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Sex without sex chromosomes: genetic architecture of multiple loci independently segregating to determine sex ratios in the copepod *Tigriopus californicus*

H. J. ALEXANDER*†, J. M. L. RICHARDSON*†, S. EDMANDS‡ & B. R. ANHOLT*†

*Bamfield Marine Sciences Centre, Bamfield, BC, Canada
†Department of Biology, University of Victoria, Victoria, BC, Canada
‡Department of Biological Sciences, University of Southern California, Los Angeles, CA, USA

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Abstract

Sex-determining systems are remarkably diverse and may evolve rapidly. Polygenic sex-determination systems are predicted to be transient and evolutionarily unstable, yet examples have been reported across a range of taxa. Here, we provide the first direct evidence of polygenic sex determination in *Tigriopus californicus*, a harpacticoid copepod with no heteromorphic sex chromosomes. Using genetically distinct inbred lines selected for male- and female-biased clutches, we generated a genetic map with 39 SNPs across 12 chromosomes. Quantitative trait locus mapping of sex ratio phenotype (the proportion of male offspring produced by an F2 female) in four F2 families revealed six independently segregating quantitative trait loci on five separate chromosomes, explaining 19% of the variation in sex ratios. The sex ratio phenotype varied among loci across chromosomes in both direction and magnitude, with the strongest phenotypic effects on chromosome 10 moderated to some degree by loci on four other chromosomes. For a given locus, sex ratio phenotype varied in magnitude for individuals derived from different dam lines. These data, together with the environmental factors known to contribute to sex determination, characterize the underlying complexity and potential lability of sex determination, and confirm the polygenic architecture of sex determination in *T. californicus*.

Introduction

Sex determination is a fundamental process in the life history of sexual organisms, and its evolution represents an important question in biology (Bull, 1983; Peichel *et al.*, 2004; Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014). Sex-determining mechanisms are diverse across taxa, revealing an evolutionary continuum from sex-chromosome systems to polygenic mechanisms, to environmental sex determination (Bull, 1983; Phillips & Edmands, 2012; Beukeboom & Perrin, 2014). That diverse sex-determining systems are found in closely related species suggests sex-determining mechanisms may evolve rapidly (Devlin & Nagahama, 2002; Peichel *et al.*, 2004; Bachtrog *et al.*, 2014) and, when interacting with intersexual conflict, may lead to rapid lineage diversification (Parnell & Streelman, 2013). In contrast, developmental pathways downstream of primary sex-determining genes are conserved among divergent organisms having very different sex-determining systems (Raymond *et al.*, 1998; Peichel *et al.*, 2004; Bachtrog *et al.*, 2014; Beukeboom & Perrin, 2014). The genetic architecture (number, location and effects of genes) contributing to variation in sex-determining mechanisms remains poorly understood, particularly in the few known examples of polygenic sex determination (Ser *et al.*, 2010; Anderson *et al.*, 2012; Parnell & Streelman, 2013). Elucidation of the genetic architecture of sex-determining systems is necessary to understand the evolution of sex determination mechanisms and their influence on processes of speciation (Kitano *et al.*, 2009; Qvarnstrom & Bailey, 2009), especially in the absence of sex chromosomes.

Correspondence: Heather J. Alexander, Bamfield Marine Sciences Centre, Bamfield, BC, Canada V0R 1B0.
Tel.: 250 728 3301; fax: 250 728 3452; e-mail: halexander@bamfieldmsc.com

Sex-determining mechanisms have direct consequences on primary sex ratio variation (Bull, 1983; Uller *et al.*, 2007). Most sexually reproducing organisms have approximately equal sex proportions. Fisher's principle provides an explanation for this through natural selection – when the population-wide sex ratio is 1 : 1, the relative value of male offspring to female offspring is equal; however, when the sex ratio differs from 1 : 1, investing in offspring of the less abundant sex increases fitness, pushing the sex ratio towards equality (Düsing, 1884; Fisher, 1930). Although this principle acts independently of sex-determining mechanisms, an evolutionarily stable state will be achieved under specific assumptions (Fisher, 1930; Hamilton, 1967; Williams, 1979; Charnov, 1982; Bull & Charnov, 1988; Carvalho *et al.*, 1998). If, however, the assumption of unrestricted competition for mates is violated or sex-linked drivers (genes controlling sex in the homogametic sex chromosome or in autosomes acting on the heterogametic sex) are present, Fisher's principle can be overridden, leading to biased sex ratios (Hamilton, 1967). Within a species, variation in sex ratio distribution can arise via polygenic mechanisms of sex determination, even in the presence of evolutionary pressures promoting equal sex ratios.

