterans (Hamilton 1967), sex is determined by an individual's ploidy, but the genes that determine whether an egg is fertilized are expressed in the mother (Orzack and Gladstone 1994). In heterogametic mechanisms, sex is determined by an individual's complement of sex chromosomes (Bull 1983). However, sex-linked genes that distort the Mendelian segregation of sex chromosomes and their autosomal suppressors are expressed in the heterogametic parent (Var-

andas et al. 1997; Carvalho et al. 1998). In haplodiploid and sex chromosome mechanisms, the genetic variation for the primary sex ratio is therefore under parental control (Bulmer and Bull 1982).

In contrast, in the threshold model, sex is determined in the offspring by the cumulative effects of one or many loci and/or an environmental deviation relative to some threshold value (Roff 1996, 1997). Individuals with a value below the threshold develop into one sex, and individuals above the threshold develop into the other sex (Bulmer and Bull 1982). Genotypes vary in their complement of sex-determining alleles at the various loci and therefore in their genetic tendency to develop into either a male or a female. In this case, the sex-determining mechanism also generates the variation in the primary sex ratio, and the latter is said to be under zygotic control (Bulmer and Bull 1982). Polygenic and/or environmental systems of sex determination are often described using the threshold model under the assumption that the genetic variation in the primary sex ratio is under zygotic control (Bull et al. 1982a; Lester et al. 1989; Janzen 1992; Premoli et al. 1996).

Variation in the primary sex ratio is intimately connected with the sex-determining mechanism. Early studies showed that sex chromosome systems contained little, if any, genetic variation for the primary sex ratio (Falconer 1954; Edwards 1970; Bar-Anan and Robertson 1975; Foster and McSherry 1980; Toro and Charlesworth 1982; Hohenboken et al. 1988). The potential for variation in the primary sex ratio in sex chromosome systems is believed to be limited due to the stability of Mendelian transmission (Williams 1979). In contrast, genetic variation for the primary sex ratio has been documented in numerous taxa with polygenic and/or environmental mechanisms of sex-determination (Ar-Rushdi 1958; Bulnheim 1978a,b; Conover and Kynard 1981; Bull et al. 1982a; Conover and Heins 1987; Lester et al. 1989; Janzen 1992; Premoli et al. 1996).

The quantification of the genetic variation in the primary

sex ratio depends on the relative number of loci involved (few vs. many) and their effects on sex-determination (major vs. minor). Mechanisms with few loci have a relatively simple characterization and their evolution can be treated exactly (Bull 1983; Basolo 1994). In contrast, polygenic mechanisms are best analyzed using quantitative genetics (Bulmer and Bull 1982). Likewise, the characterization of the genetic component in systems of ESD depends on whether it is multiple factor (Conover and Kynard 1981; Conover and Heins 1987) or polygenic (Bull et al. 1982a; Janzen 1992).

Bull (1983) lists three criteria that suggest the presence of a polygenic mechanism of sex determination: (1) a large between-family sex-ratio variance; (2) paternal and maternal effects on family sex ratio; and (3) a sex-ratio response to selection. Although these criteria are not found in systems with sex chromosomes, in practice it is difficult to distinguish between multiple factor and polygenic systems of sex determination (Bull 1983). Fortunately, quantitative genetics can still furnish a useful description of the short-term evolutionary properties of a sex-determining system as long as the loci are additive in their effects.

The application of quantitative genetics to any sex-determining system requires the investigator to know the mode of control. Under parental control, the clutch sex ratio of the offspring is a parental trait with a relatively continuous distribution between zero and one for large clutches. In contrast, under zygotic control the sex phenotype (female, male) is an offspring trait with a dichotomous distribution (0, 1) that belongs to the offspring. The distinction between the two modes of control is an important one because it dictates the experimental design and the statistical model for estimating quantitative genetics parameters. For example, under parental control, a design with multiple full-sibling clutches provides an estimate of the repeatability of the primary sex ratio (a nongenetic character; Falconer 1989). In contrast, under zygotic control, the same design allows one to estimate the heritability of the primary sex ratio (Bull et al. 1982a).

In the previous example, the “heritability of the primary sex ratio” is a confusing choice of terminology. Under zygotic control, the primary sex ratio in a family actually represents the expected sex phenotype of all the offspring in that family. By analogy, it would be similarly misleading to speak of the “heritability of the population mortality rate” because the underlying genes are the property of individuals, not populations. To avoid confusion, we will use the “heritability of the primary sex ratio” versus the “heritability of the sex tendency” to distinguish between parental versus zygotic modes of control. Likewise, when referring to systems with zygotic control we prefer the term “sex phenotype” to “clutch sex ratio” because the latter has connotations of parental control.

This study investigates polygenic variation in the primary sex ratio in the harpacticoid copepod, *Tigriopus californicus*, which inhabits the supralittoral zone of the West Coast of North America. Its splash-pool habitat is characterized by extreme fluctuations in temperature (5–35°C) and salinity (0–100 ppt) between winter and summer (Dybdahl 1995; Albert et al. 2001).

Following sexual maturity, females mate once (Burton 1985) and produce up to 12 clutches (40–100 offspring per

clutch; Haderlie et al. 1980) over their lifetime (~ 70 days in the laboratory; unpubl. data). In the field, reproduction occurs year-round and populations are capable of rapid growth (Vittor 1971; Powlik 1998). In the laboratory the average generation time is approximately three weeks at 20°C (Haderlie et al. 1980; Webb and Parsons 1988).

