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EXPERIMENTAL EVOLUTION OF THE *CAENORHABDITIS ELEGANS* SEX DETERMINATION PATHWAY

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Sex determination is a critical developmental decision with major ecological and evolutionary consequences, yet a large variety of sex determination mechanisms exist and we have a poor understanding of how they evolve. Theoretical and empirical work suggest that compensatory adaptations to mutations in genes involved in sex determination may play a role in the evolution of these pathways. Here, we directly address this problem using experimental evolution in *Caenorhabditis elegans* lines fixed for a pair of mutations in two key sex-determining genes that jointly render sex determination temperature-sensitive and cause intersexual (but still weakly to moderately fertile) phenotypes at intermediate temperatures. After 50 generations, evolved lines clearly recovered toward wild-type phenotypes. However, changes in transcript levels of key sex-determining genes in evolved lines cannot explain their partially (or in some cases, nearly completely) rescued phenotypes, implying that wild-type phenotypes can be restored independently of the transcriptional effects of these mutations. Our findings highlight the microevolutionary flexibility of sex determination pathways and suggest that compensatory adaptation to mutations can elicit novel and unpredictable evolutionary trajectories in these pathways, mirroring the phylogenetic diversity, and macroevolutionary dynamics of sex determination mechanisms.

KEY WORDS: **Experimental evolution, sex ratio, *tra-2*.**

Sexual reproduction is taxonomically widespread, and sex determination mechanisms (SDMs) can profoundly impact organismal fitness (e.g., Warner and Shine 2008) and possibly even speciation and extinction (e.g., Mitchell et al. 2008; Organ et al. 2009). One might hypothesize from these observations that purifying selection on sex determination should be strong. It is perhaps surprising, then, that SDMs, despite some deep conservation, appear to evolve at a rapid pace (on both micro- and macroevolutionary levels) and display extreme divergence among species (Haag and Doty 2005; Janzen and Phillips 2006).

A sizable body of theory has considered the selective forces that influence the evolution of SDMs, including microevolutionary dynamics. Most of this theoretical work has focused on the sex ratio (Charnov and Bull 1977; Bulmer and Bull 1982) and conflicts between the sexes (van Doorn and Kirkpatrick 2007; van Doorn 2009) or between parents and offspring (Werren et al. 2002; Uller et al. 2007). Wilkins (1995) proposed that the long, cascading sex determination pathway in *Caenorhabditis elegans* evolved via the successive addition of new upstream regulators favored by frequency-dependent sex ratio selection. Similarly,

Pomiankowski et al. (2004) developed a detailed hypothesis for the evolution of the *Drosophila melanogaster* sex determination hierarchy, based in part on selection to resolve a genomic conflict between the sexes in the expression of sex-specific genes. According to this hypothesis, new upstream sex determination regulators were recruited to reduce the deleterious mis-expression of downstream transcripts that promote the development of the alternate sex (e.g., reducing levels of the female transcript dsx^F in males), although sometimes with fitness costs for the other sex. In light of these ideas, the rapid evolution of sex determination pathways makes sense, given that genomic conflicts can lead to evolutionary "arms races" (e.g., Rice 1998).

Empirical studies yield results consistent with these hypotheses' predictions. For example, the prediction that this conflict will be resolved by the recruitment of new regulators of sex determination is supported by the observation that the upstream portions of sex determination pathways tend to be more divergent among species (e.g., Meise et al. 1998; Saccone et al. 1998; Hasselmann et al. 2008), whereas downstream sex determination and sex differentiation genes are conserved across species (e.g., Hediger et al. 2004; Pane et al. 2005) and even phyla (Zarkower 2001). Another consequence of these models is that interacting sex-determining genes should coevolve because of this intersexual conflict. This prediction, too, enjoys empirical support. For instance, *tra-2* and *fem-3* in *Caenorhabditis* nematodes have coevolved in a species-specific manner (Haag et al. 2002), as have *fem-2* and *fem-3* (Stothard and Pilgrim 2006).