Multifactorial and polygenic sex determination (PSD) systems have traditionally been considered as only an intermediate step in the evolution of sex-determining systems and theoretically evolutionarily unstable, due mainly to sex-specific natural selection (Bull, 1983; Rice, 1986; Bachtrog *et al.*, 2014). Yet, these systems have independently evolved across a range of taxa, including several species of fish, mammals, insects and plants (Charlesworth & Mank, 2010; Moore & Roberts, 2013; Beukeboom & Perrin, 2014). Multifactorial mechanisms (systems with 3 and 4 factors, with each genotype invariably giving rise to one sex) can arise through the modification of existing sex chromosomes, creating a functional third sex chromosome at the same locus (defined as 'single-locus PSD' in Moore & Roberts, 2013). Here, sex ratios vary predictably depending upon specific genotypic crosses as is reported in some populations of *Xiphophorus maculatus* (Kallman, 1973; Basolo, 1994). Similarly, multifactorial systems with an additional, independently segregating, female sex-determining locus have evolved independently in several small mammals (Fredga *et al.*, 1977; Gileva, 1987; Hoekstra & Hoekstra, 2001; Veyrunes *et al.*, 2010). Alternatively, PSD may arise from modifications of autosomal loci, leading to numerous sex-determining genes spread across multiple autosomal chromosomes; here, the additive effect of multiple, independently segregating loci or alleles determines sex (Bull, 1983; Moore & Roberts, 2013). Such a polygenic mechanism may be understood under the threshold model of quantitative genetics, where sex is a threshold trait, underlain by a continuously distributed 'sex tendency', influenced by both genetic and environmental factors. Those individuals with an underlying tendency exceeding a threshold value develop into one sex, whereas individuals below the threshold develop into the other (Falconer, 1960; Bulmer & Bull, 1982; Roff, 1996). Under such a PSD model, sex of a zygote is determined by the sum of genetic effects contributed by both parents (Bulmer & Bull, 1982). Currently, the majority of published examples suggesting PSD in a species are based on family-level, extra-binomial variation in the distribution of sex ratios beyond that expected from a single-locus sex-chromosome system, and heritability estimates of sex ratio from parent/offspring regression analyses. Here, we investigate the genetic basis of PSD, which has rarely been attempted, especially outside the vertebrates, insects and plants. Elucidating the genetic architecture of PSD through genetic mapping approaches is required to link regions of the genome associated with sex determination (Charlesworth & Mank, 2010).

Tigriopus californicus, a harpacticoid copepod inhabiting high intertidal and supra-littoral splash pools from Alaska to Baja California, appears to have a polygenic sex-determining mechanism. *T. californicus* has no heteromorphic sex chromosomes (Ar-Rushdi, 1963) and meets the three criteria outlined by Bull (1983) as necessary evidence for PSD. First, clutch sex ratios from natural populations consistently exhibit extra-binomial variation that is greater than expected through Mendelian segregation of sex chromosomes (Voordouw, 1998; Voordouw & Anholt, 2002b; Voordouw *et al.*, 2005). Second, variation in clutch sex ratio is maternally (Voordouw & Anholt, 2002b) and paternally transmitted (Voordouw *et al.*, 2005). Third, clutch sex ratio responds to artificial selection for both male- and female-biased families; realized heritability estimates in a multigenerational analysis range from 0.24 to 0.58, with response to selection detectable within the first four generations of selection (Alexander *et al.*, 2014). These outcomes are not predicted under either male or female heterogamety, due to the constraints of meiotic chromosome segregation (Williams, 1979; Toro & Charlesworth, 1982). There is no evidence for sex-biased mortality in clutches or the presence of cytoplasmic sex ratio distorters (Voordouw *et al.*, 2008), but there is evidence for elevated hybrid mortality in males (Foley *et al.*, 2013). Temperature effects are relatively weak, and high within-family genetic correlations for families reared in different temperatures support a predominantly genetic sex determination mechanism in *T. californicus* (Voordouw & Anholt, 2002a). It is currently unknown whether sex determination is under parental or zygotic control (Voordouw & Anholt, 2002b).

Extensive genetic resources have been developed for *T. californicus*, enabling further understanding of the genetic basis of phenotypic variation, including an F2 linkage map (Foley *et al.*, 2011). In a previous study, QTL mapping on reciprocal F2 hybrids between two

genetically differentiated California populations, San Diego (SD) and Santa Cruz (SC), detected five loci associated with sex in each of the two crosses, with the strongest effects mapping to chromosome 10 (Foley *et al.*, 2013). Foley *et al.* (2013) examined loci fixed between two lines, and possibly between populations to look at post-zygotic isolation, and noted sex-specific differences in non-Mendelian ratios in the F2 generation, but the study was not designed to find genotypes related to sex ratio.

In this study, our objective was to characterize the genetic architecture of PSD. We initiated four F2 mapping crosses (twice that of Foley *et al.*, 2013). We used two genetically divergent populations from strongly selected male- and female-biased clutches. Individuals from these selected lines were predicted to have multiple sex-determining alleles across the genome predominantly fixed for either 'malenesss' or 'femaleness', whereas in nature, these loci are variable within populations. Second, we used the novel approach of mapping sex ratio phenotype (SRP), the proportion of male offspring produced by an F2 female. Previous reports seeking sex-determining regions across the genome have mapped the sex of F2 individuals (Lee *et al.*, 2004; Martinez *et al.*, 2009; Bradley *et al.*, 2011; Anderson *et al.*, 2012; Foley *et al.*, 2013). Here, we implement a complementary approach to these studies, as mapping SRP should more appropriately capture the expected sex phenotype of all offspring segregating in an F2 clutch (Voordouw & Anholt, 2002b). By mapping the SRP of F2 females, we provide the first unequivocal evidence of the polygenic basis of primary sex ratio and demonstrate a unique pattern of complexity for PSD in a crustacean.

Materials and methods

Study organism

The life history and reproductive biology of *T. californicus* are well understood (Egloff, 1966; Vittor, 1971; Burton, 1985). Mature adults are sexually dimorphic, with males easily distinguishable from females by their enlarged geniculate antennules (Webb & Parsons, 1988) (Fig. 1). Reproduction occurs year round. Females mate only once, storing sperm, whereas males may mate multiple times (Burton, 1985). Females produce multiple clutches (up to 12) from stored sperm over their lifetime (~70 days in the laboratory), with the number of offspring per clutch ranging from 40 to over 100 (Haderlie *et al.*, 1980). Sexual maturity is reached ~23 days after hatching at 20 °C (Voordouw & Anholt, 2002b).

