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EFFECTS OF SPONTANEOUS MUTATION ACCUMULATION ON SEX RATIO TRAITS IN A PARASITOID WASP

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Sex allocation theory has proved extremely successful at predicting when individuals should adjust the sex of their offspring in response to environmental conditions. However, we know rather little about the underlying genetics of sex ratio or how genetic architecture might constrain adaptive sex-ratio behavior. We examined how mutation influenced genetic variation in the sex ratios produced by the parasitoid wasp *Nasonia vitripennis*. In a mutation accumulation experiment, we determined the mutability of sex ratio, and compared this with the amount of genetic variation observed in natural populations. We found that the mutability (h^2_m) ranges from 0.001 to 0.002, similar to estimates for life-history traits in other organisms. These estimates suggest one mutation every 5–60 generations, which shift the sex ratio by approximately 0.01 (proportion males). In this and other studies, the genetic variation in *N. vitripennis* sex ratio ranged from 0.02 to 0.17 (broad-sense heritability, H^2). If sex ratio is maintained by mutation–selection balance, a higher genetic variance would be expected given our mutational parameters. Instead, the observed genetic variance perhaps suggests additional selection against sex-ratio mutations with deleterious effects on other fitness traits as well as sex ratio (i.e., pleiotropy), as has been argued to be the case more generally.

KEY WORDS: **Mutation effect, mutation rate, *Nasonia vitripennis*, parasitoid wasp, sex allocation.**

Uncovering the genetic basis of adaptation remains a key objective for evolutionary geneticists, both in terms of understanding the sorts of genetic changes that occur during adaptive evolution and how genetic architecture influences the process and extent of adaptation (Orr and Coyne 1992; Orr 2005). Crucial to this objective is the use of traits for which adaptive phenotypes can be identified. The study of sex allocation has provided some of the most convincing tests of adaptive behavior. Theory predicts how organisms should adjust the allocation of resources to male and female offspring in response to environmental conditions, and there is now a sizeable body of empirical evidence supporting these predictions (Charnov 1982; Hamilton 1996; Frank 1998; West et al. 2000; Hardy 2002; West et al. 2005). The fitness consequences of sex allocation are often relatively straightforward, especially when examining the proportion of offspring that are male (sex ratio), and so there is often a very good fit between theory and data. Consequently, studies on sex-ratio adjustment have provided some of the best quantitative evidence for adaptation and the way in which natural selection acts (Charnov 1982; Herre 1987; West et al. 2000).

In contrast to theoretical and experimental advances, we have rather little knowledge about the genetics of sex-ratio adjustment (Antolin 1993; Cook 1993, 2002; Orzack 2002; West and Herre 2002; West and Sheldon 2002). Relatively few studies have examined genetic variation for sex-ratio behavior, those that have consider organisms such as parasitoid wasps (Parker

and Orzack 1985; Orzack and Parker 1986, 1990; Orzack 1990; Orzack and Gladstone 1994; Wajnberg 1994; Kobayashi et al. 2003), *Drosophila* (Toro and Charlesworth 1982; Carvalho et al. 1998), and pigs (Toro et al. 2006). Knowledge of sex-ratio genetics is likely to be important to several current debates in the field of sex allocation, such as explaining the extent of facultative sex-ratio adjustment exhibited by individuals, how variation across species varies with the mechanisms used to allocate or determine sex, and the source of variation between individuals (within populations) in sex-ratio behavior (Orzack 2002; West et al. 2002, 2005; West and Sheldon 2002). More generally, the relatively clear link between sex ratios and fitness means that the sex ratio should be an extremely useful trait for examining how factors such as genetic architecture and mutation can constrain adaptation (Barton and Partridge 2000). Here we are concerned with mutation. Mutations are important because they: (1) represent the ultimate source of novel genetic variation upon which selection acts; and (2) can shift traits away from their optimum value, which can lead to the mean value of a trait differing from its optimum, and to the maintenance of genetic variance (Barton and Turelli 1989; Lynch and Walsh 1998). However, data on mutation rates of sex-ratio traits are entirely lacking.

Our overall aims in this article are to estimate the important mutational parameters for offspring sex ratios in the parasitoid wasp *Nasonia vitripennis*, and determine the extent to which mutation can explain the genetic variation that has been observed in the offspring sex ratios produced by individuals. *Nasonia vitripennis* is a useful study organism for this purpose because we have an extremely good understanding of its sex-ratio behavior, and because it is an excellent laboratory organism (see methods). Like all wasps, *N. vitripennis* is haplodiploid, and so females can adjust the sex ratio of their offspring by choosing whether to fertilize an egg (Crozier 1971; Cook 1993, 2002; Cook and Crozier 1995). Female *N. vitripennis* adjust their offspring sex ratios in the directions predicted by Local Mate Competition (LMC) theory (Hamilton 1967). In particular, females produce extremely female-biased offspring sex ratios when laying eggs alone, and less female biased sex ratios when laying eggs on a patch with other females (Werren 1983; Orzack 1990; Shuker and West 2004). When females lay eggs alone, the offspring sex ratios predicted under LMC are subject to stabilizing selection (Green et al. 1982; West and Herre 1998). If too few males (sons) are produced, then some of the females (daughters) will remain unmated. On the other hand, if too many sons are produced, then resources are wasted that could have been used to produce more daughters. Nevertheless, despite this stabilizing selection, genetic variation has been observed for this behavior (Parker and Orzack 1985; Orzack and Parker 1986, 1990; Orzack 1990; Orzack and Gladstone 1994).

Our estimation of the importance of mutation is divided into three parts. First, we determined the mutability of offspring sex ratios in *N. vitripennis*. We define mutability (h_m^2) of a trait as the amount of genetic variance produced each generation by mutation (V_m), which is normalized by the environmental variance (V_e). We determined the mutability of sex-ratio behavior in two scenarios: when females lay eggs on a patch alone (as a single foundress) and when coparasitizing with another female (two foundresses). We estimated this by utilizing lines from three replicate mutation accumulation experiments, each starting with a different genotype. In each of these experiments, we initiated a large number of genetically identical lines, and then maintained them under relaxed selection for 20–32 generations. This allowed mutations to accumulate at approximately neutral rate, from which we could calculate: (1) the mutational variance arising per generation (V_m); (2) the mutational heritability ($h_m^2 = V_m/V_e$); (3) the diploid genomic mutation rate (U); and (4) the average mutation effect size ($\bar{a}$) (Bateman 1959; Mukai 1964; Mukai et al. 1972; Keightley 1994; Houle et al. 1996; Lynch and Walsh 1998). Second, we examined the sex-ratio behavior of individuals isolated from a natural population of *N. vitripennis*, to estimate the genetic variation in sex-ratio behavior. Third, we used our data to parameterize a theoretical model that predicts the strength of stabilizing selection on offspring sex ratios and related this to the standing genetic variation in our study population using population genetic theory (Barton, 1990). This allowed us to determine the extent to which the observed genetic variation in offspring sex ratios could be explained by mutation–selection balance (i.e., the rate at which selection can remove new mutations that shift the offspring sex ratio away from its optimal value).