Such coevolutionary patterns could emerge via compensatory adaptations to alterations to the sex determination pathway. For example, Pomiankowski et al. (2004) postulate that some mutations causing increased expression of a sex-specific transcript in one sex will correspondingly mis-express that transcript in the opposite sex. Such alleles, whose existence is supported by empirical evidence in *Drosophila* (Tarone et al. 2005), might have deleterious consequences on the opposite sex, such as partially intersexed phenotypes, leading to selection for compensation. Adaptations compensating for environmental or other nongenetic perturbations could also set this process in motion. Genomic conflicts with *Wolbachia*, an intracellular parasitic bacterium that can feminize genotypic males and cause intersexuality, for instance, may have triggered turnovers in sex chromosomes and sex determination mechanisms in terrestrial isopods (Rigaud et al. 1997). Thus, there is evidence that compensatory dynamics may play an important role in shaping the evolution of SDMs in nature. Even if evolutionary transitions in these pathways are caused by "pseudocompensation"—that is, by successive substitutions of fully cofunctional intermediate alleles rather than a single fixation of a deleterious one (Haag and Molla 2005)—identifying the intermediate alleles in this process is an essential empirical goal for understanding how developmental pathways evolve.

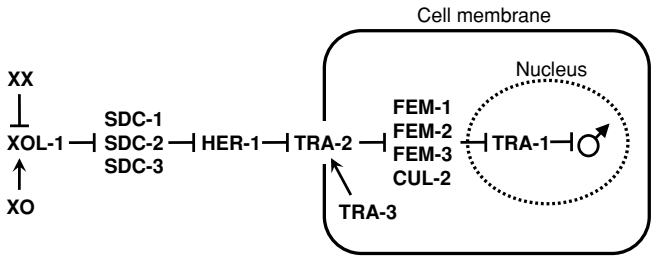

Figure 1. Overview of sex determination in *Caenorhabditis elegans*. Arrows indicate activation; bars indicate inhibitory interactions. Adapted from Ellis (2008).

Here, we use an experimental evolution approach in mutant *C. elegans* populations to provide a direct test of the hypothesis that sex determination mechanisms can evolve via compensatory adaptation, in this case to mutations causing intersexuality. This is an ideal model system because its extremely short generation times make it well suited to experimental evolution, and its sex determination pathway has been thoroughly studied (reviewed in Ellis 2008; Wolff and Zarkower 2008). Sex determination occurs through a negative regulatory cascade, triggered by the ratio of X chromosomes to autosomes (Fig. 1). In XX individuals, the double dose of X chromosomes downregulates XOL-1, and as a consequence, the SDC genes are upregulated, HER-1 is downregulated, and TRA-2 is active. TRA-2, in turn, acts in conjunction with TRA-3 to inhibit the FEM proteins, allowing for activation of TRA-1, leading to hermaphrodite development. (In *C. elegans*, populations consist of hermaphrodites and rare males; hermaphrodites are essentially somatic females that self-fertilize with a limited sperm supply produced early in development, but which can also outcross with males.) In XO worms, however, XOL-1 is upregulated, leading to the alternate activation state for each gene in the pathway and resulting in male development.

Our results indicate that compensatory adaptation can occur quickly, allowing populations to recover before extinction occurs. Surprisingly, although our evolved lines converged toward wild-type phenotypes, transcription levels of key affected sex-determining genes in the evolved lines were not restored to wild-type conditions, suggesting that deleterious phenotypes can easily be ameliorated even while gene expression patterns remain altered.

Materials and Methods

STUDY SPECIES, STRAINS, AND STRAIN CONSTRUCTION

We used strain CB5362 *tra-2*(*ar221*)II; *xol-1*(*y9*)X (Hodgkin 2002; Chandler et al. 2009), as well as strains carrying the same *tra-2* and *xol-1* mutations introgressed into four additional, relatively inbred wild genetic backgrounds (CB4856, AB1, MY2,

and JU258) to enhance genetic diversity. *tra-2(ar221)* is a missense mutation causing leucine to be substituted for proline at amino acid 127 in an extracellular loop of TRA-2A, a transmembrane receptor (Chandler et al. 2009); this mutation is responsible for the masculinization of XX worms at high temperatures. *xol-1(y9)* is a deletion of the entire coding locus (Rhind et al. 1995) that is lethal to XO animals and enhances the masculinization caused by *tra-2(ar221)*. The double-mutant combination, then, results in a temperature-dependent pattern of sex determination: all worms are XX, but they develop primarily as hermaphrodites at cool temperatures (<16°C) and as functional males at warm temperatures (>20°C). Reduced fertility and intersexuality are observed frequently at intermediate temperatures (Chandler et al. 2009). See Chandler (2010) for details of introgression and strain construction.

