Sex Ratio Theory in a Splash Pool: the Sex Ratio Trait of *Tigriopus californicus*

by

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Sex ratio theory makes predictions about how sexually reproducing organisms should allocate their reproductive efforts towards sons and daughters. Fisher predicted that the optimal strategy is one of equal investment (i.e. the 50:50 sex ratio). Subsequent analysis has shown that Fisher's equilibrium sex ratio is contingent on a number of assumptions such as autosomal inheritance of sex ratio alleles, large population size, additive offspring costs, etc. When any of these assumptions are violated the equilibrium sex ratio is not necessarily the one predicted by Fisher.

To test sex ratio theory requires systems that exhibit variation for the primary sex ratio. The harpacticoid copepod, *Tigriopus californicus* is one such system. I have repeatedly detected a large, extra-binomial variance component in the primary sex ratio among full sib families in several natural populations on Vancouver Island. Environmental factors such as temperature and larval density have a mild effect on the primary sex ratio but are not likely to drive sex ratio variation at the population level. Cytoplasmic sex ratio distorters such as *Wolbachia* are known to cause sex ratio fluctuations in the populations of other crustaceans but were not detected in *T. californicus*. In the absence of sex-biased mortality, lineage analysis revealed that the sex ratio trait in a local population of *T. californicus* was paternally transmitted. Uniparentally transmitted sex ratio factors are generally under strong selection to increase the proportion of the transmitting sex in their host population. This observation may provide an explanation as to why the population-wide primary sex ratio in *T. californicus* and other harpacticoid copepods is often male-biased.

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CHAPTER 1: A HISTORY OF SEX RATIO THEORY

Biologists have long observed that most sexually reproducing organisms produce equal numbers of sons and daughters. Darwin himself wondered why natural selection would favor a balanced sex ratio and suggested that it somehow reduced the level of intra-specific competition over the opposite sex (a group selection argument). Perhaps he knew that his explanation was incorrect as he eventually concluded that "the whole problem is so intricate that it is safer to leave its solution for the future (Darwin 1871)." In Darwin's time the problem of the balanced sex ratio was not intuitive because evolutionary biologists had yet to make the distinction between individual and group selection. The belief that individuals acted for the benefit of the species was still wide spread. With respect to the sex ratio, biologists felt that individuals ought to produce an excess of daughters in order to maximize the growth rate of the species. This is a group selection perspective because it implies that natural selection operates on species. Most evolutionary biologists are now in agreement that natural selection operates on individuals within species and has very little regard for what is "good for the species" (Williams 1966).

While Darwin's explanation of the balanced sex ratio turned out to be wrong his attempt marked the inception of a discipline of evolutionary biology that is now known as sex-ratio theory. Sex-ratio theory is concerned with the pattern of resource allocation towards male vs. female offspring (Charnov 1982). The main objective of the theory is to explain the adaptive significance of the sex ratio. Sex-ratio theory is an interesting field of inquiry because it happens to be one of the more successful quantitative branches of evolution (Bull and Charnov 1988). It has been successful because equilibria can often be

predicted without measuring fitness, and the trait of interest (sex ratio) is relatively easy to measure (Bull and Charnov 1988). Other contributions to the general theory of evolution include (1) showing the relative importance of individual versus group selection (Hamilton 1967), (2) demonstrating the potential of frequency-dependent selection in shaping evolution (Endler 1986) and (3) providing insight into traditionally nebulous concepts such as "constraints" and "irreversible evolution" (Bull and Charnov 1985; Bull and Charnov 1988). Finally, the unification of sex-ratio models and other sex allocation phenomena into a single, mathematical framework (Charnov 1982) reflects common evolutionary principles and the power and elegance of selectionist thinking. Sex ratio theory is therefore important because it has informed other areas of evolutionary biology.

The adaptive significance of the sex ratio was first proposed in 1930 by Sir Ronald Fisher (1930). Fisher's theory made predictions about the primary sex ratio, which is the proportion of male offspring at the time of sex determination (usually but not always conception). Fisher pointed out that in an outcrossed population of sexually reproducing individuals, "the collective investment by the parents in each sex ought to be equal." Natural selection ensures equal investment because each generation gets half of its autosomal complement from its fathers and the other half from its mothers. In other words, sons and daughters are equally efficient means of getting one's genes into the next generation. If one sex is in excess, parents that have a bias towards producing the rare sex will have greater fitness. If there is a genetic tendency for this bias the frequency of these genes will increase until the sex ratio is balanced in the population. The important thing to notice is that the process generates its own selection. It is the overabundance of one

sex that makes the production of the rarer sex more worthwhile. This is an example of frequency-dependent selection; i.e. the fitness of an individual's clutch sex ratio depends upon the sex ratio in the population at large.

Fisher's concept of equal investment does not necessarily imply a balanced primary sex ratio. At the end of the period of parental care, it is the ratio of the relative costs of raising sons vs. daughters that determines the primary sex ratio (Fisher 1930). If one sex costs less to rear than the other (e.g. it requires less food) it is the cheaper sex that is overproduced (Fisher 1930). Costs can manifest themselves in many different ways. If one sex is more likely to die during the period of parental care it incurs, on average, less investment (i.e. is cheaper, Charnov 1982) and the primary sex ratio will be biased towards it. Similarly, if one sex is more likely to help its parents in rearing offspring (as in many cooperatively breeding birds and mammals), it "repays" part of its own cost (i.e. is cheaper, Emlen et al. 1986) and the primary sex ratio will be biased towards "helpers". Counter-intuitively, the primary sex ratio is not affected by sex-specific mortality differences following the period of parental care (Leigh 1970; Shaw and Mohler 1953). In general, parent-offspring conflict will tend to select against large disparities in rearing costs (Trivers 1974), although there is some evidence for the latter in the nonsocial Hymenoptera (Charnov 1982).

Fisher predicted that if the cost of rearing a son equals the cost of rearing a daughter then the primary sex ratio of the population at equilibrium would be 50:50 (Fisher 1930). Hereafter I will refer to this prediction as Fisher's sex ratio principle. In the past, the ubiquity of the 50:50 primary sex ratio across a wide range of taxa has been interpreted as evidence for Fisher's sex ratio principle (Bull and Charnov 1988). Others

have pointed out that a balanced primary sex ratio is merely the consequence of heterogamety – the independent assortment of sex chromosomes during meiosis (Williams 1979). While the consensus is that the sex ratios of sex chromosome systems do not support any adaptive theory (Bull and Charnov 1988) it remains to be determined why such systems are so common in the first place (Charnov 1982). An additional limitation is that these systems typically lack genetic variation for the primary sex ratio and are therefore unable to evolve (i.e. they are constrained) which makes them unsuitable for testing Fisher's sex ratio principle (Bull 1985a; Bull et al. 1982a).

Fisher was explicit about the prediction of his model but not about its assumptions. Subsequent work has shown that there are seven critical assumptions and these were first outlined by Bull and Charnov (1988). The assumptions that are most important to Fisher's model are (1) separate sexes (Fisher 1930), (2) biparentalism (Fisher 1930) and (3) Mendelian segregation of alleles influencing the sex ratio (Hamilton 1967; Shaw 1958). The other assumptions include (4) random mating in an infinite population (Hamilton 1967), (5) additive offspring costs (Fisher 1930; MacArthur 1965), (6) no environmentally induced sex-specific fitness differences (Bull 1981b) and (7) parental control (Fisher 1930; Trivers 1974). The consequences of violating these assumptions are discussed in turn below.

Critical to Fisher's argument are the conditions of biparentalism (assumption 2) and Mendelian segregation (assumption 3). It is these two assumptions that generate frequency-dependent selection for the minority sex and their violation often results in extremely biased sex ratios (Bull and Charnov 1988). Examples include systems of cytoplasmic inheritance and systems of sex-linked inheritance (Bull 1983; Charnov

1982). Cytoplasmic inheritance refers to the transmission of extra-nuclear particles of DNA such as viruses, bacteria and protozoans (Bull 1983; O'Neil et al. 1997). Often these cytoplasmic factors are transmitted only through the mother so that from their perspective, males are an evolutionary dead end (Werren and Beukeboom 1998; Werren et al. 1988). If the cytoplasmic factor could somehow increase the number of female offspring it would increase its own transmission rate. Cytoplasmic factors affect the sex ratio of their host's offspring by a variety of mechanisms including direct control of sexual development(Hurst 1993; Rigaud 1997). In the amphipod *Gammarus duebeni* for example, mothers are infected by an obligate intracellular microsporidian parasite that converts genotypic males into fully functional phenotypic females (Dunn et al. 1993; Terry et al. 1998). In some lepidopterans and ladybird beetles there is a species of bacteria, *Wolbachia*, that kills all the male offspring in a clutch of eggs thereby enhancing the reproductive success of the female offspring (Hurst and Majerus 1993; Jiggins et al. 2000). In these systems, the equilibrium sex ratio is often 100% female (Werren 1987a) but paternally transmitted factors that result in 100% male offspring do occur (Werren et al. 1981). The extreme deviation from the Fisherian optimum is caused by the fact that inheritance is uniparental and non-Mendelian (Bull 1983; Bull and Charnov 1988; Charnov 1982).

Similar deviations from the Fisherian equilibrium are observed in sex-linked systems of inheritance (Hamilton 1967; Shaw 1958). In an XY sex chromosome system for example, a male (XY) transmits the X chromosome only to his daughters. From the perspective of a gene on the X chromosome inside the prospective father, sons do not contribute to that gene's reproductive success. If this X-linked gene could somehow bias

meiotic segregation in favor of the X chromosome it would increase its own transmission rate (Hamilton 1967; Shaw 1958). In contrast, a Y-linked gene would bias segregation in favor of the Y chrosome (Hamilton 1967). Such systems are actually known in certain species of *Drosophila* (Jaenike 1996; Jaenike 2001; Wallace 1948). In some cases segregation distortion of the X chromosome is so complete that the male produces 100% daughters (Carvalho et al. 1998; Varandas et al. 1997). These systems of meiotic drive violate the assumptions of Fisher's model because inheritance is uniparental (in the case of Y chromosomes) and because segregation is non-Mendelian (Bull 1983; Bull and Charnov 1988; Charnov 1982).

Hamilton (1967) was the first to point out that Fisher's model is dependent on random mating in an infinitely large population and that group structured matings can have drastic effects on the evolution of the primary sex ratio. In many organisms mating occurs primarily within small groups of individuals, a phenomenon known as local mate competition (LMC). LMC is common in many parasitoid wasps, fig wasps and mites where haplo-diploidy gives the mother control of the primary sex ratio (Charnov 1982). In the extreme, mating may take place only between the offspring from a single foundress. If a male is capable of mating with many females, the foundress maximizes her fecundity by producing a single son and the remainder as daughters (Bull and Charnov 1988; Hamilton 1967). As the number of foundresses increases the optimal primary sex ratio becomes increasingly less female-biased. Note that LMC also violates the assumption of additive offspring costs. This occurs because the fitness of a foundress increases linearly with each additional daughter but is relatively independent of the number of sons (Bull and Charnov 1988).

Another assumption of Fisher's model is that there are no environmentally induced sex-specific fitness differences (Bull 1981b). It is well known that the quality of the environment can have important fitness consequences for an individual but less obvious is the realization that these consequences are not necessarily the same for each sex (Charnov and Bull 1977; Trivers and Willard 1973). For example, an individual's body size is often determined by the quality of its environment. If female fecundity is limited by body size but male fertility is not, environmental quality becomes more important to female than to male reproductive success (Bull 1983; Charnov and Bull 1977). The optimal reproductive strategy is to rear only daughters in quality environments and to rear only sons in the remainder. Such a strategy requires an environmental cue that is reliably correlated with the quality of the habitat and a system of sex determination that is able to respond to this cue (Bull 1983; Charnov and Bull 1977). Bull (1981b) was the first to recognize that such life histories will tend to select for a primary sex ratio that is biased towards the sex that develops in the poorer patches. Bull's (1981b) argument is best illustrated by using our earlier example; daughters are reared in quality environments and sons are reared in sub-optimal patches. Imagine that half of the environmental patches are so poor that the males produced in them are essentially sterile. In the high quality patches frequency-dependent selection will select for a balanced sex ratio, regardless of the sex ratio in the sub-optimal patches. Because the population includes both the sterile and the quality males, the overall primary sex ratio is male biased (Bull 1981b; Bull and Charnov 1988).

The last assumption of Fisher's model is concerned with the distinction between parental vs. zygotic control of sex determination (Bulmer and Bull 1982; Fisher 1930;

Trivers 1974). Under parental control, alleles act in the parent to control offspring sex. Systems of parental control include haplo-diploidy, environmental sex determination and meiotic drive of sex chromosomes in the heterogametic sex (Bulmer and Bull 1982). In the haplo-diploid hymenopterans, females control the sex of their offspring by "deciding" whether or not to fertilize their eggs (Bulmer and Bull 1982). Similarly, in systems of environmental sex determination, females exercise control by choosing when or where to lay their eggs. In many reptiles for example, sex is determined by temperature and a female can control her clutch sex ratio by choosing a warm or a cool nesting site (Bull 1980; Bulmer and Bull 1982). The third example of parental control is sex chromosome systems with meiotic drive. In these systems the segregation distortion genes and their autosomal repressors all act in the heterogametic parent and are therefore under its control (Bulmer and Bull 1982; Carvalho et al. 1998).

Under zygotic control the alleles act in the zygote to control sex. In some species of fish for example, a zygote's sex is determined by the sum of genetic effects over many independent loci (Bulmer and Bull 1982). This is known as a polygenic system of sex determination. In other cases sex is determined in response to some environmental factor operating during development. This is known as environmental sex determination (ESD, Korpelainen 1990). The distinction between polygenic and environmental sex determination is not always clear. Most polygenic traits are affected by environmental variation to some degree and most environmentally induced traits have a polygenic basis (Bull 1983). In turtles with temperature-dependent sex determination (TSD) for example, low temperatures produce males and high temperatures produce females (an example of ESD, Bull 1980). However at intermediate (threshold) temperatures, clutch sex ratio

depends on the genetic background of the offspring (an example of polygenic sex determination, Bull et al. 1982a). The evolution of the primary sex ratio in these systems is further complicated by female nest choice. Female nest choice may be influenced by an individual's genotype (Bulmer and Bull 1982) or it may be the result of imprinting on female offspring following hatching (i.e. a learned behavior, Freedberg and Wade 2001). In other words the evolution of the primary sex ratio may be a function of maternal genotype (parental control), offspring genotype (zygotic control), nest site imprinting (cultural transmission) and environmental influences operating on all three.

As an aside, the distinction between the two modes of control necessitates a semantic dichotomy. Under parental control the clutch sex ratio is a trait of the parent and we can speak of alleles that influence the "sex ratio" of an individual's offspring. Under zygotic control, clutch sex ratio is a collective trait of a number of offspring. Each individual offspring has its own genetic tendency towards developing as either a male or a female. In this case we refer to alleles that influence the "sex tendency" or "sex predisposition" of an individual.

Fisher's original model referred exclusively to the situation where the primary sex ratio was under parental control. However, Bulmer and Bull (1982) investigated polygenic systems of sex determination and found that Fisher's sex ratio principle holds regardless of whether control is parental or zygotic. The only difference between the two modes of control was the rate of evolution towards the Fisherian equilibrium. This is half as fast under parental control because the trait is expressed in only one sex (usually the mother, Bulmer and Bull 1982). While the two modes of control are essentially equivalent under polygenic inheritance, the distinction remains important in systems

where the "sex ratio" or the "sex tendency" is determined by only one or a few genes (Carvalho et al. 1998). Under parental control, the number of genes involved in determining sex ratio is irrelevant to confirming the generality of Fisher's model when this assumption is met (Carvalho et al. 1998). However, major gene systems of sex determination under zygotic control violate the assumption of biparentalism and result in uncharacteristically fast evolution towards an equilibrium (Carvalho et al. 1998).

It is important to note that Fisher's sex ratio principle refers to a population equilibrium and makes no predictions about the optimal parental strategy (Williams 1979). Once the population sex ratio has reached its equilibrium value all family sex ratios are equally fit and the population can consist of a variety of parental strategies (Bull and Charnov 1988; Kolman 1960). For example, to achieve a population equilibrium of 50/50, half of the population could be son specialists and the other half daughter specialists. Alternatively, all of the individuals in the population could produce 50/50 clutches of sons and daughters, or any combination of these two extremes. When the population size is infinite, natural selection has no effect on the variance of the individual parental strategies (Kolman 1960).

Finite population size connects Fisher's population equilibrium argument to fitness of individual strategies (Charnov 1982; Williams 1979). 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 (Charnov 1982; Verner 1965; Williams 1979). Under the assumption that rearing a son or a daughter is equally costly, the optimal parental strategy is to produce equal

proportions of male and female offspring (MacArthur 1965; Pianka 1974; Verner 1965). In general, strong stabilizing selection for balanced sex ratios is expected to reduce genetic variation in parental strategies and increase canalization of the sex-determining mechanism. Systems with genetic variation for the primary sex ratio are therefore expected to be rare.

Adaptive evolution can proceed only in the presence of genetic variation. The absence of a model system with genetic variation for the primary sex ratio has proven to be a major stumbling block in the empirical development of the discipline. Fisher's model appeared to be so successful at eliminating variation that there was none left for nature or science to work with. All early attempts at measuring heritable variation in sex chromosome systems failed and people started to question whether these systems were even capable of evolving (Falconer 1954; Toro and Charlesworth 1982; Williams 1979). Heritability of sex tendency was first successfully measured in 1982 in a turtle with temperature dependent sex determination (TSD, Bull et al. 1982a). Other model systems were soon discovered and the first dynamic test of Fisher's sex ratio principle was conducted in 1990 in a species of fish with TSD (Conover and Van Voorhees 1990). To date there have been three dynamic tests of Fisher's prediction (Basolo 1994; Carvalho et al. 1998; Conover and Van Voorhees 1990) and only one of these (Carvalho et al. 1998) met all of the model's assumptions.

This thesis concerns itself with the discovery, or rather the rediscovery, of one such model system and its contributions to sex ratio theory. *Tigriopus californicus* is a species of harpacticoid copepod that is found in the splash pools of the Pacific coast from Alaska to Baja, Mexico (Dethier 1980). Ar-Rushdi (1958) was the first to suggest a

polygenic basis for sex determination after successfully selecting for male and female-biased primary sex ratios. His cytological studies subsequently confirmed that *T. californicus*, like other harpacticoids, lacked sex chromosomes (Ar-Rushdi 1963). Vacquier (1962) and Vacquier and Belser (1965) found that they were able to manipulate the sex ratio using hydrostatic pressure. Others found that sex determination in *T. californicus* and its congener *T. japonicus* was also influenced by environmental factors such as temperature (Egloff 1966), UV irradiation (Chalker-Scott 1995) and chemicals (Egami 1951; Takeda 1950). As early as 1959, Belser claimed that *Tigriopus* was the first example of polygenic sex determination in the literature and that it held great potential for scientists "seeking the origin of sexuality." While Belser (1959) may have overstated his case, sex determination in *T. californicus* remains enigmatic.

Chapter 2, the first data chapter of my thesis, uses the tools of quantitative genetics to measure the genetic variation in the primary sex ratio in *T. californicus*. In this chapter I use three experiments to demonstrate a polygenic basis of sex determination in *T. californicus*. Under the assumption that sex determination occurs in the zygote (zygotic control), I estimate the heritability of sex-tendency in all three experiments. I also revive two data sets from the literature and estimate, for the first time, the heritability of sex tendency in two other harpacticoid copepods, *Tisbe gracilis* (taken from Battaglia, 1958) and *Tigriopus japonicus* (taken from Igarashi, 1963). The main objective of chapter 2 is to show that *T. californicus* (and other harpacticoids) has genetic variation for the primary sex ratio. This chapter was published in the journal "Evolution"

Chapter 3 and 4 investigate the old claims of environmental sex determination (ESD) in *T. californicus*. In Chapter 3, I show that higher temperatures produce a

moderate increase in the proportion of males and that this phenomenon is not the result of differential mortality of males and females following sex determination. In Chapter 4, I show that low larval density increases the proportion of males and provide a test of Charnov and Bull's (1977) adaptive model of environmental sex determination. Chapter 4 shows that there is selection on both male and female body size and that the difference in the strength of selection between the sexes has important consequences for the evolution of ESD. However, both fecundity selection on female body size and sexual selection on male body size appear to be highly context-specific. Hence at this point, it is still not clear which sex benefits the most from large body size and whether the observed patterns of ESD in these experiments represent an adaptive mechanism. Chapter 3 was published in the "Biological Journal of the Linnean Society" and chapter 4 was submitted to the "Canadian Journal of Zoology."

Chapter 5 deals with cytoplasmic sex ratio distorters. These include bacteria such as *Wolbachia* and protozoan microsporidians that manipulate sex determination in their arthropod hosts (Rigaud 1997). Cytoplasmic sex ratio distorters generate variation in the primary sex ratio among families and their expression and transmission is often sensitive to environmental factors such as temperature (Hurst 1993). Hence cytoplasmic sex ratio distorters could account for the phenomena observed in chapters 2, 3 and 4, and it was important to rule out this alternative explanation. I am currently in collaboration with Dr. Suzanne Edmands at the University of Southern California to publish these data.

Chapter 6 investigates the paternal contribution to the sex ratio trait in *T. californicus*. This research was motivated by a recent paper that found that the sex ratio trait in the European fairy shrimp was paternally transmitted and associated with the

presence of supernumary (B) chromosomes (Beladjal et al. 2002). I likewise found that the sex ratio trait in *T. californicus* was paternally transmitted and found no evidence of maternal transmission, which contradicted our evolution paper (see discussion). This chapter has been accepted for publication in the "Journal of Evolutionary Biology" pending revisions.

Chapter 7 is a general discussion of what I believe I know about the sex ratio trait in *T. californicus*. I also give some direction about future experiments that will answer questions such as how sex is determined in *T. californicus* and what maintains variation for the primary sex ratio in this species.

CHAPTER 2: HERITABILITY OF SEX-TENDENCY IN *TIGRIOPUS*

Abstract: Sex ratio theory and empirical observation suggest that polygenic systems of sex determination are relatively rare. Evidence is presented for heritable variation of the primary sex ratio in the harpacticoid copepod *Tigriopus californicus*. Clutches of offspring were reared in the laboratory and offspring sex was determined at sexual maturity. Variation in the primary sex ratio among families is larger than expected under Mendelian segregation of sex chromosomes. The covariance of a female's replicate egg sacs and the covariance between mothers and daughters suggested that variation in the primary sex ratio is polygenic. Genetic correlations across environments (temperature treatments) indicated moderate genotype*environment interactions. There was no effect of temperature on the magnitude of the heritability. There was also no relationship between larval mortality and the magnitude of the heritability of sex tendency. It is therefore unlikely that differential mortality between the sexes biased our estimates. From five separate full-sib designs the combined broad sense heritability of sex tendency was 0.29 ± 0.068 for the original data and 0.17 ± 0.047 for the mortality-corrected data. The narrow sense heritability of sex tendency from the mother-daughter design is 0.31 ± 0.216 (not corrected for larval mortality). These heritabilities are similar to estimates that we calculate here for the first time for two other species of harpacticoid copepod.

INTRODUCTION

Evolution by natural selection can proceed only in the presence of genetic variation. Over the last 50 years, the widespread application of quantitative genetics in evolutionary biology has repeatedly shown that virtually every aspect of the phenotype has some heritable, genetic component. This realization has led to the argument that there is not much value to investigations that merely seek to confirm the existence of heritable variation (Lynch and Walsh 1998). For some traits, however, the magnitude of the heritability may be so small that its existence is far from certain. A good example of such a trait is the primary sex ratio, defined here as the proportion of male offspring at the time of sex determination. The heritability of the primary sex ratio has been notoriously elusive despite the vast body of data from domestic animals, humans and *Drosophila* (Bar-Anan and Robertson 1975; Edwards 1970; Falconer 1954; Foster and McSherry 1980; Hohenboken et al. 1988; Toro and Charlesworth 1982). This lack of evidence for a heritable component has led some authors to question whether the primary sex ratio is actually capable of evolving (Toro and Charlesworth 1982; Williams 1979). 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).

The adaptive significance of the primary sex ratio was first noted by Fisher (1930), who pointed out that in an outcrossed population of sexually reproducing individuals, "the collective investment by the parents in each sex ought to be equal." If males are rare in the population, any genetic tendency to produce sons rather than daughters will be favored by selection and the frequency of these "son-producing genes" will increase until there is no longer a shortage of males (Bull and Charnov 1988). Direct

tests support Fisher's sex-ratio principle (Carvalho et al. 1998; Conover and Van Voorhees 1990). Such observations and theory alike suggest frequency-dependent selection to be a highly efficient mechanism in shaping the evolution of the primary sex ratio, as long as there is heritable variation underlying sex determination.

In general, strong stabilizing selection for balanced sex ratios is expected to reduce genetic variation in parental strategies and increase canalization of the sex-determining mechanism. Systems of polygenic and environmental sex determination are therefore believed to be inherently unstable (Bull 1981a; Bulmer and Bull 1982; Rice 1986) and susceptible to replacement by a genotypic or chromosomal sex determination (Bull and Bulmer 1989; Bulmer and Bull 1982; Rice 1986). Organisms with systems of polygenic and environmental sex determination are therefore relatively rare. The rarity of these organisms underscores their value as model systems for studying the evolution of sex ratio.

In the genetic analysis of any quantitative trait, we typically assume that the trait of interest is under the influence of many loci with small effects. This assumption is tenuous for the primary sex ratio where a wide variety of non-polygenic sex determining mechanims are known (e.g. heterogamety, major sex genes, cytoplasmic inheritance). Bull (1983) listed three criteria that suggest the presence of a polygenic system of sex determination:

1. a large between-family sex ratio variance (that cannot be accounted for by sampling error under the assumption of male or female heterogamety)
2. paternal and maternal effects on family sex-ratio
3. a sex-ratio response to selection

These properties are not found in systems with male or female heterogamety where the primary sex ratio is constrained by meiotic segregation of sex chromosomes (Falconer 1954; Toro and Charlesworth 1982; Williams 1979).

Bulmer and Bull (1982) observed that polygenic systems of sex determination can be either under parental or zygotic control. Theory shows that Fisher's sex ratio principle holds in either case (Bulmer and Bull 1982). The distinction between parental or zygotic control is nevertheless important because it dictates the experimental design and subsequent analysis required for estimating quantitative genetic parameters.

In the classic model by Trivers and Willard (1973) and in the haplo-diploid hymenopterans (Hamilton 1967) offspring sex is determined by the parent (parental control). In these systems clutch sex ratio is a parental character and the covariance among multiple clutches provides an estimate of the repeatability of the trait (Falconer 1989). In contrast, under polygenic or environmental sex determination (ESD) sex is determined in the zygote (although for ESD the picture is potentially complicated by maternal choice of nest sites, Bulmer and Bull 1982). Under zygotic control, sex is a trait of the offspring and the same data (multiple clutches of full sibs) can be used to estimate the heritability of the primary sex ratio (Bull et al. 1982a).

When sex is determined in the offspring, "heritability of the primary sex ratio" is actually a misnomer because the clutch sex ratio is a collective trait of the brood. By analogy litter size is often believed to be a trait of the female but the weight of each newborn is a trait of the offspring and it would be erroneous to speak of the "heritability of total offspring weight". To prevent confusion and to distinguish between systems of parental and zygotic control we will use "heritability of the primary sex ratio" when referring to the former and "heritability of sex tendency" when referring to the latter.