Materials and Methods

STUDY ORGANISM

Nasonia vitripennis (Hymenoptera: Chalcidoidea) is a gregarious wasp that parasitizes large dipteran pupae (Whiting 1967). Females lay clutches of 20–40 eggs on host pupae, with adult males emerging after approximately 13 days at 25°C and females emerging soon after. Males have small wings and are unable to fly, remaining close to the emergence patch where they compete with each other for matings with emerging females. Females are fully winged and typically mate only once before dispersing to find new hosts. More than one female may oviposit in a single host, although females prefer unused hosts (Shuker et al. 2005). The mating system meets the assumptions of LMC and females adjust their sex ratio in response to both the presence of other females ovipositing on a patch (foundress number) and to the presence of eggs already laid in a host (relative clutch size) (Werren 1980, 1983; Orzack and Parker 1986; Orzack 1990; King and Skinner

1991; Orzack et al. 1991; King et al. 1995; Molbo and Parker 1996; Flanagan et al. 1998; Reece et al. 2004; Shuker et al. 2004, 2005, 2006a,b; Shuker and West 2004).

MUTATION ACCUMULATION EXPERIMENT: BASE STRAINS AND GENERATION OF EXPERIMENTAL LINES

The mutation accumulation experiments reported here were started using three *N. vitripennis* strains that originated from different localities in The Netherlands: B5 from Elspeet, HV202 and HV55 from De Hoge Veluwe (provided by L.W. Beukeboom in 2001). The strains were initiated as isofemale lines and were further inbred for 16 generations, using alternating single brother–sister and father–daughter matings. Father–daughter matings were facilitated by keeping the father alive at 4°C, until the emergence of the offspring incubated at 25°C. This resulted in base strains that are predicted to be more than 99% homozygous at all loci. From each of the three inbred strains, a single female was randomly chosen and used to generate 120 female offspring. Hundred of these females were then used to initiate the separate mutation-accumulation (MA) lines and the remaining 20 were used to generate the control lines (Fig. 1). All strains were maintained on *Calliphora vomitoria* pupae as hosts, at 25°C, 16L : 8D light conditions.

The control lines were maintained in an evolutionarily inert state by inducing them to enter diapause, which allows storage of viable larvae for up to 1.5 years (Schneiderman and Horwitz 1958). To generate the diapause control lines 20 females per genotype (mentioned above) were placed singly in vials and provided with two hosts. The vials were placed in a light proof box and kept at 15°C for 4 days. These first hosts were discarded and the female provided with a further six hosts for a period of 4 days. This was repeated twice, with the hosts being retained. The hosts were maintained at 15°C in the dark for a period of 6 weeks. After this time the pupae were checked for adult emergence and several pupae were opened to determine if diapause larvae had been

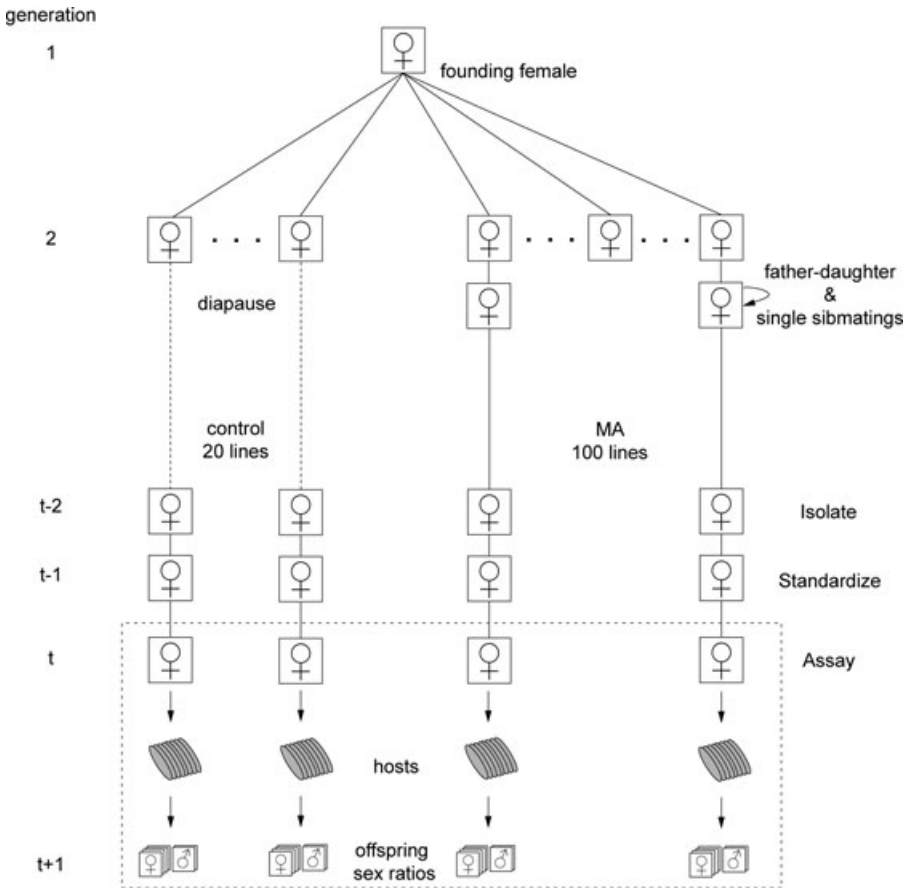

Figure 1. **The experimental design for the sex ratio mutation accumulation (MA) experiments. One hundred female offspring from a single female founded the MA lines, whereas 20 offspring from the same female founded the control lines. MA lines were propagated through single pair sib- or father–daughter matings, whereas control lines were maintained in a state of diapause. Three generations before the sex ratio assays, wasps from the control line were brought out of diapause. The sex ratio assay was performed (in generation *t*) after isolation and standardization of the wasps. Individual wasps were provided with hosts and the offspring sex ratios were counted upon offspring emergence. See main text for further details.**

produced. The opened pupae were discarded and the remaining unopened pupae were stored in sawdust in vials at 4°C in complete darkness. Some of the larvae were brought out of diapause prior to the commencement of each of the experiments. To avoid any possible carryover effects of diapause, third generation postdiapause emergence females were used as controls for all assays.

MAINTENANCE OF LINES

The MA lines were maintained by single sibling pair matings (Fig. 1). For generations 1–4, a single mated female was selected postemergence to initiate the next generation. However a substantial number of females either failed to reproduce or produced only males, suggesting the female was probably not inseminated. We therefore slightly changed the maintenance protocol for all subsequent generations (5–20 for B5, 5–22 for HV202, and 5–32 for HV55). In the new setup, male and female sibling pairs were randomly selected 1–2 days prior to the emergence of the adult wasps by opening the host puparium. Each pair was placed in a vial and kept at 25°C for 4 days to allow for emergence and mating, hence removing sexual selection on males. After mating, females were allowed to oviposit on eight large, good quality hosts for 40–48 h, thus limiting larval competition among the offspring. Three replicate pairs were set up per generation for each mutation accumulation line to reduce stochastic losses. Only one of these replicates was used to found the next generation.

ASSAY PROCEDURE FOR SINGLE FOUNDRESS EXPERIMENT

For the B5 strain, the assay was performed in generation 20 (Table 1, Fig. 1). In generation 18, an individually mated female from a single sibling pair from each MA line was allowed to oviposit on eight hosts. Forty mass-mated B5 control line females that had developed from diapause larvae were selected postemergence (generation 1 postdiapause) and treated in the same manner to generate control sublines. In generation 19 (generation 2 postdiapause for the control line), five females were randomly selected for each MA line or control subline and each was provided with a single host for 24 h as pretreatment to facilitate egg development. The pretreatment hosts were then discarded and each female was given four hosts for 24 h. The hosts were subsequently separated and placed in individual glass vials. These hosts were incubated at standard conditions to allow the wasps of generation 20 (generation 3 postdiapause for the controls) to emerge and to mate. For each MA line or control subline, 15 mated females were then selected randomly for the sex-ratio assay, where only a single female per host was used to minimize potential host environmental effects. Each female was then provided with eight hosts for a 48-h period, after which the females were removed and the pupae further incubated at 25°C. The resulting adult wasps were allowed to emerge and die prior to counting and sexing (Fig. 1).