QTL mapping was performed on adult female F2 hybrids resulting from reciprocal crosses between genetically divergent populations selected for both male- and female-biased sex ratios, followed by F1×F1 crosses (Fig. 1). We genotyped only female F2s because *T. californicus* females fertilize all their eggs from a single mating to one male (Burton, 1985), and thus, SRP is a family trait in which sire and dam effects are completely confounded. Females carry their egg sacs until eggs hatch, making the decision to genotype dams both more practical and useful given the likelihood of

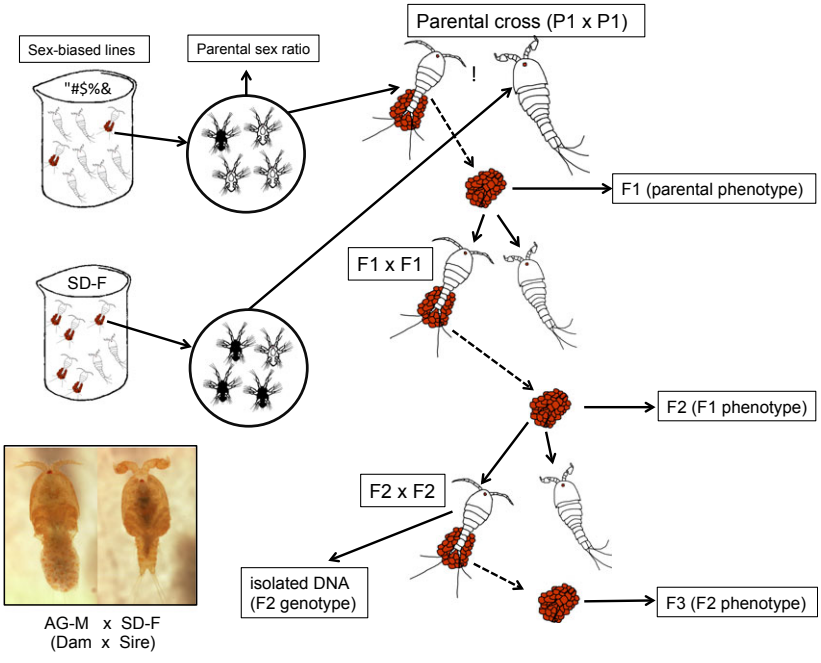

Fig. 1 Mapping cross protocol for production of F2 females and their offspring for QTL mapping showing, as an example, AG-M × SD-F (dam × sire) cross. The sex ratio phenotype (SRP) of the F2 generation, measured in females only, is the proportion of males, summing all clutches, of the F3 generation. AG-M – Aguilar Point male-biased line, SD-F – San Diego female-biased line. Copepods with an egg sac represent females, and those without an egg sac represent males. Black nauplii represent females, and white nauplii represent males.

maternal effects. Genotyping sires as well as dams while maintaining sufficient sample size was not logistically feasible in this study. Genotyping only dams may have underestimated the number of male-determining alleles. Details of laboratory culture conditions, the selection protocol for male-biased and female-biased lines and the protocol for assessment of SRP are provided in Alexander *et al.* (2014) and are summarized briefly below.

Selection lines

Adult *T. californicus* were collected from high intertidal pools at two geographically distant and genetically divergent populations: Aguilar Point, Barkley Sound, BC (AG: 48°51′28″N, 125°09′38″W) and Ocean Beach, San Diego, CA (SD: 32°44′49″N, 117°15′16″W) (Edmands, 2001). We used the Aguilar Point (AG) population because (a) we had previously established male- and female-biased lines from this population; (b) AG is genetically similar to the Santa Cruz (SC) population used in previous studies in crosses with SD (Foley *et al.*, 2011, 2013; Pritchard *et al.*, 2011; Pritchard & Edmands, 2013), having no fixed differences between AG and SC at 154 of 155 scoreable SNP markers; and (c) SD and AG are genetically distinct at 155 nuclear and two mitochondrial SNP markers (Foley *et al.*, 2011).

Samples taken from five pools at each location were combined to maximize genetic variation, although previous work indicates extensive gene flow among pools within outcrops (Burton & Swisher, 1984). Samples were collected in September 2009 (AG) and July 2010 (SD) and transported to the laboratory at the Bamfield Marine Sciences Centre, Bamfield, BC, Canada, in 500 mL Nalgene containers filled with seawater. While in the laboratory, all copepods were housed in seawater passed through a 0.5-μm filter (FSW) and fed concentrated food (0.1 g ground Spirulina and 0.1 g ground TetraminTM (Tetra Holding (US) Inc., Blacksburg, VA, USA) suspended in 20 mL of FSW).

Truncation selection for male-biased (M) and female-biased (F) families occurred prior to the mapping crosses; AG lines were selected for 14 generations and SD lines for six generations (Alexander *et al.*, 2014). In brief, to initiate selection lines for each population, 87–120 egg sacs were removed from gravid females, haphazardly selected (removed with a pipette as observed; true randomization, which would entail numbering each female and using a random number generator was not feasible) from field-collected samples, and reared to sexual maturity. From these families, three lines were created: a control line (started with 30 randomly selected families), a female-biased line (F) and a male-biased (M) line. Although we aimed to start each selection line with 30 families, we were limited by the number of families available with strongly biased sex ratios, in particular for the SD female-selection line. The control line was included to provide a baseline should clutch sex ratio be affected by the laboratory environment independent of our imposed selection. After 4 weeks (one generation), gravid females with mature egg sacs were sampled from each line and their families reared to maturity to start the next generation. For the control line, 30 families were haphazardly selected and transferred to a new container for the next generation. Truncation selection was implemented for female- and male-biased lines for this and subsequent generations, with the most female-biased clutches from the F-line and the most male-biased clutches from the M-line used to start the next generation. Truncation values (the range of clutch sex ratios included in each line), and hence selection intensities, varied depending on the number and sex ratio of clutches available for the next generation. This procedure was repeated every 4 weeks (see Alexander *et al.*, 2014 for further details).