This study investigates the sex-determining mechanism of the harpacticoid copepod, *Tigriopus californicus*. Using Bull's (1983) first two criteria, we provide three experimental lines of evidence that *T. californicus* has a polygenic system of sex determination.

Experiment 1. Populations show extra-binomial variation in the primary sex ratio of offspring (Bull's first criterion).

Experiment 2. Sex-ratio is consistent among a female's clutches; full sib heritability estimates of sex tendency (Bull's second criterion).

Experiment 3. The covariance of clutch sex ratio between mothers and daughters is positive; mother-daughter heritability estimates of sex tendency (Bull's second criterion).

Based on the assumption that sex is determined in the offspring we estimate the heritability of sex tendency from a full sib (experiment 2) and a mother-daughter design (experiment 3). In addition, we calculate here for the first time the heritability of sex tendency for two other species of harpacticoid copepod.

MATERIALS AND METHODS

Experiment1: Extra-Binomial Variation in the Primary Sex Ratio

In the summer of 1999, we reared and mated ~ 60 *Tigriopus* females (F_1) whose offspring comprised the second lab-born (F_2) generation of field-captured individuals from several locations around Victoria, British Columbia, Canada and Bamfield, British Columbia. After the F_2 generation hatched we randomly selected sibships of 20 living nauplii from each of the 60 families. We assigned each sibship of 20 siblings to a 24 well tissue culture plate. Families (the offspring of one female are referred to as a "family") were nested within plates because randomizing 1200 F_2 nauplii across 60 plates was not logistically feasible. Within this family plate, each sibling was allocated to its own well with 2.5 ml of filtered sea water and 3 drops from an *Isochrysis galbana* culture. At the first copepodite stage, we added one drop (~ 0.5 ml) of Tetramin™ flake solution (100 mg of ground up Tetramin™ flakes suspended in 50 ml of dH_2O) to each well. Plates were stored in an incubator at a temperature of 20°C and no light.

We sexed all individuals once they reached sexual maturity, about 18 days after hatching. At this stage of the life cycle, *Tigriopus* adult males are easily distinguishable from adult females by their enlarged geniculate antennae. For those cases where sex could not be identified at the time of assay (i.e. a dead or sexually immature copepodite), the individual was assigned to the rarer sex in that family. Assigning unidentified individuals to the rarer sex is a conservative approach for testing hypotheses regarding extra-binomial variation in the primary sex ratio (Bull and Vogt 1979; Bull et al. 1982b; Conover and Kynard 1981).

Experiment 2: Full-Sib Design

In the fall of 2000 we assayed the sex ratio of two consecutive egg sacs for a sample of 56 *Tigriopus* females. Gravid females were sampled from a laboratory population which had been maintained in culture for about five generations. This population had been originally collected from Arbutus Cove, Victoria (Lat. 48°28'36''N; Long. 123°18'00''W). For every female in the sample, each of her two egg sacs was split into two random groups of approximately 20 offspring. One group was assigned to a cool (15° C) and the other to a warm temperature (22° C) treatment. The four combinations of egg sac (parity) and temperature allowed us to compare the heritability of sex tendency between two different temperature regimes. Nauplii and copepodites were reared on *Isochrysis* and Tetramin™ flake solution. Offspring from the first egg sac were reared in isolation in 24-well tissue culture plates (as in experiment 1). Offspring from the second egg sac were reared together in a 30 dram Vial.

Again, to ensure that sex-specific mortality differences were not inflating the among family variance component, all unidentified individuals were assigned to the rarer sex for their family. The results from the fall experiment were compared to a pilot study conducted on 45 families in the summer of 2000 when this population was originally collected from the field. Experiment 2 and the pilot study will hereafter be referred to as the fall and summer assay, respectively. For the fall assay, survivorship at 15 and 22° C was 96.2 and 96.8 %; for the summer assay, survivorship was 95.6 and 84.1%, respectively. The correction for larval mortality in the 22° C treatment of the summer assay eliminated most of the variance among families.

Experiment 3: Mother-Daughter Design

To estimate the heritability of sex tendency in *Tigriopus* we compared the sex ratio of clutches between field-captured females and their lab-reared daughters. In the summer of 2000, we took a sample of copepods from Arbutus Cove, Victoria and selected twenty gravid females to produce the F_1 generation. For each female we isolated 3 consecutive egg sacs. Daughters from the first egg sac were reared, mated and allowed to reproduce to provide estimates of their clutch sex ratio. Offspring from the second and third egg sac were raised to provide estimates of the mother's clutch sex ratio.

To obtain the daughter generation, we reared 30 nauplii from the first egg sac in six-well tissue culture plates (5 nauplii/well) for each family. Nauplii were incubated at 15° C with no light and fed on a diet of *Isochrysis* and Tetramin™ solution. At approximately the fourth copepodite stage we introduced males from the original Arbutus Cove sample. Once the males had clasped sexually immature females we allocated each couple to a single well in a 24-well tissue culture plate. These individuals comprised the daughter generation.

For each of the twenty females in the parental generation, we isolated at least two more egg sacs. Offspring were raised in 30 dram vials at 15° C and at sexual maturity we assayed the proportion of males in each vial to obtain an estimate of each mother's sex ratio. For the daughter generation, we isolated one egg sac for each daughter and determined its sex ratio (also at 15° C). Three families were lost so that the final data set consisted of 17 mother-daughter pairs.

To obtain the best estimates of a family's sex ratio, we summed the number of males and females across all brood sacs for each mother and across all daughters for each

F_1 family. For 17 mother-daughter pairs, sex ratios were based on an average of 35 offspring (range = 14 to 53) for the mothers and 32 offspring for the daughters (range = 2 to 90). Each F_1 family was represented by an average of three sisters (range = 1 to 7).

Unlike the previous two experiments, survivorship was relatively poor. For the parental and F_1 generation, only 65% and 67% of all offspring were recovered as sexually mature adults, respectively. For this reason we did not correct the observed sex ratios for larval mortality because doing so would have eliminated most of the variation in sex ratio between families. The heritability estimate from this experiment will therefore be upwardly biased if there are sex-specific mortality differences between families prior to the time of assay.

Experiment 4: Harpacticoid Data Sets from the Literature

From the literature we obtained full-sib data sets for two other species of harpacticoid copepod, *Tisbe gracilis* (Battaglia 1958) and *Tigriopus japonicus* (Igarashi 1963a). The authors showed that variation in clutch sex ratio among families is greater than variation within families (i.e. clutch sex ratio is repeatable for females) by means of a chi-square analysis (Table VI, Battaglia 1958) and a graph (Fig. 1, Igarashi 1963a). In neither case did the author calculate a heritability of sex tendency which we do here for the first time for purposes of comparison.

Battaglia (1958) claimed that the differences between families cannot be attributed to differential mortality (i.e. he presumably minimized larval mortality). Igarashi's data (1963a) are also not complicated by differential mortality because he

discarded any replicates where survivorship to sexual maturity was less than perfect. Survivorship of nauplii to sexual maturity is 100% in both data sets.

STATISTICAL METHODS

Experiment 1: Extra-Binomial Variation in the Primary Sex Ratio

To rule out chromosomal sex determination we compared the observed distribution of clutch sex ratios with the binomial expectation using a Chi-Square Goodness of Fit test (Zar 1999). To circumvent problems with small expected frequencies and excessive Type I error, sibships with 6 or fewer males (Sex ratio ≤ 0.30) were grouped and sibships with 14 or more males (Sex ratio ≥ 0.70) were grouped (Fig. 1). We also used a randomization test to establish the statistical significance of the observed variance in clutch sex ratio. For the null distribution we generated 10,000 variance estimates. Each variance estimate was based on a random sample of sex ratios from 60 families. For each family the sex ratio was based on a trial size (N) of 20 offspring with an expected sex ratio of 50/50.

Experiment 2: Statistical Test of Family Effects

Prior to estimating the heritability of the sex tendency it is useful to determine whether there is a genetic basis to the trait. For the full-sib design, rearing two clutches in separate vials allows us to separate cage (and parity) effects from family effects (Roff 1986; Roff 1997). If there is a genetic basis for the primary sex ratio in *Tigriopus* then the proportion of males between the two clutches should be similar. This can be tested statistically using the intra-class correlation coefficient (t). Following Roff (1997) we used the one-way ANOVA and the nested ANOVA method for establishing the statistical significance of family and clutch effects. After showing that there is a genetic basis for the primary sex ratio we proceeded to estimate its heritability by pooling the two clutches for each family.

Experiment 2: Full-Sib Heritabilities of the Primary Sex Ratio

Sex can be treated as a threshold trait for quantitative genetic analysis (Bull et al. 1982a; Bulmer 1985; Bulmer and Bull 1982; Falconer 1989; Lynch and Walsh 1998; Roff 1997). The general statistical machinery for estimating the heritability of sex tendency is given by Dempster and Lerner (1950) and Bull et al. (1982a). Sex is measured on the binomial scale where females are coded as 0 and males are coded as 1. For a sample of full-sib families, the intra-class correlation coefficient (t) is calculated on this binomial scale using one of the three methods listed by Roff (1997). We used the ANOVA method to calculate t (Elston 1977; Roff 1997). Following computation of t, the heritability of sex tendency (X) on the underlying scale is estimated using the Robertson and Lerner (1949) transformation. We also used the method outlined by Bull et al.

(1982a) to estimate the heritability of sex tendency. Approximate standard errors for the full-sib heritability estimates were calculated following Roff (1997).

For the full-sib sex ratios, the statistical significance of family effects and the heritabilities were calculated for the original data and for the data following the correction for larval mortality. For further comparison, we estimated the heritability of sex tendency for the 60 families in experiment 1 under the assumption of no cage effects. In total there are five full sib 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), and experiment 1 (abbreviated as 1999). Following Mousseau and Roff (1989) we calculated a combined heritability by weighting each estimate by the inverse of its sampling variance.

Our approach for estimating the heritability of sex tendency from these full-sib data sets makes three important assumptions. The first assumption is that all offspring from the same mother are full sibs (Bull et al. 1982a; Roff 1997). This assumption is likely to be met as sperm competition and multiple paternity do not occur in *Tigriopus* (Burton 1985). The second assumption is that all variation among families is due to additive genetic effects. In other words, variation in clutch sex ratio is not influenced by epistasis, dominance, maternal effects, common environment, major genes or sex-linked loci (Bull et al. 1982a). Finally, as mentioned in the introduction, the analysis assumes that sex is determined in the offspring and not by the parent (Bull et al. 1982a; Bulmer and Bull 1982).

*Experiment 2: The Genetic Correlation among Environments and Genotype*Environment Interactions*

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* environment (G*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 which is significantly less than one indicates the existence of G*E interactions (Yamada 1962).

To evaluate the importance of the G*E interactions on the clutch sex ratio we used the correlation of family means to approximate the standard full-sib correlation.

$$r_m = cov_{m(15^\circ C,\ 22^\circ C)} / (var_{m(15^\circ C)} * var_{m(22^\circ C)})^{1/2} \quad (1)$$

where $cov_{m(15^\circ C,\ 22^\circ C)}$ = the covariance of family clutch sex ratio between the two environments (15° C and 22° C), and $var_{m(15^\circ C)}$ and $var_{m(22^\circ C)}$ = the variances of family clutch sex ratio for each of the two temperature treatments. This method is an approximation because each term in Eq. 1 contains a within-family "error" component; however, the correlation 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 two clutch sex ratios 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 as it excludes confidence limits > 1.

Experiment 3: Parent-Offspring Heritabilities of the Primary Sex Ratio

The statistical significance of the covariance in clutch sex ratio between mothers and daughters was determined on the observed scale using a standard regression analysis as well as a randomization test. In the randomization test we compared the observed correlation in clutch sex ratio to an empirical distribution of 10,000 correlations that was generated by pairing the clutch sex ratios of mothers and daughters at random. To calculate a heritability of sex tendency, the clutch sex-ratios of mothers and daughters are first transformed to the underlying scale (Roff 1986) using the standard tables in Zar (1999) or the approximation by Hamaker (1978). Analysis then proceeds as usual for a normally distributed trait (Roff 1997). The heritability of the sex tendency (h^2_X) was calculated as twice the slope of the mother-daughter regression. Standard errors of heritability estimates were calculated following Becker (1984).

Experiment 4: Heritability of the Sex Tendency for Two Other Harpacticoids

The data set for *Tisbe gracilis* was obtained from a table (Battaglia 1958, Table VI). The statistical significance of family effects and the heritability of sex tendency for *T. gracilis* were calculated as in experiment 2. The data set for *T. japonicus* was recovered from a graph (Igarashi 1963a, Fig. 1) that shows the sex ratio over nine consecutive egg sacs for eight distinct genotypes. We calculated the statistical significance of family effects for this group of eight genotypes.

The eight genotypes are representive of the three sex ratio types that Igarashi (1963a) identified; male dominance, intermediate, and female dominance. Igarashi (1963a) assayed 79 families and found that 69 families were male dominant (87.34%),

seven were intermediate in sex ratio (8.86%), and three were female dominant (3.80%). We used the frequencies of male, intermediate and female dominance to reconstruct the distribution of the eight genotypes in the original sample of 79 families.

Igarashi (1963a) only reported the percentage of males and did not indicate the number of offspring on which those percentages were based. We calculated the heritability of the sex tendency for the reported distribution of sex-ratios assuming clutch sizes of ten or 100 offspring (i.e. family sex ratios were based on 90 or 900 offspring after pooling all nine clutches).

RESULTS

Experiment 1: Extra-Binomial Variation in Clutch Sex Ratio

The population sex ratio (averaged over 60 females with 20 offspring per female) was 0.538 ± 0.0245 and was not significantly different from 0.500 ($P = 0.131$). However, the observed distribution of sex ratios had 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 sex ratios in Figure 1 reflects our conservative assignment of the dead larvae to the rarer sex. The observed mean square of the sample of sex ratios (0.0359) is almost three times larger than the binomial sampling component (0.0125). The observed among families variance component is therefore significantly larger than expected under Mendelian sex determination (randomization test; $P<0.001$).

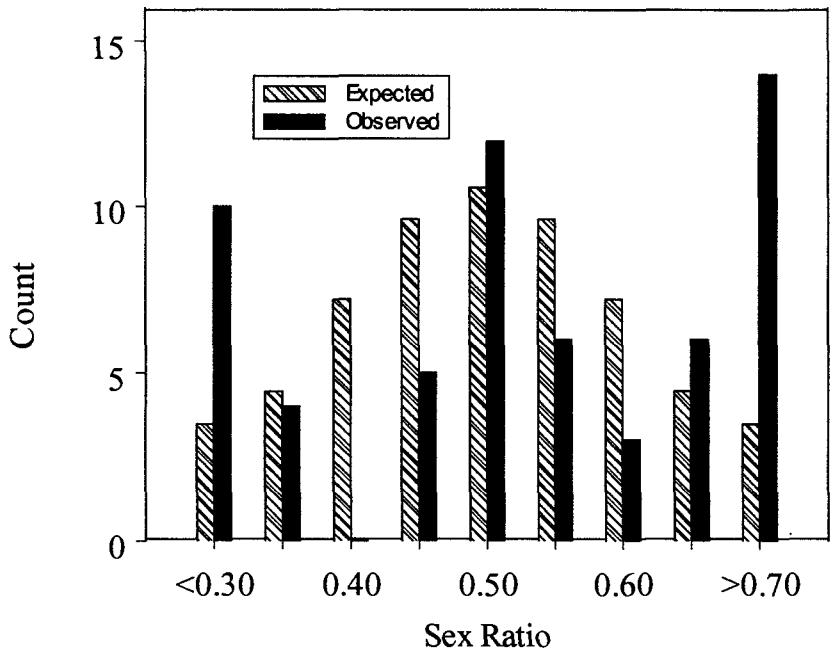

Figure 1. Proportion of males per sibship of 20 offspring. Shown are the expected and observed frequencies for 60 families. Categories with ≤ 0.3 or ≥ 0.7 males were lumped because of low expected values.

Experiment 2: Statistical Test of Family Effects

For the fall assay, the among family variance component (uncorrected for larval mortality) is significantly different from zero at both 15° C and 22° C (Table 1). For the summer assay, the among family variance component (uncorrected for larval mortality) is marginally non-significant at 15° C and not significant at 22° C (Table 1). The magnitude of the variance components and the pattern of significance are similar when the clutches are corrected for larval mortality (Table 1; Fig. 2). Hence in the fall assay there is significant variation in the primary sex ratio among families over and above the variation between successive clutches (the within family-among clutch variance component).

The within family-among clutch variance component (hereafter referred to as the parity variance component) is statistically significant for all four season-temperature combinations for both the uncorrected and corrected data (Table 1; Fig. 2). The contribution of parity to the total variance in clutch sex ratio is in some cases much larger than the between family variance component (Fig. 2).

We also found significant family effects in *Tisbe gracilis* ($P < 0.001$) and *Tigriopus japonicus* ($P < 0.001$). From these significant among family variance components we conclude that there is a genetic basis for the primary sex ratio in *Tigriopus* and *Tisbe*.

Table 1. Among family, within family (among clutches), and within clutch (among siblings) variance components of the sex phenotype for three species of harpacticoid copepod. "Description" refers to the experiment and "Corrected" refers to whether the clutches were adjusted for larval mortality. Variance components were obtained from the nested ANOVA. Statistical significance of the among and within family effects (P-values) were obtained from a nested ANOVA (nAOV) and a one-way ANOVA (AOV). Note that the statistical significance of within family effects is not estimable from a one-way ANOVA. For the data set by Igarashi (1963a), we performed the analysis on the eight distinct genotypes assuming a clutch size of ten nauplii per egg sac.

			nAOV Variance Components				P-values		
							nAOV	nAOV	AOV
			Among	Within	Within	Total	Among	Within	Among
Species	Description	Corrected	Family	Family	Clutch		Family	Family	Family
T. californicus	Fall 15° C	No	0.019	0.009	0.223	0.251	**<0.001**	**0.001**	**<0.001**
T. californicus	Fall 22° C	No	0.013	0.024	0.213	0.250	**0.029**	**<0.001**	**0.036**
T. californicus	Summer 15° C	No	0.010	0.024	0.215	0.249	0.067	**<0.001**	0.082
T. californicus	Summer 22° C	No	0.000	0.032	0.202	0.234	0.522	**<0.001**	0.177
T. californicus	Fall 15° C	Yes	0.016	0.006	0.230	0.251	**<0.001**	**0.021**	**<0.001**
T. californicus	Fall 22° C	Yes	0.010	0.018	0.221	0.248	**0.035**	**<0.001**	**0.047**
T. californicus	Summer 15° C	Yes	0.008	0.015	0.227	0.249	0.053	**<0.001**	0.050
T. californicus	Summer 22° C	Yes	0.000	0.022	0.224	0.246	0.839	**<0.001**	0.827
Tisbe gracilis	Battaglia 1957	No	0.022	0.002	0.216	0.240	**<0.001**	0.094	**<0.001**
T. japonicus	Igarashi 1963	No	0.023	0.000	0.228	0.251	**<0.001**	0.979	**<0.001**

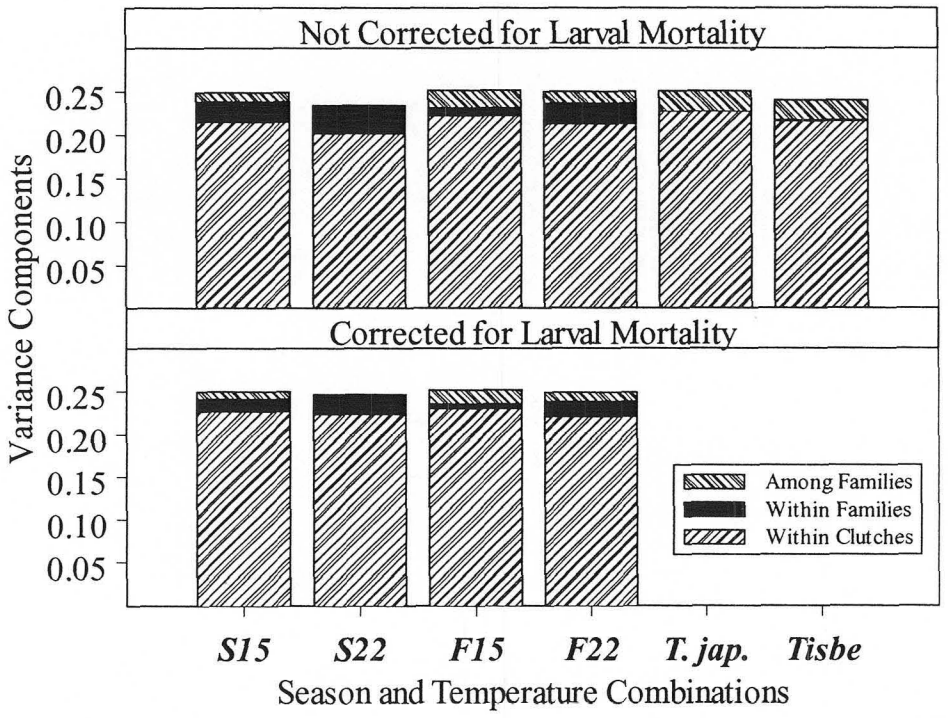

Figure 2. Between and within family variance components of the clutch sex ratio for the summer (S) and fall (F) assays at 15 and 22° C for *Tigriopus californicus* and for *T. japonicus* (Igarashi 1963a) and *Tisbe gracilis* (Battaglia 1958). The top and bottom panels show the variance components for the original and the larval mortality-corrected data, respectively. Legend; S15 = summer assay at 15° C, S22 = summer assay at 22° C, F15 = fall assay at 15° C, F22 = fall assay at 22° C, *T. jap.* = *Tigriopus japonicus* (Igarashi 1963a) and *Tisbe* = *Tisbe gracilis* (Battaglia 1958).

Experiment 2: Full Sib Heritability of Sex Tendency

We pooled the egg sacs for each full-sib family to obtain the best heritability estimate for each season*temperature combination in experiment 2 (Roff 1997). The method by Roff (1997) and by Bull et al. (1982a) produced similar results (Table 2). The heritabilities of the uncorrected sex ratios are very similar for all four data sets in experiment 2 and for the data from experiment 1 (Table 2). The combined heritability of sex tendency for these five estimates (weighted by the inverse of the sampling variance) is 0.29 ± 0.068. Following the correction for larval mortality the heritabilities are substantially smaller and the combined estimate is 0.17 ± 0.047. For the corrected data there is a correlation between survivorship to sexual maturity and the magnitude of the heritability estimate (Fig. 3). This relationship is an artefact of the larval mortality correction which reduces most of the variation among families. There is no relationship between survivorship and the uncorrected heritabilities which suggests that these estimates are not biased by differential mortality.

Table 2. Heritabilities of the sex tendency for three species of harpacticoid copepod. Shown are the uncorrected and the corrected estimates for the ANOVA method outlined by Roff (1997) and for the method by Bull et al. (1982a). "Description" refers to the experiment, N = number of families, c = average number of offspring per family, % Surv = percent survival to sexual maturity, h^2 = heritability. NA = larval correction is Not Applicable because survivorship = 100%; *** = larval correction was not performed because survivorship was too low.

					Uncorrected h^2		Corrected h^2	
Species	Description	N	c	% Surv	Roff (1997)	Bull et al. (1982)	Roff (1997)	Bull et al. (1982)
T. californicus	Fall 15° C	56	36.0	96.0	0.29 ± 0.065	0.30	0.22 ± 0.054	0.22
T. californicus	Fall 22° C	56	36.1	97.1	0.30 ± 0.067	0.31	0.24 ± 0.057	0.25
T. californicus	Summer 15° C	45	49.5	93.8	0.27 ± 0.065	0.26	0.19 ± 0.050	0.19
T. californicus	Summer 22° C	45	49.2	81.9	0.29 ± 0.071	0.34	0.10 ± 0.035	0.10
T. californicus	Experiment 1	60	20.0	100	0.31 ± 0.077	0.31	NA	NA
	Combined				0.29 ± 0.068	0.30	0.17 ± 0.047	0.17
T. californicus	Parent-Offspring	17	34.0	66.0	0.31 ± 0.216		***	***
Tisbe gracilis	Battaglia 1957	14	111	100	0.30 ± 0.118	0.31	NA	NA
T. japonicus	Igarashi 1963	79	~90	100	0.10 ± 0.021	0.09	NA	NA
T. japonicus	Igarashi 1963	79	~900	100	0.12 ± 0.020	0.11	NA	NA

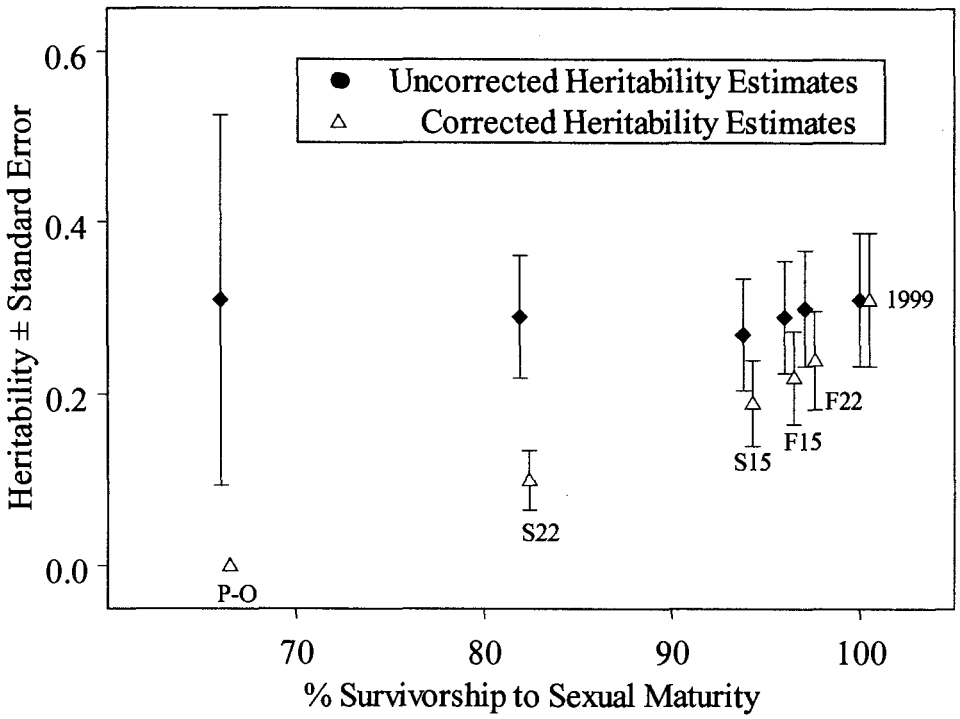

Figure 3. The heritability (± standard error) of sex tendency as a function of the larval mortality correction and survivorship to sexual maturity. Shown are the estimates from experiment 1 (1999), the four season-temperature combinations (S15, S22, F15, F22) from experiment 2, and the parent-offspring (P-O) estimate from experiment 3.

*Experiment 2: The Genetic Correlation among Environments and Genotype*Environment Interactions*

For the uncorrected data, the genetic correlation of clutch sex-ratio across the two temperature treatments is positive and significantly different from zero in both the fall and summer assay (Table 3, Fig. 4). However, in both assays, the upper 95% confidence limit of r_m does not include +1 (Table 3) indicating that there are moderate genotype*environment interactions. After correcting for larval mortality the results remain essentially unchanged (Table 3). For the uncorrected data of the fall and summer assay, approximately 35% of the genetic variation in clutch sex ratio across temperatures can be attributed to pleiotropy (Table 3). Finally, the correlation across temperature treatments lends further support to polygenic sex determination in *Tigriopus* (by Bull's second criterion).