For the HV202 and the HV55 strains, the assays were done in generation 22 and involved slightly different numbers of hosts to ensure sufficient replication (Table 1). In generation 20, six individually mated females per mutation accumulation line were allowed to oviposit on five large hosts for a period of 24 h. Thirty mass-mated control line females (HV202 or HV55) that had developed from diapause larvae were selected postemergence (generation 1 postdiapause) and treated in the same manner to generate control sublines. In generation 21 (generation 2 postdiapause for the control line), one replicate female per MA line or control subline was randomly chosen and from her offspring six male–female pairs were selected from three randomly chosen hosts, selecting two pairs per host. After emergence and mating, each mated female was given five hosts for 24 h. These parasitized hosts were subsequently separated and placed in individual glass vials. The hosts were incubated at standard conditions to allow the wasps of generation 22 (generation 3 postdiapause for the control line) to emerge and to mate. For each MA line or control subline, 15 mated females were selected out of the offspring of three randomly chosen females for use in the sex-ratio assay. Again, only a single female was used per host to minimize potential host environmental effects. Each female was then provided with 10 hosts for a 48-h period, after which the females were removed and the pupae were further incubated at 25°C. As before, the resulting adult wasps were allowed to emerge and die prior to counting and sexing (Table 1, Fig. 1).

ASSAY PROCEDURE FOR TWO FOUNDRESS EXPERIMENT

The two foundress sex-ratio assay was performed using the HV55 strain in generation 32 (Table 1). In this experiment, the laboratory red-eye mutant marker strain STDR was used to follow the sex ratio of focal wild-type females when parasitizing together with a red-eye foundress. In generation 30, three individually mated

Table 1. Experimental setup for the sex ratio mutation accumulation experiments. For further details see the main text.

Line	Foundress number	Hosts provided	Replicate/line	Lines tested
B5	1	8	15	59
HV202	1	10	15	47
HV55	1	10	15	65
HV55	2	2	15	35

females per mutation accumulation line were provided with a single host for 24 h as pretreatment. These hosts were discarded and each female was allowed to oviposit on five large hosts for a period of 24 h. Thirty mass-mated HV55 control line females that had developed from diapause larvae were selected postemergence (generation 1 postdiapause) and treated in the same manner to generate control sublines. In generation 31 (generation 2 postdiapause for the control line), one replicate female per MA line or control subline was randomly chosen and from her offspring five male–female pairs were selected, each from a different host. After emergence and mating, each mated female was provided with a single host for 24 h as pretreatment after which she was given five hosts for 24 h for oviposition. These parasitized hosts were subsequently separated and placed in individual glass vials to produce the wasps of generation 32 (generation 3 postdiapause of the control line). For each MA line or control subline, 15 male–female pairs were collected as pupae out of the offspring of three randomly chosen females. After emergence and mating, the males were removed and a single mated STDR female was introduced. The two females were given a single host for 24 h as pretreatment, after which they were allowed to oviposit together on two hosts for 24 h. These parasitized hosts were then separated and incubated under standard conditions. The adult wasps were allowed to emerge and die prior to counting and sexing (Table 1, Fig. 1).

ESTIMATION OF MUTATIONAL PARAMETERS

In contrast to many morphological and life-history traits, the direction of deleterious mutation for sex-ratio traits is hard to predict a priori, but mutations are expected to increase variance. To account for this, we obtained estimates of the mutational parameters using two different methods. Both use the variance between MA lines to estimate the mutational parameters: if very many mutations of small effect are involved, then the variance between lines would be small; conversely, a few mutations of large effect would lead to large variance between lines. We used the Bateman-Mukai (BM) method (Bateman 1959; Mukai 1964; Mukai et al. 1972) that assumes constant mutational effects, both in size and direction (Lynch and Walsh 1998), and a maximum-likelihood (ML) method that allows for both positive and negative mutational effects (Keightley 1994).

Because the control line sex-ratio observations are not approximately normally distributed (which is assumed by the ML method) we transformed the data using a Box–Cox transformation (Box and Cox 1964). Data were transformed using a Box–Cox transformation after adding 1 to all datapoints (because the transformation is only defined for strictly positive values and the sex ratio may be zero). The transformation is defined as

$$y_t = \begin{cases} ((y+1)^{\lambda} - 1)/\lambda & (y \geq 0, \lambda \neq 0) \\ \log(y+1) & (y \geq 0, \lambda = 0) \end{cases},$$

where y is an untransformed sex ratio and y_t is a transformed sex ratio. For each experiment we estimated the power term of the Box–Cox transformation (λ) that yielded the best fit of the transformed control line sex-ratio data to a normal distribution by ML. This transformation could then be applied to both the control and mutant line data. The ML estimates of λ obtained were -14.7, -11.9, -13.0, and -3.5 for the B5 single foundress, HV202 single foundress, HV55 single foundress, and HV55 two foundress datasets, respectively. However, it was noted that, for the three single foundress experiments, the three different estimates of λ provided as good a fit as any of the others for all datasets (e.g., the ML estimates of λ obtained for the HV55 and HV202 single foundress control line data were not a significantly worse fit than the ML estimate obtained for the B5 single foundress data, and this was true for comparisons made using fits to the HV55 and HV202 data as well). On the other hand, all estimates of λ obtained for the single foundress experiments were found to be significantly worse than the ML estimate for the two foundress experiment and vice versa. We therefore decided to use two different transformations, one for the single foundress experiments (obtained by finding the ML estimate of λ for all the single foundress control lines together: $\lambda = -12.2$) and one for the two foundress experiment ($\lambda = -3.5$). We then applied this transformation to control and mutant line sex-ratio data from the experiments. To allow for comparison between both methods of estimating mutational parameters, we used the Box–Cox transformed sex ratios for both the BM and ML approaches.

We obtained estimates of the different variance components in the single foundress experiment by fitting linear mixed models with the transformed sex ratios, with MA line modeled as a random variable. Because offspring sex ratio of superparasitizing *N. vitripennis* wasps is negatively correlated with relative clutch size (Werren 1980), we fitted a more extensive linear mixed model for the two foundress experiment, with relative clutch size modeled as a fixed effect and MA line as random effect. The components of variance between lines (V_l) and within replicates (the environmental variance V_e) were estimated by restricted maximum likelihood (REML). Because we did not account for line effect in the controls, it was not possible to estimate the residual variance in the controls. We therefore assume the residual variance in the control lines to be zero. The change in genetic variation per generation due to mutation was estimated as $V_m = V_l/(2t)$, where t is the number of generations of mutation accumulation (Lynch and Walsh 1998). The mutational heritability (h_m^2) was estimated as the ratio of the mutational variance (V_m) over the average environmental variance (V_e) (Lynch and Walsh 1998). The mutational coefficient of variation (CV_m) was estimated as $CV_m = \sqrt{V_m}/Mean_{Control}$.