Belser (1959) claimed that *T. californicus* was the first reported example of polygenic sex determination in the literature (Ar-Rushdi 1958). A cytological assay found no evidence of sex chromosomes (Ar-Rushdi 1963). Later work showed that the primary sex ratio of this organism was also affected by temperature (Egloff 1966; Vittor 1971; Voordouw and Anholt 2002). Egloff (1966) pointed out that seasonal variation in splash-pool temperatures may cause fluctuations in the primary sex ratio of natural populations of *T. californicus*. The response of the population to these fluctuations is dependent on the supply of genetic variation for the primary sex ratio. A proper characterization of this variation is therefore a necessary point of departure for understanding its role in shaping the evolution of the primary sex ratio in *T. californicus*.

In this study we define the primary sex ratio as the proportion of males at the time of sex determination. Our primary objective is to provide additional evidence for polygenic variation in the primary sex ratio in *T. californicus* using the criteria outlined by Bull (1983). In the first experiment we show that families of *T. californicus* exhibit extrabinomial variation in the proportion of males. In the second experiment we show that family accounts for a substantial portion of the total variance in sex phenotype and that the proportion of males is correlated between split clutches (of full-siblings) reared at two different temperature treatments. In the third experiment we show that the proportion of males in families of full-siblings is correlated between generations.

Our second objective is to estimate heritabilities and genetic correlations of the sex phenotype in *T. californicus*. Assuming zygotic control, we use a polygenic threshold model (Bulmer and Bull 1982; Roff 1997) to estimate the full-sibling and the mother-offspring heritability of sex tendency in experiments 2 and 3, respectively. In experiment 2, we also estimate the full-sibling genetic correlation in sex phenotype across the two temperature treatments.

MATERIALS AND METHODS

Experiments 1–3: Sex Identification and the Larval Mortality Correction

In all three experiments we sexed individuals once they reached sexual maturity (20–30 days after hatching). At this stage of the life cycle, males are easily distinguishable from females by their enlarged geniculate antennae. In cases where an individual’s sex could not be identified at the time of assay (i.e., dead, missing, or sexually immature individuals), the individual was assigned to the less common sex for that family. Assigning unidentified individuals to the less common sex is a conservative approach for detecting variation in the primary sex ratio among families (Bull and Vogt 1979; Conover and Kynard 1981; Bull et al. 1982a). This protocol will hereafter be referred to as the “larval mortality correction.”

Experiment 1: Variation in the Primary Sex Ratio

In the summer of 1999, we reared and mated 60 *T. californicus* females (F_1) whose offspring comprised the second laboratory-born (F_2) generation of field-captured individuals from several locations around Victoria and Bamfield, British Columbia. The F_2 offspring of these F_1 females will hereafter be referred to as a “family.”

After the F_2 generation hatched we randomly selected groups of 20 full-siblings from each of the 60 families. We assigned each family of 20 full-siblings to a 24-well tissue culture plate. Families were nested within plates because randomizing 1200 F_2 individuals across 60 plates was not logistically feasible. Within this family plate, each full-sibling was allocated to its own well with 2.5 ml of filtered seawater and reared on a diet of *Isochrysis galbana* cells and Tetramin (TetraSales) flakes. Plates were stored in an incubator at a temperature of 20°C with no light. Families were assayed for the proportion of males after 18 days.

Mortality in experiment 1.—There was almost no mortality in the first experiment (mortality < 1%), therefore we did not use the larval mortality correction.

Experiment 2: Full-Sibling Design

General outline.—Experiment 2 consists of two separate experiments that are similar in design but separated in time. One experiment was conducted in the summer of 2000 and will hereafter be referred to as the “summer assay”. The other experiment was conducted in the fall of 2000 and will hereafter be referred to as the “fall assay”. A more detailed description of the experimental protocol is given in Voordouw and Anholt (2002), where the summer and fall assay are referred to as experiment 1 and 2, respectively.

Summer assay.—We collected samples of *T. californicus* from splash pools located in Arbutus Cove (48°28'36" N, 123°18'00" W), Victoria, British Columbia, on 31 May 2000. We selected 60 gravid females from our splash-pool samples and tried to obtain two clutches for each female. For the summer assay we successfully removed two clutches from 45 females.

Each clutch (two per female) was split into two random groups of 20 nauplii (full-siblings). One group was assigned to a cool (15°C) and the other to a warm (22°C) treatment. These temperatures are representative of field conditions and also reflect a compromise between slow development and high mortality at cool and warm temperatures, respectively.

Each group of 20 nauplii was reared in a plastic 30-dram vial on a diet of *I. galbana* cells and Tetramin flakes. Vials were placed in a constant temperature incubator (either 15°C or 22°C) with no light. Individuals were assayed for their sex phenotype after 20 to 30 days (depending on the temperature).

Fall assay.—We used the offspring from the summer assay to create two laboratory populations on 19 July 2000 (discussed in more detail in Voordouw and Anholt 2002). Each population was stocked with offspring from 20 different females. Both populations were cultured in incubation refrigerators on a diet of Tetramin flakes without light for 4 months. Over this period of time the two populations went through four to six generations.

We obtained a sample of 50 gravid females from each

population on 26 October 2000. As in the summer assay, we tried to isolate two clutches for each female. We obtained two clutches for 36 females in one population and for 20 females in the other population. We combined the two populations for a total sample size of 56 females (with two clutches per female) in the fall assay.

As in the summer assay, each clutch (two per female) was split into two groups of 20 nauplii (full-siblings). Each group was subsequently assigned to either the 15°C or the 22°C treatment. Individuals were assayed for their sex phenotype after 20 to 30 days (depending on the temperature).

Mortality in experiment 2.—For the summer assay, survivorship was 95.6% at the 15°C treatment and 84.1% at the 22°C treatment. The larval mortality correction in the 22°C treatment of the summer assay eliminated most of the variance in sex phenotype among families. For the fall assay, survivorship at 15°C and 22°C was 96.2% and 96.8%, respectively. Hence, the larval mortality correction had little effect on the variance in sex phenotype among families in the fall assay.