Table 3. The full sibling genetic correlation (r_m) of the clutch sex ratio across the two temperature treatments (15° C and 22° C) in *T. californicus* for the summer and fall assay. Shown are the uncorrected and the corrected estimates, the sample size (N), the 95% confidence interval of the genetic correlation (95% C. I.), the P-value for the null hypothesis that $r_m = 0$ (P), and the percent of the genetic variation in clutch sex ratio across the two temperature treatments that can be attributed to pleiotropy ($\%V_G$).

Assay	Corrected	N	r_m	95% C. I.	P	$\%V_G$
Fall	No	76	0.58	0.38 – 0.79	**<0.001**	34
Summer	No	55	0.60	0.42 – 0.78	**<0.001**	36
Fall	Yes	77	0.61	0.43 – 0.78	**<0.001**	37
Summer	Yes	57	0.42	0.20 – 0.64	**0.001**	18

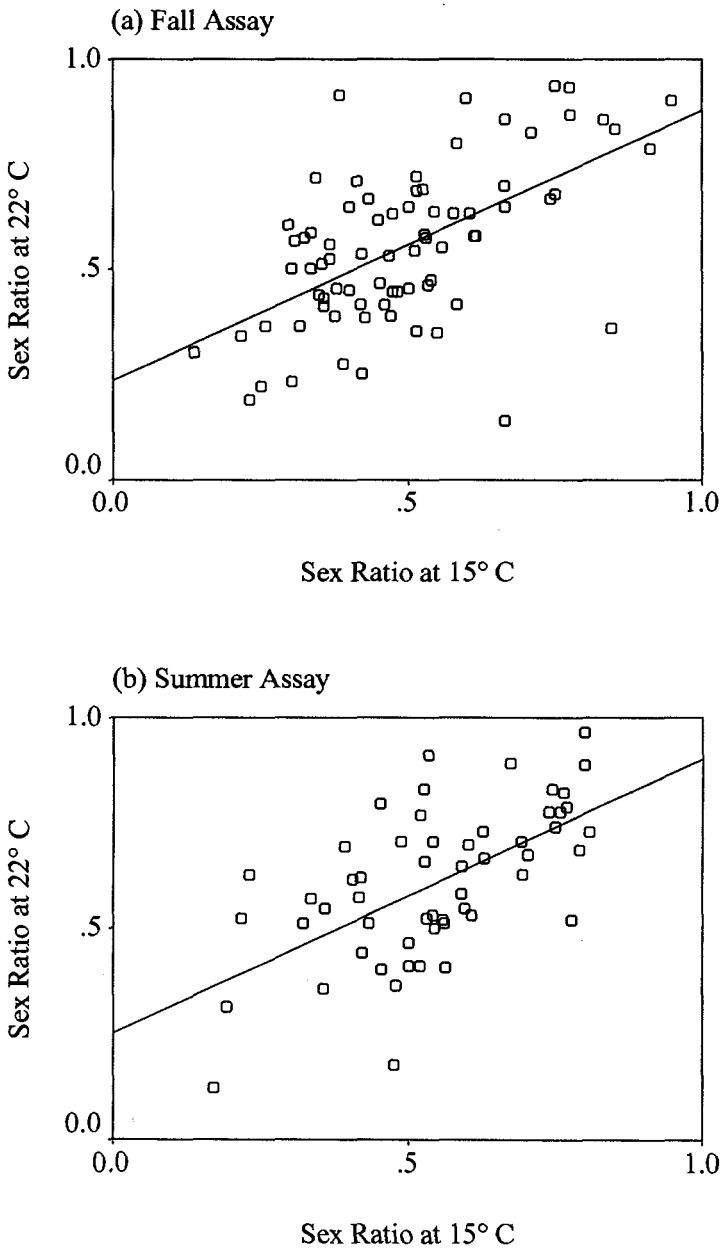

Figure 4. Family correlation (r_m) of clutch sex ratio at 15° C and 22° C for (a) the fall and (b) the summer assay. The clutch sex ratios are not corrected for larval mortality. Shown is the line of best fit.

Experiment 3: Mother-Daughter Heritability of Sex Tendency

The clutch sex ratio of mothers accounted for 37.2% of the variation in the clutch sex ratio of their daughters ($F_{1, 15} = 8.884$, $P = 0.009$; randomization test; $P < 0.005$; Fig. 5). If the regression is weighted by the square root of the trial size ($\sqrt{N_i}$) the clutch sex ratio of the mothers accounts for 43.9% of the variation in the clutch sex ratio of the daughter generation ($F_{1, 15} = 11.750$, $P = 0.004$; Fig. 6). However, upon transformation to the underlying scale, the mother-daughter regression is no longer significant for both the weighted ($F_{1, 15} = 2.031$, $P = 0.1746$) and the unweighted analysis ($F_{1, 15} = 2.591$, $P = 0.1283$). On this scale the heritability of sex tendency is 0.31 ± 0.216 (Table 1) and 0.36 ± 0.225 for the weighted and unweighted analysis, respectively.

Experiment 4: Heritability of Sex Tendency in Tisbe gracilis and Tigriopus japonicus

The heritability of sex tendency in *Tisbe gracilis* (0.31 ± 0.216) is similar to our uncorrected estimates in *T. californicus*. After adjusting the eight genotypes for their observed frequency in Igarashi's (1963a) sample of 79 individuals and under the assumption that he sampled ten offspring per clutch (90 offspring per family) the heritability of the sex tendency in *Tigriopus japonicus* is 0.10 ± 0.021 (Table 2). If we assume that Igarashi sampled 100 offspring per clutch (900 offspring per family) the estimate increases to 0.12 ± 0.020 (Table 2). The magnitude of these estimates is therefore relatively insensitive to whether Igarashi sampled 90 or 900 offspring per family.

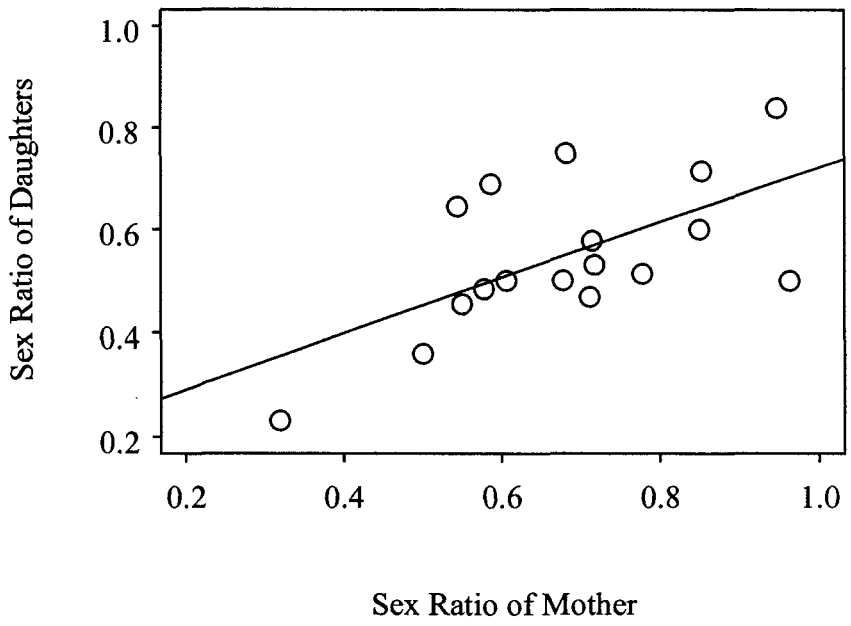

Figure 5. Relationship between the sex ratio (proportion of males) of mothers (X) and their daughters (Y). Shown is the line of best fit, $Y = 0.183 + 0.540X$.

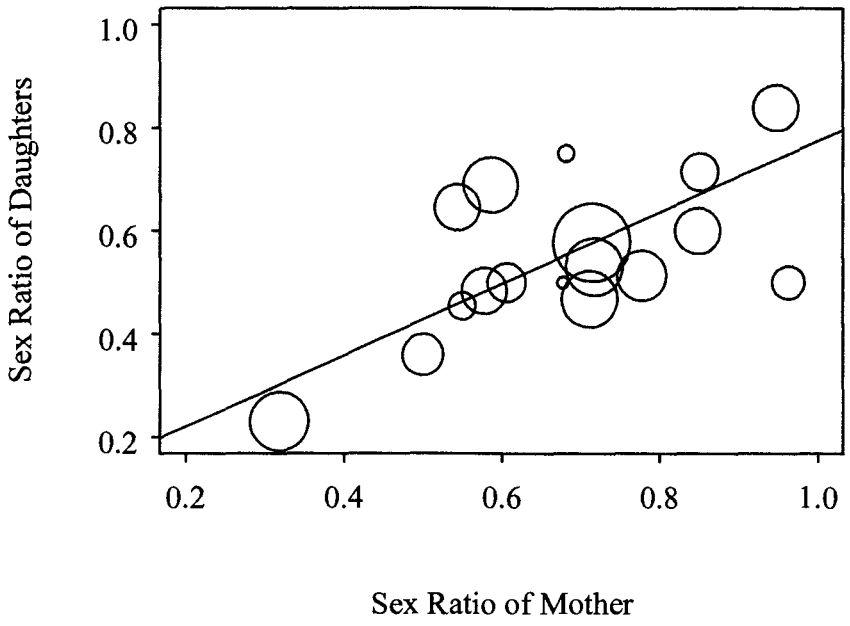

Figure 6. Relationship between the sex ratio (proportion of males) of mothers (X) and their daughters (Y). Sex ratios of the daughters are weighted by the square root of the trial size ($\sqrt{N_i}$). Shown is the line of best fit, $Y = 0.152 + 0.580X$.

DISCUSSION

Evidence for Polygenic Sex Determination in Tigriopus californicus

These experiments suggest that there is polygenic variation for sex determination and therefore heritable genetic influences on the primary sex ratio in *Tigriopus*. In the first experiment, extra-binomial variation rules out Mendelian sex determination and shows that there is considerable variation in the primary sex ratio of *Tigriopus*. In the second experiment, the significant among family variance component in the fall assay suggest that there is a genetic basis for the primary sex ratio. This interpretation is further supported by the significant correlation between the clutch sex ratios of mothers and daughters in the third experiment. Finally the correlation of family means across temperature treatments suggest that the clutch sex ratio is generally determined by the same set of genetic factors but that there are moderate genotype*environment interactions (reaction norms).

Further support for polygenic sex determination in *Tigriopus* comes from the work by Egloff (1966) and from the selection experiments and cytological assays of Ar-Rushdi (1958; 1963). Egloff (1966) observed extra-binomial variation in the primary sex ratio in his laboratory experiments with *T. californicus*. Ar-Rushdi (1958) selected for high and low male lines in *T. californicus* and succeeded in shifting the original sex ratio to as high as 99.5% male and as low as 20% male (Bull's third criterion for polygenic sex determination; see introduction). In his cytological assays he found no evidence of heteromorphic sex chromosomes (Ar-Rushdi 1963; Lecher et al. 1995). The absence of heteromorphic sex chromosomes is considered to be a necessary precondition for polygenic or environmental sex-determination (Bull 1980; Janzen and Paukstis 1991).

Similarly, Battaglia (1958) selected the harpacticoid copepod, *Tisbe gracilis*, for highly male-biased lines (selection for female bias was not successful) and concluded that sex determination was polygenic in this organism.

Despite the fact that *Tigriopus* meets three of Bull's criteria for polygenic sex determination, there are alternative explanations. Major sex determining genes have been reported in silverside fish (Conover and Heins 1987b; Conover and Kynard 1981), lemmings and house flies (Bull 1983). Such systems also show extra-binomial variation in the primary sex ratio, paternal and maternal effects on family sex ratio and a response to selection. Unfortunately, the design of our experiment is unable to address the number of loci responsible for sex determination in *Tigriopus*.

Our data also cannot distinguish between nuclear or cytoplasmic inheritance. Under cytoplasmic inheritance sex is determined by the presence or absence of intra-cellular parasites that are transmitted from mother to daughter via the cytoplasm. In general, infection by these parasites results in sex ratio biases which are much greater than those observed in our experiments (i.e. 100% female clutches are common). Cytoplasmic inheritance has been well documented in crustaceans including two species of amphipod (Bulnheim 1978b), terrestrial isopods (Bull 1983) and *Tigriopus japonicus* (Igarashi 1964).

Evidence for Zygotic Control of Sex Determination

Our heritability estimates implicitly assume that sex determination is under zygotic control. Evidence for zygotic control of sex determination is obvious in organisms with environmental sex determination (ESD) where sex is determined after

conception by an environmental factor. For example in turtles (Bull and Vogt 1979), or the Atlantic silverside (Conover and Heins 1987b; Conover and Kynard 1981) offspring sex is largely determined in response to temperature. There is evidence to suggest that sex is similarly labile in *Tigriopus californicus* (Egloff 1966; Vacquier 1962; Vacquier and Belser 1965; Voordouw and Anholt 2002a) and in its congener, *T. japonicus* (Egami 1951; Igarashi 1960; Takeda 1950).

Bulmer and Bull (1982) pointed out that under ESD, female choice of nest sites is probably an important aspect of sex ratio evolution in nature. However, the random assignment of offspring to growth chambers removes maternal behavior from the experimental design. As a result most quantitative investigations of organisms with ESD treat sex as a function of the offspring (Bull et al. 1982a; Janzen 1992; Lester et al. 1989). Following Bull et al. (1982a), Lester et al. (1989) and Janzen (1992) we assume that sex is determined in the zygote.

The Heritability of Sex Ratio and the Heritability of Sex Tendency

Heritability estimates of the primary sex ratio (parental control) or of the sex tendency (zygotic control) are relatively rare in the literature. In species with chromosomal sex-determination the magnitude of such estimates is often very small (Bar-Anan and Robertson 1975), or non-existent (Falconer 1954; Foster and McSherry 1980; Hohenboken et al. 1988; Toro and Charlesworth 1982). In other cases the data do not distinguish between variation in the primary sex ratio and sex-specific differences in mortality (King 1918; Weir 1962). In general, the potential for genetic variation in these

heterogametic systems is believed to be extremely limited as a consequence of the stability of Mendelian transmission (Williams 1979).

More success has been met in species with environmental or polygenic sex determination. Heritabilities of sex tendency have been reported in the map turtle (h^2 = 0.82, Bull et al. 1982a), the snapping turtle (h^2 = 0.56, Janzen 1992) and tilapia (h^2 = 0.26, Lester et al. 1989). These estimates were calculated using the approach outlined by Bull et al. (1982a) which gives similar estimates to the methods outlined by Roff (1997). Premoli et al. (1996) demonstrated polygenic variation for the primary sex ratio in a species of polychaete worm. They assumed that sex determination was under zygotic control but in their statistical analysis Premoli et al. (1996) treated clutch sex ratio as a parental trait (i.e. they estimated the heritability of the sex ratio).

Here we report the first heritability estimates of sex tendency in *Tigriopus* and two other harpacticoid copepods. The heritability of sex tendency in Tigriopus is ~ 0.30. For the original data (not corrected for larval mortality) the broad-sense heritability from the full-sib design and the narrow-sense estimate from the mother-daughter design are very similar (Table 2, Fig. 3). In addition we are the first to calculate the heritability of sex tendency for two other species of harpacticoid copepod; *Tisbe gracilis* (h^2 ~ 0.30) and *T. japonicus* (h^2 ~ 0.10).

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; Bull et al. 1982b; Conover and Kynard 1981) this protocol allows us to eliminate differential mortality of the sexes as an alternative explanation (especially where sex cannot be determined for those larvae that died). Hence in experiments 1 and 2 we have

conservative evidence that the observed results are caused by variation in the primary sex ratio. In experiment 3 we did not correct for larval mortality and so it is possible that our mother-daughter heritability of sex tendency is upwardly biased if there are sex-specific viability differences between families. However, the absence of any relationship between the uncorrected heritabilities and survivorship (Fig. 3) argues against such a bias and suggests that larval mortality is random with respect to sex.

Likewise, there does not appear to be an effect of temperature on the heritability estimates. This was unexpected for three reasons. First, the test of family effects (Table 1) suggests that clutch sex ratio is more repeatable at the lower temperature (especially in the fall assay). This observation suggests that the genetic expression of the primary sex ratio is more canalized at lower temperatures, yet this canalization is not reflected in the magnitude of the heritability estimates. Second, the magnitude of the genetic correlations (Table 3) indicates the existence of moderate genotype*environment interactions (Yamada 1962). The r^2 value from these correlations suggests that only 35% of the genetic variation in clutch sex ratio at each temperature can be attributed to common or linked factors (Via 1984). Finally, temperature is believed to affect sex determination in *Tigriopus* (Egloff 1966; Voordouw and Anholt 2002a) and from other organisms with such systems it is well known that heritable variation for the primary sex ratio is often expressed over a narrow range of temperatures, often less than a few degrees (Baker and Ridge 1980; Bull et al. 1982a; Janzen 1992).

The stability of polygenic sex determination in *Tigriopus* and *Tisbe* is enigmatic given the volatile nature of the splash pool habitat. Temperature dependent sex determination coupled with seasonal extremes of hot and cool weather are expected to

generate cyclical changes in the population sex ratio. Such temporal fluctuations in sex-specific reproductive success would tend to select for more stable systems of sex determination (i.e. heterogamety). This study shows that there is genetic variation for the primary sex ratio and that it is theoretically capable of evolving to Fisher's equilibrium. However, the conditions which maintain polygenic sex determination in splash pool copepods have yet to be resolved.

CHAPTER 3: ENVIRONMENTAL SEX DETERMINATION IN *TIGRIOPUS*

Abstract: The sex determining mechanism has important demographic and genetic consequences by virtue of its effect on the population sex ratio. Here we investigate the effect of temperature dependent sex determination (TSD) on the primary sex ratio of the harpacticoid copepod, *Tigriopus californicus*. At the two experimental temperatures (15° and 22° C) used in this study, the primary sex ratio is almost always biased in favor of males. Higher temperatures induce masculinization and the change in sex ratio is not caused by differential mortality of the sexes. The mean level of TSD in the population is small (proportion of males increases by ~ 5% between 15° and 22° C) because only one-third of the families actually exhibit a significant sex-ratio response while the rest of the population is insensitive to temperature. A comparison of the primary sex ratio and the level of TSD between two locations reveals few differences among populations. Finally, individuals still exhibited TSD after having been maintained under constant temperature conditions in the lab for several generations. In addition the proportion of temperature-sensitive individuals remained unchanged. This suggests that the observed level of TSD is not an artefact of testing field-captured individuals in a novel laboratory environment. At this point the adaptive significance of temperature-dependent sex determination in *T. californicus* remains unknown.

INTRODUCTION

In sexually reproducing organisms there is a wide variety of sex-determining mechanisms (Bull 1983). The significance of this diversity becomes evident when we consider how sex-determining mechanisms affect population processes. The sex-determining mechanism drives the population sex ratio and influences the effective population size. These in turn have important consequences for the demographic and genetic properties of sexually reproducing populations (Bull and Charnov 1988; Bulmer and Bull 1982). This is particularly true of organisms with environmental sex determination (ESD) where sex is determined sometime after conception by an environmental factor (Bull 1983; Korpelainen 1990). In these systems, the population sex ratio is driven by the sensitivity of the sex-determining mechanism to the environmental factor and by the range of environmental variation (Bulmer and Bull 1982). Extreme environmental fluctuations can result in highly biased sex ratios that may predispose the population towards extinction (Bulmer and Bull 1982). This raises the question of why such sex-determining mechanisms exist and how they manage to persist over time. The fact that these mechanisms have persisted suggests that ESD may have some adaptive benefit.

Charnov and Bull (1977) were the first to propose an adaptive explanation for environmental sex determination. They pointed out that if the environment is patchy and if one sex has a higher fitness in certain patches than the other sex, selection would favor those individuals that develop into the optimum sex for that particular patch. Two other conditions of their model are that individuals have no control over the patch in which they are born and mating takes place among individuals from all patches (Charnov and

Bull 1977). The term "patch" suggests that the different environments are separated on a spatial scale but the stratification may also be temporal . The distinction between spatial and temporal patches is useful because the two types of ESD tend to be associated with different life histories. ESD based on spatial patches is relatively common in parasitic organisms where the size or quality of the host has major fitness consequences for the parasite (Blackmore and Charnov 1989; Hipeau 1988). In contrast, temporally-based ESD appears to be restricted to organisms living in seasonal habitats where the recruitment date determines the potential for future growth (Conover and Heins 1987b; Naylor et al. 1988a; Naylor et al. 1988b; Watt and Adams 1994).

ESD is found across a wide range of taxa but is relatively rare (Bull 1983). One group in which ESD is potentially widespread is the harpacticoid copepods. The harpacticoid copepods lack sex chromosomes (Lecher et al. 1995), a necessary precondition for the evolution of ESD (Bull 1980), and systems of ESD have been reported for a variety of species (Hicks and Coull 1983).

Field workers have found substantial variation in the adult sex ratio of natural populations of the harpacticoid *Tigriopus* (Egloff 1966; Igarashi 1963b; Powlik 1998; Vittor 1971). Differences in the population sex ratio occur on both a spatial (between pools) and a temporal scale (between seasons). To date it remains unclear whether this variation is the result of biased sampling protocols, differential mortality of the sexes in the field or the sex determining mechanism.

Our study organism, *Tigriopus californicus* (Baker) is found in the splash pools on the Pacific coast from Alaska to Baja, California. Population densities of *T. californicus* are highest in the summer (Powlik 1998) and lower in the winter but

reproduction occurs year round (Burton 1985; Haderlie et al. 1980). Females mate once and produce up to a dozen egg sacs over their life-time, each egg sac containing 40 to 80 offspring (Burton 1985; Haderlie et al. 1980). In the lab, individuals can live up to 70 days and we have found no evidence of sex-specific mortality differences (unpublished data).

The environment in which *T. californicus* lives is characterized by extreme variation in temperature, salinity and irradiation. Between winter and summer, splash pool temperatures and salinities range between 5° C to 35° C and from 0 to over 100 ppt, respectively (Albert et al. 2001; Dybdahl 1995; Egloff 1966). Out of the collection of relevant environmental factors we chose temperature as it changes predictably across seasons and has met with some success in earlier studies (Egloff 1966; Vittor 1971). Hence one objective of this study is to determine whether temperature-dependent sex determination (TSD) does in fact exist in *T. californicus*. Another is to measure the primary sex ratio of *T. californicus* over a range of relevant temperatures because of its effect on demography.

In most studies concerned with variation in the primary sex ratio (including ours), reliable identification of the sex phenotype is only possible at some later stage of development (often sexual maturity). Inevitably, some individuals die before sexual maturity and so their sex phenotype is unknown. It therefore becomes important to rule out differential mortality of the sexes as an alternative explanation. Following the example of Bull & Vogt (1979), Conover & Kynard (1981), and Bull, Vogt & Bulmer (1982a), we assign dead, missing or unidentified individuals (e.g. juveniles) to the rarer sex. This protocol, hereafter referred to as the "larval mortality correction" is

conservative because it biases the observed sex ratio towards the null expection (50:50 sex ratio).

An important point to consider in the study of ESD (or any plastic response) is the distinction between the individual versus the population response. From quantitative genetics it is well known that different genotypes may not respond in the same way to changed environments (Falconer 1989). In the extreme case, genotypes may exhibit opposite phenotypes across a range of environments. Averaged across the population there is no response to the environment when in fact the opposite is true. In this study we show that the sex-ratio response differs among families and this had not been shown in previous ESD studies on *Tigriopus* (Chalker-Scott 1995; Egloff 1966; Vacquier 1962; Vacquier and Belser 1965; Vittor 1971).

Another objective of this study was to determine whether there was variation in the sex-ratio response to temperature between populations from different locations. Although we only use two locations, this study marks the first attempt to quantify variation in TSD among populations of *Tigriopus*.

Most studies investigating ESD collect gravid females from the field and rear their offspring in a laboratory setting (Bull et al. 1982a; Bull et al. 1982b; Janzen 1992). It is possible that the observed level of ESD is influenced by rearing offspring from field-captured individuals in a novel environment. In this study we address this problem by comparing the sex ratio response between field-captured and lab-reared individuals.

MATERIAL AND METHODS

General Overview of Experiments 1 and 2

In the summer and fall of 2000 we conducted two separate experiments to investigate temperature-dependent sex determination (TSD) and its effect on the primary sex ratio in *Tigriopus* at two experimental temperature treatments. In both experiments we quantified the variation among families in the sex ratio response to temperature. In the first experiment we compared the primary sex ratio and the level of TSD between populations from two different locations. In the second experiment we determined whether populations still exhibited TSD after having been maintained under constant temperature conditions in the lab for several generations.

In experiments 1 and 2 we quantified TSD by comparing a female's clutch sex ratio between 15° C and 22° C temperature treatments. Each female and her offspring represent a family and the difference in clutch sex ratio between the two temperature treatments is an estimate of the level of TSD for that family. The 15 and 22° C treatments were chosen as representative of field conditions (see intro). The choice of these two treatments also reflects a compromise between increased larval mortality at higher temperatures and slow development at lower temperatures. At 22° C, *Tigriopus* develops almost one week faster than at 15° C (Haderlie et al. 1980; Webb and Parsons 1988).

For a sample of gravid females we estimated the clutch sex ratio of each individual by raising her offspring in the lab and counting the number of males and females at sexual maturity (sixth copepodite stage). At this stage males are easy to distinguish from females by their large, geniculate antennae. For every female (family) at

each temperature, estimates of clutch sex ratio were based on approximately forty offspring.

Experiment 1: Variation in TSD Among Locations

To determine if there is variation in the sex ratio response to temperature among locations we compared the primary sex ratio and the level of TSD between a population from Arbutus Cove (48°28'36'', 123°18'00''), Victoria, British Columbia, Canada and one from Low Island (52°55'00'', 131°32'30''), Haida Gwaii, British Columbia, Canada. We obtained samples of *Tigriopus* from Arbutus Cove on May 31st, 2000; the Low Island samples were collected by T. Reimchen on May 25th, 2000. Samples were taken by haphazardly scooping a small quantity of splash pool water into a 250 ml nalgene bottle. We selected 60 gravid females from each population and individual females were isolated in their own well within 24 well tissue culture plates. Wells were stocked with 2.5 ml of filtered sea water and one drop (~ 0.5 ml) of a Tetramin™ flake solution (100 mg of ground up Tetramin™ flakes suspended in 50 ml of dH_2O).

From each of the 60 females we collected two egg sacs. Egg sacs were removed from the female's urosome with the use of a thin needle under a dissecting scope (Vittor 1971). Removing egg sacs in this way prevents females from cannibalizing their offspring. Egg sacs were left to hatch at room temperature in glass spot plates stocked with 2 ml of filtered sea water and 3 drops of an *Isochrysis galbana* culture. In the majority of cases, the transfer of recently hatched nauplii from the spot plates to a temperature treatment was accomplished within 24 hours.

For every female in the experiment, each of her two egg sacs was split into two groups of approximately 20 nauplii (no group contained less than 18 nauplii). The two groups, hereafter referred to as "sibships," were randomly assigned to either the 15° C or the 22° C treatment. Sibships were placed in 100 ml plastic vials containing 40 ml of filtered sea water and 10 ml of the *Isochrysis* culture. These vials were capped with lids to prevent contamination and reduce evaporation and were incubated without light. We added 5 mg of Tetramin™ flakes and 2.5 ml of *Isochryis* every five days to ensure that the sibships had sufficient food for maximal development.