To obtain estimates of the diploid genomic mutation rate and the mutational effect for the sex-ratio traits, we first used the BM

method that compares the rate of change in the mean of the mutant lines (R_m) to that in the among-line variance (V_m). Because of its assumption of constant mutational effects, the Bateman–Mukai method yields downwardly biased estimates of the diploid genomic mutation rate (U_{BM}) and upwardly biased estimates of the average mutation effect size ($\bar{a}_{\max}$) (Lynch and Walsh 1998). The formulae are $U_{BM} = 2R_m^2/V_m$, and $\bar{a}_{\max} = V_m/2R_m$. Confidence intervals for the variance components, h_m^2, U_{BM}, and $\bar{a}_{\max}$ were calculated as the 2.5 and 97.5 percentiles from 1000 bootstrapped datasets sampled at the level of MA line. Estimates of the mutational parameters were done using *R* statistical software (ver. 2.6.1; Ihaka and Gentleman 1996).

We then used the ML method to estimate the mutation parameters under a model allowing both positive and negative effect mutations. The model assumes that the control line trait estimates are normally distributed with mean μ and variance σ^2, whereas the distributions of the mutant lines are affected by mutations. The number of new mutations appearing per generation (the diploid mutation rate) is assumed to be Poisson distributed with mean (U_{ML}) and each mutation is assumed to have the same magnitude of effect ($\bar{a}$), a fraction P of which have a positive effect and $1 - P$ a negative effect. We used the program mlgenomeu (Keightley 1994; Keightley and Ohnishi 1998) to jointly estimate μ, σ^2, U_{ML}, $\bar{a}$ and P for each experiment by ML. We did not attempt to fit the gamma parameter (allowing for variable mutational effects) because of computational limitations involved when including positive effect mutations in the model. Confidence intervals on parameter estimates were estimated using a profile likelihood of the parameter of interest (see Keightley 1994 for details).

We then back transformed the ML estimated mutation effect sizes from the Box–Cox transformed sex ratios using the following transformation:

$$y = \begin{cases} ((y_t\lambda + 1)^{1/\lambda} - 1)/\lambda & (y \geq 0, \lambda \neq 0) \\ e^{y_t} - 1 & (y \geq 0, \lambda = 0). \end{cases}.$$

However, because the effects of mutations after back transformation depend on the sex ratio, we calculated what affect a positive- and negative-effect mutation would have for sex ratios varying between 0 and 1 (see Fig. 2). An average effect size on the untransformed scale was obtained by adding or subtracting the best-fitting effect size to the mean on the transformed scale, which was then back transformed after which the untransformed mean was subtracted to give the effect size on the untransformed scale.

FIELD ESTIMATES OF GENETIC VARIATION

We estimated the genetic variation in sex-ratio behavior among *N. vitripennis* strains collected from a single population (De Hoge Veluwe in The Netherlands). Strains were collected in 2003 by L.W. Beukeboom (strain labels starting with "C") and in 2004 by T. Koevoets, M. N. Burton-Chellew and E. M. Sykes (strain labels starting with "HV"; for collection details see Burton-Chellew et al. 2008). Strains were setup as isofemale lines and maintained on *C. vomitoria* pupae as hosts, at 25°C, 16L : 8D light conditions.

For each strain, we isolated 2-day-mated-old females in glass vials and provided them with a single host for 24 h as pretreatment to facilitate egg development. We then discarded the pretreatment hosts and gave each female access to honey solution for 24 h. We then provided pretreated females with a single host for oviposition. After 1 h, we placed one-way escape tubes on the glass vials to allow females to disperse away from the patches, to limit unnatural superparasitism (Werren 1983; Godfray 1994). After 24 h, we transferred the females from the hosts and escape tubes to clean glass vials, provided them with honey solution, and stored the wasps at 25°C for 24 h. Subsequently, we provided the females with a single host that had previously been parasitized by a single redeye STDR female for 24 h. After 1 h, we fitted escape tubes on the glass vials and the females were allowed to parasitize the host for a further 24 h. After this period, we removed the females and further incubated the parasitized hosts at 25°C. We randomly distributed vials with hosts across test tube racks, so that hosts from the same strain were distributed across racks and shelves in the incubator. We allowed the resulting adult wasps to emerge and die prior to genotyping (wildtype or STDR), counting, and sexing. Using a similar setup, we confirmed the differences in single foundress sex ratio for two lines at the ends of the sex-ratio distribution (lines HV06 and C222a). This setup was identical to that described above, except that the females in this setup were not provided with a previously parasitized host, but were removed after ovipositing on the first host.

We analyzed the variation for sex-ratio behavior in two steps. First, we fitted a generalized linear model with a binomial error structure, which included line, foundress number, and the interaction between line and foundress number as effects. This allowed us to analyze possible differences in sex-ratio adjustment between the isofemale lines. Second, we estimated genetic variation for sex-ratio behavior in the single and two foundress situations separately by determining the between-isofemale-line variation using linear mixed-effect models on arcsine-transformed sex-ratio data. Isofemale line was fitted as a random effect and variance components were estimated by REML. This isofemale line analysis estimates the genetic variation as the broad-sense heritability H^2 (Hoffmann and Parsons 1988; Wajnberg 1994, 2004; Lynch and Walsh 1998). All statistical analyses were done using R statistical software (ver. 2.6.1; Ihaka and Gentleman 1996).

FITNESS CONSEQUENCES OF SEX-RATIO VARIATION

We estimated the form of selection on single foundress sex ratio following West and Herre (1998). If one female lays eggs on a patch (single foundress), and all mating occurs on the patch before