Experiment 3: F_1 - F_2 Family Design

General outline.—In experiment 3 there are three generations: the field, F_1 , and F_2 . We assayed the proportion of males in full-sibling families in the F_1 and F_2 generations. We refer to the females that produced the F_1 and F_2 generation as “field females” and “mothers,” respectively.

Field females and the assay of F_1 families.—In the summer of 2000, we took a sample of copepods from Arbutus Cove, Victoria, and selected 20 gravid females. For each field female we isolated three clutches of F_1 offspring. Nauplii from the second and third clutch were reared in separate 30-dram vials at 15°C with no light. At sexual maturity, we assayed the vials to estimate the proportion of males in each F_1 family of full-siblings.

Rearing and mating of the mothers.—For each field female we used nauplii from the first clutch to obtain the mothers. Thirty nauplii were reared in six-well tissue culture plates (five nauplii/well) at 15°C with no light. When individuals had reached the fourth copepodite stage, we introduced the males (i.e., the fathers of the F_2 generation). These males were taken from the original field sample. Once the fathers had clasped a sexually immature mother we allocated each couple to a single well in a 24-well tissue culture plate. From each field female, we obtained an average of three mothers (range = 1–7). Mothers obtained from the same field female represent a set of full-sibling sisters.

Mothers and the assay of F_2 families.—For each mated mother we obtained at least one clutch of F_2 offspring. These clutches were reared in separate 30-dram vials at 15°C with no light. At sexual maturity, we assayed the vials to estimate the proportion of males in each F_2 family of full-siblings.

Mortality in experiment 3.—Unlike experiments 1 and 2, survivorship was relatively poor. For the F_1 and F_2 families, only 65% and 67% of all nauplii were recovered as adults. We did not use the larval mortality correction, because it would have eliminated most of the variation in the proportion of males among families.

TABLE 1. The family, clutch, and offspring variance components of the sex phenotype in *Tigriopus californicus* in experiment 2. “Corrected” refers to whether the data were adjusted for larval mortality. “Description” refers to the combination of season (S, summer; F, fall) and temperature (°C). “1° SR” denotes the primary sex ratio for all the individuals in the sample. Variance components and *P*-values were obtained from a nested ANOVA.

| Corrected | Description | 1° SR | Variance components | | | | P-values | |
|-----------|-------------|-------|---------------------|--------|-----------|-------|----------|--------|
| | | | Family | Clutch | Offspring | Total | Family | Clutch |
| no | S15 | 0.545 | 0.010 | 0.024 | 0.215 | 0.249 | 0.067 | <0.001 |
| no | S22 | 0.602 | 0.000 | 0.032 | 0.202 | 0.234 | 0.522 | <0.001 |
| no | F15 | 0.482 | 0.019 | 0.009 | 0.223 | 0.251 | <0.001 | 0.001 |
| no | F22 | 0.548 | 0.013 | 0.024 | 0.213 | 0.250 | 0.029 | <0.001 |
| yes | S15 | 0.533 | 0.008 | 0.015 | 0.227 | 0.249 | 0.053 | <0.001 |
| yes | S22 | 0.582 | 0.000 | 0.022 | 0.224 | 0.246 | 0.839 | <0.001 |
| yes | F15 | 0.494 | 0.016 | 0.006 | 0.230 | 0.251 | <0.001 | 0.021 |
| yes | F22 | 0.550 | 0.010 | 0.018 | 0.221 | 0.248 | 0.035 | <0.001 |

Statistical Methods

Experiment 1: variation in the primary sex ratio

To compare the observed distribution of the primary sex ratio among families with the binomial expectation we used a chi-square goodness-of-fit test. To circumvent problems with small expected frequencies and excessive Type I error, families with six or fewer males (proportion of males ≤ 0.30) were grouped and families with 14 or more males (proportion of males ≥ 0.70) were grouped.

Experiment 2: full-sibling design

Statistical test of family effects and the estimation of variance components.—Prior to estimating the heritability of sex tendency it is useful to determine whether there is a genetic basis to the sex phenotype (Roff 1997). For the full-sibling design in experiment 2, rearing two clutches in separate vials allows us to separate clutch effects (and all the sources of variation contained therein) from family effects (Roff 1986, 1997). Family should account for a significant proportion of the total variance in sex phenotype if this variance has a genetic component in *T. californicus*.

Following Roff (1997) we used nested ANOVA to establish the statistical significance of family and clutch effects on the sex phenotype. Family and clutch are modeled as random factors and female and male individuals are coded as ‘0’ and ‘1’, respectively. Individual offspring were nested inside clutches and clutches were nested inside families. The nested ANOVA generated three variance components of sex phenotype: (1) among-family; (2) among-clutch (within-family); and (3) among-offspring (within-clutch) variance component. These three will hereafter be referred to as the “family,” “clutch,” and “offspring” variance components of the sex phenotype, respectively. In the next section we use these variance components to calculate the heritability.

Full-sibling heritability of sex tendency.—Under zygotic control, the sex phenotype can be analyzed using the polygenic threshold model (see introduction; Bull et al. 1982; Bulmer and Bull 1982; Roff 1997). The first step is to calculate the heritability on the observed scale (0, 1 data for females vs. males), the heritability of sex phenotype ($h_{0,1}^2$). We used the variance components from our nested ANOVA and equation 2.30 in Roff (1997) to calculate $h_{0,1}^2$:

$$h_{0,1}^2 = 2[V_{\text{fam}}/(V_{\text{fam}} + V_{\text{clutch}} + V_{\text{offspring}})]. \quad (1)$$

Here V_{fam} , V_{clutch} , and $V_{\text{offspring}}$ correspond to the family, clutch, and offspring variance components of sex phenotype. Next, we used equation (2.44) in Roff (1997) to calculate the heritability on the underlying scale, the heritability of sex tendency (h^2).

$$h^2 = h_{0,1}^2 p(1 - p)/z^2. \quad (2)$$

Here p is primary sex ratio for all the individuals in the sample (see Table 1) and z is the ordinate on the standardized normal curve that corresponds to a probability of p . Approximate standard errors for the full-sibling heritability estimates were calculated using equation (2.50) in Roff (1997).