Experiment 2: TSD in Lab-Reared Populations

At the end of experiment 1 we used the offspring from the sex ratio assay to create several laboratory stocks on July 19th, 2000. Two of these stocks, hereafter referred to as the cold line and the hot line, were initiated with offspring from the Victoria population (originally sampled from Arbutus Cove on May 31st, 2000). Both the hot and cold line were stocked with 20 different sibships from the Victoria population. The cold and hot lines were maintained in culture for four months and were kept in incubation fridges without light at 15° C and 22° C, respectively. We estimate that over this period of time the cold line went through four generations and the hot line went through six generations.

We obtained a haphazard sample of 50 gravid females from each line on October 26th, 2000. As in the first experiment, we isolated two egg sacs per female, split each egg sac into two groups (sibships) and subsequently assigned these sibships to either the 15° C or the 22° C temperature treatment. For the hot line we obtained 36 gravid females of

which 20 produced a second egg sac. For the cold line I obtained 42 females of which 36 produced a second egg sac.

To eliminate mortality (which was a problem in the first experiment) nauplii from the first egg sac were reared in isolation in 24-well tissue culture plates. Unfortunately, the method is time-consuming and so nauplii from the second egg sac were reared in vials (as in the first experiment). Hence nauplii from the first egg sac developed in isolation (1 nauplius /2.5 ml well) while those from the second egg sac developed in groups (20 nauplii /50 ml vial). In both rearing methods the density was the same (~ 1 nauplius /2.5 ml).

We were concerned that the difference in rearing methods between the first and second egg sac might have an effect on clutch sex ratio in experiment 2. To determine whether this was in fact the case we used a paired t-test to compare clutch sex ratio between egg sacs reared in tissue culture plates (first egg sac) versus those reared in vials (second egg sac). This analysis showed that there was no effect of rearing method (and/or parity) on clutch sex ratio (at 15° C, $t = 1.594$, $df = 49$, $P = 0.117$; at 22° C, $t = 1.484$, $df = 49$, $P = 0.144$).

STATISTICAL METHODS

Temperature Effects on Sex and Survivorship

For every family*temperature combination we pooled the number of sons and daughters across the two egg sacs. Sex ratio is defined here as the proportion of males and was calculated for each family (at each temperature) by dividing the number of sexually mature males by the total number of sexually mature adults (males and females).

Survivorship (l_x) was calculated as the percentage of live individuals recovered relative to the number of nauplii with which the vial(s) had been stocked.

To determine if the primary sex ratio of *T. californicus* is significantly biased (in either direction) we used a one sample t-test where the null expectation was 0.5 (a balanced sex ratio). To investigate differences in the primary sex ratio between locations (at each temperature treatment) we used a two sample t-test.

The family clutch sex-ratios at 15° and 22° C represent paired observations and are therefore not independent (Zar 1999). For each family, we can subtract the sex ratio at 15° C from the sex ratio at 22° C and use this difference as an independent estimate of the level of TSD in that population (Conover et al. 1992; Watt and Adams 1994). Similarly, the change in survivorship (Δl_x) was calculated as $l_{x\ 22°C} - l_{x\ 15°C}$. To determine whether temperature affected sex ratio we performed a one sample t-test on the sample of TSD's (equivalent to a paired t-test on the original data). We used a two sample t-test to determine whether the mean level of TSD differed between locations in experiment 1, and between the hot and cold line in experiment 2. We used a similar approach to test for temperature-dependent variation in survivorship among locations. The averaged pair-wise differences were normally distributed for both the sex ratio and the survivorship data in experiments 1 and 2.

To investigate the intrapopulation variation in the sex ratio response to temperature we classified families as either temperature insensitive or temperature sensitive. For each family we used the trial size and sex ratio at 15° C to determine whether the increase in the proportion of males observed at 22° C could have occurred by chance alone (i.e. perform a one-sided test). Families were classified as temperature

insensitive if the p-value > 0.05 and temperature sensitive if the p-value < 0.05. If temperature has no effect on sex-determination, the expected frequency of temperature sensitive genotypes is equal to the type I error rate ($\alpha = 0.05$). Families were classified as having 'reverse temperature sensitivity' if the proportion of males observed at 22° C represented a statistically significant decrease from that at 15° C.

We analyzed both the original and the larval mortality-corrected data and present both. For the uncorrected data we excluded all families where the sex ratio at 15° or 22° C was based on fewer than ten offspring. All means are reported with their standard error.

RESULTS

Experiment 1: Differences in Survivorship Among Locations

Survivorship in the Haida Gwaii population is much higher at 15° C than at 22° C (Table 1). In comparison, survivorship in the Victoria population is relatively insensitive to temperature (Table 1). In both sites, the change in survivorship (Δl_x) between temperature treatments is significantly different from zero (Table 1). The change in survivorship (Δl_x) was also significantly different between the two locations ($t = 9.17$, $df = 98$, $P < .001$). This difference between locations was mostly the result of poor survivorship of Haida Gwaii nauplii at 22° C (Table 1).

Differences in survivorship complicate the comparison of the primary sex ratio and TSD among locations. This is because the larval mortality correction essentially removes all the variation in clutch sex ratio at 22° C for the Haida Gwaii population. Hence we emphasize from the outset that the results from the comparison among locations are weak.

Table 1. Survivorship for populations from Haida Gwaii (HG) and Victoria (VIC) in experiment 1 and for the hot and cold lines in experiment 2. In each experiment families are reared at two different temperatures (15° and 22° C). Shown are the number of families (N) and the mean survivorship ± the standard error (S.E.). The paired sample t-statistic tests whether survivorship is significantly different between temperature treatments for each population (Pop); t = t-statistic, df = degrees of freedom, P = P-value.

Expt	Pop	Temp	N	Survivorship ± S.E.	t	df	P
1	HG	15° C	56	86.4 ± 2.15	14.3	55	**<0.001**
		22° C	56	31.1 ± 3.31			
	VIC	15° C	57	93.3 ± 1.41	4.7	56	**<0.001**
		22° C	57	81.0 ± 2.15			
2	Hot	15° C	36	96.7 ± 1.23	-0.1	35	0.895
		22° C	36	96.8 ± 0.97			
	Cold	15° C	42	95.8 ± 0.93	-0.8	41	0.424
		22° C	42	96.5 ± 0.83			

Experiment 1: Differences in the Primary Sex Ratio and TSD Among Locations

Primary Sex Ratio: The mean clutch sex ratio of both the Haida Gwaii and the Victoria population are significantly male-biased at both temperature treatments (Table 2). The primary sex ratio remains significantly male biased after correcting the data for larval mortality for all location*temperature combinations except the Victoria population at 15° C (Table 2).

At 15°C, *T. californicus* from Haida Gwaii produced a clutch sex ratio (0.59 ± 0.038; mean ± standard error) that is slightly but not significantly more male-biased than that of Victoria (0.55 ± 0.022; t = 1.133, dof = 83, P = 0.261). Similarly, at 22° C, the clutch sex ratio from Haida Gwaii (0.68 ± 0.028) is slightly but not significantly more male-biased than that of Victoria (0.61 ± 0.024; t = 1.914, dof = 83, P = 0.059). After correcting the clutches for larval mortality (Table 2) the differences are even smaller. Hence we have no evidence that the primary sex ratio differs among locations at either temperature treatment.

Temperature-dependent Sex Determination: In both sites, significantly more males were produced at higher temperatures (Table 3; Fig. 1). After correcting the clutches for larval mortality, there is no longer a significant increase in the proportion of males in the Haida Gwaii population but the response remains significant in the Victoria population (table 3). The increase was slightly, but not significantly, larger in Haida Gwaii (0.09 ± 0.038) than in the Victoria population (0.06 ± 0.021; t=0.690, dof = 83, P = 0.492; Fig. 1).

Table 2. The primary sex ratio (proportion of males) for populations (Pop) from Haida Gwaii (HG) and Victoria (VIC) in experiment 1 and for the hot and cold lines in experiment 2. In each experiment families are reared at two different temperatures (15° and 22° C). Shown are the number of families (N) and the mean primary sex ratio ± the standard error (S.E.). The one sample t-statistic tests whether the primary sex ratio is significantly different from 0.5; t = t-statistic, df = degrees of freedom, P = P-value. 'Correct' refers to whether the data were adjusted for larval mortality.

Correct	Expt	Pop	Temp	N	Mean ± S.E.	t	df	P
No	1	HG	15° C	30	0.59 ± 0.038	2.435	29	**0.021**
			22° C	30	0.68 ± 0.028	6.503	29	**<0.001**
		VIC	15° C	55	0.55 ± 0.022	2.057	54	**0.045**
			22° C	55	0.61 ± 0.024	4.562	54	**<0.001**
	2	Hot	15° C	36	0.49 ± 0.027	-0.390	35	0.699
			22° C	36	0.53 ± 0.032	0.928	35	0.360
		Cold	15° C	40	0.50 ± 0.029	0.150	39	0.882
			22° C	40	0.58 ± 0.029	2.789	39	**0.008**
Yes	1	HG	15° C	56	0.56 ± 0.022	2.703	55	**0.009**
			22° C	56	0.53 ● 0.008	3.335	55	**0.002**
		VIC	15° C	57	0.53 ± 0.019	1.589	56	0.118
			22° C	57	0.58 ± 0.016	5.164	56	**<0.001**
	2	Hot	15° C	36	0.49 ± 0.024	-0.498	35	0.621
			22° C	36	0.54 ± 0.029	1.365	35	0.181
		Cold	15° C	41	0.52 ± 0.026	0.841	40	0.405
			22° C	41	0.59 ± 0.026	3.354	40	**0.002**

Table 3. Temperature-dependent sex determination (TSD) for populations (Pop) from Haida Gwaii (HG) and Victoria (VIC) in experiment 1 and for the hot and cold lines in experiment 2. For each family, TSD is calculated as the proportion of males in a clutch at 22° C minus the proportion of males in a clutch at 15° C. Shown are the number of families (N) and the mean level of TSD ± the standard error (S.E.). The one sample t-statistic tests whether the proportion of males increases at 22° C; t = t-statistic, df = degrees of freedom, P = P-value. 'Correct' refers to whether the data was adjusted for larval mortality.

Correct	Expt	Pop	N	Mean TSD ± S.E.	t	df	P
No	1	HG	30	+ 0.09 ± 0.038	2.411	29	**0.022**
		VIC	55	+ 0.06 ± 0.021	3.067	54	**0.003**
	2	Hot	36	+ 0.04 ± 0.030	1.334	35	0.191
		Cold	40	+ 0.08 ± 0.024	3.238	39	**0.002**
Yes	1	HG	56	- 0.03 ± 0.022	1.427	55	0.159
		VIC	57	+ 0.05 ± 0.018	2.876	56	**0.006**
	2	Hot	36	+ 0.05 ± 0.025	2.034	35	0.050
		Cold	41	+ 0.06 ± 0.022	2.964	40	**0.005**

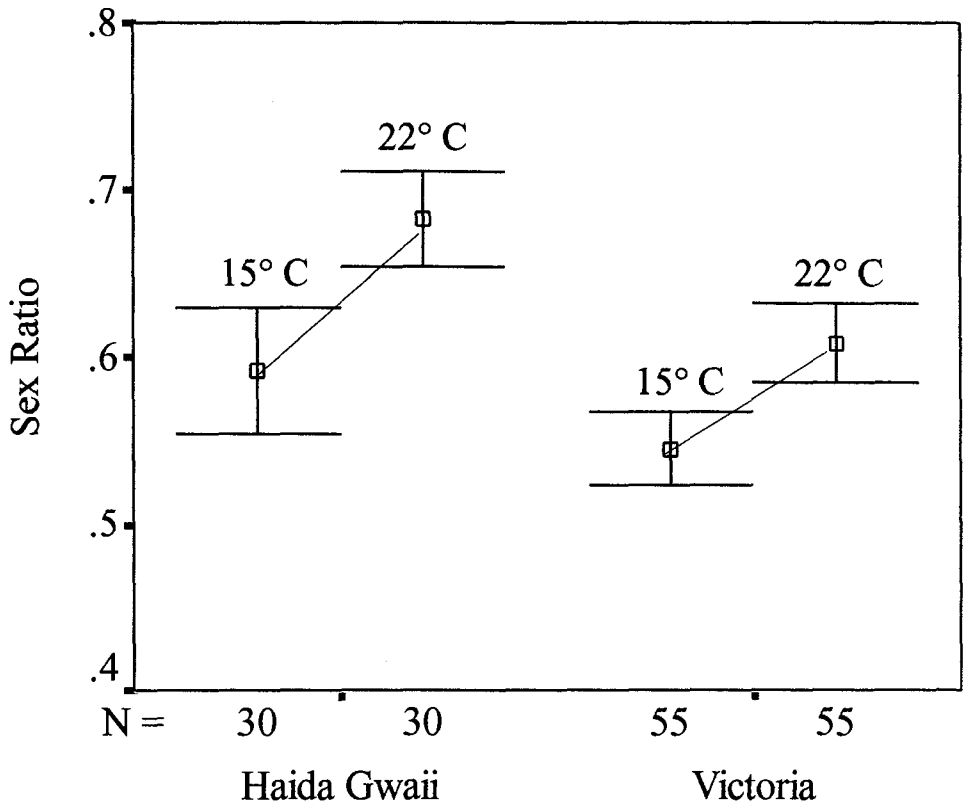

Figure 1. Temperature-dependent sex determination (TSD) in two different populations; Haida Gwaii and Victoria. Clutch sex ratios were not corrected for larval mortality. Shown are the mean and standard error.

Experiment 1: Variation in TSD Among Families

Compared to the sex ratio observed at 15° C, 10 out of 30 families from the Haida Gwaii population were significantly more male-biased (i.e. exhibited temperature-sensitivity) and 3 out of 30 were significantly less male-biased at 22° C (i.e. exhibited reverse temperature sensitivity). For the Victoria population, 18 out of 55 families were significantly more male-biased at 22° C (temperature-sensitive) compared to the sex ratio observed at 15° C. Only 2 out of 55 Victoria families were significantly less male-biased. Across both populations, approximately one-third of the families (28/85) were clearly temperature-sensitive (i.e. exhibited statistically significant male-biased TSD). Even with the larval mortality correction, 25 out of 85 families were still temperature-sensitive.

Experiment 2: TSD in Lab-Reared Populations

Primary Sex Ratio: The mean clutch sex ratio in the cold line is significantly male-biased at 22° C but not at 15° C (Table 2). The hot line is not significantly different from 0.5 at either temperature (Table 2). Survivorship in both lines (hot and cold) at each temperature treatment is close to 100% (Table 1). The results are therefore unchanged by the larval mortality correction (Table 2).

Temperature-dependent Sex Determination: After four months of inhabiting a constant temperature environment both the hot and the cold line still exhibit TSD in the predicted direction (proportion of males increases at 22° C). However the level of TSD is only statistically significant for the cold line (Table 3; Fig. 2). The mean level of TSD was larger in the cold line (0.08 ± 0.024) than in the hot line (0.04 ± 0.030), but this

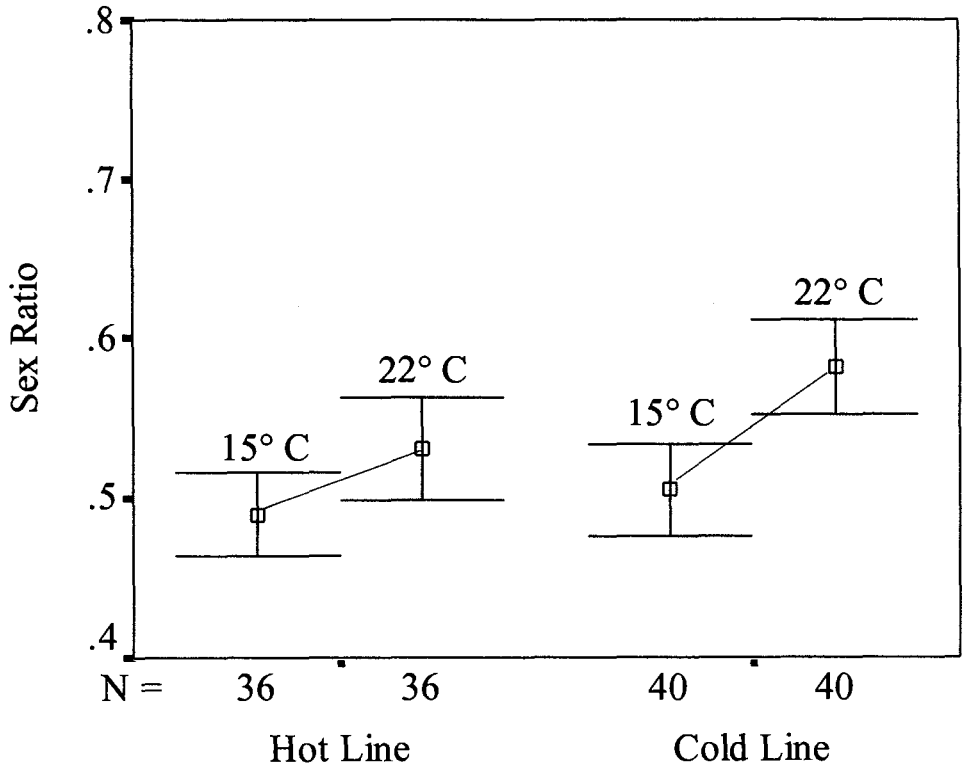

Figure 2. Temperature-dependent sex determination (TSD) in two laboratory populations; hot line and the cold line. Clutch sex ratios were not corrected for larval mortality. Shown are the mean and standard error.

difference between lines was not statistically significant (t=0.961, dof = 74, P = 0.340; Fig. 2). These results are unchanged by the larval mortality correction (Table 3). The level of TSD in the Victoria population in experiment 1 (Table 3, Fig. 1) was comparable to that observed in the hot and cold line in experiment 2 (Table 3, Fig. 2).

Experiment 2: Variation in TSD Among Families

Across both lines (hot and cold), 22 out of 76 families (28.9%) were significantly more male-biased at 22° C compared to 15° C (i.e. exhibited temperature sensitivity). In contrast, only 3 out of 76 families (3.9%) were significantly less male-biased at 22° C relative to 15° C (exhibited reverse temperature sensitivity). When corrected for larval mortality only one of the 22 families with male-biased TSD lost its statistical significance. Hence the proportion of temperature-sensitive families remained relatively constant over four to six generations in the laboratory environment.

DISCUSSION

The Primary Sex Ratio in Tigriopus

Out of eight population*temperature combinations in both experiments only one was not male biased (hot line at 15° C; Table 2). In general the primary sex ratio of *T. californicus* under laboratory conditions is male-biased (Fig. 1 & 2). Egloff (1966) also obtained a male-biased primary sex ratio in his work on *T. californicus* and similar observations have been made for *T. japonicus*. In Egami's (1951) work on sex conversion in *T. japonicus* only six out of 62 control samples were female biased and the same is true for Takeda's (1950) work. Igarashi (1963b) showed that the primary sex ratio in *T.*

japonicus is always male biased and that this is true regardless of the geographic origin of the stock.

In systems of ESD, biased sex ratios are frequently observed and are expected from theory (Charnov 1982). ESD models predict a primary sex ratio that is biased towards the sex that develops in the poorer patches (Bull 1981b; Charnov 1982; Charnov and Bull 1989a; Charnov and Bull 1989b). Hence the observation that the primary sex ratio is generally male-biased in *T. californicus* suggests that males are making the best of a bad lot at warmer temperatures. This assumes however, that adaptive TSD exists in *T. californicus* for which we have no evidence. To address these questions we need information on sex-specific fitness curves as a function of temperature.

TSD and ESD in Crustaceans

Environmental sex determination is relatively common in crustaceans (Korpelainen 1990; Legrand et al. 1987) and has been reported for a variety of harpacticoid copepods (Hicks and Coull 1983). Many of these studies fail to account for differential mortality of the sexes and are therefore ambiguous examples of ESD (Bergmans 1981; Gaudy et al. 1982; Hagiwara et al. 1995; Miliou 1993). In contrast our use of the larval mortality correction allows us to rule out differential mortality of the sexes. The disadvantage of the larval mortality correction is that it increases Type II error. However, we still detected statistically significant TSD in half of the location*temperature combinations (Victoria population in experiment 1 and the cold line in experiment 2).

These experiments show that sex determination in *T. californicus* is affected by temperature and that high temperatures induce masculinization in this species. The level of plasticity in the sex-ratio response is much smaller than the systems of TSD observed in reptiles (Bull 1985b; Bull and Vogt 1979; Ferguson and Joanen 1982) and fish (Conover and Heins 1987a; Lagomarsino and Conover 1993) where high temperatures typically increase the proportion of one sex by more than 50%. In comparison, higher temperatures increased the proportion of males by only five to ten percent in *T. californicus* (Table 2, Fig. 1 & 2). The mean population response is weak because only 30% of the families had statistically significant TSD. In contrast, photoperiod has a statistically significant effect on brood sex ratio in more than 90% of the families examined in *Gammarus duebeni* (Bulnheim 1978a). Hence compared to other examples both the magnitude and the incidence of TSD are relatively low in *T. californicus.*

Egloff (1966) found that high temperatures increased the proportion of males by as much as 30% in *T. californicus*. Survivorship was 98% or higher so that he was able to rule out differential mortality. Vittor (1971) also found that the proportion of males increased with temperature in 100% seawater but that the relationship was reversed in 50% seawater. However, Vittor (1971) points out that differential male and female survivorship may have influenced his results. Other environmental factors that influence sex determination in *T. californicus* include UV-B irradiation (Chalker-Scott 1995), salinity (Egloff 1966), light (Egloff 1966) and hydrostatic pressure (Vacquier 1962; Vacquier and Belser 1965). Application of hydrostatic pressure treatments (500 atmospheres) to different developmental stages indicated that sex was irreversibly determined at the first copepodite stage (Vacquier and Belser 1965).

Other apparent examples of ESD are caused by cytoplasmic factors such as bacteria and viruses. Cytoplasmic inheritance has been well documented in crustaceans including *Gammarus duebeni* (Bulnheim 1978a; Bulnheim 1978b), terrestrial isopods (Rigaud 1997) and *Tigriopus japonicus* (Igarashi 1963a; Igarashi 1964). In these systems thelygenous (100% female) broods are common (Bull 1983); however, in some cases the penetrance of these pathogens depends on temperature and other environmental conditions (Bulnheim 1978a; Bulnheim 1978b; Hurst and Jiggins 2000). Unfortunately, our experimental design does not allow us to rule out this phenomenon as an alternative explanation of our results.

Differences in Survivorship, the Primary Sex Ratio and TSD Among Locations

The comparison between the Haida Gwaii and Victoria population was a preliminary attempt to detect latitudinal variation in the primary sex ratio and the level of TSD among populations of *Tigriopus*. Such comparisons have given insight into the adaptive significance of ESD in other systems (Blackmore and Charnov 1989; Bull et al. 1982b; Conover and Heins 1987a; Naylor et al. 1988b; Watt and Adams 1994). We recognize that because we only sampled one pool for each location (HG vs. VIC), the effect of latitude is confounded with all other sources of variation (e.g. shade, aspect, pool size, etc) that contribute to differences between two splash pools (Hurlbert 1984).

Geographic clines in thermal tolerance are well known in marine animals (Spicer and Gaston 1999; Vernberg and Vernberg 1972). Southern populations of the supratidal fiddler crab, *Uca rapax*, are more tolerant of higher temperatures than individuals from northern latitudes (Vernberg and Tashian 1959). Similar patterns have been observed in

bivalve mollusks (Ansell et al. 1986) and beachfleas (Gaston and Spicer 1998). The pattern of survivorship observed in our experiments is consistent with this cline in thermal tolerance. Survivorship of nauplii from the northern population (Haida Gwaii) was substantially lower at the warmer temperature (22° C) compared to that of the southern (Victoria) population (Table 3). Still, the magnitude of the difference in survivorship was surprising for two reasons. First, *T. californicus* has been shown to tolerate a wide range of temperatures (Huizinga 1971) and second, mean daily minimum and maximum temperatures are very similar between Haida Gwaii and Victoria (Environment Canada 1982).

If the environmental cue varies across the range of the organism we would expect the sex-determining response (i.e. the threshold) to be adjusted accordingly (Blackmore and Charnov 1989; Bull et al. 1982b). For example, under TSD individuals from northern populations are expected to differentiate into the high temperature sex at lower threshold temperatures than individuals from southern (warmer) locales (Bull et al. 1982b). At both temperatures, the northern Haida Gwaii population produced more males than the more southern Victoria population. Although this pattern is consistent with Bull et al.'s (1982b) prediction it is not statistically significant. Similarly, the magnitude of TSD is not significantly different between the two populations although such variation has been detected in other systems (Conover and Heins 1987a; Naylor et al. 1988a; Naylor et al. 1988b; Watt and Adams 1994).

Maintenance of TSD in Lab-Reared Populations

After several generations of exposure to constant temperature conditions in the lab the cold line still produced significantly more males at higher temperatures but the hot line did not. The level of TSD in both lines (in experiment 2) was similar to that observed in the Victoria population in experiment 1 (Table 3). Similarly, the proportion of temperature-sensitive families remained unchanged between experiment 1 and 2. Hence both the level and the incidence of TSD remained stable over the four months that the two lines inhabited their constant temperature environments. This suggests that the observed response is not simply an artefact of testing field-captured genotypes in the lab.

CONCLUSIONS

We show that the primary sex ratio of *T. californicus* is almost always male-biased and provide evidence for moderate TSD in this species. Only a third of the families actually exhibited TSD; the rest did not respond to temperature. This is one reason why the mean level of TSD in the population is so low. There is no difference in the primary sex ratio or the level of TSD between populations from Haida Gwaii and Victoria. Populations reared under constant temperature conditions for several generations still exhibited a sex ratio response to temperature. At this point the adaptive significance of a temperature-based cue is not clear and it is possible that other factors (e.g. photoperiod, maternal condition, cytoplasmic factors) are more important in the sex determination of this organism.

CHAPTER 4: LARVAL DENSITY AND THE CHARNOV-BULL MODEL OF ADAPTIVE ENVIRONMENTAL SEX DETERMINATION IN *TIGRIOPUS*

Abstract: The Charnov and Bull theory suggests that environmentally-induced variation in adult body size coupled with sex-specific differences in the strength of selection can select for the evolution of adaptive environmental sex determination (ESD). In this study we determine whether larval density affects sex determination in the copepod, *Tigriopus californicus*, in the manner predicted by Charnov and Bull. Individuals reared at low density were larger and significantly more likely to differentiate into a male than their high-density siblings (although the second result was not repeated in a follow-up experiment). For these lab-reared individuals, sexual selection on male body size was stronger than fecundity selection on female body size but this pattern of sex-specific selection on adult body size was reversed in a field sample. Differences in food availability (for females) and the mode of competition (for males) may account for the conflicting results between lab and field. While larval density plays an equivocal role in the sex determination of *T. californicus*, our sex-specific estimates of selection on adult body size will inform future models of adaptive ESD in this and other copepods.

INTRODUCTION

In animals with separate sexes, sex determining mechanisms are often classified into genotypic versus environmental (Bull 1983). In genotypic sex determination (GSD) an individual's sex phenotype is predominantly determined by its genotype (Bull 1983). By contrast, in environmental sex determination (ESD) an individual's sex phenotype is predominantly determined by an environmental factor at some point during development (Adams et al. 1987; Bull 1983; Korpelainen 1990). GSD mechanisms can be influenced by environmental factors (Baker and Ridge 1980) and conversely, ESD mechanisms often exhibit a genetic component (Bull et al. 1982a; Conover and Heins 1987b; Janzen 1992; Rhen and Lang 1998); hence the distinction between these two is not always clear (Bull 1983).