EXPERIMENTAL EVOLUTION

We evolved experimental populations of temperature-sensitive worms at 16 and 18°C, two temperatures that yield highly intersexed and low-fertility animals in the ancestral strains (Chandler et al. 2009; Chandler 2010), thus imposing selection to improve fertility and fecundity; we then assayed phenotypes and gene expression after 50 generations (Fig. 2). To generate genetically variable populations, we raised the starting strains at 24 and 13°C to produce robust males and hermaphrodites, respectively, ensuring that at least one round of initial outcrossing could occur. From these stocks, we placed 10 hermaphrodites and 12 males of each strain onto a 10-cm petri plate seeded with a lawn of OP50 *Escherichia coli* at each experimental temperature (16 and 18°C). Both mating per se and hermaphrodites with sperm plugs (Hodgkin and Doniach 1997) were observed on the plates at both temperatures (C. H. Chandler, pers. obs.). When the F1 worms reached the L4 stage, the agar on the plate at each temperature was divided into 10 symmetrical "pie-slice" shaped pieces, and each piece placed onto a fresh plate, thus splitting each original five-way "cross" into 10 replicate populations.

Subsequently, we transferred worms to fresh plates when the *E. coli* lawn was exhausted and worms began to starve, roughly once per generation (hundreds of worms transferred at a time), by cutting a fragment of worm media with a flame-sterilized spatula and placing it facedown on the new plate (i.e., "chunking"). Worms remaining on the original plate after chunking each generation were frozen. Chunking intervals were typically approximately four days at 18°C and approximately five days at 16°C. Occasionally, to combat the growth of bacterial and fungal contaminants, we bleached populations following standard protocols (Stiernagle 2006) rather than chunking. The experiment was terminated at the end of generation 50 in each rearing environment.

PHENOTYPIC ASSAYS

We phenotyped the five ancestral strains used to initialize the experimental populations, as well as evolved worms, at six temperatures (13, 16, 18, 20, 22, and 24°C). We chose tail morphology as the somatic sexual trait of interest because the tail is an easily observed sexually dimorphic structure, shows varying degrees of intersexuality in these strains at these temperatures, and has been

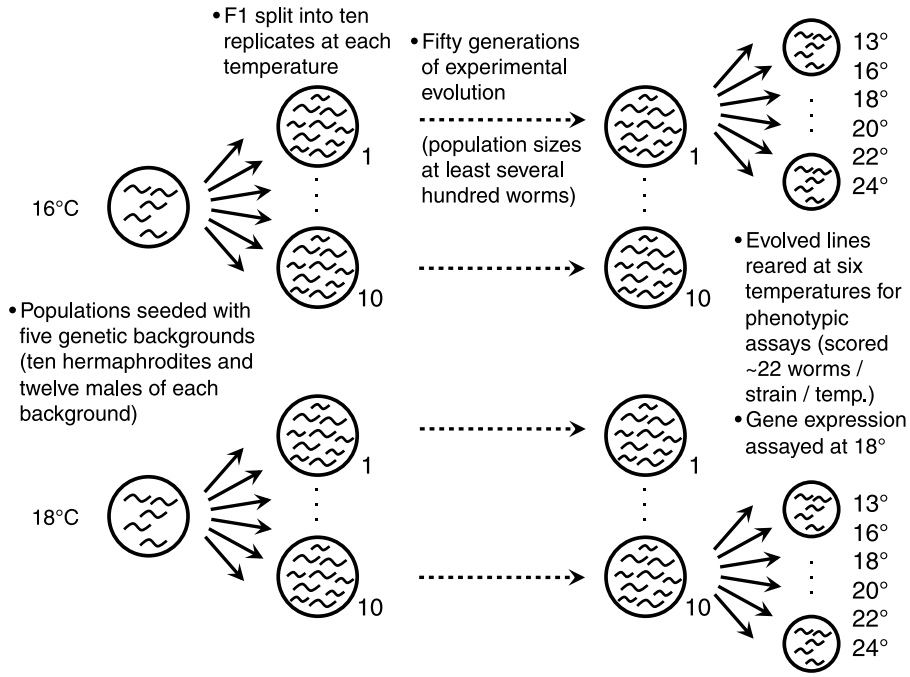

Figure 2. Summary of experimental design.