In both the summer and fall assay, our split-clutch design allows us to estimate the full-sibling heritability of sex tendency in *T. californicus* at each temperature treatment. Thus, we have a total of four full-sibling heritability estimates: summer assay at 15°C (S15), summer assay at 22°C (S22), fall assay at 15°C (F15), and fall assay at 22°C (F22). We calculated a combined heritability of sex tendency by taking the average of these four estimates and their standard errors.

The genetic correlation in sex phenotype across two environments.—The same character expressed in two different environments can be thought of as two characters that are genetically correlated (Falconer 1952). If there are no genotype-by-environment ($G \times E$) interactions, the character is determined by the same set of genes in both environments and the genetic correlation is expected to be highly positive. Conversely, any genetic correlation across environments that is significantly less than one indicates the existence of $G \times E$ interactions (Yamada 1962).

In experiment 2, for both the summer and fall assay we used a design where groups of 20 full-siblings from the same clutch were reared at two different temperature treatments (15°C and 22°C). This split-clutch design allows us to evaluate the importance of genotype-by-temperature interactions on the offspring’s sex phenotype. We used the correlation of family means (i.e., the proportion of males for a group of 20 full-siblings; method 1 of Via 1984) between temperature treatments to approximate the standard full-sibling genetic correlation in sex phenotype (r_m):

$$r_m = \text{cov}_{m(15^\circ\text{C}, 22^\circ\text{C})}/[(\text{var}_{m(15^\circ\text{C})})(\text{var}_{m(22^\circ\text{C})})]^{1/2}, \quad (3)$$

where $\text{cov}_{m(15^\circ\text{C}, 22^\circ\text{C})}$ is the covariance of the family proportion of males between the two environments (15°C and 22°C) and $\text{var}_{m(15^\circ\text{C})}$ and $\text{var}_{m(22^\circ\text{C})}$ are the variances of the family

proportion of males for each of the two temperature treatments (Via 1984). This method is an approximation because each term in equation (3) contains a within-family error component; however, the observed correlation in sex phenotype approaches the true genetic correlation as family size increases (Via 1984).

The r^2 -value from these correlations can be used to estimate the proportion of genetic variation in the sex phenotype that is due to pleiotropy (Via 1984). Confidence limits of the genetic correlation were calculated using Tukey's jackknife method (Sokal and Rohlf 1981). We did not use the z -transformation because it excludes confidence limits greater than 1.0. (Janzen 1992). Note, we did not transform r_m to the underlying scale of sex tendency; therefore, our estimate measures the genetic correlation in the sex phenotype between the two temperature environments.

The larval mortality correction.—For the full-sibling design in experiment 2, the statistical significance of family and clutch effects on sex phenotype were calculated for the original and for the larval mortality corrected data. Likewise, we recalculated the four heritability estimates (S15, S22, F15, F22), and the genetic correlations following the larval mortality correction.

Experiment 3: F_1 - F_2 family design

Pooling in F_1 and F_2 families.—For each field female we pooled the second and third clutch (full-siblings) so that the average F_1 family contained 35 offspring (range = 14–53). Mothers obtained from the same clutch (i.e., sisters) are not independent. In addition, we have only one estimate of the F_1 proportion of males for each set of sisters. To avoid pseudo-replication, we pooled all the F_2 offspring for each set of sisters. After pooling, the average F_2 family contained 23 offspring (range = 2–90). Note, because F_2 offspring were produced by a set of sisters, each F_2 family contains a mix of full-siblings and cousins. Three families were lost, so the final dataset consisted of the proportion of males for 17 pairs of F_1 - F_2 families.

Covariance in the proportion of males between F_1 and F_2 families.—We expect the proportion of males to covary between the F_1 and F_2 families if it has a genetic component. The statistical significance of the covariance in the proportion of males between F_1 and F_2 families was determined using a standard regression analysis as well as a randomization test. In the randomization test we paired the proportion of males at random between F_1 and F_2 families and calculated Pearson's correlation coefficient. We generated an empirical distribution of 10,000 of such correlations for comparison with the observed correlation.

Mother-offspring heritability of sex tendency.—For each full-sibling family (F_1 or F_2), the observed proportion of males is an estimate of the expected sex phenotype for that family. In other words, the proportion of males in a family estimates an individual's probability of developing into a male (assuming zygotic control). The proportion of males in each pair of F_1 - F_2 families are therefore estimates of the expected sex phenotype of the mothers and their offspring, respectively.