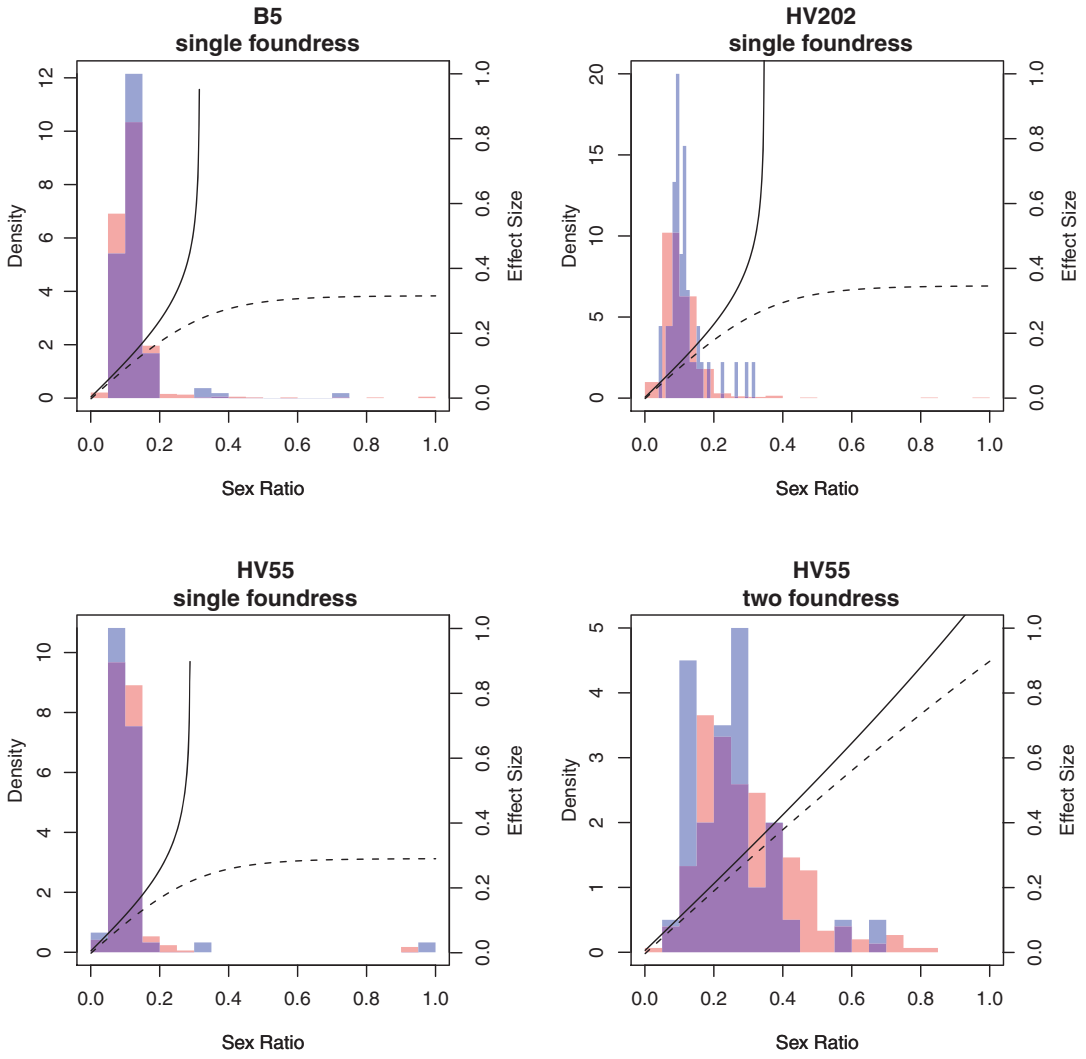

Figure 2. **Sex ratio distributions for the control (blue) and mutation accumulation (red) lines, using untransformed sex ratio data. Plots of the magnitude of the most likely mutation effect size inferred from the Box–Cox transformed data on the untransformed scale are also shown on a separate scale (right-hand axis). The solid line shows the magnitude of a positive effect mutation and the dotted line shows the magnitude of a negative effect mutation. Untransformed positive and negative effect sizes can be read from the graph using the average sex ratio from each experiment. Sex ratio is given as proportion male.**

the females disperse, then the optimal sex ratio is to produce just enough sons to mate the daughters (Hamilton 1967). If there is a chance that developmental mortality can result in the males dying, then this can favor the production of extra "insurance" males (Green et al. 1982; Nagelkerke and Hardy 1994; West et al. 1997). If we make the simplifying assumption that one male is sufficient to inseminate all the females in a brood, then the relative fitness return (W) in terms of mated female offspring from a brood of size b and a sex ratio y (proportion males) will be

$$W = b(1 - y)(1 - d)(1 - d^{yb}), \quad (1)$$

where d is the probability of an individual dying before it reaches maturity (defined as the developmental mortality rate) (West and Herre 1998).

Equation (1) illustrates that there is stabilizing selection on sex ratio (Green et al. 1982; West and Herre 1998). This stabilizing selection arises because if there are too few males (sons) produced, then the females (daughters) will remain unmated. However, if too many sons are produced, then resources are wasted that could have been used to produce more daughters. The strength of this stabilizing selection on sex ratio can be quantified as the second differential of log fitness ($\partial^2 \log(W)/\partial y^2$ following Lande and Arnold 1983; Turelli and Barton 1994), which gives

$$\partial^2 \log(W)/\partial y^2 = -\frac{(bd^y - 1)^2 + [d^y b(y - 1)^2(\log[d])^2]}{(bd^y - 1)^2(y - 1)^2} \quad (2)$$

(West and Herre 1998). The reciprocal ($1/[\partial^2 \log(W)/\partial y^2]$), termed V_s, is a quantity used in population genetics theory (e.g., Barton

Table 2. Means and the rates of change in means for sex ratio characters in control and mutation accumulation lines of *Nasonia vitripennis*.

Experiment	t	$Mean_{Control}$	n	$Mean_{MA}$	n	$R_m \times 10^3$	% change
B5, single foundress sex ratio	20	0.129 (0.007)	107	0.119 (0.003)	764 from 59 lines	−0.483 (0.378)	−0.37
HV202, single foundress sex ratio	22	0.119 (0.009)	45	0.107 (0.003)	545 from 47 lines	−0.532 (0.411)	−0.45
HV55, single foundress sex ratio	22	0.113 (0.016)	61	0.109 (0.005)	339 from 65 lines	−0.182 (0.734)	−0.16
HV55, two foundress sex ratio	32	0.255 (0.019)	40	0.302 (0.008)	301 from 35 lines	+1.474 (0.652)	+0.58

Number of generations of mutation accumulation (t), mean sex ratio on the untransformed scale (SE in parentheses) for each experiment in the control ($Mean_{Control}$) and mutation accumulation lines ($Mean_{MA}$), the rate of change in the mean of the mutant lines (R_m), and R_m standardized by the control mean (% change).

1990), for instance to estimate the reduction in mean fitness as a result of a given amount of environmental variance (V_e; Barton 1990 and see below).

Results

MUTATION ACCUMULATION EXPERIMENTS

Across the mutation accumulation experiments, from the initial 300 lines, between 40 and 60 lines per experiment were scored for sex ratio, with in total 1949 females tested (complete dataset is available as online Supplementary Table S1). The single foundress sex-ratio experiments all showed a decline in sex ratio with the accumulation of mutations (average change relative to control mean = −0.3% per generation, or an average sex-ratio change of −0.004 per generation), but this was not significant for any of the experiments (Table 2, Fig. 2). Conversely, the second foundress experiment did show a significant change in sex ratio, which increased by 0.6% per generation, i.e., in increase in sex ratio of +0.002 per generation ($F_{1,339} = 6.08$, $P < 0.05$; Table 2, Fig. 2). All experiments showed an increase in genetic variance (V_m) due to mutation (Table 3, Fig. 3), which was significant for the single foundress experiments (likelihood-ratio test; B5: $\chi^2_1 = 14.71$, $P < 0.001$; HV202: $\chi^2_1 = 5.50$, $P < 0.05$; HV55: $\chi^2_1 = 3.83$, $P = 0.05$), but not for the two foundress experiment ($\chi^2_1 = 2.60$, $P = 0.11$). The mutational heritabilities (h^2_m) for the single foundress experiments were higher than that of the two foundress experiment (average transformed single foundress sex ratio $h^2_m = 0.0016$, transformed two foundress sex ratio $h^2_m = 0.0008$, Table 3). The mutational coefficient of variation (CV_m) did show comparable values for both types of experiment (average transformed single foundress sex ratio $CV_m = 0.64\%$, transformed two foundress sex ratio $CV_m = 0.72\%$, Table 3).

The Bateman–Mukai (BM) minimum estimates of the total genomic mutation rate (U_{BM}) for single foundress sex ratio on the transformed scale were in the range 0.02–0.19 per generation, with the average estimate over all experiments being 0.099 (Table 4). The ML estimates (U_{ML}) ranged from 0.02 to 0.06 per generation on the transformed scale, with the average estimate being 0.037. The BM maximum homozygous mutational effects ($\bar{a}_{\max}$) on single foundress sex ratio are in the range of 0.002–0.005 on the transformed scale, with an average over all experiments of 0.003, corresponding to an average change in sex ratio of 0.014 (proportion males) per mutation on the untransformed scale. The ML homozygous mutational effects ($\bar{a}$) for single foundress sex ratio have a similar range (0.002–0.004), and an average effect of 0.003 on the transformed scale, which

Table 3. Variance components, mutational coefficients of variation, and mutational heritability for sex ratio characters in control and mutation accumulation lines of *Nasonia vitripennis*.