used successfully to measure variation in sexual differentiation in prior work (Chandler 2010). We also scored hermaphrodites for the presence or absence of eggs/oocytes (hereafter "fertility") because earlier work (Chandler et al. 2009) indicated that the reduction in fitness in the mutant genotype is partially driven by the occurrence of sterile worms. Each ancestral strain or evolved replicate population was bleached, incubated in SBasal buffer overnight at each temperature, and pipetted onto 10-cm plates seeded with OP50 at a density of 100–200 worms/plate. As soon as worms reached adulthood, they were killed by heat shock, fixed in 4% formalin, transferred to glycerol, and mounted onto slides for later phenotypic scoring. Pilot tests indicated that tail structures and eggs are well preserved by this method for at least several weeks (C.H. Chandler, pers. obs.).

We took digital photographs of mounted worms using a Leica (Buffalo Grove, IL) DM2500 camera-equipped microscope, cropped individual worms from images, and scored them blindly with respect to strain, treatment (ancestral or evolved), and rearing temperature. Tail phenotypes were rated on a scale from 1 to 6, 1 indicating a wild-type hermaphrodite, 6 being a wild-type male, and 2–5 designated as varying degrees of intersexuality (Chandler 2010). To obtain sex ratio and fertility data, we scored the gonads as either male, hermaphrodite without eggs, or hermaphrodite with eggs. Only hermaphrodites were considered in fertility analyses, and in sex ratio analyses, both categories of hermaphrodites were pooled together.

To test whether reaction norms for sex ratio, fertility, and tail phenotypes had evolved throughout the course of the experiment, we compared sets of generalized linear mixed models using the deviance information criterion (DIC) to test the effects of temperature, treatment (ancestral vs. each evolved line), and temperature-by-treatment interaction on these traits. All analyses were performed in R version 2.12 (R Development Core Team), with model fitting using the MCMCglmm package, version 2.10 (Hadfield 2010). For fertility and sex ratio, we used mixed logistic regression models, with a fixed residual variance of 1 (MCMCglmm documentation). Formally, the full model we fitted was:

$$\text{Fertility} \sim \mu = \beta_0 + \beta_1\,\text{Temp} + \beta_2\text{Treatment} + \beta_2\text{Temp} \times \text{Treatment}.$$

We tested different random effect structures and found that including a random effect of line (i.e., strain) on the slope of temperature (i.e., accounting for among-strain variance in the relationship between temperature and fertility) best explained the observed data:

$$\beta_1 \sim N\left(\mu = 0, {\sigma_{\text{line}}}^2\right).$$

Fertility and sex ratio models used a logit link function on the response variable, because these were scored as binary traits (fertile/sterile or male/female). The prior for the random effect was $\sim\text{IW}(V = 1, \nu = 0.002)$, and for the fixed effects, $\sim N(\mu = 0, \sigma^2 = 1 + \pi^2/3)$, which is relatively flat on the probability scale, and thus uninformative (in other words, the model fitting procedure does not have a priori information about the expected values of the parameters, and thus, parameter estimates are largely derived from the current experimental data). To test the effects of temperature, treatment, and their interaction on tail scores, we fitted similar models but used an ordinal response variable (because tail scores can take on integer values between one and six), fixing the residual variance of the latent variable at 1. The ordinal tail models used the same prior for the random effects as the fertility and sex ratio models. However, for the fixed effects, we used a normal prior with a variance of 10^8 because of the different scale of the response variable; this high variance on the prior ensures that it is uninformative (and, again, that our conclusions are therefore driven by the data rather than the prior). For all analyses, we ran chains for 3.3×10^5 iterations, with a burn-in of 3×10^4 iterations and a thinning interval of 100, for a total posterior sample size of 3×10^3. Results were similar across a range of different priors, as well as when bootstrapped (with model fitting performed by restricted maximum likelihood).