The adaptive significance of ESD was elucidated by Charnov and Bull (1977) who suggested that differences in the strength of selection on adult body size between males and females can select for ESD. Their logic is as follows: (1) the environment is patchy, (2) individuals developing in high quality patches reach a larger body size at sexual maturity than individuals developing in low quality patches, (3) adult body size has greater fitness consequences for one sex than the other, hence, (4) individuals that find themselves in high quality patches should develop into the sex that benefits the most from being large (Charnov and Bull 1977). One consequence of this type of ESD is sexual size dimorphism (SSD) in adult males and females (Bull 1983).

A classic example of adaptive ESD is photoperiod-dependent sex determination in the amphipod, *Gammarus duebeni* (Bulnheim 1967; Bulnheim 1972). In annual populations of *G. duebeni*, the environment is patchy with respect to the duration of the

growing season and individuals that are born early in the season have more time to grow large than individuals that are born late (Naylor et al. 1988a; Watt and Adams 1994). The precopulatory mate-guarding and assortative mating habits of *G. duebeni* exert strong selection on male body size because large males are capable of mating with females of all sizes whereas small males are unable to mate with larger females (McCabe and Dunn 1997). While females also benefit from large body size (due to increased egg production), females that are too large have difficulty finding a suitable partner (Hatcher and Dunn 1997). Because males benefit more from being large, they are produced early in the season under long day light hours whereas females are produced late in the season under short day light hours. Other classic examples of adaptive ESD include temperature-dependent sex determination in the fish *Menidia* spp. (Conover 1984; Conover and Heins 1987a; Lagomarsino and Conover 1993; Yamahira and Conover 2003) and density-dependent sex determination in mermithid nematodes (Blackmore and Charnov 1989).

Recent work on calanoid copepods (Irigoien et al. 2000) and the parasitic copepod *Pachypygus gibber* (Becheikh et al. 1998) has shown that food levels can affect sex-determination during larval development. These studies motivated us to investigate the effect of larval density-induced food limitation on the primary sex ratio of the harpacticoid copepod, *Tigriopus californicus*. Although Egloff (1966) conducted this experiment almost 40 years ago (and found no effect), we decided to repeat his experiment for a number of reasons. First, it is not clear whether his three larval density treatments actually limited per capita food availability. Second, he measured ESD as a population level response instead of quantifying among family variation in degree of sex ratio plasticity (Rhen and Lang 1998; Voordouw and Anholt 2002a). Third, the level of

ESD varies among populations (Watt and Adams 1994; Yamahira and Conover 2003) and its absence in Oregon does not preclude its presence on Vancouver Island.

In copepods, larval density-induced variation in per capita food availability can have important consequences for adult body size as this trait is often fixed upon reaching sexual maturity (Gilbert and Williamson 1983). The importance of female body size on egg production in copepods is well established from taxonomic surveys (Hopcroft and Roff 1998; Kiorboe and Sabatini 1995). Likewise, the prevalence of precopulatory mate-guarding in this group suggests that body size also plays a role in male reproductive success (Jormalainen 1998). While several workers have compared the strength of selection on adult body size between male and female amphipods (Bertin and Cezilly 2003; McCabe and Dunn 1997; Ward 1988), to our knowledge, this has never been done in a species of copepod. Manipulating larval density increases the range in adult body size and therefore the probability of detecting selection (Anholt 1991). Even if larval density has no effect on the primary sex ratio, our sex-specific estimates of selection on adult body size will inform future models of adaptive ESD in this and other copepods.

This study consists of two experiments. In the first experiment, we reared paired sibships at high and low density and compared their primary sex ratio to investigate the effect of density on the primary sex ratio. We also compared high and low-density siblings with respect to adult body size, female fecundity, and male mating success. Our density manipulation created two unnatural conditions: (1) females were mated after reaching sexual maturity, and (2) males competed over virgin, adult females instead of virgin copepodites. Hence in the second experiment we quantified fecundity selection on female body size and sexual selection on male body size in field-sampled copepods.

In most harpacticoid copepods, females are generally larger than males (Hicks and Coull 1983). According to Gilbert and Williamson (1983), female-biased SSD in copepods suggests that selection is stronger on female than male body size (but see Fairbairn 1997). From Gilbert and Williamson (1983), we predict that low-density environments will produce a female-biased primary sex ratio and that individuals developing in high density environments will make the best of a bad lot by differentiating into small males. We also predict that low-density individuals will be larger, more fecund (in the case of females), and more competitive (in the case of males) than their high density siblings and that the slope of the relationship between fitness and adult body size (i.e. the selection coefficient) will be steeper for females than for males.

MATERIALS AND METHODS

Experiment 1: Charnov-Bull model of adaptive ESD

General overview: In the summer of 2001, 2002 and 2003 we conducted experiments 1A, 1B and 1C, respectively. The purpose of experiments 1B and 1C was to test the Charnov-Bull model of adaptive ESD in *T. californicus* with respect to larval density. Specifically, experiments 1B and 1C were designed to answer the following three questions: (1) does larval density affect adult body size? (2) how does larval-density induced variation in adult body size affect female fecundity and male reproductive success? and (3) does larval density affect the primary sex ratio in a manner consistent with the Charnov-Bull model? The original purpose of experiment 1A was to investigate sexual selection on male body size. However, because the design of the male mating trials was the same for all three experiments, we were able to combine the results.

Larval density treatments: We used a low and high density treatment to test the effect of density on the primary sex ratio and to create large and small individuals. The low density treatment ranged between 200 – 500 nauplii per liter and the high density treatment ranged between 1000 – 3000 nauplii per liter (Table 1). Our density treatments (200 – 3000 nauplii per liter) were similar to the ones used by Egloff (200 – 2000 individuals per liter,1966) and fall in the lower range of observed field conditions (200 to 20 000 individuals per liter, Powlik 1998). The choice of this conservative effect size was chiefly governed by our desire to minimize larval mortality.

For experiments 1B and 1C we used a paired design in which nauplii from the same full-sib family were reared under both low and high density. The advantage of this paired design is that it allows one to control for among-family effects in the primary sex ratio (Cook 2002; Yusa 2004). In experiment 1B we removed egg sacs from 50 field-captured females (sampled from Arbutus Cove, Victoria, British Columbia, Canada 48°28'36'', 123°18'00''), obtained 20 F_1 offspring from each female and haphazardly assigned them to either the low or the high density treatment. In experiment 1C we removed egg sacs from 72 lab-reared females (originally sampled from Arbutus Cove), obtained 48 F_1 offspring from each female and assigned them to either density treatment. For experiment 1A, nauplii in the high and low treatments were obtained from different females (sampled from Gordon Head, Victoria, B.C., Canada 48°29'48'', 123°18'24'').

In all three experiments the nauplii were initially reared on a diet of cultured *Isochrysis galbaena* cells. In experiments 1A and 1B, the density of the *Isochrysis galbaena* solution was not known. For experiment 1C we controlled algal density ($2*10^6$ cells/ml) and, from previous grazing experiments (unpublished data), calculated daily,

Table 1. Larval density manipulation for experiments 1A, B and C. Shown are the source populations (field vs. lab; GH = Gordon Head, AC = Arbutus Cove) from which the copepods were sampled, the volume of filtered sea water and the type of tissue culture plate (24 well vs. 6 well) in which the nauplii were reared, the food levels that each well received (the volume of cultured *Isochrysis galbana* solution and the amount of Tetramin flakes), the sample size (N), the well density and the absolute density for the low and high density treatments in all three experiments.

Expt	Source	Vol. & Plate	Food	N	Well Densities	Abs. Densities
1A	Field	2 ml	12 drops	23	1 nauplii / well	500 nauplii / L
	(GH)	24-well	---		4 nauplii / well	2000 nauplii / L
1B	Field	10 ml	2 ml	50	2 nauplii / well	200 nauplii / L
	(AC)	6-well	~ 0.2 mg		10 nauplii / well	1000 nauplii / L
1C	Lab	2 ml	$4*10^6$ cells	72	1 nauplii / well	500 nauplii / L
	(AC)	24-well	~ 0.2 mg		6 nauplii / well	3000 nauplii / L

per capita algal consumption rates for each stage (Appendix A). At 20° C, development from an N1 nauplius to C6 adult takes ~16 days during which the average individual consumes $1.9*10^6$ cells of *I. galbaena*. Hence, in experiment 1C, the initial food supply of $4.0*10^6$ cells was not enough for the six siblings in the high density treatment to complete development (Table 1) and we calculate that they ran out of algae sometime during the third copepodite stage. To prevent high-density individuals from starving to death, we added Tetramin flakes at the third copepodite stage (for consistency this was also done in the low density treatment). We are confident that this tetramin supplement did not affect the primary sex ratio because sex is determined before the third copepodite stage in *T. californicus* (Burton 1985). Plates were stored at 20° C with a 12 hour day/night cycle. In experiments 1B and 1C, we sexed all individuals at adulthood to estimate the primary sex-ratio.

Larval density, female body size and female fecundity: In experiments 1B and 1C we investigated the effect of larval density on female fitness by comparing fecundity (number of eggs per egg sac) between high and low-density sisters from the same full-sib family.

Larval density, male body size and male mating success: In experiments 1A, 1B and 1C we investigated the effect of larval density on male mating success through a competition experiment. In experiment 1A, competition occurred between males from different egg sacs: one from the low density treatment and one from the high density treatment to ensure size asymmetry between competitors. In experiments 1B and 1C, we controlled for family effects by pairing high and low-density brothers from the same full-sib family. The two brothers were randomly selected from each treatment which allowed

us to test whether our density manipulation affected adult body size. In experiments 1B and 1C, we randomly dyed one of the males with a drop of red food coloring to facilitate identification and placed them in a mating arena. In a pilot experiment, we determined that this dye protocol had no effect on the outcome of the mating trial.

In all of the experiments, we placed the two males in a mating arena (one well stocked with 10 ml of sea water and *Isochrysis galbaena* in a 6-well tissue culture plate) and allowed them to acclimatize for a few minutes before adding one adult virgin female. Once the trial began, we scanned the mating arena every five minutes. When a male had clasped the female for more than two successive scans (> 5 minutes) we terminated the trial and measured the body size of each male (see below).

Adult body size measurements: To obtain adult body size measurements, copepods were mounted on a slide in three drops of water and immobilized with a coverslip. We viewed the slides with a Leica inverted microscope (magnification = 2.5x) and after taking a photograph with a Nikon D1 digital camera, we placed the slide in a petri dish filled with salt water. We flushed the coverslip off the slide using a pipette and recovered the individual (generally unharmed). This procedure allowed us to mount some individuals at least twice to estimate the repeatability of the mounting and measurement procedure.

For males and females in both experiments 1 and 2, adult body size was defined as the distance between the rostrum and the end of the last abdominal segment. In experiment 1A, male body size was measured as the width of the cephalosome. In a series of pilot experiments, the repeatability of female adult body length and male cephalosome width were found to be 0.52, and 0.62, respectively. This suggests that

variation in these measurements was due to actual differences between individuals and not due to the mounting procedure.

Experiment 2: Selection on adult body size in females and males

Fecundity selection on female body size: On July 20, 2004, we sampled 80 gravid females from a pool in Arbutus Cove (48°28'36'', 123°18'00''), Victoria, British Columbia, Canada. We immediately removed all egg sacs and hatched these in glass spot plates. We placed each female in her own well in a 24-well tissue culture plate stocked with 2 ml of filtered sea water and abundant unicellular algae (*Isochrysis galbaena*) for food. Once the female produced a second egg sac, we removed it as well and then measured the female's body size. The first and second egg sac represent egg production in the field vs. the lab environment, respectively.

Sexual selection on male body size: On July 30, 2004, we obtained a field sample from Arbutus Cove that contained 112 adult males, 85 gravid females and 163 single copepodites. Of the 112 adult males, 94 were in a precopula with a female copepodite and 18 were single. To quantify sexual selection on male body size, we measured the body size of all 112 males after separating the 94 precopula pairs with a thin, metal needle.

In the field sample, the proportion of single males was low (18/112 = 16.1%), so that sexual selection on male body size (if any) was relatively weak. To increase the probability of detecting sexual selection, we conducted a lab experiment where the operational sex-ratio was more male-biased. We isolated 140 single males from several field samples and placed them in a 4.2 liter plastic box containing 0.5 l of filtered sea

water. After allowing the males to acclimatize for one hour we added 50 virgin female copepodites (obtained by separating 50 precopula pairs). Seventeen hours later, we recovered 47 precopula pairs, 93 single males and 3 single female copepodites. The high proportion of single males in the lab sample (93/140 = 66.4%) means that sexual selection was much stronger in the lab than in the field sample.

STATISTICAL METHODS

Experiment 1: Charnov-Bull model of adaptive ESD

We used a paired sample t-test to determine whether density had a significant effect on adult body size, female fecundity and the primary sex-ratio (1B and 1C) and whether the difference in body size between mated and unmated males was significantly different from zero (1A, 1B and 1C). We used a binomial test to determine whether the density treatment had a significant effect on male mating success (1B and 1C). We were unable to use this test in experiment 1A because we did not color-dye the two competitors and therefore cannot unambiguously assign treatment identity to the winner. Whenever possible we combined p-values using Fisher's (1954) method as outlined in Quinn and Keough (2002).

Larval mortality correction: We used the larval mortality correction to ensure that differential mortality did not bias our estimates of the primary sex ratio. For each plate of full sibs, unidentified individuals were assigned to the less common sex for that plate. In experiment 1 for example, if a plate consisted of seven brothers, two sisters and one unidentified individual the latter would be treated as a female (proportion of males = 7/10

= 0.70). Throughout this paper we will refer to the larval-mortality corrected estimates of the primary sex ratio unless otherwise specified.

Experiment 2: Selection on adult body size in females and males

Fecundity selection on female body size: We measured selection on female body size as the slope of the linear regression of fecundity on female body length. We conducted separate fecundity vs. body size regressions for the field and lab-based egg sacs. However, we did not use ANCOVA to compare the slopes and/or intercepts because the two regressions are not independent (i.e. both regressions contain the same 80 females). We used a paired t-test to determine whether fecundity changed between the first, field egg sac and the second, lab egg sac.

Sexual selection on male body size: We measured selection on male body size as the slope of the logistic regression of precopula success (precopula male = 1, single male = 0) on male body length for both the field and the lab sample. We also used an independent two sample t-test to compare the difference in size between precopula and single males in both samples.

RESULTS

Experiment 1: Charnov-Bull model of adaptive ESD

Larval density and adult body size: In experiment 1B, males reared at low density (0.89 ± 0.017 mm; mean ± s.e.) were significantly larger than their brothers in the high density treatment (0.84 ± 0.011 mm; $t = 2.78$, $df = 34$, $P = 0.009$). In experiment 1C, males reared at low density (0.77 ± 0.005 mm) were the same size as their high-density

brothers (0.77 ± 0.005; t = 0.595; df = 58; P = 0.554), but low-density females (0.82 ± 0.007 mm) were significantly larger than their high-density sisters (0.79 ● 0.008 mm; t = 3.092; df = 53; P = 0.003). Taken together, these three results suggest that the density manipulation worked and that low-density adults are generally larger than their high-density siblings. Furthermore, delayed development of high-density individuals (M.J.V. personal observation) lends additional support that our density manipulation achieved the desired effect.

Larval density and female fecundity: In experiment 1B, the mean fecundity of low-density females (38.3 ± 3.49 offspring) was not significantly greater than that of their high-density sisters (32.4 ± 4.36 offspring; t = 1.50, d.f. = 18, P = 0.151). In experiment 1C, the mean fecundity of the low-density females (47.3 ± 1.52 offspring) was actually significantly less than that of their high-density sisters (53.3 ± 2.07 offspring; t = 2.593; df = 41; P = 0.013). Hence, there was no support that low larval density increased female fecundity even though it increased female body size. This may be because resources were not limiting in the lab environment.

Larval density and male-mating success: In experiment 1A, the larger male (based on cephalosome width) outcompeted the smaller male in 17 of the 23 trials (binomial test; P = 0.0327). In experiment 1B, the low-density male mated in 20 out of 30 trials (binomial test; P = 0.099) and in experiment 1C, the low-density male was successful in 37 out of 54 trials (binomial test; P = 0.009). Assuming that the 17 large winners in experiment 1A were taken from the low density treatment, these three experiments suggest that low-density males have greater mating success than their high-density brothers ($\chi^2 = 20.887$, d.f. = 6, P = 0.002).

Male body size and male-mating success: In experiment 1A, the mean cephalosome width for mated males (0.32 ± 0.027 mm) was significantly larger than that of the unmated males (0.29 ± 0.031 mm; t = 3.792, d.f. = 22, P = 0.001). In experiment 1B, the body length of the mated (0.90 ± 0.019 mm) males was significantly longer than that of their unmated brothers (0.82 ± 0.014 mm; t = 2.985, d.f. = 22, P = 0.007). In experiment 1C, the difference in body length between the mated (0.77 ± 0.005 μm) and unmated brothers (0.78 ± 0.006 μm) was not significantly different from zero (t = 0.110, d.f. = 51, P = 0.913). These three experiments taken together suggest that larger males have greater mating success than their smaller brothers.

Survivorship and the larval mortality correction: In experiment 1B, mean survivorship for the 50 F_1 families at the low and high density treatment was 81.6 ± 1.72% and 82.8 ± 2.23%, respectively. In experiment 1C, mean survivorship for the 72 F_2 families at low and high density was 89.4 ± 2.28% and 85.8 ± 2.45%, respectively. The correlation between the raw and larval mortality-corrected sex ratios was high in both experiments 1B ($r = 0.851, n = 50, p < 0.001$) and 1C ($r = 0.874, n = 72, p < 0.001$).

Larval density and the primary sex-ratio: In experiment 1B, the mean sex ratio (not corrected for larval mortality) for the low density treatment (0.71 ± 0.027) was significantly more male-biased than that of the high density treatment (0.64 ± 0.030; t = 2.500, d.f. = 49, P = 0.016). After correcting for larval mortality, the low-density proportion of males (0.62 ± 0.018) was still significantly greater than the high-density proportion of males (0.57 ± 0.018; t = 2.201, d.f. = 49, P = 0.032). In experiment 1C, the pattern of ESD was reversed with the low density treatment (0.52 ± 0.024) slightly less

male-biased than the high density treatment (0.54 ± 0.023) although this difference was not statistically significant ($t = 1.178$, d.f. = 71, $p = 0.243$).

Family effects on the primary sex-ratio: In experiment 1B, there is a strong correlation in the raw proportion of males between the low and high density treatments (r = 0.470, n = 50, P < 0.001). After correcting the data for larval mortality, this full-sib correlation in the primary sex-ratio is marginally non-significant (r = 0.273, n = 50, P = 0.055). There is also a strong full-sib correlation in the proportion of males (corrected for larval mortality) in experiment 1C ($r = 0.727$, $n = 72$, $p < 0.001$; Figure 1). These full-sib correlations indicate the presence of family effects on the primary sex-ratio.

Experiment 2: Selection on adult body size in females and males

Fecundity selection on female body size: For females, body length ranged between 0.74 and 0.97 mm (mean = 0.83 ± 0.005 mm, N = 70) and average fecundity (for the two consecutive egg sacs) ranged between 26.0 and 80.0 eggs per clutch (mean = 52.9 ± 1.25 eggs per clutch, N = 80). The linear regression equation is $Y = b_0 + b_1 * X$, where Y = average fecundity of the two eggsacs, X = female body length (mm), $b_0 = 4.1 \pm 24.03$, $b_1 = 59.7 \pm 28.94$. The positive slope (b_1) is statistically significant ($F_{1, 68} = 4.249$, P = 0.043) suggesting the presence of fecundity selection for larger female body size.

The size of the first field clutch (47.3 ± 1.40 eggs per clutch, N = 66) was much smaller than that of the second lab clutch (61.0 ± 1.75 eggs per clutch, N = 66) and a paired t test found that this difference was statistically significant (t = 8.265, df = 66, P < 0.001). This observation suggests that egg production in *Tigriopus* females is resource-limited in the field.

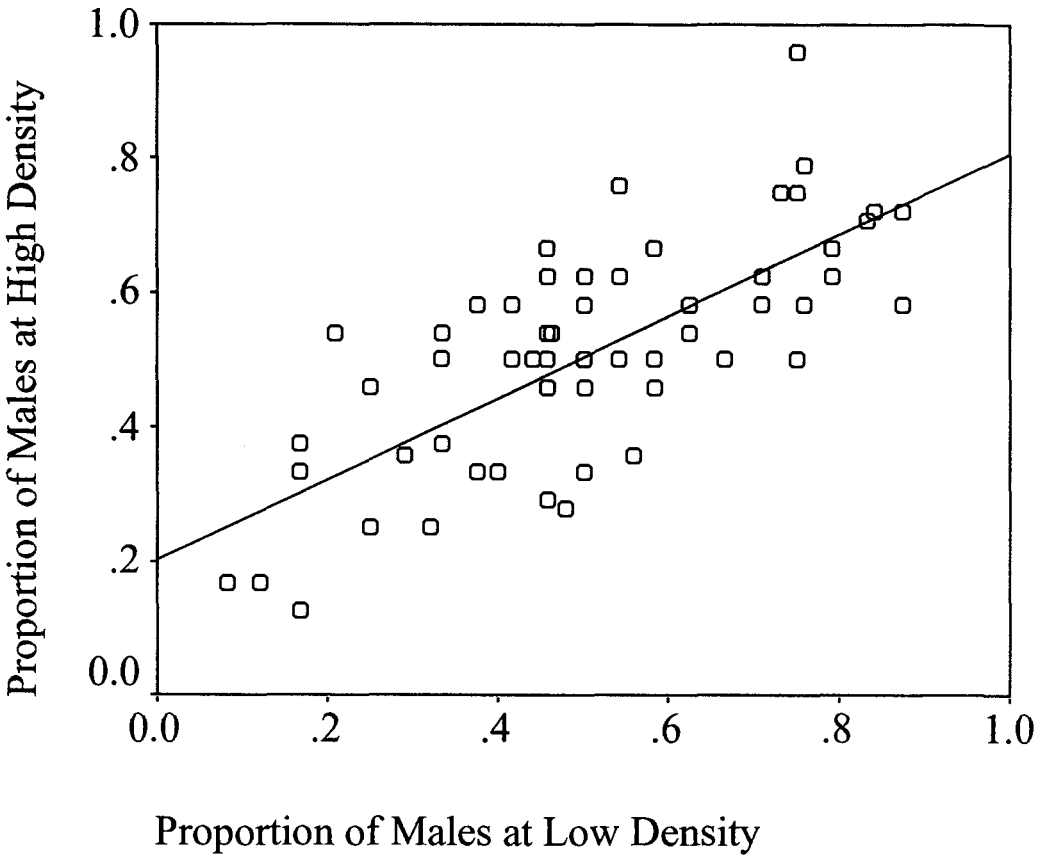

Figure 1. The correlation in the primary sex ratio (corrected for larval mortality) between full sibs (from the same egg sac) exposed to the high and low density treatments in experiment 1C. For each family (data point) there are 24 offspring in the high and 24 offspring in the low density treatment.

The regression of the first, field clutch on body size is statistically significant ($F_{1, 68} = 5.414$, $P = 0.023$, $b = 66.6 \pm 28.62$ eggs/mm, Figure 2) but the regression of the second, lab clutch on body size is not statistically significant ($F_{1, 60} = 2.199$, $P = 0.143$, $b = 56.9 \pm 38.39$ eggs/mm, Figure 2). This observation suggests that fecundity selection on female body size is stronger in the field than in the lab which is expected if egg production is resource-limited.

Sexual selection on male body size: In the field sample, the mean body length of the single males (0.74 ± 0.013, $N = 18$, range = 0.66 – 0.89) was larger than that of the precopula males (0.72 ± 0.006, $N = 94$, range = 0.53 – 0.85), although the difference was not statistically significant ($t = 1.260$, $df = 110$, $P = 0.210$, Figure 3). The logistic regression coefficient for male body size was negative ($b = -5.891 \pm 4.693$) but not statistically significant (Wald statistic = 1.575, $df = 1$, $P = 0.209$).

Likewise for the lab sample, the mean body length of the single males (0.74 ± 0.004, $N = 93$, range = 0.65 – 0.82) was larger than that of the precopula males (0.72 ± 0.008, $N = 47$, range = 0.62 – 0.88) and in this case the difference was statistically significant ($t = 2.383$, $df = 138$, $P = 0.019$, Figure 3). The logistic regression coefficient for male body size was negative ($b = -9.894 \pm 4.281$) and statistically significant (Wald statistic = 5.341, $df = 1$, $P = 0.021$) suggesting that smaller males are more likely to enter the precopula state than large males.

Combining the field and lab samples, the mean body length of single males (0.74 ± 0.004, $N = 111$) was significantly larger than that of the precopula males (0.72 ± 0.005, $N = 141$, $t = 2.435$, $df = 250$, $P = 0.016$). The logistic regression equation is given by $Y = 1/(1 + e^{-Z})$, where Y = probability of a male being in the precopula state, X = male body

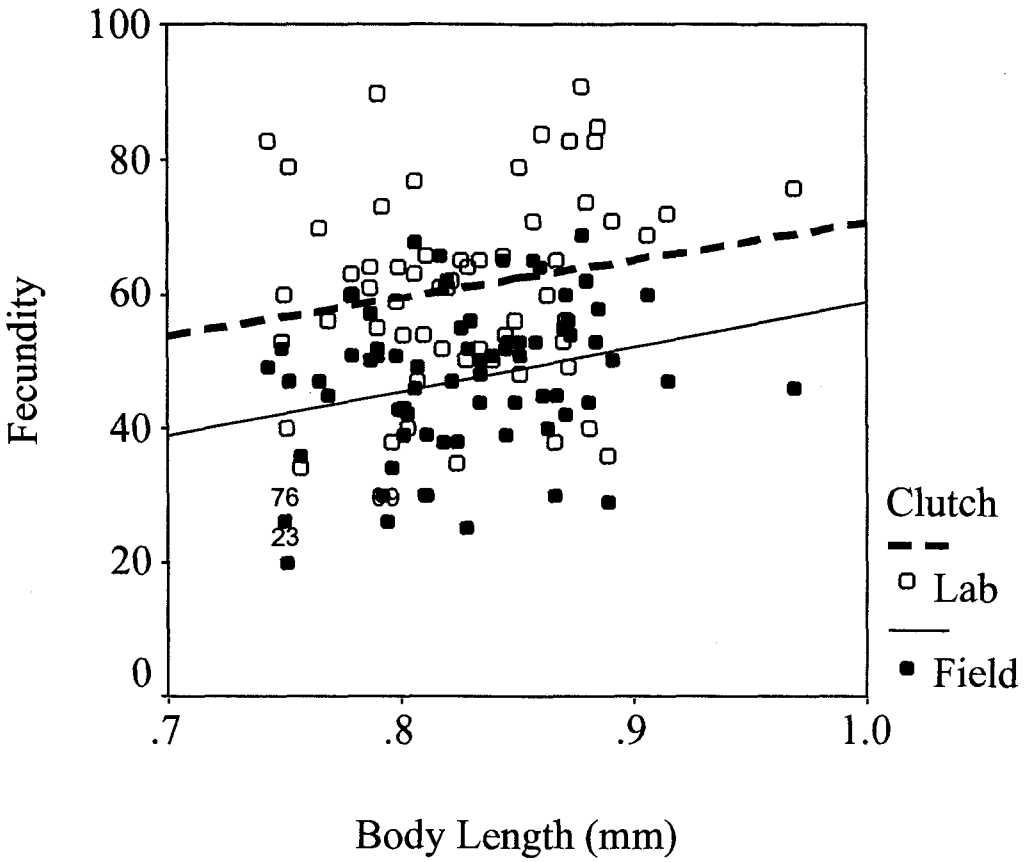

Figure 2. The relationship between fecundity (number of eggs per clutch) and body length in *T. californicus* females for the first, field-born clutch and the second, lab-born clutch.