Mapping crosses

Mapping crosses were initiated from females collected from generation 14 of the AG sex-biased lines and generation 6 of the SD sex-biased lines (Alexander *et al.*, 2014). Females with mature egg sacs were haphazardly sampled from each of the four selection lines (AG-F, $n = 60$; AG-M, $n = 79$; SD-F, $n = 60$; SD-M, $n = 53$), and hatchlings from egg sacs reared to produce virgin females and males from known parental SRP to initiate mapping crosses.

The initial parental crosses were established by placing a mature virgin female and a mature virgin male together in one well of a six-well tissue culture plate. From one to five within-family replicate crosses were established, with each (dam × sire) pair placed in a separate six-well tissue culture plate; wells contained filtered seawater and concentrated food, and were kept at 20 °C with a 12 : 12 (L : D) photoperiod. Once the female produced a red (mature) egg sac, the male was discarded and the mature egg sac placed into the next well of the plate for rearing. Subsequent mature egg sacs were similarly placed in new wells, so that each clutch was reared separately.

The P1 × P1 cross was conducted between virgin females from each of the parental male- and female-biased lines, and males of the opposite population. From the male-biased lines, parents were chosen from the most male-biased clutches, and from the female-biased lines, parents were chosen from the most female-biased clutches. Reciprocal crosses between populations (AG and SD) and between male-biased (M) and female-biased (F) selected lines were performed to obtain the F1 generation (dam × sire; AG-M × SD-F, SD-F × AG-M; AG-F × SD-M, SD-M × AG-F). The four crosses are denoted by parental dam line (e.g. AG-M × SD-F is denoted as AG-M; see Fig. 1, Tables S1 and S2 for details). These crosses were established to examine

selection line and population, ensuring the identification of the alleles in the F2 generation in terms of the originating population/selection line from which it came.

In subsequent crosses, full-sib mating was allowed to produce the next generation. When females from a clutch had mated and produced a red egg sac, females were removed to a new six-well plate to start the next generation. As above, each mature egg sac was placed in its own well for rearing. Between one and five clutches (mean (± SE) number of clutches, 3.8 ± 0.03; Table S2) from each female for each generation were reared in order to obtain a robust estimate of the female's SRP, and to maximize within-family replication for the next generation. Parental sex ratio phenotype is based on the offspring sex ratio of pure parentals, the parental cross (PX) phenotype is the clutch sex ratio of the F1 generation, the F1 phenotype is the clutch sex ratio of the F2 generation, and the F2 phenotype is the clutch sex ratio of the F3 generation (Fig. 1). Clutch sex ratios were recorded at maturity, and in all clutches, any copepodids remaining (< 5, maximum proportion of brood = 0.15) were assigned to the less common sex, following Voordouw & Anholt (2002a,b).

All F2 females were retained for genotyping. F2 females were briefly rinsed in dH_2O and blotted dry on filter paper, then placed in a 1.5-mL Eppendorf tube and stored in a −80 °C freezer until DNA isolation. Sexually mature copepods in the F3 generation were briefly anesthetized in 10% $MgCl_2$ (in FSW) and identified as males, females or juveniles. A total of 1538 F2 females from the four mapping crosses were reared to maturity and phenotyped for SRP (dam × sire; AG-M × SD-F, n = 354, SD-F × AG-M, n = 369; AG-F × SD-M, n = 408, SD-M × AG-F, n = 407).

Genotyping

To extract DNA from frozen F2 females, 50 μL lysis buffer (10 mM Tris pH 8.3, 50 mM KCl, 0.5% Tween 20) with 200 μL mL^{-1} proteinase K (final concentration) was added to each tube. Approximately 10–15 1.0-mm-diameter zirconia/silica beads (Biospec Products Cat. No. 11079110z) were then added to each tube and agitated in a Mini-Beadbeater-16 (Biospec Products Cat. No. 607) for 20 s. The tubes were briefly centrifuged at 15 871 g and incubated at 65 °C for 1 h followed by 100 °C for 15 min. Aliquots of 20 μL were taken, dried down at 60 °C for 4 h and sent to the Roswell Park Cancer Institute for genotyping.

From the 190 mapped markers of Foley *et al.* (2011), we selected 46 markers for genotyping. Seven loci were dropped from the analysis; four markers did not pass validation using parental and F1 genotypes; two markers showed extreme segregation distortion; and one marker had a large number of missing values; thus, we ultimately retained 39 markers for QTL mapping. An effort was made to select markers at the ends of chromosomes, and at approximately even distances between (Fig. S1). Two mtDNA markers were used as controls to test for contamination in the samples. Primers and extension sequences were developed by Jeffrey Conroy using the iPLEX GOLD software (Sequenom, San Diego CA), and products were scored using the iPLEX GOLD Assay on the MASSARRAY Compact (Sequenom) mass spectrometer. The accuracy of marker scoring was assessed by screening 25 loci on males and females from both populations and screening the remaining 21 loci on males and females from reciprocal F1 hybrids in addition to the two parental populations.

Statistical analysis

For QTL analysis, only F2 females with > 9 offspring were considered; a total of 1538 F2 females were phenotypically scored and genotyped at 39 loci (Tables S3 and S4). The sex ratio phenotype (SRP) of each F2 female was calculated as the logit-transformed proportion of male offspring (all clutches combined). Marker segregation distortion was assessed using the GLM method of Xu (2012), which allows a conditional test of multiple loci. Quantitative trait loci data were analysed using the packages QTL (Broman *et al.*, 2003) and QTLBIM (Yandell *et al.*, 2007) in R v 3.06 (R Development Core Team, 2014). The recommended methods found in Broman (2003) and Yandell *et al.* (2013) were followed. We chose to use Bayesian interval mapping of QTL (qtlbim) because it allows us to simultaneously compare many models of genetic architecture (Yandell *et al.*, 2007). First, genotypic conditional probabilities were estimated using the Kosambi map function at 1 cM intervals, and one-dimensional standard interval mapping was performed to provide a preliminary assessment of possible QTL. A subsequent two-dimensional scan function using Haley–Knott regression was used to estimate main and epistatic effects. As these preliminary results suggested the presence of at least five QTL, qtlbim was then used to estimate the number and position of QTL, with the starting values of five main QTL and mean number of QTL equal to seven. Based on these results, which suggested two QTL on chromosome 10 (Chr. 10), Chr. 10 was split for the analysis and QTL effects re-estimated. We used the qb.bf and qb.best routines of R/qtlbim to ascertain the most likely QTL pattern and the step.fitqtl routine to select the most appropriate model of genetic architecture. The R/qtlbim function step.fitqtl calls the R/qtl routine fitqtl multiple times (Yandell *et al.*, 2013).