RT-POLYMERASE CHAIN REACTION (PCR)

We sought to answer four questions regarding the expression levels of five key sex-determining genes (*her-1*, *tra-2*, *tra-3*, *fem-3*, and *tra-1*): (1) is there genetic variation among wild isolates for transcript levels of these genes?; (2) how does this pair of mutations alter these genes' expression levels?; (3) did expression patterns evolve during this experiment?; and (4) do expression patterns follow the patterns predicted by sexual and somatic phenotypes (e.g., higher levels of genes involved in male development in lines with more masculinized phenotypes, or restoration of wild-type transcript levels in evolved lines)? In particular, we expected to observe the strongest differences for *her-1*, because this gene shows strong differences between the sexes in its transcript levels (Trent et al. 1991). There is also evidence for sex-specific differences in *tra-2* transcript levels (Okkema and Kimble 1991), but other types of regulation are also critical for its gene function (Wolff and Zarkower 2008). Finally, although transcriptional control does not appear to be the primary mode of regulation for *fem-3*, *tra-1*, and *tra-3* in wild-type worms (Ahringer et al. 1992; Barnes and Hodgkin 1996; Starostina et al. 2007), we nonetheless chose to assay them due to their importance in sex determination.

We selected six evolved lines (three from 16°C and three from 18°C) showing variable degrees of evolutionary change in tail phenotypes for gene expression analysis, along with wild-type and ancestral mutant lines. To obtain RNA samples (three biological replicates per line), we bleached populations of worms that had been reared at 13°C, incubated the eggs overnight at 18°C in

SBasal buffer, and plated the resulting starved L1 worms approximately 24 h after bleaching. Although we did not specifically control male frequencies in the parent wild-type populations that we bleached, our observations revealed them to be negligible (<5%); thus, we believe that differences in transcript levels among their offspring reflect heritable variation in expression rather than differences in sex ratio. After 48 h of rearing at 18°C (approximately late L3 stage, near the end of the thermosensitive period; Chandler et al. 2009), worms were rinsed from plates in water, washed several times to remove excess *E. coli*, and transferred to RNAlater® (Ambion). RNA was extracted using a MagMax™-96 Total RNA Isolation Kit (Ambion) following the manufacturer's instructions.

We designed RT-PCR primers for our five target sex-determining genes and used *cdc-42* as a reference/control gene (Hoogewijs et al. 2008). See Supporting information Text S1 and Table S1 for experimental details. For each target gene, we computed ΔCT, a relative measure of expression levels, by subtracting the threshold cycle value (CT) for the target gene from the corresponding CT value for the control gene for the same sample (Yuan et al. 2006). All amplification efficiencies were >1.9 (indicating a near-doubling of the amount of product with each PCR cycle).

We tested for differences in ΔCT values between evolved and ancestral genotypes using linear mixed models. First, to test whether the *tra-2* and *xol-1* mutations jointly affect the expression of each gene and, if so, whether their effects depend on the genetic background in which they occur, we considered only the wild-type and ancestral mutant lines, and fitted models with genotype, genetic background, and the genotype-by-background interaction as fixed effects:

$$\Delta\text{CT} \sim N(\mu = \beta_0 + \beta_1 \text{Genotype} + \beta_2 \text{Background} + \beta_3 \text{Genotype} \times \text{Background}, \sigma^2).$$

Next, to test whether relative transcript levels differed between wild-type, ancestral mutant, and evolved mutant worms, we fitted models using treatment (wild-type, ancestral mutant, or evolved mutant) as a fixed effect, and line as a random effect:

$$\Delta\text{CT} \sim N(\mu = \beta_0 + \beta_1 \text{Treatment}, \sigma^2)$$

$$\beta_0 \sim N(\mu = 0, {\sigma_{\text{line}}}^2).$$

For all of these models, we used the default MCMCglmm priors (normal distribution with mean 0 and variance 10^{10} for fixed effects, and inverse Wishart with $V = 1$ and $\nu = 0$ for random effects, chosen to be uninformative), running chains for a total of 4.1×10^5 generations with a burn-in of 10^4 and thinning interval of 20 iterations. Finally, to examine qualitatively whether evolved lines are more similar to wild-type worms or ancestral mutant worms in their overall gene expression profiles (or different from both), we performed linear discriminant function analysis using ΔCT values for each gene for the wild-type and ancestral mutant lines. We then applied those loadings to the ΔCT values from the evolved lines to see whether they clustered more closely with wild-type worms or ancestral mutant worms. We also performed a second discriminant analysis including all three treatment groups. These analyses were performed using the lda function in the MASS package in R.

Results

All data are available from the Dryad data repository (doi:10.5061/dryad.cg872).