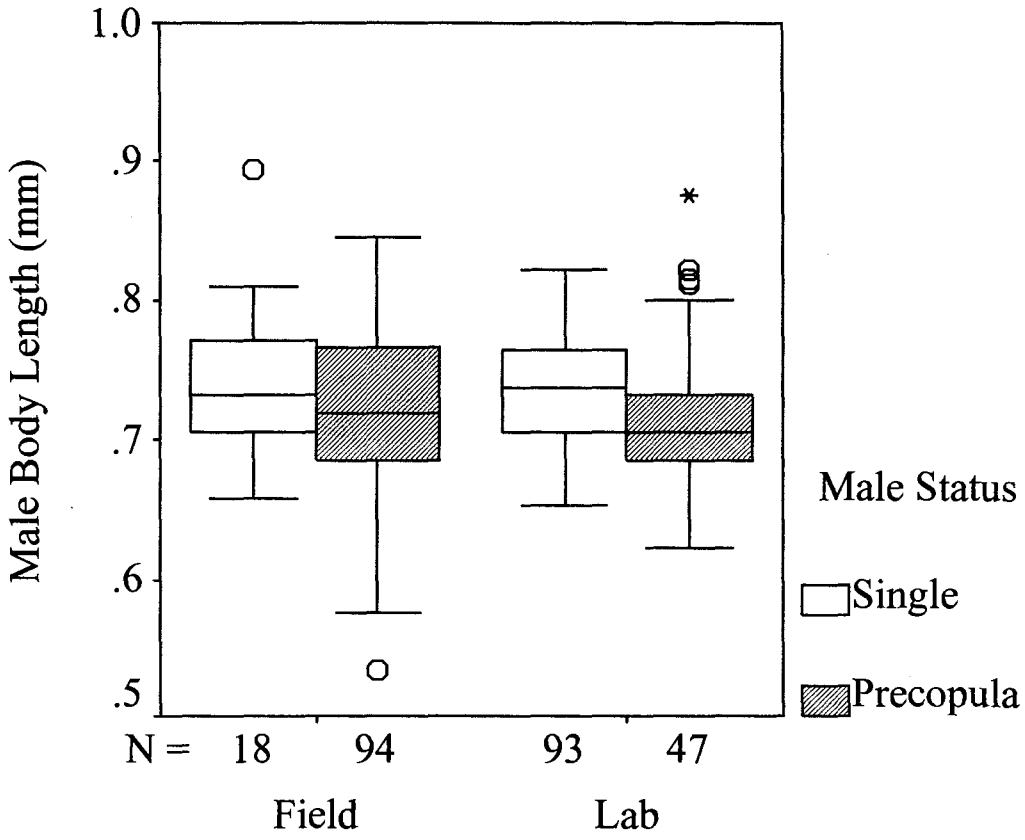

Figure 3. Male body length vs. precopula status (single male vs. precopula males) in the field and lab sexual selection experiments. The box represents the interquartile range which contains 50% of the values. A line across the box indicates the median. The whiskers represent the highest and lowest values, excluding outliers. Outliers (o) are values that are between 1.5 and 3 box lengths from the upper or lower edge of the box. Extremes (*) are values that are more than 3 box lengths from the upper or lower edge of the box.

length, $Z = b_0 + b_1X$, $b_0 = 4.769 \pm 1.906$ (Wald statistic = 6.262, df = 1, P = 0.012) and $b_1 = -6.217 \pm 2.607$ (Wald statistic = 5.688, df = 1, P = 0.017). Again, the negative slope of the logistic regression coefficient for male body size (b_1), suggests that smaller males are more likely to be found in precopula than larger ones.

Differential selection on body size in males and females: To compare the strength of selection on adult body size between the sexes, we used the female fecundity selection (Average Fecundity = 4.1 + 59.7*Female Body Length) and the male sexual selection (Probability of Precopula = $1/(1 + e^{-(4.8 - 6.2*\text{Male Body Length})})$ equations to generate fitness values (average fecundity for females, probability of precopula for males) for a range of body lengths (0.5 – 1.0 mm). For each sex, we standardized these fitness values to relative fitness (absolute fitness divided by the population's mean absolute fitness). As body size increases, relative fitness increases for females but decreases for males (Figure 4). With respect to larval density and the Charnov-Bull model of adaptive ESD, these data suggest that large, low-density individuals should differentiate as females.

DISCUSSION

The primary objective of this study was to determine whether larval density affects sex determination in *T. californicus*. Although the high density treatment produced significantly more males in experiment 1B, there was no effect of larval density on the primary sex ratio in experiment 1C. In terms of absolute density, the effect size in experiment 1C (high density = 3000 nauplii/ liter) was three times that of experiment 1B (high density = 1000 nauplii/ liter; Table 1) and cannot account for the lack of a density effect in experiment 1C. A post-hoc power analysis found that experiment 1C had a 60%

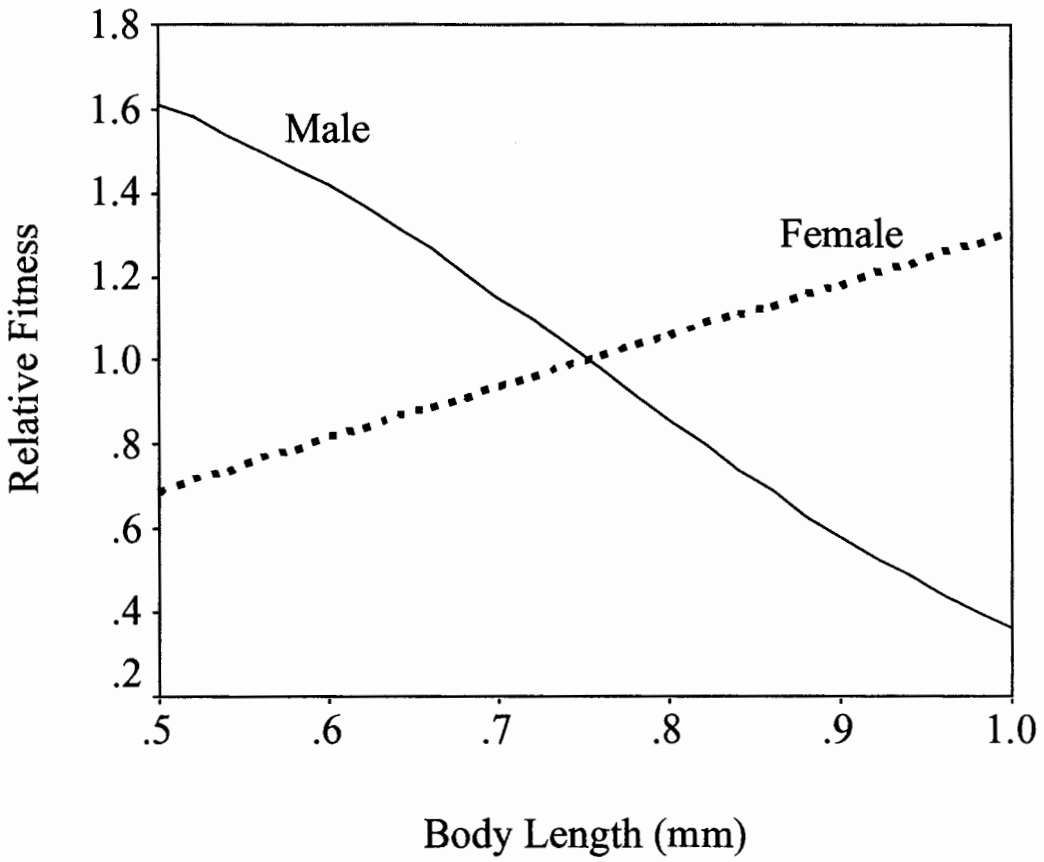

Figure 4. Relative fitness vs. body size for males (solid line) and females (stippled line).

chance of detecting the level of ESD found in experiment 1B and a 90% chance of detecting a difference in the proportion of males between the high and low density treatments > 0.10. Across the two experiments (1B and 1C) and weighted by sample size, the mean difference in the proportion of males between the low and high density treatments was < 0.02. This small effect size suggests that larval density does not play a biologically significant role in the sex-determination of *T. californicus*.

Our results are similar to Egloff (1966) who also found that larval density did not affect the primary sex ratio in *T. californicus*. Egloff (1966) used a similar range of densities (low = 200, mid = 1000 and high = 2000 nauplii/ml) and had high survivorship (> 96%); however, he had no data to show that larval density-induced food limitation actually occurred. Our calculations of the daily per capita algal consumption rates during development (Appendix A), indicate that high-density individuals were food limited in experiment 1C. Further evidence for density-induced food limitation comes from the observation that high-density individuals took longer to reach sexual maturity and were significantly smaller than their low-density siblings. Hence, our density treatments provided a relevant manipulation of the opportunity for future growth as required by the Charnov-Bull model of adaptive ESD.

Egloff (1966) used nauplii from 80 egg sacs and assigned them at random to one of three density treatments. In contrast, we used a paired design where nauplii from the same full-sib family were reared under both low and high density. The advantage of this paired design is that it allows one to assess the among-family variation in the primary sex ratio (Cook 2002; Yusa 2004), which is clearly significant in *T. californicus* (i.e. in figure 1 the probability of differentiating into a male ranges between <0.20 to >0.90). The

results of this study are similar to Voordouw and Anholt (2002a) who also found that differences among families accounted for much more of the variation in the primary sex ratio than differences among temperatures. With respect to the rearing methodology in future experiments, one encouraging conclusion that can be drawn from Voordouw and Anholt (2002a) and the present study is that the sex ratio trait in *T. californicus* appears to be robust to substantial variation in temperature and food availability.

The secondary objective of this study was to determine whether larval density-induced variation in adult body size has different fitness consequences between the sexes. In lab-reared copepods (experiments 1B & 1C), and in contrast to our predictions, large, low-density females did not produce significantly more eggs than their smaller, high-density sisters but large, low-density males had significantly higher mating success than their smaller, high-density brothers. This pattern of selection was reversed in the field sample (Figure 4) where large females produced significantly more eggs and small males were significantly more likely to be found in the precopula state. What accounts for these contradictory results between the lab and the field?

In the field-sampled females (experiment 2), the first, field-born clutch was much smaller than the second, lab-born clutch suggesting that egg production is food-limited in the field (Figure 2). That a statistically significant relationship was found between female body size and the first, field-born clutch but not the second, lab-born clutch, further suggests that fecundity selection on female body size is more likely to be detected in food-limited environments. Similarly, while lab-reared females (experiment 1B & 1C) in the high density treatment were initially food limited, the Tetramin flake food supplement (at the third copepodite stage) may have subsequently obscured differences in body size

related egg production. With respect to the lab-reared females, another possible confounding factor is that the high-density females may have benefitted from cannibalizing their dead sisters (i.e. for some families < 20 individuals were recovered from the high density treatment).

The conflicting role of male body size in this study may reflect the possibility that experiments 1 and 2 are actually measuring different aspects of sexual selection. In the first experiment, larger, low-density males were more than twice as likely to win the mating competition as their high-density competitors. These results are consistent with other studies that have shown the importance of male body size in crustaceans with precopulatory mate-guarding (Jormalainen 1998). Sexual selection on male body size has been reported in a calanoid copepod (Grad and Maly 1988; Grad and Maly 1992) and several species of amphipod (Bertin and Cezilly 2003; Ward 1988). In amphipods and isopods, large males are capable of displacing smaller males from their mates (called a take-over, Jormalainen et al. 1994) and are better at subduing females that actively struggle against male clasping attempts (Jormalainen and Merilaita 1993; Jormalainen and Merilaita 1995).

By contrast, in experiment 2 we found that smaller males were more likely to be in the precopula state than larger males (in both the field and lab sample). Several authors have pointed out that when sexually receptive females are rare, male mating success may depend more on encounter rates than male-male interactions (Ghiselin 1974; Reiss 1989; Trivers 1972). Under this type of scramble competition, selection will favor small, mobile males over large, dominant ones (Fairbairn and Preziosi 1994). However, the mating success of these smaller males is not necessarily guaranteed if larger males are

capable of take-overs (Jormalainen 1998). Recent trials using color-dyed males have shown that take-overs do in fact occur in *T. californicus*, although they are not common (Gabe Stebbins personal observation). Our results therefore suggest conflicting selection pressures on male body size: small males more likely to find and clasp sexually, immature females but larger males are more likely to win mating competitions.

In conclusion, this study finds no compelling evidence that larval density plays a biologically significant role in the sex-determination of *T. californicus*. Hence the enigmatic sex-ratio trait in this species and in harpacticoid copepods in general is still without ecological explanation. To our knowledge, this study is the first attempt at measuring sex-specific differences in the strength of selection on adult body size in any copepod and these data can be used to inform models of ESD, sexual size dimorphism, protandry and other phenomena that play a prominent role in role in the biology of copepods and other crustaceans.

APPENDIX A

In previous grazing experiments, we determined that the average stage I nauplius consumes 9,000 algal cells per day and that the average *T. californicus* consumes 455,000 algal cells per day at 20° C. We modelled the stage-specific daily algal consumption rate (Q) at 20° C as an exponential growth equation: $Q_{n+i} = Q_n * e^{ri}$, where Q_{n+i} and Q_n are the daily consumption rates of the $(n+i)^{th}$ and the n^{th} stage, r = the instantaneous rate of increase (in the daily consumption rate) per stage, and i is the number of stages between the $(n+i)^{th}$ and the n^{th} stage. The stage-specific instantaneous rate of increase in the daily consumption rate at 20° C, $r = \ln(Q_{12}/Q_{01})/ (Q_{12} - Q_{01}) = \ln(455{,}000/9{,}000)/(11 \text{ stages}) =$ 0.3566/stage. To calculate the cumulative number of algal cells consumed from birth to adulthood at 20° C, we assumed that an average individual spent one day in each of the six naupliar stages and 2 days in each of the five copepodite stages for a total of 16 days from egg to adulthood. The average individual consumes $1.9*10^6$ cells of *Isochrysis galbaena* from birth to adulthood (Table A1).

Table A1. Stage-specific daily consumption rates (1000 *Isochrysis galbaena* cells/day), the duration of each stage (days), and the total number of algal cells consumed for the duration of that stage. Daily stage-specific consumption rates for stage 1 nauplii (N1) and stage 6 copepodites (C6) were obtained from grazing experiments.

Stage	Consumption Rate (10^3 cells/day)	Time (days)	Total Consumption (10^3 cells)
N1	9.0	1.0	9.0
N2	13.0	1.0	13.0
N3	18.0	1.0	18.0
N4	26.0	1.0	26.0
N5	37.0	1.0	37.0
N6	54.0	1.0	54.0
C1	76.0	2.0	153.0
C2	109.0	2.0	219.0
C3	156.0	2.0	312.0
C4	223.0	2.0	446.0
C5	318.0	2.0	637.0
C6	455.0		
Total			1924.0

APPENDIX B

STATISTICAL METHODS

Logistic regression: To separate body size effects from the density manipulation, we used binary logistic regression to test whether these two variables influenced the outcome of the male mating competition trial (1B and 1C). The outcome of the mating trial was defined with respect to whether the low-density male won (outcome = 1) or lost (outcome = 0). The probability that the low-density male wins the mating contest, $P(\text{success}) = 1/(1 + e^{-(\beta 0 + \beta 1 * X + \text{error})})$, where X = the difference in body length between the low and high-density male, β_0 = the natural logarithm of the odds of the low-density male winning the contest and β_1 = the slope relating X to the probability of success.

RESULTS

Logistic regression of male-mating success: According to Akaike's Information Criterion (AIC), the best model included the difference in body size between the high and low-density males in experiments 1A and 1B but not in 1C (Table B1). Likelihood ratio tests confirmed that body size was important in experiment 1A but not in 1B (Table B1). These three analyses suggest that body size does in fact contribute to male mating success after accounting for density treatment ($\chi^2 = 14.607$, d.f. = 6, P = 0.024).

In the null models ($Y \sim \beta_0$) of all three experiments, the log odds of the low to the high-density male is significantly different from zero in experiments 1A ($\beta_0 = 1.04 \pm 0.475$, t = 2.193, d.f. = 22, P = 0.039), 1B ($\beta_0 = 1.28 \pm 0.506$, t = 2.534, d.f. = 22, P = 0.011), and 1C ($\beta_0 = 0.81 \pm 0.300$, t = 2.699, d.f. = 51, P = 0.009). These three analyses confirm that low-density males have a higher chance of winning the mating competition (54/75 = 0.72) than their high-density brothers ($\chi^2 = 24.906$, d.f. = 6, P < 0.001).

Table B1. Analysis of deviance of the mating trial outcome for three different experiments (1A, 1B, 1C). Part I; Maximum likelihood estimates of the deviance of models incorporating parameters for the difference in cephalothorax width (X_1) and body length (X_2). The best model for each experiment is chosen by minimizing Akaike's Information Criterion (AIC) and is marked in boldface. Part II; Likelihood ratio tests of the effect of X_1 and X_2. Part III; Parameter estimates from the best model; the intercept of the null model (B_0) is the log odds of the low-density male winning the contest.

Part	Expt	Number	Model	np	Deviance	AIC
I	1A	1	$Y \sim B_0$	1	26.4	28.4
		2	**$Y \sim B_0 + B_1(X_1)$**	**2**	**20.6**	**24.6**
	1B	3	$Y \sim B_0$	1	24.1	26.1
		4	**$Y \sim B_0 + B_1(X_2)$**	**2**	**20.5**	**24.5**
	1C	**5**	**$Y \sim B_0$**	**1**	**64.2**	**66.2**
		6	$Y \sim B_0 + B_1(X_2)$	2	64.2	68.2

II	Expt	Effect	Comparison	df	χ^2	P-value
	1A	B_1	1 v 2	1	5.8	0.016
	1B	B_1	3 v 4	1	3.5	0.060
	1C	B_1	5 v 6	1	0.0	0.883

III	Expt	model	Estimate	df	T	P-value
	1A	2	$B_0 = -0.504 \pm 0.801$	21	-0.630	0.536
			$B_1 = 0.049 \pm 0.025$	21	1.995	0.059
	1B	4	$B_0 = 0.824 \pm 0.555$	21	1.486	0.152
			$B_1 = 11.939 \pm 7.932$	21	1.505	0.147
	1C	5	$B_0 = 0.811 \pm 0.300$	51	2.699	0.009

CHAPTER 5: *WOLBACHIA* AND *TIGIROPUS*

Crustaceans exhibit a wide range of sex-allocation strategies including sequential sex change in pandalid shrimp (Charnov and Anderson 1989), male dimorphism in parasitic copepods (Becheikh et al. 1998; Hipeau 1988), female dimorphism in calanoid copepods (Fleminger 1985), and environmental sex-determination in amphipods (Naylor et al. 1988b; Watt and Adams 1994). In addition to this diversity of sex-allocation strategies, a number of crustacean taxa have been found to host cytoplasmic feminizers (Legrand et al. 1987). These are maternally-transmitted microorganisms that convert genetic males into functional females (Stouthamer et al. 2002).

To date, cytoplasmic sex-determination has been reported in at least 22 species of isopod (Bouchon et al. 1998) and in several species of amphipod (Bulnheim 1978b; Ginsburger-Vogel and Desportes 1979). In isopods, the causal agent is the bacterium *Wolbachia* (Rousset et al. 1992) whereas amphipods are feminized by a microsporidian protozoan (Dunn et al. 1993; Terry et al. 1998). Infected hosts generally produce an excess of daughters (Dunn and Hatcher 1997b; Juchault et al. 1993). However, the sex-ratio of the host is not necessarily female-biased because abiotic factors (Dunn and Hatcher 1997a; Rigaud et al. 1997), host resistance (Rigaud and Juchault 1992; Rigaud and Juchault 1993), and population structure (Hatcher et al. 2000) can limit parasite prevalence under natural conditions (Kelly et al. 2001; Rigaud et al. 1999).

The widespread occurrence of cytoplasmic feminizers in isopods and amphipods begs the question whether other crustacean taxa are infected with these parasites. Highly female-biased sex ratios have been reported in field samples of calanoid copepods (Irigoien et al. 2000), vent copepods (Tsurumi et al. 2003) and harpacticoid copepods

(Hicks and Coull 1983). Laboratory experiments on the easy-to-culture harpacticoid copepods (*Tigriopus californicus*, *Tigriopus japonicus* and *Tisbe gracilis*) have repeatedly found extra-binomial variation in the primary sex-ratio (Ar-Rushdi 1958; Battaglia 1958; Battaglia and Malesani 1959; Egloff 1966; Igarashi 1963a; Igarashi 1963b). Forty years ago, Igarashi (1964) suggested that the sex-ratio trait in *T. japonicus* was cytoplasmically inherited. In our work on *T. californicus*, we recently reported a correlation in the clutch sex-ratio between mothers and daughters and mentioned cytoplasmic inheritance as one potential explanation (Voordouw and Anholt 2002b). In view of this present state of affairs, a search for cytoplasmic feminizers in *T. californicus* seemed timely.

Most of our work on the sex-ratio trait in *T. californicus* has focussed on a population from Arbutus Cove, Victoria, British Columbia, Canada (48°28'36'', 123°18'00''). Prior to testing for the presence of cytoplasmic feminizers, we were interested to determine whether other populations of *T. californicus* had the sex-ratio trait. After finding the sex-ratio trait in several populations of *T. californicus*, we assayed these populations for feminizing *Wolbachia* and microsporidia using standard PCR protocols (and a DAPI staining technique in the case of the microsporidia).

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, Canada: Arbutus Cove (AC), Victoria (48°28'36'', 123°18'00''), Cattle Point (CP), Victoria (48°26'14'', 123°17'22''), East Sooke Park (ES), Sooke (48°18'52'', 123°38'57''), Ross Bay Cemetery (RB), Victoria (48°24'28'', 123°20'00''), Crystal Cove (CC), Tofino (49°08'07'', 125°54'41''), and Botanical Beach (BB), Port Renfrew (48°31'45'', 124°26'54'').

In the lab, 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 haphazardly selected 24 live and vigorous nauplii (i.e. large size and 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 sea water, ~ four 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 hour 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 haphazard selection of the 24 nauplii for each family might inadvertently bias our estimates of the primary sex-ratio if sex-biased mortality occurs prior to hatching. To test whether this was the case, we conducted a separate experiment (i.e. a protocol check)

where we chose 24 nauplii using our haphazard selection criteria (see above) and reared them (as above) for a sample of 65 families. 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 ± 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 haphazardly selected sample (0.539 ± 0.0251) was not significantly different ($t = -0.3418$, $d.f. = 55$, $p = 0.7338$) from that of the unselected sample (0.547 ± 0.0239) demonstrating that pre-hatching sex-biased mortality 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 PCR: 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 contained 5 egg sac carrying females for a total of 60 gravid females per population. We used the mitochondrial primer ($679^f - 1241^r$), to determine if our DNA extraction was successful (Bouchon et al. 1998). For detecting *Wolbachia*, we used the 16S rDNA primer (O'Neil et al.), and *Wolbachia*-infected *Drosophila simulans seychellia* females as a positive control. For detecting microsporidia, we used the SSU rRNA microsporidian-specific primers $V1f - 0530^r$ and $18^f - 1492^r$ (Terry et al. 1999), and used microsporidia-infected *Gammarus rosilini* females as a positive control. We also tried to

visualize microsporidia nuclei in *T. californicus* embryos using DAPI staining and fluorescence microscopy (Dunn et al. 1993).

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 (at the plate level). This larval mortality correction (Voordouw and Anholt 2002a; Voordouw and Anholt 2002b) is statistically conservative as it tends to reduce extra-binomial variation in the primary sex ratio as well as the full-sib correlation in the proportion of males. Throughout this paper we will refer to both the uncorrected (raw) and the larval mortality-corrected data.

Geographic distribution of the sex-ratio trait: For the six populations, we tested whether the mean primary sex-ratio was significantly biased (towards either males or females) using a randomization protocol (similar to but more sensitive than a one sample t-test). We used a second randomization protocol (similar to but more sensitive than an F-test) to determine whether the observed variance in the primary sex-ratio was greater than expected under Mendelian segregation of sex-chromosomes and estimated the full sib correlation in the proportion of males between plates 1 and 2. Finally, we tested whether the mean proportion of males was significantly different among populations using a simple one-way ANOVA.

Wolbachia and microsporidia PCR: The probability of detecting *Wolbachia* or microsporidia for any *T. californicus* population is given by the binomial equation

$$P = 1 - (1 - \rho)^n \qquad \text{Eq. 1}$$

Where P is the probability that at least one of the 'n' samples will test positive and ρ is the parasite prevalence in the population (i.e. the proportion of infected hosts). If we assume that the DNA extraction protocol was successful for every processed copepod then n = 60 for each of the five populations. If we assume that DNA extraction was successful for each sample of five copepods, then n = 12 for each of the five populations. Equation 1 also assumes that all infected hosts have an equal probability of testing positive (i.e. there is no effect of parasite load/density within a host on detection levels).

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 very strong (range = 0.883 – 0.996) and highly statistically significant (Table 1) indicating that these two measures of the primary sex-ratio are virtually interchangeable.

Mean proportion of males: The mean proportion of males was generally male-biased (5 out of 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 proportion of males among the six populations ($F_{5, 387}$ = 0.822, p = 0.5344). The observed lack of female bias does not lend strong support to the cytoplasmic sex-ratio distortion hypothesis. However, across all six populations, 14.8%

Table 1. Mean survivorship (expressed as a %) ± standard error, the correlation between the raw proportion of males and survivorship (r_1) and the correlation between the raw and larval mortality-corrected proportion of males (r_2) for all six populations. Also shown are the sample size (N) and the statistical significance (p_1 and p_2) of the two correlations (r_1 and r_2). Statistically significant correlations are outlined in boldface type.

Pop.	N	Survivorship + s.e.	r_1	p_1	r_2	p_2
AC	112	85.3 + 1.43	0.175	0.066	**0.883**	<0.001
CP	40	91.5 + 2.06	-0.038	0.817	**0.962**	<0.001
ES	47	87.9 + 1.95	0.151	0.312	**0.933**	<0.001
RB	34	94.5 + 1.49	0.262	0.134	**0.968**	<0.001
CC	92	96.6 + 0.69	-0.055	0.601	**0.987**	<0.001
BB	68	98.8 + 0.44	0.076	0.537	**0.996**	<0.001

of the families were strongly female biased (family proportion of males ≤ 0.300) for the uncorrected data (9.9% for the corrected data; Table 3). The frequency of female-biased families was highest in Crystal Cove (21.7%) and lowest in Botanical Beach (8.8%). Male-biased families (family proportion of males ≥ 0.700) were slightly more common (19.6% and 15.5% for the raw and corrected data) with the highest and lowest frequency of male-biased families in East Sooke Park (25.5%) and Ross Bay (8.8%), respectively.

Variance in the proportion of males: For the raw proportion of males, the observed variance was, on average, 2.8 times larger than the Mendelian expectation (Table 2; range = 1.9 – 3.9). For the corrected proportion of males, the average ratio of the observed to the expected variance was 2.3 (Table 2; range = 1.5 – 3.4). Irrespective of whether the data were corrected or not, the observed variance in the primary sex ratio was always significantly greater than the binomial expectation (Table 2). The full sib correlation between plates 1 and 2 was statistically significant for all the populations except Ross Bay.