For each full-sibling family (F_1 or F_2), we calculated the

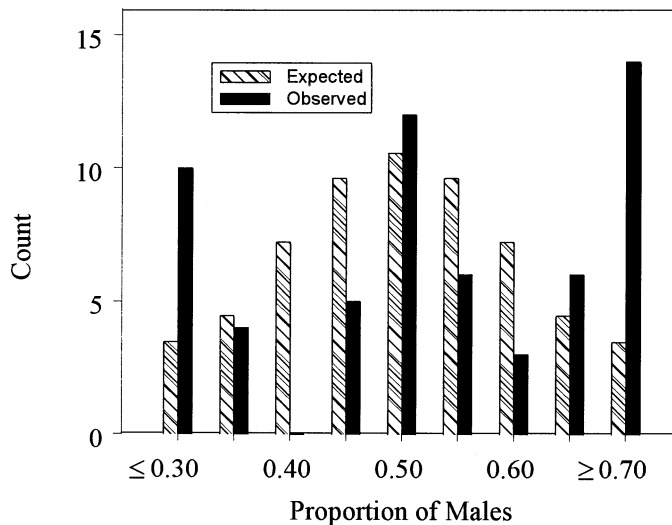


FIG. 1. The distribution of the proportion of males for 60 families; each family consists of 20 offspring. Shown are the expected and observed counts. Families with a proportion of males ≤ 0.3 or ≥ 0.7 were lumped because of low expected values.

ordinate on the standardized normal curve (z_i) that corresponds to the proportion of males in that family (p_i). The transformed value (z_i) approximates an individual's phenotypic value on the underlying scale of sex tendency (Roff 1986, 1997). These phenotypic values (sex tendencies) are normally distributed. After calculating the sex tendencies (z_i -values) for the mothers (F_1 families) and their offspring (F_2 families), analysis proceeds as usual for a normally distributed trait (Roff 1986, 1997).

The heritability of sex tendency (h^2) was calculated as twice the slope of the mother-offspring regression of sex tendency. Because the number of F_2 offspring varied considerably between families (range = 2–90), we repeated the analysis after weighting the F_2 sex tendency by the square root of its family size. Standard errors of heritability estimates were calculated following Becker (1984).

RESULTS

Experiment 1: Variation in the Primary Sex Ratio

In experiment 1, the primary sex ratio of the population (averaged over 60 families with 20 offspring per family) was 0.54 ± 0.025 and was not significantly different from 0.50 ($P = 0.131$). However, the average family contained significantly more sons or more daughters than expected from a binomial distribution ($\chi^2 = 58.5$, $df = 8$, $P < 0.001$; Fig. 1). The small excess of 50/50 families (i.e., proportion of males = 0.5) in Figure 1 reflects the larval mortality correction (i.e., unidentified individuals assigned to the less common sex in that family).

Experiment 2: Full-Sibling Design

Variance components.—For the summer assay, the family variance component in sex phenotype is marginally nonsignificant at 15°C and not significant at 22°C (Table 1, Fig. 2). For the fall assay, the family variance component in sex

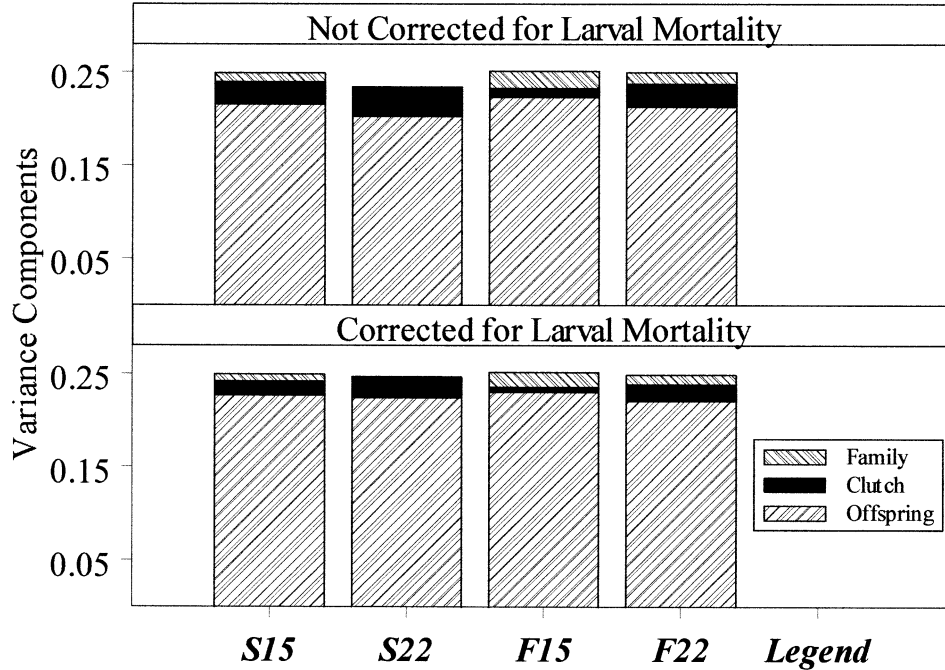


FIG. 2. The family, clutch, and offspring variance components of the sex phenotype in *Tigriopus californicus* in experiment 2. The top and bottom panels show the original and the larval mortality-corrected data, respectively. S15, summer assay at 15°C; S22, summer assay at 22°C; F15, fall assay at 15°C; F22, fall assay at 22°C.

phenotype is significantly different from zero at both 15°C and 22°C (Table 1, Fig. 2).

The clutch variance component in sex phenotype is statistically significant for all four season and temperature combinations (Table 1, Fig. 2). In the summer assay the clutch variance component is much larger than the family variance component (Table 1, Fig. 2). We did not pool clutches for each family because this would have added a substantial non-genetic component to our heritability estimates (see Discussion). After correcting for larval mortality, the family and clutch variance components are slightly smaller but the pattern of significance remains the same (Table 1, Fig. 2).