ANCESTRAL AND EVOLVED PHENOTYPES

No extinctions of any replicate lines occurred throughout the entire experiment. Instead, we observed a shift toward more strongly hermaphrodite-biased sex ratios, an increase in hermaphrodite fertility rates, and a shift toward more natural hermaphrodite-like tails (Figs. 3 and 4). In a few evolved lines, reaction norms for tail morphology were almost completely flat, suggesting the rapid evolution of thermal insensitivity in these cases; however, there were no detectable differences between lines evolved at 16 and 18°C, so they were treated identically in all analyses. The observed trends were supported by statistical analysis. For sex ratio, models including effects of temperature and treatment (ancestral vs. each evolved line), and effects of temperature and a temperature-by-treatment interaction were virtually indistinguishable from one another (ΔDIC = 0.52), but substantially outperformed a null model assuming a common reaction norm between ancestral and evolved lines (ΔDIC >30), and nearly all lines individually differed from the ancestral population (Table S2). Similarly, variation in hermaphrodite fertility was best explained by a model including temperature, treatment, and a temperature-by-treatment interaction (ΔDIC to second-best model = 5.3; ΔDIC to null model assuming no among-line variation = 25.5). Variation in tail phenotypes was best explained by a model including temperature and a temperature-by-treatment interaction (ΔDIC 37.4), meaning that evolved lines differed from the ancestral population in the slopes of their reaction norms for tail morphology.

GENE EXPRESSION

We found strong evidence of variation in *her-1* and *fem-3* transcript levels among wild isolates (i.e., a significant genetic background term; ΔDIC >25), but weak or no evidence for such variation within *tra-2*, *tra-3*, and *tra-1* (ΔDIC between models <3; Table 1). The *xol-1* and *tra-2* mutations jointly affected transcript levels of *her-1*, *fem-3*, and *tra-1*, with little to no support for such an effect for *tra-2* and *tra-3* (ΔDIC between models <3; Table 1). On average, mutant worms displayed decreased expression of

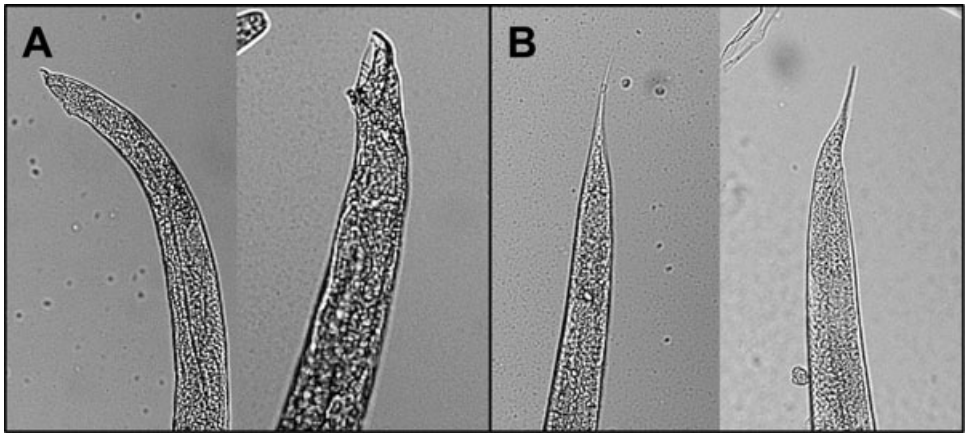

Figure 3. Examples of typical tail phenotypes seen in (A) ancestral mutant worm strains reared at 16 and 18°C, and (B) evolved mutant worm strains reared at 16 and 18°C.

Table 1. DIC scores of models testing the effects of genotype (wild-type or *tra-2(ar221)*II;*xol-1(y9)*X), genetic background (N2, CB4856, MY2, AB1, or JU258), and their interaction on relative expression levels (ΔCT) of five core sex determination genes. Asterisks indicate the best-fitting model for each gene.

Gene	Genotype + background + interaction	Genotype + background	Genotype	Background	Null
her-1	30.6*	56.3	83.5	76.4	90.1
tra-2	29.2	32.0	27.2*	34.5	29.4
tra-3	7.38	8.06	6.83	6.13	5.00*
fem-3	13.2*	68.6	76.4	74.3	79.1
tra-1	−3.00	−7.66*	−5.24	11.2	7.73

her-1 and increased expression of *fem-3* and *tra-1* (Tables S5–S9). However, for *her-1* and *fem-3*, the presence of an interaction between genotype and genetic background influencing transcript levels (Tables 1, S5–S9; Fig. 5) indicates that the effects of these mutations depend on the genetic background in which they occur. Finally, there was no strong evidence of an evolutionary response in any of the tested genes. However, there was weak evidence of an evolutionary increase in *tra-2* levels: the full model performed slightly better (ΔDIC <2) than a null model assuming equal expression levels across treatment groups (wild-type, ancestral, and evolved), and the difference between mean ancestral and evolved transcript levels in the full model was near the significance threshold (P <0.1). Models considering only the