Wolbachia and microsporidia PCR: None of the samples from the 5 *T. californicus* populations and none of the negative controls tested positive for *Wolbachia* DNA whereas all of the *Drosophila simulans seychellia* positive controls tested positive for *Wolbachia*. If we assume that the DNA extraction protocol was successful for all 60 copepods for each population, it is safe to conclude that *Wolbachia* prevalence is negligible (i.e. < 0.05; Figure 1). If we assume that the DNA extraction was only successful for the twelve samples for each population, it is possible that *Wolbachia* exists at low levels (Figure 1), although across all five populations it would still suggest that *Wolbachia* prevalence is negligible. For the microsporidia PCR using primer $V1^{f} - 0530^{r}$,

Table 2. The mean proportion of males (P.male) ± standard error for all six populations. We conducted randomization tests to determine if (1) a population's mean proportion of males was significantly different from 0.500 (p_1) and (2) whether the observed variance (Obs. Var) in the proportion of males was significantly greater than the expected variance (Exp. Var) under Mendelian segregation of sex chromosomes (p_2). The ratio is the observed variance in the proportion of males divided by the expected variance. Also shown is the full sib correlation in the proportion of males between plates 1 and 2 (r) and the statistical significance of this correlation (p_3). For each population, the top and bottom row show the raw and larval mortality-corrected data, respectively. Means and variances that are signifcantly different from the Mendelian expectation and significant full-sib correlations are shown in boldface type.

Pop.	N	P.male + s.e.	p_1	Obs. Var.	Exp. Var.	Ratio	p_2	r	p_3
AC	112	**0.52 + 0.017**	0.040	**0.0318**	0.0129	2.5	<0.001	**0.318**	<0.001
		0.53 + 0.012	<0.001	**0.0160**	0.0105	1.5	<0.001	**0.326**	<0.001
CP	40	0.48 + 0.029	0.199	**0.0328**	0.0120	2.7	<0.001	**0.575**	<0.001
		0.48 + 0.025	0.216	**0.0246**	0.0106	2.3	<0.001	**0.583**	<0.001
ES	47	**0.55 + 0.029**	0.001	**0.0384**	0.0125	3.1	<0.001	**0.395**	0.006
		0.55 + 0.023	0.002	**0.0254**	0.0107	2.4	<0.001	**0.554**	<0.001
RB	34	0.52 + 0.026	0.282	**0.0234**	0.0120	1.9	<0.001	0.312	0.072
		0.52 + 0.022	0.247	**0.0164**	0.0112	1.5	0.040	0.196	0.266
CC	92	0.52 + 0.023	0.160	**0.0489**	0.0126	3.9	<0.001	**0.670**	<0.001
		0.51 + 0.021	0.249	**0.0410**	0.0121	3.4	<0.001	**0.633**	<0.001
BB	68	**0.54 + 0.021**	0.001	**0.0309**	0.0110	2.8	<0.001	**0.588**	<0.001
		0.54 + 0.021	<0.001	**0.0292**	0.0109	2.7	<0.001	**0.572**	<0.001

Table 3. The number of male-biased (MB; proportion of males ≥ 0.700) and female-biased (FB; proportion of males ≤ 0.300) families for each of the six populations for the uncorrected (raw) and the larval mortality-corrected data. Shown are the number of families for each population (N) and the frequencies of male-biased (Freq. MB) and female-biased (Freq. FB) families in each of the six populations.

		Uncorrected Data				Larval Mortality-Corrected Data			
Pop.	N	MB	FB	Freq. MB	Freq. FB	MB	FB	Freq. MB	Freq. FB
AC	112	19	14	0.170	0.125	11	6	0.098	0.054
CP	40	5	7	0.125	0.175	4	5	0.100	0.125
ES	47	12	7	0.255	0.149	8	3	0.170	0.064
RB	34	3	4	0.088	0.118	2	3	0.059	0.088
CC	92	23	20	0.250	0.217	21	17	0.228	0.185
BB	68	15	6	0.221	0.088	15	5	0.221	0.074
Total	393	77	58	0.196	0.148	61	39	0.155	0.099

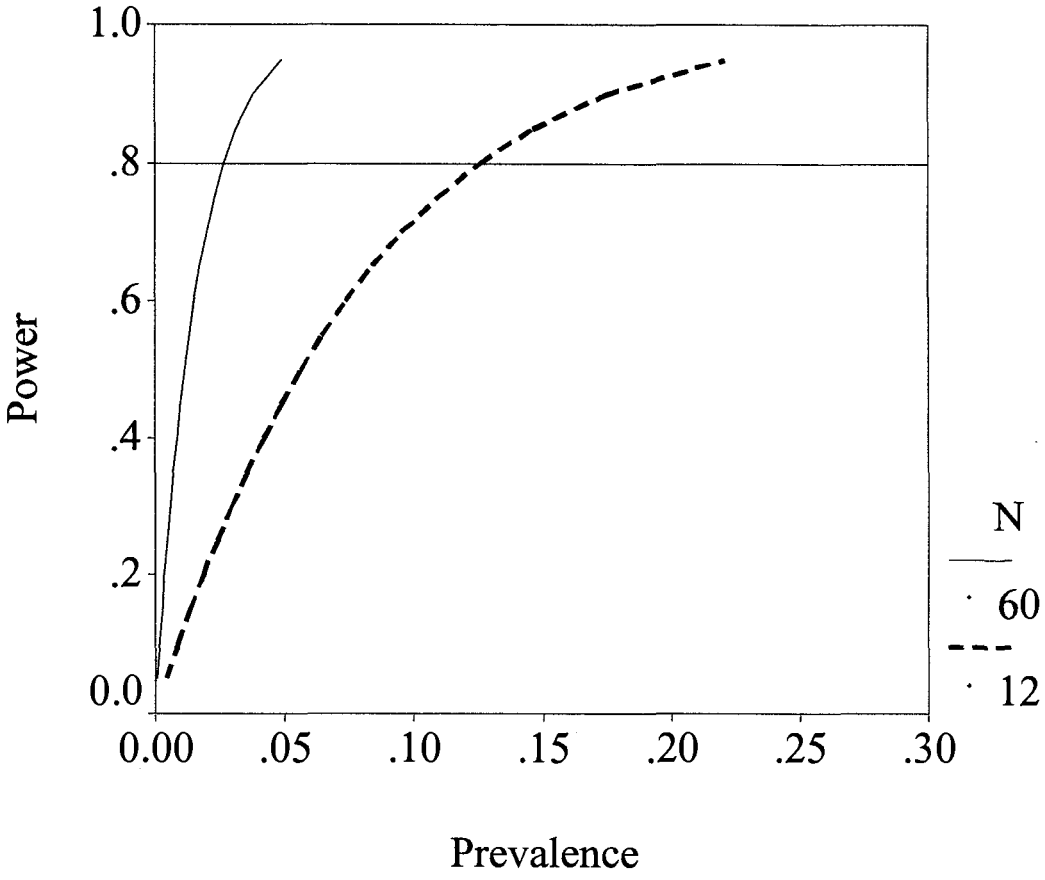

Figure 1. The probability of detecting cytoplasmic feminizers for a given parasite prevalence for each population assuming that the DNA extraction worked for all processed copepods (N = 60) or assuming that the DNA extraction worked for all the samples (N = 12).

two samples (RB01 and ES06) from two populations (Ross Bay Cemetery and East Sooke Park), tested positive. We obtained a lot of non-specific, background amplification for the microsporidian primer $18^{f} - 1492^{r}$ and so we stopped using this primer. Using DAPI staining and fluorescence microscopy we did not find microsporidian nuclei in any of the *T. californicus* embryos sampled.

DISCUSSION

Our protocol check demonstrates that the sex ratio of our haphazardly selected sample of 24 nauplii is representative of all of the nauplii in the egg sac. We are confident that the observed variation in the proportion of males reflects variation in the primary sex ratio because (1) larval mortality was low and (2) there was a strong correlation between the raw and the larval mortality-corrected estimates. The absence of male-biased mortality allows us to rule out male-killers as a potential explanation for the sex-ratio trait in *T. californicus* (Hurst 1993).

Our geographic survey of *T. californicus* populations on Vancouver Island found the sex ratio trait in five out of six populations. Previous work in our lab had primarily focused on a population from Arbutus Cove (Voordouw and Anholt 2002a; Voordouw and Anholt 2002b) but this study shows that the sex ratio trait is potentially widespread on Vancouver Island. In general, the populations were male-biased and the frequency of female-biased families was low (~10%). Theory predicts a female biased sex ratio in host populations infected with cytoplasmic sex ratio distorters (Werren 1987a). *Wolbachia*-infected isopod populations are often female-biased (Rigaud 1997) but microsporidia-infected amphipod populations are sometimes male-biased (Kelly et al. 2001).

Our PCR tests suggest that *Wolbachia* prevalence in *T. californicus* is low or non-existent. Likewise, PCR tests and antibiotics experiments conducted on *T. californicus* populations in California found no evidence of *Wolbachia* (Dr. Suzanne Edmands, personal communication). While two samples (one from Ross Bay the other from East Sooke Park) tested positive for the microsporidian primer $V1^f - 0530^r$, we were unable to detect microsporidian nuclei in the embryos of these animals. Finally, a subsequent lineage experiment on the Arbutus Cove population (see Chapter 6) found no maternal transmission of the sex ratio trait contradicting the results from Voordouw and Anholt . Hence, while the sex ratio trait appears to be maternally transmitted in *T. japonicus* (Igarashi 1964), cytoplasmic feminizers do not appear to play a role in the sex ratio trait of *T. californicus*.

CHAPTER 6: PATERNAL INHERITANCE OF THE PRIMARY SEX RATIO IN *TIGRIOPUS*

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

INTRODUCTION

"Because each sex must supply half the ancestry of all future generations of the species... (Fisher 1930)."

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

Sex-ratio distorters include sex chromosomes with meiotic drive (Jaenike 2001), maternally inherited microorganisms (O'Neil et al. 1997; Stouthamer et al. 1999; Weeks et al. 2002; Werren 1997) and supernumerary 'B' chromosomes (Werren and Stouthamer 2003). Some populations of fruitflies have an X chromosome with meiotic drive that causes males (the heterogametic sex) to produce predominantly X-bearing sperm resulting in a female-biased sex-ratio (Carvalho et al. 1998; Varandas et al. 1997). In several species of isopod, a cytoplasmically (maternally) inherited bacterium, *Wolbachia*, feminizes genetic males into phenotypic females (Rigaud 1997). In two species of

parasitoid wasp, *Nasonia vitripennis* and *Trichogramma kaykai*, a small, paternally-transmitted B chromosome converts diploid female embryos into haploid male embryos by destroying the other paternal chromosomes (Werren and Stouthamer 2003). In each of the above examples, an asymmetric inheritance pattern selects for genetic elements that skew the primary sex-ratio towards the transmitting sex.

Traditional lineage analysis of family sex ratios is often the first step in establishing the presence of a uniparentally inherited sex ratio distorter (Hurst 1993). For example, the maternal inheritance of a feminizing genetic element in the isopod, *Armadillidium vulgare*, had been characterized for several decades (Legrand et al. 1987) prior to its molecular identification as the bacterium *Wolbachia* (Rousset et al. 1992). Likwise, the paternal sex ratio (PSR) factor of *Nasonia vitripennis* was accidentally discovered during a selection experiment (Werren et al. 1981) and its mechanism was subsequently elucidated (Werren and Stouthamer 2003). While lineage experiments can not pinpoint the mechanism per se, the inheritance pattern (i.e. maternal vs. paternal) can narrow the field of potential candidates. For example, a paternally transmitted sex ratio trait would eliminate *Wolbachia* (or other maternally inherited microorganisms) and would suggest the presence of a male heterogametic system with Y-drive.

Over the last five years we have studied the sex ratio trait in the harpacticoid copepod, *Tigriopus californicus*. We have repeatedly found that this splash pool copepod has a large among family component in the primary sex ratio; i.e. full sibs covary in their tendency to develop into a male (Voordouw and Anholt 2002b). Experiments investigating environmental sex determination (ESD) suggest that temperature (Voordouw and Anholt 2002a) and density (unpublished data) do not play a significant

role in structuring the sex ratio variance in *T. californicus*. The sex-determining mechanism for *T. californicus* remains unknown. Karyotypes have found no heteromorphic sex chromosomes (Ar-Rushdi 1963) and several authors have claimed a polygenic sex-determining mechanism (Ar-Rushdi 1958; Belser 1959; Egloff 1966; Voordouw and Anholt 2002b).

We reported a parent-offspring correlation in the sex ratio between mothers and daughters but this experiment had poor survivorship in both generations so that we cannot rule out the presence of sex-specific mortality (Voordouw and Anholt 2002b). In addition, the mother-offspring regression is potentially confounded by maternal effects which is not a problem for the father-offspring regression (Falconer 1989). The recent demonstration that the primary sex ratio is paternally inherited in the European fairy shrimp, *Branchipus schaefferi* (Beladjal et al. 2002), motivated us to investigate the paternal contribution to the sex ratio trait in *T. californicus*. Like *B. schaefferi*, we found that the sex ratio trait was paternally transmitted in *T. californicus*, although the mechanism that generates this inheritance pattern is currently unknown.

MATERIALS AND METHODS

General Overview: The lineage experiment consists of three generations: the F_1, the F_2 and F_3 generation (Figure 1). There are 7 different types of relatives and all of them are defined in relation to the F_3 generation. The F_1 generation consists of the paternal grandfathers, the paternal grandmothers, the maternal grandfathers and the maternal grandmothers. The F_2 generation consists of the fathers and mothers and the F_3 generation consists of offspring.

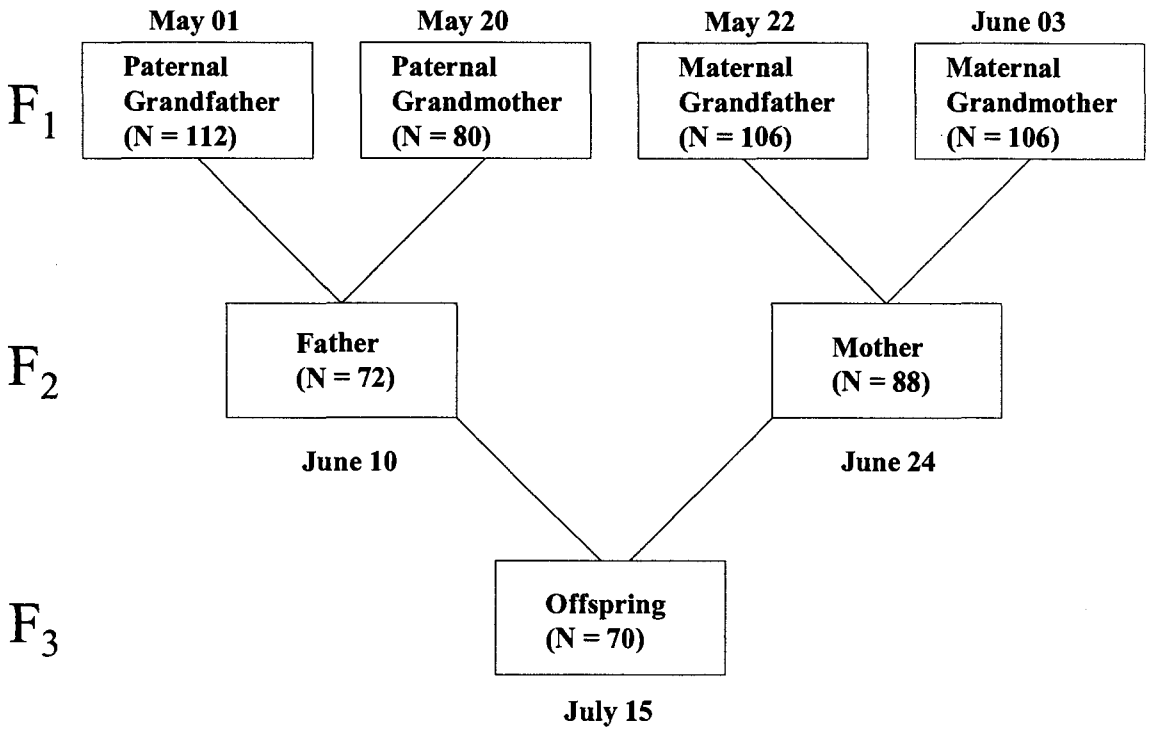

Figure 1. Experimental design of the lineage experiment showing the three generations (F_1, F_2 and F_3), the seven relatives (paternal grandfathers, paternal grandmothers, maternal grandfathers, maternal grandmothers, fathers, mothers and offspring), the approximate dates on which each relative was born and the sample sizes (N).

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

Rearing Protocol: Each nauplius was reared in its own well in a 24-well tissue culture plate. Each well was stocked with 2.5 ml of filtered sea water, ~ $4.0*10^6$ cells of *Isochrysis galbaena* and ~ 0.2 mg of Tetramin flakes. Tissue culture plates were stored on top of moist paper towels in covered plastic containers to prevent evaporation. Containers were stored in incubators set at a constant temperature of 20° C with a 12 hour day/night cycle.

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

We collected ~ 100 gravid females from the field on each of four sampling dates (May 1st, May 20th, May 22nd and June 3rd, 2003) and used the egg sacs from these field-captured females to create the paternal grandfathers (N = 112), the paternal grandmothers (N = 80), the maternal grandfathers (N = 106) and the maternal grandmothers (N = 106), respectively (Figure 1). The 19 day delay (May 1st – May 20th) between the paternal

grandfathers and grandmothers and the 12 day delay (May 22nd – June 3rd) between the maternal grandfathers and grandmothers ensured that the sires reached adulthood well before the dams. Likewise, the 14 day delay (May 20th – June 3rd) between the paternal and maternal grandmothers ensured that the F_2 fathers reached adulthood prior to the F_2 mothers.

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

F_1 Mating Protocol: For each randomly assigned pair of sire and dam families, we used a mating arena to introduce three haphazardly selected adult males (brothers A, B & C) from the sire family to 12 full-sib copepodites of unknown sex phenotype from the dam family. The mating arena consisted of a single well in a 6-well tissue culture plate stocked with 5 ml of filtered sea water and 5 to 10 million cells of *Isochrysis galbaena*. Once a sexually mature male from the sire family (i.e. a future grandfather) had clasped a female copepodite from the dam family (i.e. a future grandmother), we removed that couple from the mating arena. We moved the remaining, unclasped copepodites from the mating arena to a 24-well tissue culture plate and reared them to sexual maturity to estimate the dam family sex ratio. The F_1 mating protocol simulated natural courtship in that all female dams were guarded by the sires prior to mating. However, this protocol was very time consuming and so we changed it for the F_2 cross.

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

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

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

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

first cousins (r = 1/4). The genetic coefficient of relatedness among F_3 families whose fathers were full sibs or double first cousins is 1/8 and 1/16, respectively. In the results we account for this genetic covariance by aggregating the sex phenotype data across paternal grandfathers. Hence the average number of offspring per family is often greater than 24 individuals

For the paternal F1 cross, 72 of the 80 couples (112 paternal grandfathers*80 paternal grandmothers) produced F_2 offspring (the fathers). For the maternal cross, 88 of the 106 couples (106 maternal grandfathers*106 maternal grandmothers) produced F_2 offspring (the mothers). For the F_2 cross (after aggregating the four and five F_2 crosses whose fathers were full sibs and double first cousins, respectively), 70 of the 72 couples (72 fathers*88 mothers) produced F_3 offspring (Figure 1).

STATISTICAL METHODS

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

Mean and variance in the proportion of males: For each relative, we used binomial randomization tests to determine whether the observed mean and variance in the proportion of males was significantly different from the Mendelian expectation (i.e.

independent assortment of sex chromosomes). For each parent-offspring pair, we used a bootstrap procedure to determine the statistical significance of any differences in the sex ratio mean and variance between parents and offspring.

To compare the distribution of the proportion of males among relatives, we only included those 70 families that actually produced F_3 offspring. Excluded families include those families that were not selected in the mating protocol and those families that failed to produce either sons or daughters due to low survivorship and/or extreme sex ratios. Excluding the latter type of family (low survivorship and/or extreme sex ratios) will tend to reduce the variance and/or the skew in the population sex ratio (i.e. if sex-determination and/or larval-mortality is sex-biased). Our estimates of the sex ratio mean and sex ratio variance for each population of relatives should therefore be conservative.

Paternal inheritance of the primary sex ratio: To determine whether fathers have a stronger effect than mothers on the primary sex ratio of their offspring (or vice versa), we used multiple regression to compare the partial regression coefficients of the father-offspring and the mother-offspring regressions for all three crosses (F_1 paternal cross, F_1 maternal cross, F_2 cross) separately and combined.

To compare this study to Voordouw and Anholt , we treated sex as a quantitative threshold trait (Bulmer and Bull 1982) and estimated the heritability of sex tendency following the method by Roff (1986; 1997) with one important exception. For an individual from the i^{th} family, Roff (1986; 1997) calculates the phenotypic value of that individual (on the underlying scale) as the ordinate on the standardised normal curve that corresponds to p_i, the proportion of males in that family. This calculation is wrong because families with 10% or 90% males both have the same probability density (0.175).

We used the cumulative standardised normal curve instead so that the above two families have a cumulative probability density of 0.035 and 0.965, respectively.

To estimate the heritability of sex tendency (h^2), we doubled the slope (b) and standard error from each of the six parent-offspring regressions: (1) paternal grandfathers-F_2 fathers, (2) paternal grandmothers-F_2 fathers, (3) maternal grandfathers-F_2 mothers, (4) maternal grandmothers-F_2 mothers, (5) F_2 fathers-F_3 offspring, (6) F_2 mothers-F_3 offspring (for the three midparent-offspring regressions, $b = h^2$). Again, we only included those 70 families that actually produced F_3 offspring.

RESULTS

Survivorship, male-biased mortality and the larval mortality correction: For the seven types of relatives across three generations we reared 18094 individuals from 608 different families, sexed 15754 adults (average survivorship = 87.1%) of which 8179 individuals were males (proportion of males = 0.519). For the seven types of relatives, survivorship ranged between 78.5 and 97.9% (Table 1). Survivorship was lowest for the F_3 offspring (78.5%) and the maternal grandmothers (81.0%). For the maternal grandmothers, the correlation between survivorship and the raw proportion of males was positive ($r = 0.485$) and highly statistically significant ($p < 0.001$) suggesting that mortality was male-biased in this relative. The correlation between the raw and the larval-mortality corrected proportion of males was lowest for the maternal grandmothers ($r = 0.744$) and this is expected under low survivorship and/or sex-biased mortality. For all other relatives, there was little or no evidence that mortality was sex-biased and the correlation between the raw and corrected proportion of males was high (Table 1). Hence

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

			Raw sex ratio vs. Survivorship		Corrected vs. Raw sex ratio	
Relative	N	Survivorship ± s.e.	r	p	r	p
Paternal Grandfather	112	85.3 ± 1.43	0.175	0.066	**0.884**	<0.001
Paternal Grandmother	80	96.4 ± 0.88	0.023	0.843	**0.978**	<0.001
Maternal Grandfather	106	97.9 ± 0.36	**0.200**	0.040	**0.991**	<0.001
Maternal Grandmother	106	81.0 ± 2.16	**0.485**	<0.001	**0.744**	<0.001
F_2 Father	72	89.8 ± 2.19	-0.065	0.590	**0.948**	<0.001
F_2 Mother	88	90.8 ± 0.99	0.157	0.144	**0.963**	<0.001
F_3 Offspring	70	78.5 ± 1.92	-0.081	0.508	**0.899**	<0.001

we are confident that our results are not biased by sex-linked lethal alleles and that they reflect the segregation of genetic factors that affect the primary sex ratio.

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

Mean proportion of males among generations: Multiple independent two sample t-tests found that the balanced sex ratio in the F_2 fathers was not significantly different from the male-biased sex ratio in either the paternal grandfathers or the paternal grandmothers (Table 3). In contrast, the mildly female-biased sex ratio in the F_2 mothers was significantly different from both the highly male-biased sex ratio of the maternal grandfathers and the highly female-biased sex ratio of the maternal grandmothers (Table 3). Finally the proportion of males in the F_3 offspring was significantly more male-biased than either that of their F_2 mothers or their F_2 fathers (Table 3). Hence the bias in the primary sex-ratio decreased from the F_1 to the F_2 generation and increased slightly from the F_2 to the F_3 generation (Figure 2). From our random mating protocol, we expect the

Table 2. The mean proportion of males (P.male) ± standard error and the observed variance in the proportion of males (Obs. Var.) for all seven types of relatives. We only included those families that actually produced F_3 offspring (N = 70 for each relative). We conducted randomization tests to determine if (1) a relative's mean proportion of males was significantly different from 0.500 (H_0: P.male = 0.50) and (2) whether the observed variance (Obs. Var) in the proportion of males was significantly greater than the expected variance (Exp. Var) under Mendelian segregation of sex chromosomes (H_0: Obs. Var. ≤ Exp. Var.). The ratio is the observed variance in the proportion of males divided by the expected variance. For each type of relative, the top and bottom row show the raw and larval mortality-corrected (L.M.C.) data, respectively (as indicated in the 'Data' column). Means and variances that are signifcantly different from the Mendelian expectation are shown in boldface type.

			H_0: P.male = 0.50		H_0: Obs. Var. ≤ Exp. Var.			
Relative	N	Data	P.male ± s.e.	p	Obs. Var.	Exp. Var.	Ratio	p
Paternal	70	Raw	**0.55 ± 0.020**	<0.001	**0.028**	0.011	2.5	<0.001
Grandfather		L.M.C.	**0.55 ± 0.017**	<0.001	**0.020**	0.010	1.9	<0.001
Paternal	70	Raw	**0.56 ± 0.019**	<0.001	**0.025**	0.011	2.3	<0.001
Grandmother		L.M.C.	**0.55 ± 0.018**	<0.001	**0.022**	0.010	2.1	<0.001
Maternal	70	Raw	**0.60 ± 0.025**	<0.001	**0.042**	0.010	4.2	<0.001
Grandfather		L.M.C.	**0.59 ± 0.024**	<0.001	**0.039**	0.010	3.9	<0.001
Maternal	70	Raw	**0.33 ± 0.021**	<0.001	**0.032**	0.013	2.5	<0.001
Grandmother		L.M.C.	**0.42 ± 0.015**	<0.001	**0.015**	0.009	1.6	<0.001
F_2 Father	70	Raw	0.51 ± 0.024	0.497	**0.039**	0.012	3.2	<0.001
		L.M.C.	0.50 ± 0.021	0.799	**0.030**	0.010	3.0	<0.001
F_2 Mother	70	Raw	**0.46 ± 0.024**	<0.001	**0.041**	0.010	4.2	<0.001
		L.M.C.	**0.47 ± 0.021**	0.016	**0.030**	0.009	3.4	<0.001
F_3 Offspring	70	Raw	**0.58 ± 0.021**	<0.001	**0.030**	0.008	3.8	<0.001
		L.M.C.	**0.54 ± 0.014**	<0.001	**0.015**	0.006	2.5	<0.001

Table 3. Comparing the sex ratio mean and the sex ratio variance between parents and offspring. We only included those families that actually produced F_3 offspring (N = 70 for each parent-offspring pair). For each of the six parent-offspring pairs, we compared the sex ratio mean between parents and offspring using an independent two sample t-test (t_{138}, p_1) and a bootstrap test (p_2). We compared the sex ratio variance between parents and offspring using an F-test ($F_{69, 69}$, p_3) and a bootstrap test (p_4). For each type of relative, the top and bottom row show the raw and larval mortality-corrected (L.M.C.) data, respectively (as indicated in the 'Data' column). Statistically significant t-tests and F-tests are shown in boldface type.