Full-sibling heritability of sex tendency.—Averaged over the four combinations of season and temperature, the heritability of sex tendency in *T. californicus* is 0.13 ± 0.040 (range = 0.00–0.24). The heritability estimates were higher in the fall than in the summer assay and were also higher at the lower temperature treatment (Table 2). After correcting

TABLE 2. The full-sibling heritability ($h^2 \pm$ standard error) of sex tendency in *Tigriopus californicus* in experiment 2. “Corrected” and “uncorrected” refers to whether the data were adjusted for larval mortality or not. “Description” refers to the combination of season and temperature. *N*, number of families; *n*, average number of offspring per family; % surv, percent survival to sexual maturity.

| Description | <i>N</i> | <i>n</i> | % surv | Uncorrected $h^2 \pm$ SE | Corrected $h^2 \pm$ SE |
|-------------|----------|----------|--------|-----------------------------|---------------------------|
| Summer 15°C | 45 | 49.5 | 93.8 | 0.12 ± 0.039 | 0.10 ± 0.033 |
| Summer 22°C | 45 | 49.2 | 81.9 | 0.00 ± 0.017 | 0.00 ± 0.014 |
| Fall 15°C | 56 | 36.0 | 96.0 | 0.24 ± 0.058 | 0.19 ± 0.050 |
| Fall 22°C | 56 | 36.1 | 97.1 | 0.16 ± 0.045 | 0.12 ± 0.039 |
| Average | | | | 0.13 ± 0.040 | 0.10 ± 0.034 |

the data for larval mortality the heritabilities are somewhat smaller for all combinations of season and temperature, and the combined estimate is 0.10 ± 0.034 (Table 2).

The genetic correlation in sex phenotype across two environments.—For both the fall and summer assay the genetic correlation (r_m) in sex phenotype across the two temperature treatments is positive and significantly different from zero (Table 3). In both assays, the upper 95% confidence limit of r_m does not include +1, indicating the presence of genotype-by-temperature interactions. That only 35% of the genetic variance in sex phenotype can be attributed to pleiotropy ($r^2 = 0.35$) suggests that these genotype-by-temperature interactions are large. Correcting the data for larval mortality does not affect this conclusion (Table 3).

Experiment 3: F_1 - F_2 Family Design

Covariance in the proportion of males between F_1 and F_2 families.—The proportion of males in the F_1 family accounts for 37.2% of the variation in the proportion of males in the F_2 families ($F_{1,15} = 8.884$, $P = 0.009$; randomization test, $P < 0.005$). If the regression is weighted by the square root of the F_2 family size, the F_1 proportion of males accounts for 43.9% of the variation in the F_2 proportion of males ($F_{1,15} = 11.750$, $P = 0.004$; Fig. 3).

Mother-offspring heritability of sex tendency.—Following transformation to the underlying scale of sex tendency, the mother-offspring regression is no longer significant for both the weighted ($F_{1,15} = 2.009$, $P = 0.177$) and the unweighted analysis ($F_{1,15} = 2.562$, $P = 0.130$). On the scale of sex tendency, the heritability estimates are 0.31 ± 0.216 and 0.36 ± 0.225 for the weighted and unweighted analyses, respec-

TABLE 3. The genetic correlation (r_m) in sex phenotype across the two temperature treatments (15°C and 22°C) in *Tigriopus californicus* in experiment 2. “Assay” refers to the summer and fall assay. “Corrected” refers to whether the clutches were adjusted for larval mortality. Shown are the sample size (N), the 95% confidence interval of r_m , the P -value for the null hypothesis that $r_m = 0$, and the percentage of the genetic variation in sex phenotype due to pleiotropy (i.e., the r^2 -value).

| Assay | Corrected | N | Correlation (r_m) | 95% confidence interval | P | % genetic variance |
|--------|-----------|-----|-----------------------|-------------------------|--------|--------------------|
| Summer | no | 55 | 0.60 | 0.42–0.78 | <0.001 | 36 |
| Fall | no | 76 | 0.58 | 0.38–0.79 | <0.001 | 34 |
| Summer | yes | 57 | 0.42 | 0.20–0.64 | 0.001 | 18 |
| Fall | yes | 77 | 0.61 | 0.43–0.78 | <0.001 | 37 |

tively. These h^2 estimates will be upwardly biased if there are sex-specific mortality differences among families because we did not use the larval mortality correction in experiment 3.

DISCUSSION

Evidence for Polygenic Variation in the Primary Sex Ratio in Tigriopus californicus

Our experiments provide additional evidence for the existence of polygenic variation in the primary sex ratio in *T. californicus*. In the first experiment, the observed variation in the primary sex ratio among families cannot be accounted for by Mendelian segregation of sex chromosomes. In the second experiment, the significant variance component among families (fall assay) suggests a polygenic basis for the variance in sex phenotype. This conclusion is further supported by the covariance in the proportion of males between temperature treatments (experiment 2) and between the F_1 and F_2 families (experiment 3).

Further support for polygenic variation in the primary sex ratio in *T. californicus* comes from the work by Egloff (1966) and from the selection experiments of Ar-Rushdi (1958). Egloff (1966) observed extrabinomial variation in the primary sex ratio but did not distinguish between genetic and environmental effects. Ar-Rushdi (1958) selected the proportion of males for high and low values and succeeded in shifting the original proportion of males to 0.995 and 0.200, respectively. Unfortunately, Ar-Rushdi (1958) did not estimate realized heritabilities and his report is incomplete in several respects. For example, it is not clear whether he ruled out sex-specific mortality differences, how many generations of selection were done, and what sample sizes were used (Ar-Rushdi 1958).

An essential aspect of our analyses is our use of the larval mortality correction. Following the example set by other sex-ratio workers (Bull and Vogt 1979; Conover and Kynard 1981; Bull et al. 1982a) this protocol allows us to eliminate differential mortality of the sexes as an alternative explanation. Thus, in experiments 1 and 2 (but not experiment 3) we have conservative evidence that the observed results are caused by variation in the primary sex ratio.