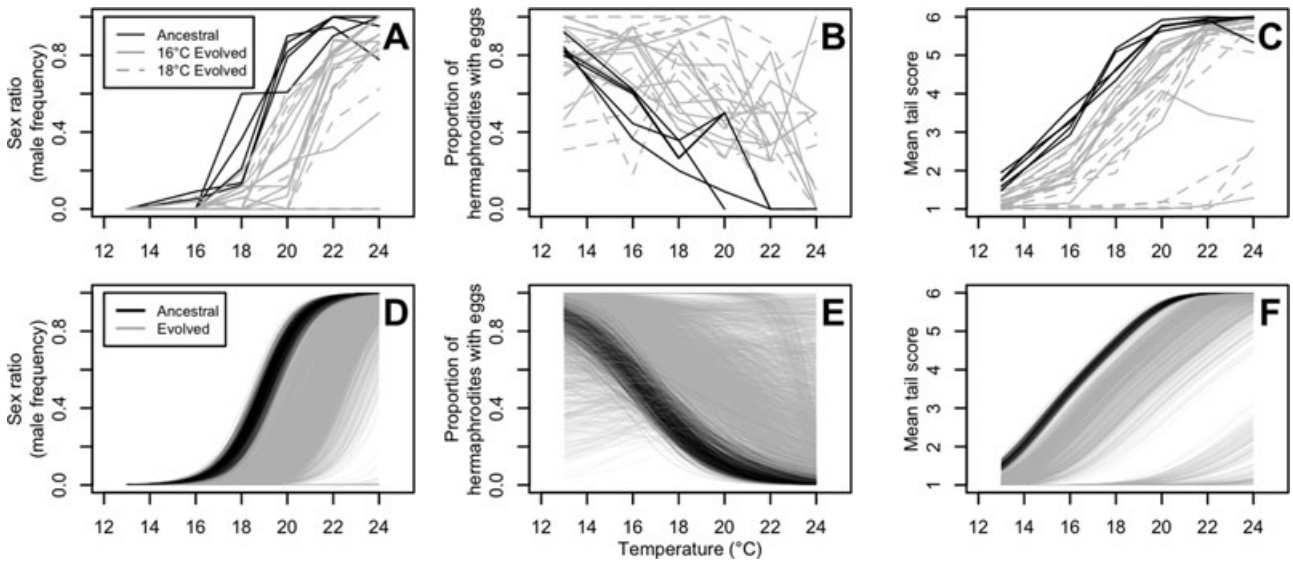

Figure 4. Thermal reaction norms for ancestral and evolved mutant lines, showing the relationship between temperature and (A) sex ratio expressed as the frequency of males in the population, (B) the proportion of hermaphrodites carrying visible eggs/oocytes, and (C) the mean tail score. (D–F) Plots of the corresponding statistical models fitted to the data. Line densities represent the posterior probabilities of parameter values. For clarity, all evolved lines are depicted in the same color, but each evolved line was modeled as a separate treatment level. Ancestral lines refer to the five wild-type strains into which the mutations were introgressed.