Experiment	$V_I \times 10^3$	$V_m \times 10^3$	$V_e \times 10^3$	CV_m	h^2_m
B5, single foundress sex ratio	0.0051 (0.0028–0.0110)	0.00013 (0.00007–0.00027)	0.0663 (0.0514–0.0785)	0.0059 (0.0044–0.0087)	0.0019 (0.0012–0.0042)
HV202, single foundress sex ratio	0.0055 (0.0023–0.0146)	0.00012 (0.00005–0.00033)	0.1132 (0.0943–0.1270)	0.0061 (0.0039–0.0100)	0.0011 (0.0005–0.0030)
HV55, single foundress sex ratio	0.0056 (0.0054–0.0206)	0.00013 (0.00012–0.00047)	0.0699 (0.0470–0.0869)	0.0065 (0.0064–0.0125)	0.0018 (0.0017–0.0082)
HV55, two foundress sex ratio	0.0717 (0.0069–0.2136)	0.0011 (0.0001–0.0033)	1.4865 (1.2176–1.6294)	0.0072 (0.0022–0.0124)	0.0008 (0.0001–0.0026)

The among-mutation accumulation-line variance (V_I), the rate of change in the among-line variance (V_m), the average environmental variance (V_e), mutational coefficient of variation (CV_m), and mutational heritability (h^2_m). Estimates are based on Box–Cox transformed data and given with their 2.5 and 97.5 percentiles from 1000 bootstraps of the dataset.

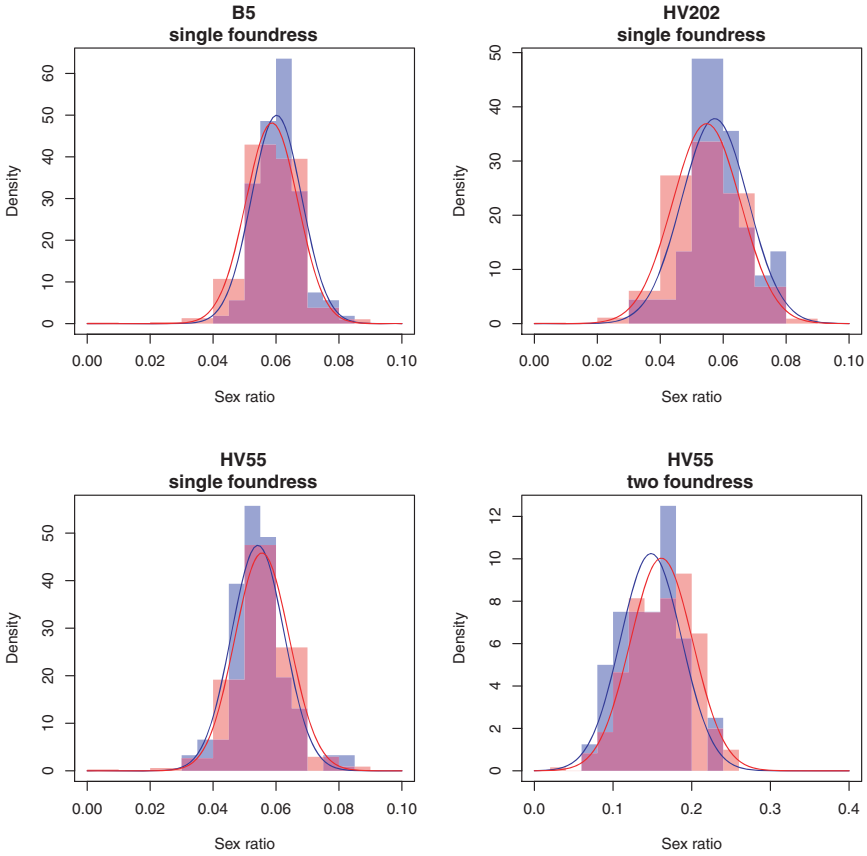

Figure 3. Sex ratio distributions for the Box–Cox transformed data for control lines (blue) and mutation accumulation lines (red), for each of the four experiments. The curves represent the expected control (blue) or mutation (red) distributions inferred by maximum likelihood. Sex ratio is given as proportion male.

corresponds to an average change in sex ratio of 0.011 (proportion males) per mutation on the untransformed scale, with 90% of these mutations reducing sex ratio (i.e., a greater production of female offspring).

With a minimum of 0.18 (BM) and 0.12 (ML) mutations per generation, the total genomic mutation rate (U) for two foundress sex ratio is at the top end of the range found for single foundress sex ratio. The maximum homozygous mutational effect on two

Table 4. Bateman–Mukai (BM) and Maximum-Likelihood (ML) estimates of genomic mutational parameters for sex ratio characters in *Nasonia vitripennis*.

	BM estimates		ML estimates		
Experiment	U_{BM}	$\bar{a}_{\max}$	U_{ML}	$\bar{a}$	P
B5, single foundress	0.0833 (0.0250–0.1601)	−0.002 (−0.006 to −0.001)	0.0316 (0.0009–∞)	0.002 (0.000–0.010)	0.05 (0.000–0.400)
HV202, single foundress	0.1923 (0.0522–0.4755)	−0.002 (−0.005 to −0.001)	0.0562 (0.0003–∞)	0.002 (0.000–0.006)	0.00 (0.000–0.745)
HV55, single foundress	0.0186 (0.0002–0.0380)	+0.005 (0.004–0.064)	0.0178 (0.0005–∞)	0.004 (0.000–0.009)	1.00 (0.164–1.000)
HV55, two foundress	0.1812 (0.0450–1.4129)	+0.005 (0.006–0.016)	0.1210 (0.0004–∞)	0.005 (0.000–0.039)	1.00 (0.428–1.000)

BM estimates of the diploid genomic mutation rate (U_{BM}) and the average homozygous mutational effect ($\bar{a}_{\max}$). ML estimates of the diploid genomic mutation rate (U_{ML}), the average homozygous mutational effect ($\bar{a}$), and the fraction positive effect mutations (P). Estimates are based on Box–Cox transformed data. BM estimates are given with their 2.5 and 97.5 percentiles from 1000 bootstraps of the dataset. ML estimates are given with their 95% confidence intervals.

Table 5. Field estimates of genetic variation for offspring sex ratio in *Nasonia vitripennis*.

		Variance				
	Heritability	Genetic	Environmental	Method	Material	Source
Single foundress	0.16	NA		artificial selection differential [narrow -sense h^2]	5 isofemale lines, 5 populations, USA	Parker and Orzack (1985)
	0.12[1]	NA		parent-offspring regression [narrow-sense h^2]	119 isofemale lines, single population, Södertälje Sweden	Orzack and Gladstone (1994)
	0.02	0.001	0.034	isofemale analysis [broad-sense h^2]	18 isofemale lines, single population, Hoge Veluwe, The Netherlands	This study
Two foundress	0.15[1]	NA		parent-offspring regression [narrow-sense h^2]	119 isofemale lines, single population, Södertälje Sweden	Orzack and Gladstone (1994)
	0.05	0.002	0.039	isofemale analysis [broad-sense h^2]	18 isofemale lines, single population, Hoge Veluwe, The Netherlands	This study

[1]Regression of arcsine-transformed data.
NA= Data not available.

foundress sex ratio is 0.005 on the transformed scale (both BM and ML estimates), or a sex-ratio increase of 0.013 (proportion males) on the untransformed scale.