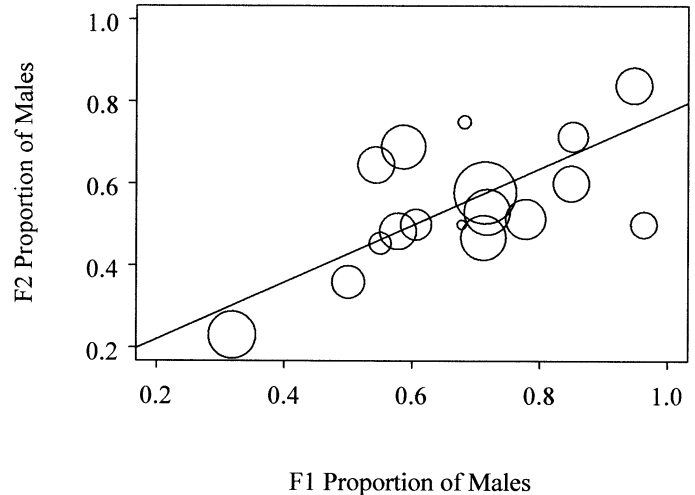


FIG. 3. The correlation in the proportion of males between the F_1 and the F_2 families. The proportion of males in each F_2 family is weighted by the square root of the number of individuals in that family. Shown is the line of best fit, $y = 0.152 + 0.580x$.

Assumptions of Our Heritability of Sex Tendency Estimates

Our approach for estimating the heritability of sex tendency makes three important assumptions. The first is that all offspring from the same mother are full-siblings (Bull et al. 1982a; Roff 1997). This assumption is likely to be met, because sperm competition and multiple paternity do not occur in *Tigriopus* (Burton 1985).

The second assumption is that the covariance among relatives is the result of additive genetic effects. Violation of this assumption includes dominance, epistasis, maternal effects, environmental effects, major genes, and sex-linked loci. With the exception of dominance and environmental effects (see below), we are unable to address these problems in the current study.

Finally, as mentioned in the introduction, the analysis assumes that the genetic variation in the primary sex ratio is under zygotic control (Bull et al. 1982a; Bulmer and Bull 1982). Our rationale for choosing the polygenic threshold model as a putative description of the sex-determining mechanism in *T. californicus* was primarily based on reports of ESD in this species (Vacquier 1962; Vacquier and Belser 1965; Egloff 1966; Chalker-Scott 1995; Voordouw and Anholt 2002). Although ESD mechanisms in no way preclude the presence of parental control (Bulmer and Bull 1982), the genetic analysis of these systems typically assigns responsibility to the zygote (Conover and Kynard 1981; Bull et al. 1982a; Conover and Heins 1987; Lester et al. 1989; Janzen 1992).

Heritability of Sex Tendency in Tigriopus californicus

Here we report the first heritability estimates of sex tendency in *T. californicus*. The mother-offspring heritability estimate ($h^2 = 0.31$) is comparable to the full-sibling estimates from the fall assay (Table 2). From theory, we would expect the mother-offspring h^2 to be lower than the full-sibling estimates (Falconer 1989). The mother-offspring covariance is not affected by dominance and is less likely to

be influenced by maternal effects than the full-sibling covariance (Falconer 1989). Error in the estimate of the mother's phenotypic value (sex tendency) should result in a conservative mother-offspring h^2 (Roff 1986, 1997).

We did not correct for larval mortality in experiment 3 and so it is possible that the mother-offspring h^2 is inflated by sex-specific viability differences between families. However, the association between low heritabilities and low survivorship in experiment 2 (S15, S22; Table 2) argues against such a bias and suggests that larval mortality is random with respect to sex.

The standard error of the mother-offspring h^2 (± 0.216) is more than five times larger than that of the combined full-sibling h^2 (± 0.040). This lack of precision is partly due to reduced sample size ($n = 17$) in the mother-offspring design and again because the estimate of the mother's phenotypic value is relatively imprecise (Roff 1986, 1997). In addition, the standard error may be large because each F_2 family contains a mix of full-siblings and cousins (due to pooling) and this increases the variance in the phenotypic values of the offspring.

In experiment 2, the families in the summer assay were obtained from field-captured females. In contrast, families from the fall assay were sampled from two populations that had been cultured under constant laboratory conditions for four to six generations. A more variable maternal environment in the field may have lowered the full-sibling estimates in the summer assay, although other studies have found no consistent differences between field and laboratory estimates (Weigensberg and Roff 1996). More likely, the nonexistent h^2 of the summer assay at 22°C was caused by the low survivorship of nauplii (81.9%) in this replicate.

As recommended by Roff (1997), experiment 2 demonstrates the importance of rearing offspring in separate cages to estimate the contribution of cage (clutch) to the total phenotypic variance. Clutch and all the nongenetic components included therein (e.g., maternal age, maternal condition, parity, larval density, food levels) accounted for a significant proportion of the total variance in sex phenotype (Table 1). If we had pooled the clutches for each family, as is commonly done (Roff 1986, 1997; Mousseau and Roff 1989), the average heritability of sex tendency (for the original data) would be 0.29 ± 0.068 (range = 0.27–0.31). This pooled estimate is higher than the nonpooled estimate (Table 2) because it includes a substantial nongenetic component of variation.

Heritability of the Primary Sex Ratio Versus Heritability of the Sex Tendency

Heritability of the primary sex ratio.—The early studies on heritable variation for the primary sex ratio typically treated this variation as being under parental control. The heritability of the primary sex ratio in most of these studies (all sex chromosome systems) was indistinguishable from zero (Falconer 1954; Edwards 1970; Bar-Anan and Robertson 1975; Foster and McSherry 1980; Toro and Charlesworth 1982; Hohenboken et al. 1988). Heritabilities in the primary sex ratio have now been reported in a parasitic wasp (realized $h^2 = 0.15$ – 0.17 , Parker and Orzack 1985; parent-offspring $h^2 = 0.05$ – 0.15 , Orzack and Gladstone 1994) and in populations

of *Drosophila mediopunctata* (parent-offspring $h^2 = 0.41$, Varandas et al. 1997; realized $h^2 = 0.20$, Carvalho et al. 1998) that have sex-linked segregation distortion.