Results

Overview

Our results estimate that at least six loci may be significantly associated with sex ratio phenotype (SRP) in

T. californicus F2 females (Fig. 2, Table 1). Log posterior density (LPD) results are from the qb.scanone() function of the qtlbim package in R (Yandell *et al.*, 2007) (threshold = 2.85 based on 1000 permutations). LPD is analogous to typical LOD scores (logarithm (base 10) of odds) but has the advantage of averaging over all unknowns.

A conditional test of all 39 markers for segregation distortion found that 7 of 12 chromosomes had significant deviations from expected 1 : 2 : 1 ratios (Table S3). To assess whether the QTL we observed were driven by segregation distortion (especially distortion due to sex-specific viability), we compared marker LPD results (from the function qb.scanone(), as above) with LRT (likelihood ratio test) of segregation distortion from the conditional test. If sex-specific mortality is generating the patterns of SRP with QTL that we observe (as opposed to loci that directly affect sex determination), we expect to find a correlation between marker QTL LPD and marker segregation distortion. We found no such correlation ($r = 0.112$, $P = 0.50$; Fig. S2). To further convince ourselves that no such correlation exists, we also considered correlations between LRT of segregation distortion from a standard geno.table() output that uses chi-square analyses to test for segregation distortion on each locus independently. None of the analyses we tried to compare segregation distortion and LPD gave a significant correlation.

Bayesian analysis (based on a two-dimensional scan of the genome using qb.scantwo in the 'QTLBIM' package (Yandell *et al.*, 2013)) suggests that the most likely pattern of QTL for SRP (logit(pmale)) has six QTL with one QTL on each of chromosomes 2, 4, 6 and 11 and two QTL on Chr. 10 (Bayes Factor = 11.80 ± 0.35), although a model including an additional QTL on Chr. 12 was nearly as good (Bayes factor = 9.83 ± 0.48). Analysis of the best pattern (using function qb.best() with the option includes = 'all'), which takes into consideration locus position as well as presence confirmed the presence of six QTL (Table 1). A stepwise analysis dropping one QTL at a time for this best-fit model indicated that all QTL significantly improve model fit (using multiple imputation methods, all $P < 0.01$; Table 1).

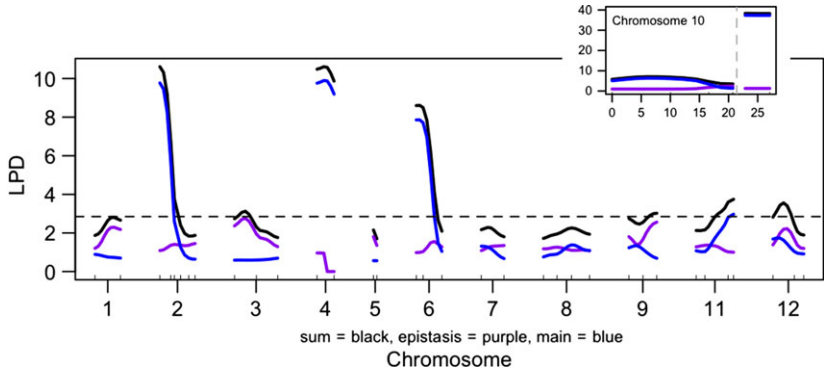

Fig. 2 Log posterior density (LPD) results from qb.scanone() function of the qtlbim package in R. LPD is analogous to typical LOD scores but averages over all unknowns. Dashed horizontal line shows LOD threshold (2.85) calculated from 1000 permutations using scanone() from the qtl package in R, run on genome without chromosome 10 (Chr. 10) split into two QTL. LPD for Chr. 10 shown in inset plot to accommodate its much larger values; dashed vertical grey line in inset plot shows position of split in Chr. 10.

Table 1 ANOVA table results from analysis comparing full model for QTL best fit against model with each predicted QTL dropped. Results suggest all six postulated QTL should be kept in model. Note that chromosome and locus position are for estimated QTL position, not marker position.

	d.f.	Type III SS	LOD	% var explained	*F* value	*P*-value (χ^2)	*P*-value (F)
Full model	12	259.54	69.51	18.81	29.4	0	0
By QTL							
2@3.82	2	31.82	9.34	2.31	21.64	0.000	< 0.0001
4@0.43	2	31.34	9.20	2.27	21.31	0.000	< 0.0001
6@2.19	2	22.82	6.73	1.65	15.51	0.000	< 0.0001
10@6.03	2	11.04	3.27	0.80	7.51	0.001	0.0006
10@27.1	2	88.59	25.39	6.42	60.23	0.000	< 0.0001
11@23.7	2	10.30	3.05	0.75	7.00	0.001	< 0.001

Phenotype, genotype and parental dam-line interactions

Allele effects on SRP varied in size and direction among chromosomes; at Chr. 10 and 11 markers, AG homozygotes tended to produce male-biased clutches, whereas SD homozygotes tended to produce female-biased clutches (Fig. 3, Fig. S3). Additionally, an opposite, though weaker effect was detected for Chr. 2, 4 and 6; at these loci, AG homozygotes tended to produce female-biased clutches and SD homozygotes tended to produce male-biased clutches. There was a moderate amount of variance across all markers in allele effect depending on parental dam line, but these differences were not statistically significant (comparison of linear mixed model with and without dam line included as fixed effect: $\chi^2_3 = 4.41$, $P = 0.22$).