FIELD ESTIMATES OF GENETIC VARIATION

For each of the 18 isofemale lines, we estimated the sex ratio of between 50 and 60 females per line, with in total 950 females tested. The field estimates of the genetic variation for sex-ratio behavior showed significant genetic variation for offspring sex ratio ($F_{17,1630} = 4.36$, $P < 0.0001$, Table 5, Fig. 4), as well as a significant change in sex ratio in response to foundress number ($F_{1,1630} = 93.02$, $P < 0.0001$). The results did not show a significant interaction between isofemale line and foundress number ($F_{17,1630} = 1.1902$, $P = 0.26$), indicating there is no

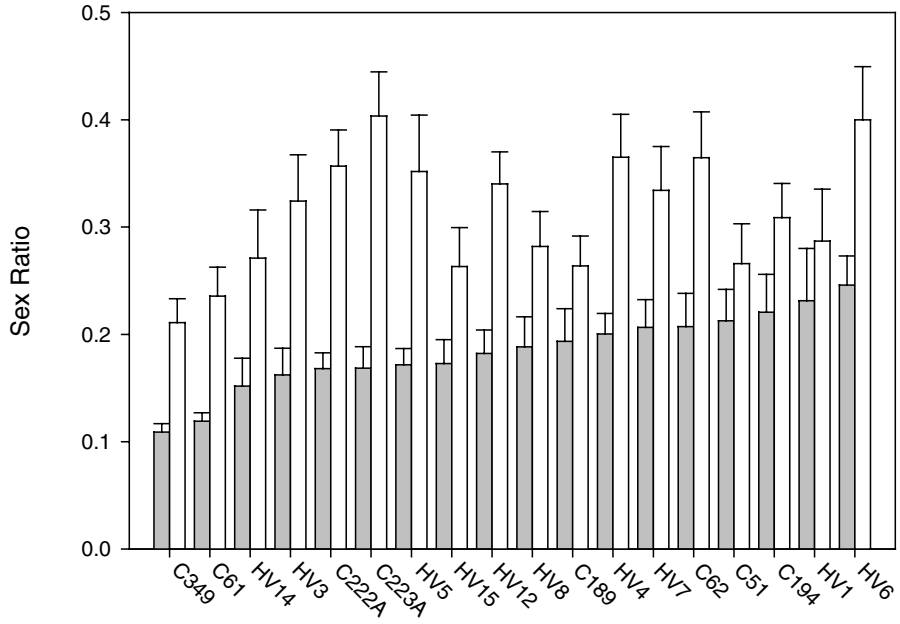

Figure 4. Field estimates of offspring sex ratio (proportion male) of isofemale lines from the Hoge Veluwe, The Netherlands. Gray bars represent single foundress sex ratio, open bars two foundress sex ratio. Error bars represent standard errors.

difference among lines for adjustment of sex ratio between single and two foundress situations. Estimated over 18 isolines, the broad-sense heritability of single foundress sex ratio was $H^2 = 0.02$ (likelihood-ratio test; $\chi_1^2 = 5.61$, $P = 0.02$), and for two foundress sex ratio $H^2 = 0.05$ (likelihood-ratio test; $\chi_1^2 = 6.49$, $P = 0.01$). When repeated for a subset of two lines at the ends of the distribution (Fig. 4; C222A: 0.13 ± 0.01, HV6: 0.23 ± 0.03 (mean single foundress sex ratio $\pm$ SE), the presence of genetic variation for offspring sex ratio was confirmed ($F_{1,89} = 21.33$, $P < 0.0001$).

FITNESS CONSEQUENCES OF SEX-RATIO VARIATION

We used our data and estimates from the literature to parameterize equation (2) and estimate the strength of stabilizing selection on single foundress offspring sex ratios. In the single foundress case ($n = 950$), we found that the average brood size b ($\pm$ SE) was 33.70 ± 0.35, with an average sex ratio y ($\pm$ SE) of 0.183 ± 0.006, or a transformed sex ratio y_t ($\pm$ SE) of 0.062 ± 0.0004. In *N. vitripennis*, developmental mortality rate d has been estimated by Werren (1980) to be 0.06. Using these data, the strength of stabilizing selection on single foundress sex ratio on the transformed scale can estimated as $\partial^2 \log(W)/\partial\, y_t^2 = -1.22$, or alternatively expressed as $V_s = 1/(\partial^2 \log(W)/\partial y_t^2) \sim -0.82$.

Discussion

Mutation generates variation in the offspring sex-ratio behavior of the parasitoid wasp *N. vitripennis*. For single foundress sex ratios, we estimated on average approximately one mutation every 20 generations, shifting the sex ratio by 0.011 (proportion males). For the two foundress sex ratio the mutation rate was higher, with approximately one mutation every eight generations, shifting sex ratio by 0.013 (proportion males). Although the mutational effect sizes for both single and two foundress sex ratios are comparable, the direction of the effects is opposite, with single foundress sex ratio being decreased and two foundress sex ratio being increased by the mutations we have uncovered. These experiments show that for both sex-ratio traits mutation can generate variation that natural selection can act upon. In a separate experiment, we found significant genetic variation in offspring sex ratios in a natural population of *N. vitripennis*. Our analyses, described in detail below, suggest that this genetic variation in offspring sex ratios cannot be explained by mutation–selection balance around an optimal value.

SEX-RATIO MUTABILITY

Our results provide a lower bound on the mutation rates and an upper bound on the mutational effect size for sex ratio. This is because the Bateman–Mukai method assumes constant mutational effects (Lynch and Walsh 1998), whereas sex ratio is under stabilizing selection (Orzack 1990; Greeff 1998; West and Herre 1998), and hence the direction of deleterious mutation for sex-ratio traits is hard to predict a priori. Even though most of the observed mutations will be deleterious for overall fitness, not all of them will contribute to a unidirectional change in the mean sex ratio. This is reflected in our single foundress sex-ratio experiments, which failed to show a significant change in mean sex ratio. The estimates of U and $\bar{a}$ for single foundress sex ratio were therefore nonsignificant, although the experiments did show a significant mutational variance (V_m). These issues are inherent to the mutational analysis of traits such as these under stabilizing selection (e.g., total progeny production in *Caenorhabditis elegans*, Vassilieva and Lynch 1999). Despite this, the mutational parameters are significant for the two foundress sex ratio, and they provide the first ever estimates of mutation rate and mutational effect size for sex-ratio behavior. They are also comparable to those reported for life-history traits in other organisms (Table 6, for an extensive review see Houle et al. 1996; Roff 1997; Lynch and Walsh 1998; Lynch et al. 1999). This is to be expected given the close link between offspring sex ratio and fitness (Charnov 1982; Frank 1998).

Table 6. Comparison of mutational parameters between offspring sex ratio in *Nasonia vitripennis* and life-history traits in other well studied organisms.