			H_0: $\mu_{parent} = \mu_{offspring}$			H_0: $\sigma^2_{parent} = \sigma^2_{offspring}$		
			t-test		Boot	F-test		Boot
Parent	Offspring	Data	t_{138}	p_1	p_2	$F_{69, 69}$	p_3	p_4
Paternal	F_2	Raw	1.364	0.175	0.175	1.406	0.159	0.103
Grandfathers	Fathers	L.M.C.	1.671	0.097	0.097	1.513	0.088	0.098
Paternal	F_2	Raw	1.646	0.102	0.101	1.577	0.061	0.039
Grandmothers	Fathers	L.M.C.	1.826	0.070	0.070	1.380	0.184	0.198
Maternal	F_2	Raw	**3.970**	<0.001	<0.001	0.957	0.857	0.809
Grandfathers	Mothers	L.M.C.	**3.888**	<0.001	<0.001	0.769	0.278	0.194
Maternal	F_2	Raw	**-4.158**	<0.001	<0.001	1.282	0.305	0.265
Grandmothers	Mothers	L.M.C.	**-2.252**	0.026	0.029	**1.961**	0.006	0.016
F_2	F_3	Raw	**-2.330**	0.021	0.024	0.754	0.244	0.171
Fathers	Offspring	L.M.C.	-1.621	0.107	0.109	**0.490**	0.004	0.010
F_2	F_3	Raw	**-3.850**	<0.001	<0.001	0.728	0.191	0.109
Mothers	Offspring	L.M.C.	**-2.828**	0.006	0.005	**0.487**	0.003	0.008

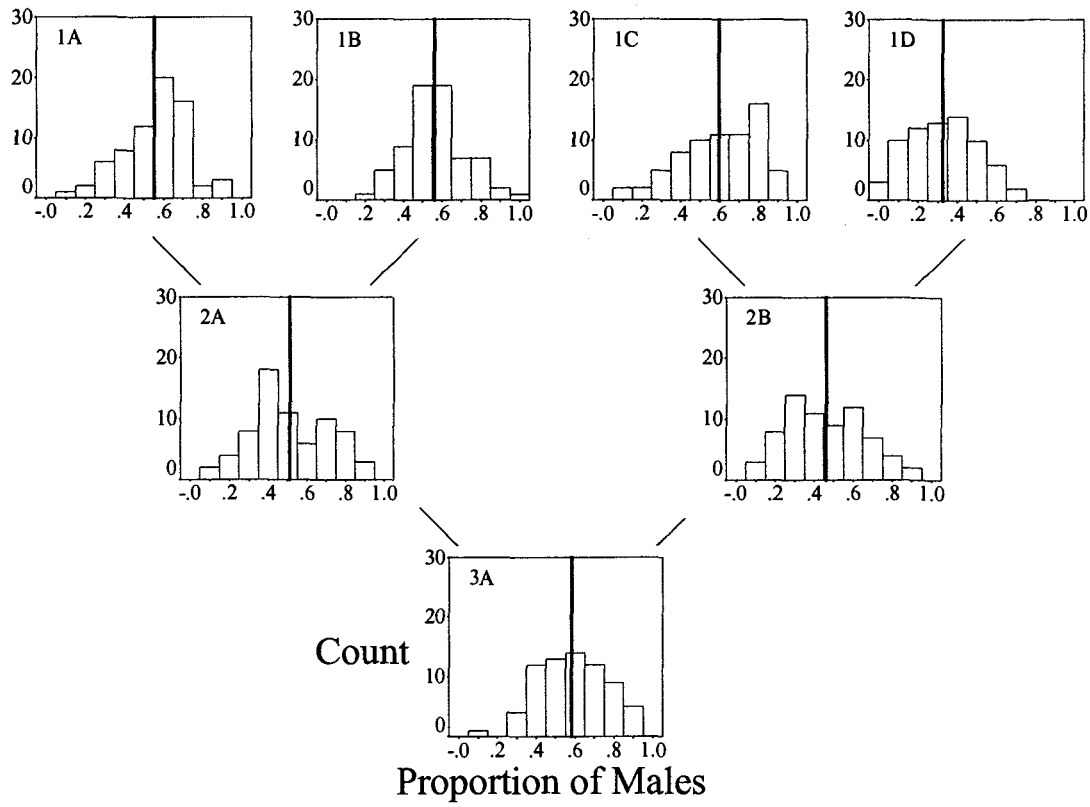

Figure 2. Distribution of the raw proportion of males for three generations (F_1, F_2 and F_3) and seven relatives (1A = paternal grandfathers, 1B = paternal grandmothers, 1C = maternal grandfathers, 1D = maternal grandmothers, 2A = F_2 fathers, 2B = F_2 mothers and 3A = F_3 offspring). We only included those families that actually produced F_3 offspring (N = 70 for each relative). For each relative, the mean proportion of males (uncorrected for larval mortality) is depicted by the bold black line.

primary sex ratio to fluctuate at random around the mean of 0.500. With only two observations it is impossible for us to determine whether the fluctuations are random or directional.

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

Variance in the proportion of males among generations: For the uncorrected data, multiple F-tests found that the change in sex-ratio variance between parents and offspring was not statistically significant for any of the six parent-offspring pairs (Table 3). For the larval mortality-corrected data, the sex-ratio variance in the F_2 mothers is almost twice as great as that of the maternal grandmothers. Conversely, the variance in the F_3 offspring is half as great as that of their F_2 fathers and mothers (Table 3). In both cases this change in variance between generations appears to be the result of differences in survivorship (Table 1; the maternal grandmothers and the F_3 offspring both had low survivorship, compared to their F_2 offspring and F_2 parents, respectively). Hence, there do not appear to be any meaningful changes in sex-ratio variance between generations suggesting, again, that it was conserved in this experiment.

Paternal inheritance of the primary sex ratio: For the paternal F_1 cross (larval mortality-corrected data), the partial regression coefficient for the paternal grandfathers ($b = 0.41 \pm 0.143$) is highly statistically significant ($t = 2.842$, $p = 0.006$) and is much larger than the partial regression coefficient for the paternal grandmothers ($b = -0.01 \pm 0.137$) which is not statistically significant ($t = -0.063$, $p = 0.950$). For the maternal F_1 cross (larval mortality-corrected data), the highly significant partial regression coefficient for the maternal grandfathers ($b = 0.34 \pm 0.100$, $t = 3.379$, $p = 0.001$) is almost 8 times larger than the non-significant partial regression coefficient for the maternal grandmothers ($b = 0.04 \pm 0.159$, $t = 0.272$, $p = 0.787$). For the F_2 cross (larval mortality-corrected data), the partial regression coefficient for the fathers ($b = 0.08 \pm 0.088$) is not statistically significant ($t = 0.922$, $p = 0.360$) but still larger than the non-significant partial regression coefficient for the mothers ($b = -0.02 \pm 0.088$, $t = -0.207$, $p = 0.837$). When all three crosses are combined (larval mortality-corrected data), the highly significant partial regression coefficient for the father ($b = 0.22 \pm 0.062$, $t = 3.463$, $p = 0.001$) was almost 8 times larger than that of the mother ($b = 0.03 \pm 0.068$, $t = 0.410$, $p = 0.682$). Hence fathers appear to have a stronger effect than mothers on the offspring sex ratio.

The estimates of the heritability of sex tendency were calculated from parent-offspring regressions (i.e. not from the multiple regression involving both parents) and reflect the pattern of transmission of the sex ratio trait (Table 4; Figure 3). The heritability estimates are high for the paternal grandfather-F_2 father regression and the maternal grandfather-F_2 mother regression. For the F_2 father-F_3 offspring

Table 4. The relationship in the proportion of males between parents and offspring. We only included those families that actually produced F_3 offspring (N = 70 for each parent-offspring pair). For each of the six parent-offspring regressions, we show the correlation coefficient (r), the F-statistic ($F_{1, 68}$), the statistical significance (p) and the heritability of sex tendency and its standard error ($h^2 \pm$ s. e.). For each type of relative, the top and bottom row show the raw and larval mortality-corrected (L.M.C.) data, respectively (as indicated in the 'Data' column). Significant parent-offspring correlations and heritabilities are outlined in boldface type.

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

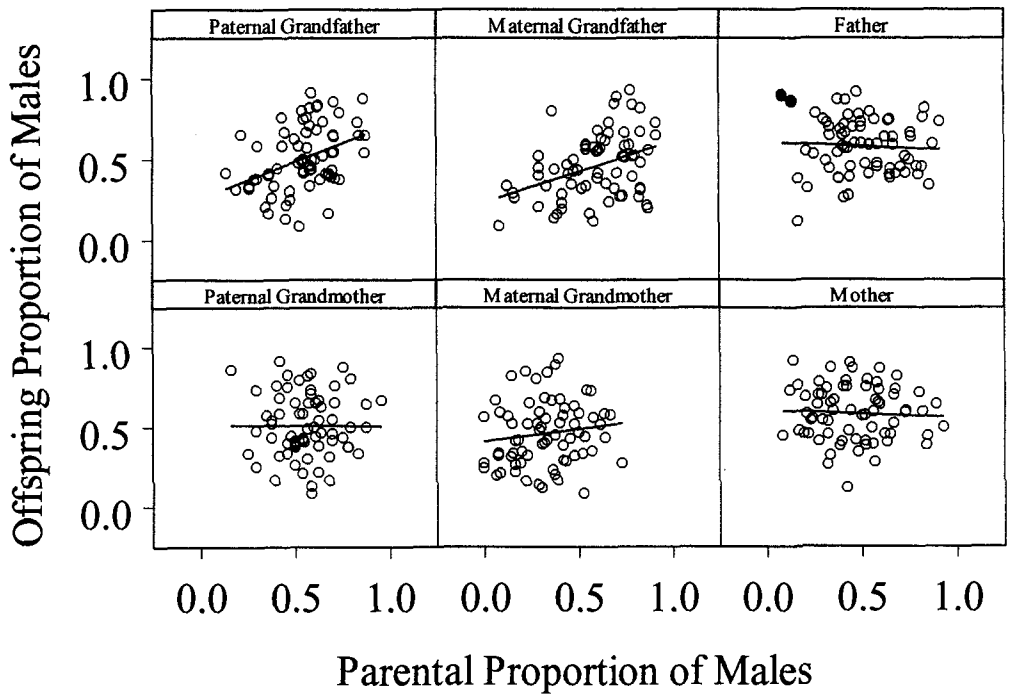

Figure 3. The relationship in the raw proportion of males for the six parent-offspring pairs (paternal grandfathers-F_2 fathers, maternal grandfathers-F_2 mothers, F_2 fathers-F_3 offspring, paternal grandmothers-F_2 fathers, maternal grandmothers-F_2 mothers, F_2 mothers-F_3 offspring). We only included those families that actually produced F_3 offspring (N = 70 for each parent-offspring pair). Shown is the line of best fit from the linear regression of the offspring proportion of males vs. the parental proportion of males.

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

DISCUSSION

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

and is similar among generations (Table 3), and (3) this extra-binomial variation in the primary sex-ratio appears to be paternally transmitted (at least from the F_1 to the F_2).

In the present study, the primary sex ratio in two successive field samples dropped from 0.60 ± 0.025 (maternal grandfathers) to 0.33 ± 0.021 (maternal grandmothers) in less than two weeks and changed to 0.46 ± 0.024 in the next generation (F_2 mothers). In the summer of 2002, the mean primary sex ratio in a sample of 47 field-captured females and 167 of their lab-reared daughters changed from 0.59 ± 0.027 to 0.46 ± 0.015 (unpublished data). We have previously suggested that the sex phenotype in adult copepods is influenced by environmental factors operating during naupliar development (Voordouw and Anholt 2002a). However, the fact that all of these copepods were reared under similar conditions (i.e. 20° C, 12 hours light:12 hours dark cycle, abundant *Isochrysis galbaena* food source) argues against this interpretation.

The primary sex ratio is often male-biased in *T. californicus*. In Voordouw and Anholt (2002a), all eight independent estimates of the primary sex ratio (uncorrected for larval mortality) were ≥ 0.50 (range = 0.50 – 0.68). In a recent experiment where we manipulated larval density, the mean primary sex ratio in the low (0.71 ± 0.027) and high (0.64 ± 0.030) density treatment was highly male-biased (unpublished data). In the present study, male bias was less obvious but still more common than female bias (Table 2). Igarashi (1963b) reported highly male-biased sex ratios (range = 0.50 – 0.91) among families in the closely related *T. japonicus*. However, his practice of excluding any family with less than perfect survivorship may have left him with an unrepresentative sample, especially considering that female bias and mortality appear to be correlated in this species (Igarashi 1963a). To date, the male bias in *Tigriopus* and other closely related

copepods (Battaglia 1958; Battaglia and Malesani 1959) has not been satisfactorily explained.

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

In this study, the sex ratio trait was paternally transmitted for both the paternal and the maternal F_1 cross but not for the F_2 cross (Figure 3). Differences in the mating protocol between the F_1 and F_2 crosses included precopulatory mate-guarding, age of fertilization, and parity of the egg sac. In an earlier study, we found no effect of precopulatory mate-guarding on the primary sex ratio (unpublished data) but it is possible that the lack of mate-guarding in the F_2 cross somehow inhibited paternal transmission of the sex ratio factor (although we have no idea how this would occur). Volkmann-Rocco (1972) found that delayed fertilization caused females to increase the proportion of males

in three species of *Tisbe* (a closely related genus of harpacticoid copepods). If this behavior exists in *T. californicus* females, variance in the age of fertilization could compromise the father-offspring covariance in the primary sex ratio. If there are parity effects on the primary sex ratio (i.e. due to sperm or maternal aging), differences in egg sac parity between the three generations would likewise lower the parent-offspring covariance. For all the grandmothers in the F_1 cross, we used their first egg sac to produce the F_2 generation. In the F_2 cross by contrast, some of the mothers had already produced a sterile egg sac before they were mated and so we used their second egg sac to produce the F_3 generation. Although parental age and clutch parity were more variable in the F_3 than the F_2 generation these variables were, presumably, the most variable in the F_1 generation where they comprised a haphazard sample from the field population. More likely, paternal transmission in the F_2 cross was compromised by poor survivorship in the F_3 generation (Table 1).

Paternal transmission of the sex-ratio is well-known from two species of parasitoid wasp (Werren and Stouthamer 2003); it was first discovered in *Nasonia vitripennis* (Werren et al. 1981) and was subsequently characterized in *Trichogramma kaykai* (Stouthamer et al. 2001). In these two species of parasitoid wasp, a supernumerary chromosome (B chromosome) in the male's sperm destroys the paternal genome (except themselves) shortly after fertilization. In the haplo-diploid Hymenoptera, males develop from unfertilized eggs and females develop from fertilized eggs. The destruction of the paternal genome by the B-chromosome results in a haploid embryo that then develops into a male (Werren and Stouthamer 2003). Haploid males produce sperm via mitosis whereas diploid females produce eggs via meiosis. B-chromosomes are lost during

meiosis but not during mitosis (Werren and Stouthamer 2003). Hence from the perspective of the B-chromosome, destruction of the paternal genome is adaptive because it creates a mitotic lineage through which it can be transmitted to the next generation.

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

Associations between B chromosomes and sex ratios have been found in other organisms (Henderson 1988; Lopez-Leon et al. 1996; Vicente et al. 1996). In the ladybird beetle, *Exochomus quadripustulatus*, B chromosome frequency was positively correlated with the adult proportion of females across 14 populations (Henderson 1988). In the fish, *Astyanax scabripinnis*, B chromosomes were more common in females than males and populations with a high prevalence of these B chromosomes had female-biased sex ratios (Vicente et al. 1996). In the grasshopper, *Eyprepocnemis plorans*, high B chromosome load in field-mated parents was associated with a male-biased sex ratio in the offspring

but this sex ratio distortion was not detected in lab-mated individuals (Lopez-Leon et al. 1996). Again, for all of these examples, it is not clear how B-chromosomes and the observed sex ratio bias are related.

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

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

CHAPTER 7: FUTURE AVENUES OF INVESTIGATION

The first contribution of this thesis to sex ratio theory is the rediscovery of extra-binomial variation in the primary sex ratio of *T. californicus*. All of the chapters in my thesis repeatedly show a strong among family variance component in the primary sex ratio that is not reconcilable with Mendelian segregation of sex chromosomes. In addition, this sex ratio trait appears to be transmitted across generations (Chapter 2 and 6), although the pattern of transmission is different between experiments (maternal vs. paternal) and the mechanism of sex determination remains unknown.

Copepods can only be sexed once they are sexually mature which can take up to several weeks at 20° C. Hence differences in the mortality rate between the sexes can exert substantial bias on the estimates of the primary sex ratio in my experiments. The use of the larval mortality correction is ubiquitous throughout this thesis and is crucial for preventing sex-specific lethal alleles from biasing our variance estimates of the primary sex ratio. The presence of these sex-specific alleles is illustrated by the correlation between survivorship and the proportion of males (i.e. in the maternal grandmothers of Chapter 6) suggesting that males have higher mortality rates than females during development. Poor survivorship and sex-biased mortality may have contributed to the correlation in sex ratio between mothers and daughters in Chapter 2. Hence one way to reconcile the contradictory results between Chapters 2 and 6 is to conclude that factors affecting the primary sex ratio in *T. californicus* are paternally transmitted (Chapter 6) but that genetic variation in sex-biased mortality rates can be expressed in poor environments and that this genetic variation is maternally transmitted. However, I

emphasize that the sample size for the mother-daughter sex ratio correlation in Chapter 2 (N = 17) was over a dozen times smaller than the sample size in Chapter 6 (N = 3*70 = 210). In addition, the mother-daughter correlation in Chapter 2 was not corrected for larval mortality whereas all of the data in Chapter 6 was corrected for larval mortality. Therefore, with respect to the transmission of the trait between generations, I place much more confidence on the results from Chapter 6 than from Chapter 2.

A further consequence of concluding that the sex ratio is paternally transmitted is that it nullifies the predictive ability of the estimates of the heritability of sex tendency in Chapter 2. Quantitative genetics theory is based on the assumption that the trait in question (i.e. the sex ratio) is affected by many loci and that these loci are in Hardy-Weinberg equilibrium (Falconer 1989). The observation that the sex ratio trait in *T. californicus* is paternally transmitted clearly violates Hardy-Weinberg equilibrium. Furthermore, from the perspective of a paternally transmitted gene, the optimal sex ratio is 100% male and not the Fisherian equilibrium (Bull and Charnov 1988). If natural populations of *T. californicus* are in a coevolutionary arms race between paternally transmitted genetic elements that increase the proportion of males in the population and autosomal genes that are under Fisherian selection, the trajectory of the sex ratio in such populations might show a lot of fluctuations depending on the state of the system and whether it has reached equilibrium or not (Werren 1987b).

A second contribution of Chapter 2 was the realization that it was crucial to distinguish between parental vs. zygotic modes of control. While this dichotomy was first pointed out by Bulmer and Bull in 1982 there appears to be a lack of clarity on this issue in the literature (Conover, 1990; Basolo, 1994; Premoli et al., 1996). I hope that my

attempts to distinguish between these two modes of control will inform future investigation and discussion of sex ratio theory.

While there appears to be some effect of temperature (Chapter 3) and density (Chapter 4) on the primary sex ratio, these are weak and I do not believe that they represent an adaptive response. For one, the two environmental factors appear to be in opposition to each other. High temperatures generally induce rapid development and growth with a concomitantly small body size at sexual maturity. High temperatures produce a male biased primary sex ratio suggesting that males lose less from growing fast and maturing at smaller body sizes. In contrast, individuals developing at lower densities were larger and were more likely to develop into males suggesting that density-induced variation in body size had greater fitness consequences for males than females. Hence, the temperature reaction norm appears to favor smaller males, whereas the density reaction norm appears to favor larger ones. In addition, the differences in the strength of fecundity selection on female body size between the lab and the field environment and the differences in the strength of sexual selection on male body size between the paired mating trials and the scramble competition underscored the context-specific nature of body size and fitness and made it difficult to predict which sex benefits the most from large body size.

My thesis also suggests that cytoplasmic sex ratio distorters such as *Wolbachia* and protozoan microsporidia are unlikely to play a role in structuring the primary sex ratio in populations of *T. californicus*. As mentioned before, the observation that these microorganisms are relatively common in other crustaceans such as isopods and amphipods (Rigaud 1997) was a motivating factor for investigating this alternative

conclusion. Although my results do not irrevocably refute the possibility of cytoplasmic sex ratio distortion in *T. californicus*, the observation that in high survivorship environments, the sex ratio trait is paternally and not maternally transmitted (Chapter 6) in conjunction with Chapter 5 and the antibiotics experiments of Dr. Suzzanne Edmands has convinced me to the point where I would no longer dedicate any more time and effort to investigating the possibility of cytoplasmic sex ratio distortion in *T. californicus*.

In summary, the most important contribution of this thesis to sex ratio theory is to show that extra-binomial variation in the primary sex ratio is found in a number of populations of *T. californicus* and is paternally transmitted. If I were to continue sex ratio research on *Tigriopus* I would direct my energies to the following areas: more quantitative genetics work, molecular and chromosomal basis of sex determination in *Tigriopus* and the ecological context that maintains this system.

Paternal Transmission: The discovery that the sex ratio trait is paternally transmitted in the Arbutus Cove population (Chapter 6) leads to the question of whether paternal transmission also occurs in other *Tigriopus* populations exhibiting the sex ratio trait. The simplest approach is to conduct a half-sib design where a field-sampled sire is mated to two (or more) virgin, field-sampled dams. Temporal separation between dams of one or more weeks might be informative with respect to paternal age effects on the sex ratio. If there are paternal age effects, I would expect a negative association between the half-sib covariance and the temporal separation between dams. Likewise, if a sire was mated to three (or more) dams, I would expect the half-sib covariance to be greater between adjacent dams (i.e. dams 1 & 2, dams 2 & 3) then betweens separated dams (dams 1 & 3). The only problem with the half-sib design is that it does not rule out

maternal transmission. A more thorough approach would be to repeat the experimental design in Chapter 6 for several populations, but this would be a lot of work.

Maternal Effects: In several species of harpacticoid copepod from the genus *Tisbe*, Volkmann-Rocco (1972) found that the clutch sex ratio was male-biased for females where fertilization had been artificially delayed. Charnov (1982) suggested that increasing the proportion of sons in response to delayed fertilization is adaptive because the latter suggests a paucity of adult males. In *Tigriopus* the operational sex ratio is generally highly male-biased and so I have trouble envisioning how natural selection would shape this adaptive reaction norm but this phenomenon might be worth investigating. In addition, it is also necessary to determine if maternal age has any effect on sex ratio. As females store sperm from a single mating, parity effects might reflect aging of the sperm, aging of the maternal environment, or both and these two would be difficult to separate. These maternal age/parity experiments would be complementary to the paternal age experiments described above.

Selection and the Trajectory of the Primary Sex Ratio: Someone should repeat Ar-Rushdi's (1958) selection experiment. After creating populations with male-biased and female-biased primary sex ratios one could relax selection and observe whether the primary sex ratio returns to 50:50 (i.e. perform a Fisherian sex ratio selection experiment). The steps are as follows:

1. Select for high, intermediate, and low sex ratio in three independent lineages for several generations. Estimate the realized heritability of sex tendency.

2. Establish male-biased and female-biased lineages and subsample each replicate over time with respect to the adult and/or the primary sex ratio. One could either have discrete or continuous population dynamics.
3. The trajectory of the adult and/or primary sex ratio measures both the strength of Fisherian selection and the genetic response allowing one to calculate realized heritabilities.
4. The predictions are that the primary sex ratio in both the male and female-biased lineage will return to 50/50 and that the 50/50 lineage will not exhibit any change in the primary sex ratio.

Proximate Mechanisms: Quantitative genetics can only take us so far. Eventually, someone will have to determine the molecular mechanism of sex determination in *Tigriopus*. Karyotyping might prove informative if B-chromosomes are involved (Chapter 6). Dr. Suzanne Edmands is currently creating a map of molecular markers and has yet to discover any sex-linkage (personal communication).

Field Work: A crucial question is how the variation in the primary sex ratio is related to the adult sex ratio in the field. One very important experiment in this regard would be to repeatedly sample a collection of splash pools and measure both the adult sex ratio and the primary sex ratio. The adult sex ratio would simply consist of the number of adult males and females in the sample (or subsample). One problem with estimating adult sex ratios in the field is that large sexually immature copepodites are often classified as females. One solution to this problem is to rear all field-captured individuals to sexual maturity. The primary sex ratio would have to be determined in the lab under standard conditions by rearing offspring from a number of gravid females. To avoid age/parity

composition effects on the primary sex ratio, I suggest only sampling couples to ensure that you are measuring the sex ratio of the first egg sac for each female.

One problem with using natural field populations is that the population densities fluctuate drastically between winter and summer. In the winter time, estimates of the adult and primary sex ratio might only be based on a few individuals compared to the summer when hundreds of individuals are available for sampling. One possible solution is to create artificial *Tigriopus* populations in the lab.

Population Size and Sex Ratio Variance: In an infinite population, there is no selection on the sex ratio variance. In small, subdivided populations, random, demographic fluctuations in the sex ratio have strong consequences for individual reproductive success. I would expect a positive correlation between sex ratio variance and population size. However, such a pattern might easily be obscured in the field where migration connects large and small pools. It would be interesting to set up several replicates of a large (i.e. 200 individuals) and a small (i.e. 20 individuals) population size and monitor the sex ratio variance over several generations. I am not sure whether it is possible to separate inbreeding effects from population size and demographic uncertainty. Perhaps one way is to have some migration between the small populations. In this way, there would be strong selection on individuals to produce a balanced sex ratio in each small population but periodic mixing between these small populations would avoid inbreeding effects.

Effect of Discrete vs. Continuous Population Dynamics on Sex Ratio Variance: Bull and Charnov (1989) predicted that longevity and generation overlap would reduce selection for a canalized sex-determining mechanism. *Tigriopus* naturally has continuous,

population growth with overlapping generations. One could simulate discrete population dynamics by straining the water in an aquarium with a sieve, removing egg sacs from all of the gravid females and allowing these to form the next generation. I would expect the sex ratio variance in this discrete generation population to be smaller than that of the continuously reproducing populations.

Outcrossing Effects on Sex Ratio: Investigate the effect of crossing genetically distant (geographically separated) populations on the subsequent F_1 and F_2 sex ratios. Recent work on autosomal suppressors of meiotic drive *Drosophila* found that crossing genetically distant populations breaks up these autosomal, co-adapted suppressor complexes and the subsequent hybrids exhibit sex ratio distortion. Similar phenomena might be found in *Tigriopus*. Alternatively, if major sex-determining genes are located on different chromosomes in different *Tigriopus* populations, epistatic interactions might be important in structuring sex ratio variation among crosses and the observed patterns in the F_1 and F_2 sex ratios might not be readily interpretable.

Sexual Size Dimorphism and Sex Determination: Rice (1984) argued that the evolution of sex chromosomes is an important step for the evolution of sexual size dimorphism (SSD). SSD requires a genetic correlation between the sex-determining genes and the genes that enhance or suppress a sexually dimorphic trait. Under polygenic sex determination, the sex determining genes are scattered through-out the genome and it is therefore difficult to evolve such genetic correlations. Organisms with polygenic sex determining mechanisms are therefore expected to have low levels of SSD. I would therefore predict a negative association/correlation between the degree of SSD and the variance in the primary sex ratio.

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