The SRP of offspring arising from crosses between strongly selected male- and female-biased clutches meets quantitative genetic expectations for an additive genetic trait; the parental cross sex ratios (F1 clutches) had intermediate means and higher variances than the parentals (paired *t*-test on SE for each dam line: $t_3 = 6.42$, $P < 0.01$; Fig. 4, Table S1A), further supporting the hypothesis that SRP in *T. californicus* is affected by multiple loci with different alleles contributing some additive genetic value of 'maleness' or 'femaleness'. There was no indication of a maternal effect detected in parental cross SRP (comparison of generalized linear mixed models with and without dam line included: $\chi^2_3 = 5.6$, $P = 0.13$). There was also no effect of parental dam line or mapping cross generation on mean clutch size (comparison of generalized linear mixed models with and without dam line included: $\chi^2_3 = 5.17$, $P = 0.16$).

Discussion

Overview

We provide unequivocal evidence that sex determination is a polygenic trait in *T. californicus*; at least six quantitative trait loci (QTL) with variable effect sizes on five separate chromosomes contribute to variation in the proportion of male offspring produced by a female (sex ratio phenotype, SRP). Using a Bayesian model selection approach (Yandell *et al.*, 2007), we show that the six loci we identified account for an estimated 19% of the variance in F2 SRP (Table 1).

The rapid response to selection for male- and female-biased sex ratios (Alexander *et al.*, 2014) implies a relatively simple basis to sex determination; however, the per cent phenotypic variance explained was relatively low. The estimated per cent variance explained in the model fit (Table 1) is a simple transformation of conditional LOD scores, provided by R/QTL because no alternative method exits; it is also only reliable with unlinked QTL (Broman *et al.*, 2003). As proportion sex ratio is analysed using a logistic regression, and due to the dichotomous nature of the data, it is not possible to

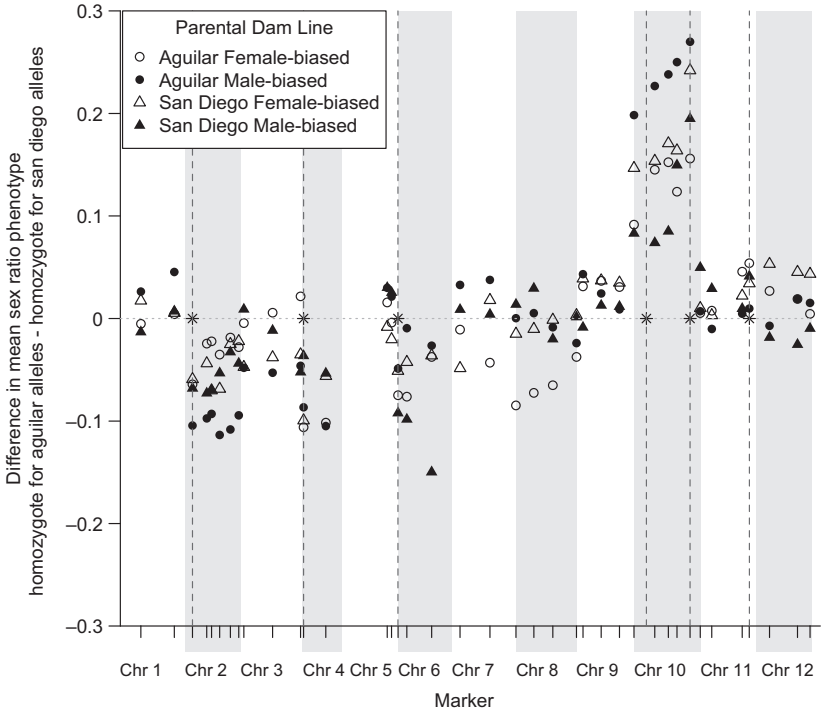

Fig. 3 The difference in mean sex ratio phenotype for each marker, homozygote for Aguilar alleles minus homozygote for San Diego alleles. Positive values indicate AG homozygotes tend to be more male biased than SD homozygotes. Negative values indicate AG homozygotes more female biased than SD homozygotes. Each tick on the *x*-axis represents a marker position. Grey shading delineates chromosomes. Dashed vertical lines indicate estimated QTL positions.