Species	Trait	h_m^2	CV_m	U	$\lvert\bar{a}\rvert$	References
Nasonia vitripennis	offspring sex ratio	0.0014	0.0064	0.02–0.18	0.01	This study
Drosophila melanogaster	egg-to-adult viability	0.0003	0.002	0.6	0.06	Mukai (1964); Mukai et al. (1972); Cardellino and Mukai (1975); Ohnishi (1977)
Daphina pulex	survival to maturity	0.0012	0.013	1.946	0.008	Lynch et al. (1998)
Caenorhabditis elegans	lifetime reproductive output	0.0012	0.012	0.0026	0.21	Keightley and Caballero (1997)
Arabidopsis thaliana	germination proportion	0.0032	NA	0.0024	0.62	Schultz et al. (1999)

Mutational heritability (h_m^2), mutational coefficient of variation (CV_m), the diploid genomic mutation rate (U), and the absolute homozygous mutational effect size ($|\bar{a}|$). NA, data not available.

There have been two previous attempts to study the mutational parameters of sex ratio. In *Drosophila melanogaster*, both sib-analysis and artificial selection failed to reveal genetic variation for sex ratio (Toro and Charlesworth 1982). In contrast, in *Drosophila mediopunctata*, sex ratio increased by 0.005 (proportion males) per generation in populations that were perturbed to be female biased and then allowed to evolve toward a Fisherian sex ratio (50% males, Carvalho et al. 1998). However, this increase in sex ratio was not due to mutation, but rather reflected an increase in the frequency of autosomal suppressors of the sex-ratio trait, an X–Y meiotic drive system that was used to generate the female-biased populations in the first place (Carvalho et al. 1998).

GENETIC VARIATION IN OFFSPRING SEX RATIOS

The genetic variation for sex ratio in a field population of *N. vitripennis* was low, with the broad-sense heritabilities of single and two foundress sex ratio based on 18 lines being 0.02 and 0.05, respectively. As for other fitness-related traits, this low heritability is probably due to the large environmental variance (Houle 1992; Merila and Sheldon 1999). When the single foundress sex ratio was measured over a subset of two lines at the ends of the distribution, the environmental variance was artificially reduced (giving a notional broad sense heritability of 0.17). Previous measurements of sex-ratio heritability in *N. vitripennis* are 0.16 for single foundress sex ratio (Parker and Orzack 1985) and 0.15 for two foundress sex ratio (Parker and Orzack 1985). Large environmental variance has also been found to be a major component of the heritability of sex ratio in the parasitoid wasp *Heterospilus prosopidis*, which ranged from 0.19 to 0.32 (Kobayashi et al. 2003).

The offspring sex ratio in species subject to LMC, such as *N. vitripennis*, is subject to stabilizing selection (Orzack 1990; Greeff 1998; West and Herre 1998). This has two implications. First, as discussed above, the direction of change by mutation is hard to predict a priori, and might just result in an increase in variance, in contrast to most fitness related traits where mutation accumulation results in a clear decline of trait values (Lynch and Walsh 1998). Hence, although single and two foundress sex ratio showed opposite mutational effects (and increased variance), in both cases the mutations are likely to have decreased fitness. Second, traits that are subject to stabilizing selection are expected to show an erosion of genetic variance. Indeed, the observation of genetic variance in the offspring sex ratios of *N. vitripennis* has been argued to pose a problem for sex-ratio theory (e.g., Orzack 2002)

Our estimate of the strength of stabilizing selection on single foundress sex ratio can be: (1) used to estimate the reduction in mean fitness due to variation around the optimum (Barton 1990); (2) combined with our estimate of mutational variance to predict how much genetic variance we should see in a population. In terms of (1), our results suggest that the strength of stabilizing selection on our observed single foundress sex ratios implies a reduction in mean fitness due to random variation around the optimum of $V_e/2V_s \sim -0.0001$ (p. 774 in Barton, 1990; $V_e = 0.0002$, based on Box–Cox transformed data, $V_s = -0.82$). This means that stabilizing selection on our single foundress sex ratios is quite weak, as is typical for traits measured in natural populations (Kingsolver et al. 2001), although we give the caveat that our estimate assumes a Gaussian distribution and selection on the sex ratio can follow a skewed distribution (see Fig. 1 of West and Herre, 1998). Our estimates of the mutational variance and the heritability of single foundress sex ratio in the field also are within the range found for other life-history traits (Table 6). Perhaps the most important open question in quantitative genetics is why such a variety of traits, in such a variety of species, show these typical parameter values (Johnson and Barton 2005). We must not only explain how quantitative genetic variation is maintained, but also why the amount of genetic variation and the amount of mutational variance falls within a fairly narrow range when scaled relative to the environmental variance.

In terms of predicting how much genetic variance we should see in a population (2), if variation is maintained by a balance between mutation and selection, then the typical selection on each allele must be $s = V_m/V_g$ (p. 775 in Barton, 1990), which for sex ratio is about 0.01 (mean single foundress sex ratio $V_m = 1.27 \times 10^{-7}$, single foundress sex ratio $V_g = 9.01 \times 10^{-6}$, both estimates based on Box–Cox transformed data). This is a robust relationship: essentially, the fraction of deleterious alleles that are removed by selection in each generation must balance the fraction of genetic variance that is contributed by new mutations (Barton 1990). A key question is whether this selection is due to stabilizing selection on sex ratio, or is instead due to selection on the pleiotropic effects of the alleles involved on other traits. For example, mutations that impair female reproduction might reduce the fraction of eggs that are fertilized, and hence the sex ratio—yet their deleterious effects might arise primarily from reduced female fertility. We estimate that the effect of new mutations on sex ratio is at most $\bar{a} \sim 0.005$. Such alleles would have fitness reduced by $s \sim a^2/2V_s \sim -1.52 \times 10^{-5}$ (p. 774 in Barton, 1990)—which is several orders of magnitude less than is implied by the ratio of mutational to genetic variance (V_m/V_g). This suggests that genetic variance for sex ratio is not maintained by mutation–selection balance, but instead by a balance between mutation and the pleiotropic effects that new alleles can have in other traits, as has been suggested to be the case generally (Turelli 1984).

If pleiotropy is the primary cause of genetic variation for sex ratio, we would expect the level of genetic variance to be independent of the strength of stabilizing selection. However, West and Herre (1998) showed that in fig wasp species in which stabilizing

selection on sex ratio is stronger, the phenotypic variance in sex ratio is lower. It may be that this correlation reflects a lower environmental variance for sex ratio in species subject to stronger selection, which arises from selection for stronger canalization (Stearns and Kawecki 1994; Stearns et al. 1995). Our results suggest that levels of genetic variance would not vary across species in this way. It would be interesting to test this hypothesis by measuring genetic variance for sex ratio across species that experience varying degrees of stabilizing selection. Although at the molecular level, the level of variation is primarily determined by the strength of selection (compare, for example, variation at synonymous and nonsynonymous sites), we are not aware of any examples in which such a relation between selection and genetic variance has been demonstrated for a quantitative trait.

In conclusion, our results show that in *N. vitripennis*, mutation generates variation in sex ratio, and our estimates of mutational parameters for sex ratio are comparable to other life-history traits in other species. However, genetic variation for sex ratio in natural populations is low. A higher genetic variance would be expected given these mutational parameters if sex ratio is at mutation–selection balance. Instead, the observed genetic variance suggests additional selection against sex-ratio mutations, with deleterious effects on other fitness traits as well as sex ratio (i.e., pleiotropy). By determining the mutational parameters and their effects for the standing genetic variation in sex ratio in *N. vitripennis*, our results provide the first step toward understanding the genetic basis of sex ratio, and how genetic architecture might constrain adaptive sex ratio behavior.

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Supplementary Material

The following supplementary material is available for this article:

Table S1. Datasets for *Nasonia vitripennis* mutation accumulation experiments for sex ratio traits.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2008.00434.x
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