Heritability of the sex tendency.—The heritabilities of sex tendency have been reported in the map turtle (full-sibling $h^2 = 0.82$, Bull et al. 1982a), the common snapping turtle (full-sibling $h^2 = 0.56$, Janzen 1992), and tilapia (full-sibling $h^2 = 0.26$, Lester et al. 1989). Both species of turtle exhibit temperature-dependent sex determination (TSD). The sex-determining mechanism is unknown in tilapia (Lester et al. 1989). In addition, we calculated the heritability of sex tendency in the harpacticoid copepod *Tisbe gracilis* (full-sibling $h^2 = 0.29 \pm 0.115$) from a published dataset in the literature (Battaglia 1958). Volkmann-Rocco (1972) has suggested that sex in *T. gracilis* is determined by a multiple-factor system.

In their study of a polychaete worm, Premoli et al. (1996) presented the polygenic threshold model in the introduction but subsequently estimated the heritability of the primary sex ratio (thereby implicitly assuming that clutch sex ratio was controlled by the parent). As it turned out, parental control seems more likely in this system because only the father had an effect on the sex ratio of the offspring (father-offspring $h^2 = 0.54$, mother-offspring $h^2 = 0$, Premoli et al. 1996).

Bulmer and Bull (1982) pointed out that in natural populations, the heritability of sex tendency in systems with ESD is affected by variation in the relevant (sex-determining) environmental factor. In turtles with temperature-dependent sex determination, the effective heritability (h_{eff}^2) was found to be an order of magnitude lower than the laboratory estimates ($h_{\text{eff}}^2 = 0.06$, Bull et al. 1982b; $h_{\text{eff}}^2 = 0.05$, Janzen 1992; see above for laboratory h^2). The response of natural populations to environment-induced fluctuations in the primary sex ratio may therefore be limited (Bull et al. 1982a).

Maternal Control in the Threshold Model: Whose Trait Is It?

The naive dichotomy of maternal versus zygotic control breaks down in many polygenic/environmental systems of sex determination. In reptiles with TSD, mothers can influence the sex of their offspring via nest choice (Bull et al. 1988), thermoregulatory behavior (Robert and Thompson 2001), maternal condition (Roosenburg 1996), and through the seasonal allocation of yolk hormones (Bowden et al. 2000). Theory suggests that the genetic variation for these maternally expressed traits may be more important in shaping the evolution of the primary sex ratio than the sex tendency of the zygote (Bulmer and Bull 1982).

*G × E Interactions and Temperature-Dependent Sex Determination in *Tigriopus californicus**

The primary sex ratio in *T. californicus* is influenced by temperature (Egloff 1966; Vittor 1971; Voordouw and Anholt 2002), although the effect is not strong. Between 15°C and 22°C, the proportion of males increases from 0.53 to 0.58 (Voordouw and Anholt 2002). The adaptive significance of TSD in *T. californicus*, if any, is not known.

The genetic correlations in sex phenotype across the two temperature treatments (15°C and 22°C) suggest that there are substantial genotype-by-temperature interactions. Ap-

proximately one-third of families increase the proportion of males in response to higher temperatures but the rest do not (Voordouw and Anholt 2002). Populations of *T. californicus* appear to consist of a mix of temperature-insensitive (GSD) and temperature-sensitive (TSD) genotypes. Similar systems have been detected in *Menidia menidia* (Conover and Kynard 1981; Conover and Heins 1987).

Cytoplasmic Inheritance of Sex Factors

An alternative explanation for the variation in the primary sex ratio in *T. californicus* is cytoplasmic inheritance. Under cytoplasmic inheritance, sex is determined by intracellular parasites (or other selfish genetic elements) that distort the sex ratio toward the transmitting sex, usually the female (Bull 1983). Theoretical models indicate that cytoplasmic sex factors can eventually cause the extinction of the nontransmitting sex (Hamilton 1967; Hatcher et al. 1999) and extremely female-biased sex ratios have been found in natural populations (Jiggins et al. 2000).

Cytoplasmic inheritance has been reported in a number of crustaceans including *Gammarus duebeni* (Dunn et al. 1995; Dunn and Hatcher 1997; Kelly et al. 2001), terrestrial isopods (Bull 1983; Rigaud et al. 1997), and *Orchestia gammarellus* (Ginsburger-Vogel and Desportes 1979). Igarashi (1964) inferred a cytoplasmic mode of sex determination in *Tigriopus japonicus* after showing that the primary sex ratio in the F₁ offspring is exclusively determined by the maternal genotype. Igarashi (1964) only reared two generations and so his results could have been caused by maternal control of the primary sex ratio, nevertheless, his findings in *T. japonicus* have important implications for the present study.

In this study, the variation in the primary sex ratio in *T. californicus* may be under zygotic, parental or cytoplasmic control. For each mode of control, we expect a different pattern of covariance in the primary sex ratio among relatives. These patterns are outlined below.

In the polygenic threshold model, parents make an equal genetic contribution to the sex phenotype of their F₁ offspring. In the absence of maternal effects, the father-offspring and the mother-offspring heritabilities are expected to be equal (Roff 1986). In contrast, if variation in the primary sex ratio is under maternal control the father-offspring heritability will be zero. However, the maternal grandparent-offspring heritability is expected to be equal for the two sexes (grandmother vs. grandfather) because both contributed equally to the genotype of their daughters. Under maternal transmission of sex factors, the covariance in sex phenotype between males and their descendants (F₁, F₂, etc.) is expected to be zero. Future experimental designs need to distinguish between these alternative mechanisms (zygotic, parental, cytoplasmic) of sex determination in *T. californicus*.

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