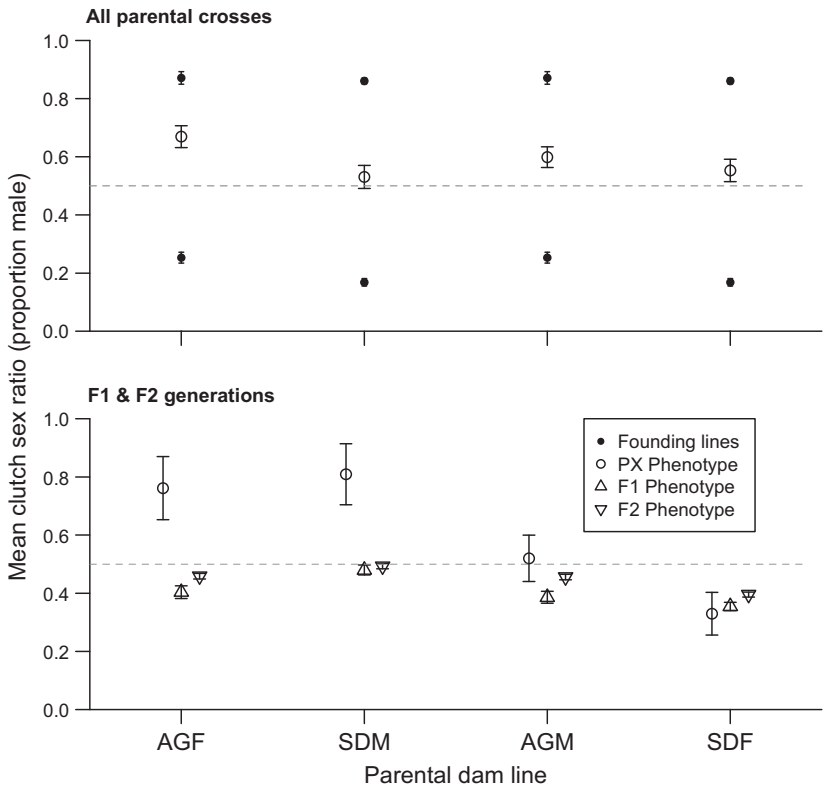

Fig. 4 Mean clutch sex ratio (proportion male ± SE) for each generation of the QTL mapping cross by parental dam line. All parental crosses: All clutches initially reared for mapping crosses from the male- and female-biased lines and genetically divergent populations (AG and SD) and the resultant parental cross (PX) phenotypes. F1 and F2 generations: A subset of those crosses that provided sufficient progeny to continue on to next generation and the F2 generation. PX phenotypes here are a subset of the PX phenotypes presented in the top panel. See Fig. 1 and Table S1 for more details.

explain 100% of the variance: even if the data fit the model perfectly and residuals are zero, the maximum $r^2 = 0.75$ (Mittlbock & Schemper, 1996); thus, we can account for about one-fourth of the phenotypic variance that can possibly be explained. Mapping crosses were initiated using lines that were strongly selected for SRP and thus started with little genetic variation; the San Diego male-biased line (SD-M) also appeared to have suffered from a bottleneck at some point (Alexander *et al.*, 2014), further reducing the proportion of the total variance that could possibly be explained (assuming the remaining variance is unchanged). The phenotypic variance arising from genetic differences may also be lower than expected because (1) little opportunity for recombination in F2 females exists; recombination occurs only in gametes passed on by males (Burton *et al.*, 1981); (2) a relatively small number of markers were used (a deliberate choice because of the limited recombination); (3) evidence suggests environmental factors also influence sex determination (Voordouw & Anholt, 2002a,b); (4) allelic contributions from the F2 males were not assessed, and this potentially reduced our power to detect additional sex-determining loci; and (5) segregation distortion caused by genotype-specific viability effects in the F2 generation may have reduced variation (Pritchard *et al.*, 2011; Foley *et al.*, 2013). In a previous study of F2 mapping crosses between inbred *T. californicus* lines (San Diego × Santa Cruz; SD × SC), 5 QTL explained 23.3% of variance in sex of F2 individuals in one cross (SD dam × SD sire), and a somewhat different set of 5 QTL explained 24.8% of the variance in the reciprocal cross (Foley *et al.*, 2013), a slightly higher per cent variance explained than in our study. In Lake Malawi cichlids, a larger number of markers (353 informative SNPs) identified 5 QTL and 3 epistatic effects, accounting for 51.5% of variance explained (Parnell & Streelman, 2013).

Deviations from expected F2 segregation ratios were found in more than half of the chromosomes. Although distorted ratios can reduce power to detect QTL, generally it should not produce spurious evidence for QTL (Xu, 2008; Broman & Sen, 2009; Zhang *et al.*, 2010). Segregation distortion can result from viability selection, and this has been observed in several previous *T. californicus* studies (Willett & Berkowitz, 2007). These studies found segregation distortion was not present in F2 nauplii, but occurred in F2 adults, suggesting that distorted ratios are caused by viability selection rather than meiotic drive or genotyping error. If viability selection in our crosses is sex specific, this would affect our estimates of sex ratio, and consequently, our purported QTL would be related to viability selection as opposed to sex determination. We addressed this issue

by examining the correlation between the segregation distortion probabilities for each marker and the LPD for the SRP at the same markers. Although it is difficult to completely tease the two possibilities apart, the lack of correlation between the LDP for phenotypic values and segregation distortion probabilities supports our contention that the QTL we have detected are in fact QTL for determination of clutch sex ratio and not solely a reflection of sex-specific mortality (Fig. S2).

Genomic region of large effect but no sex chromosome

Chromosome 10 (Chr. 10) was identified as having the strongest association with SRP (Fig. 2). Further examination of Chr. 10 revealed two independently segregating loci have significant associations with SRP, accounting for nearly 7% of the SRP variation in F2 females (Table 1). Strong associations between sex and markers on Chr. 10 have been identified in previous QTL mapping studies in three interpopulation crosses for *T. californicus* (all of which involve the SD population), leading Pritchard & Edmands (2013) and Foley *et al.* (2013) to posit a large-effect sex ratio distortion locus on Chr. 10 with alternate alleles fixed in the SD and SC lines, balanced by other opposing loci in the same population to maintain an equal sex ratio overall. In those studies, SD Chr. 10 homozygotes were female biased and in our study, SD Chr. 10 homozygotes had female-biased SRP. Likewise, SC homozygotes were male-biased in those studies and in our study, Aguilar (AG) Chr. 10 homozygotes had male-biased SRP (SC is genetically similar to AG used in our study). Here, we show that Chr. 2, 4 and 6 had striking, although not significant, population-specific locus effects in the opposite direction of the population effect on Chr. 10.

It is unlikely that Chr. 10 is acting as a sex chromosome, that is a single segregating genomic region on a chromosome with a major sex-determining function. First, two SRP-associated loci on Chr. 10 segregate independently. If the QTL on Chr. 10 were not segregating independently, they would act as a single locus; knowing the genotype at one locus would determine the genotype at the second locus. However, we see various combinations of heterozygotes and homozygotes at both loci among F2 females. Second, sex associations with Chr. 10 do not occur in all interpopulation hybridizations (Pritchard & Edmands, 2013). We cannot rule out possible epistasis between QTL on chromosomes 2, 4, 6 and 10 (@27.0); 25 models clustered together with equal squared-attenuation scores to the best model and 7 of these included an epistatic term. Although epistatic variation in our results was not statistically significant, this may reflect low power in our study to detect such effects (Fig. S4).

Phenotype, genotype and parental dam-line interactions

Sex ratio phenotype and allele effect varied in size and direction among chromosomes (Fig. 3, Fig. S3). At Chr. 10 markers, AG homozygotes tended to produce more male-biased clutches, whereas an opposite, although less strong effect towards more female-biased clutches in AG homozygotes occurred at Chr. 2, 4 and 6. Our results suggest that the SRP effects of Chr. 10 are modified to some degree by Chr. 2, 4 and 6. SRP effects varied among parental dam lines, particularly between populations, implying variation in SRP is associated with the underlying genetic architecture. Evidence that higher temperatures increase male bias in some families (Voordouw & Anholt, 2002a) and variation in temperature tolerance across the southern latitudinal range (Willett, 2010; Kelly *et al.*, 2012) raises the question of whether variation in SRP is adaptive, and associated with variation in genetic architecture across a latitudinal temperature gradient.

The AG male-biased parental dam line alleles hint at larger effects on SRP, especially on Chr. 10 and 2 (Fig. 3); however, the differences among dam lines were not significant. In this parental dam line, it is possible that numerous sex-determining alleles of small effect, not detectable by our analysis, may have been fixed across the genome. Our study provides evidence for, and a further understanding of, a polygenic sex-determining mechanism operating under the threshold model of quantitative genetics, where the combined effects of alleles at numerous loci determine SRP. That these loci explain only 19% of the variance in SRP, and evidence that temperature affects sex ratio (Voordouw & Anholt, 2002a), implies that the genetic architecture of sex determination is highly complex in *T. californicus*.

Genome-wide linkage analyses seeking multiple sex-determination regions are rare. In the African cichlid fishes, some families of *Metriaclima pyrsonotus* exhibit a multifactorial system of independently segregating XY and ZW alleles on separate chromosomes with strong epistatic interactions between the loci, giving rise to predictable sex ratios (Ser *et al.*, 2010). QTL analysis of F2 hybrids arising from a cross between two Lake Malawi cichlid species, *Cynotilapia afra* and *Pseudotropheus elongatus*, revealed a complex epistatic sex system spanning four chromosomes, consisting of a ZW locus on one chromosome, two separate XY loci on another chromosome, two additional interacting loci on additional chromosomes, and associated with nuptial coloration, the interaction of which is hypothesized to contribute to rapid evolutionary diversification (Parnell & Streelman, 2013). One of the most comprehensively studied systems suggesting PSD is in laboratory strains of zebrafish (*Danio rerio*), where the primary sex determination mechanisms are complex and the details

remain elusive. Zebrafish in nature possess a single sex-linked region, with a WZ/ZZ sex-determining mechanism, whereas the laboratory strains are believed to have evolved variant sex-determining mechanisms during domestication (Wilson *et al.*, 2014). The location of sex-determining loci among laboratory strains vary across the genome depending on families and strains (Bradley *et al.*, 2011; Anderson *et al.*, 2012; Howe *et al.*, 2013; Wilson *et al.*, 2014) and are complicated by the influence of environmental factors such as temperature, hypoxia and density on sex ratios (Uchida *et al.*, 2004; Abozaid *et al.*, 2011). These examples illustrate the potential for diversity, complexity and lability of sex-determining systems in fish lineages. Our data show a similar level of sex determination complexity in a crustacean.

Sex-determination systems are expected to influence rates and patterns of speciation. In polygenic systems, we expect reduced speciation rates in the absence of sex chromosomes and incompatibilities arising from the effects of Haldane's rule and large-X effects (Coyne & Orr, 1989; Turelli & Moyle, 2007); however, speciation in the absence of sex chromosomes remains understudied (Phillips & Edmands, 2012; Beukeboom & Perrin, 2014). In anticipation of the sequenced *T. californicus* genome, the next step is to identify potential genes contributing to sex determination in genomic regions identified as associated with F2 sex and SRP. Most challenging, however, will be to account for the remaining variance component of SRP not attributed to the six sex-associated loci. Evidence of environmental influences on sex ratio variation such as temperature (Voordouw & Anholt, 2002a) and whether sex tendency, associated with genetic architecture, responds to selection across a latitudinal gradient provide challenging future research directions.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 (A and B) Mean clutch sex ratio (proportion male ± SE) for each parental dam line and each generation of the mapping cross.

Table S2 The mean number of clutches, the mean total number of offspring for each F2 female (all clutches combined), and the number of F2 females genotyped with total family size > 9 for each mapping cross.

Table S3 Allele segregation proportions for each the 39 SNP markers used.

Table S4 The 39 nuclear markers used for our QTL analysis with the abbreviations we used for the markers in this paper.

Figure S1 Linkage map used in QTL analysis of *T. californicus*.

Figure S2 Marker QTL LPD values plotted against the segregation distortion LRT, calculated using the hwdm.glm() function of the R package hwd.glm (Xu, 2012).

Figure S3 Mean clutch sex ratio phenotype (proportion male) at each marker for each parental dam line.

Figure S4 Plot of 2logBF (2log(Baye's Factor)) for putative QTL for sex ratio phenotype in *Tigriopus californicus*.

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