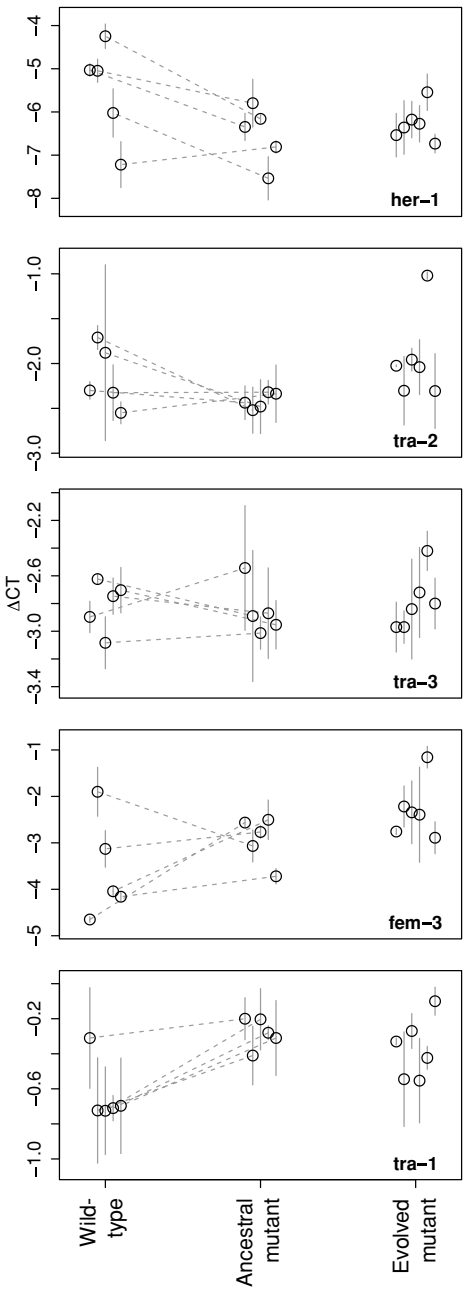

Figure 5. **Relative transcript levels (ΔCT values) for *her-1*, *tra-2*, *tra-3*, *fem-3*, and *tra-1* in five different wild-type strains, mutant lines with the same wild genetic backgrounds into which the *tra-2(ar221)* and *xol-1(y9)* mutations were introgressed, and six different experimental evolution lines. Dashed lines connect wild-type and ancestral mutant lines with the same genetic background. Points represent line means ± two standard errors.**

differences between ancestral and evolved mutants also yielded similar results.

Considering transcript levels across all genes simultaneously, discriminant function analysis revealed that overall expression patterns of evolved lines are qualitatively more similar to ancestral mutant lines than to wild-type lines (Fig. 6). The treatment groups were all distinguishable from one another in pairwise multivariate analysis of variances (wild-type vs. ancestral mutant: $P = 1.5 \times 10^{-4}$; wild-type vs. evolved mutant: $P = 6.1 \times 10^{-6}$; ancestral vs. evolved: $P = 0.038$). However, even though evolved mutant worms were phenotypically similar to wild-type worms and dissimilar to ancestral mutant worms, we found the opposite pattern in distances between treatment groups in gene expression "space" (Mahalanobis distance between evolved mutant and wild-types: 3.89; evolved mutant and ancestral mutant: 1.59; wild-type and ancestral mutant: 4.07). Thus, although morphological phenotypes of evolved lines converged on those exhibited by wild-type worms, expression patterns of targeted genes did not evolve concordantly in those same lines.

Discussion

Sex determination mechanisms (SDMs) display abundant phylogenetic diversity and evolve rapidly. Although there is excellent theory aiming to explain this diversity, empirically addressing this problem at the microevolutionary level is much more difficult because we have relatively few examples of functional polymorphisms in SDMs in nature. In this study, we circumvented this problem by using experimental evolution in an engineered laboratory model system, specifically temperature-sensitive mutant strains of *C. elegans*, to investigate how compensatory adaptations to mutations alter SDMs on microevolutionary time scales.

SEXUAL PHENOTYPES

These *C. elegans* populations fixed for a pair of mutations in *tra-2* and *xol-1* initially displayed low-fertility rates and high levels of intersexuality in tail morphology, a sexually dimorphic somatic structure, at the two experimental temperatures. However, within just 50 generations of laboratory evolution, we observed a clear recovery of more wild-type like somatic sexual phenotypes, more hermaphrodite-biased sex ratios, and an increase in the frequency of worms carrying clearly visible oocytes/eggs. Although each population experienced only a single temperature during the experiment, phenotypes were "improved" at all temperatures, and the effect of temperature (i.e., slope) differed in the evolved lines, suggesting a loss or dampening of the novel phenotypic plasticity originally induced by this pair of mutations. Finally, the phenotypic changes observed here cannot be explained easily by changes in overall transcript levels of candidate sex-determining genes, suggesting that compensatory adaptation to sex determination mutations can alter these pathways in unpredictable ways.

The magnitude of the evolutionary response is striking. Although Chandler (2010) identified variation among these wild genetic backgrounds in the joint phenotypic effects of these mutations, the phenotypes of the evolved lines generally exceed the

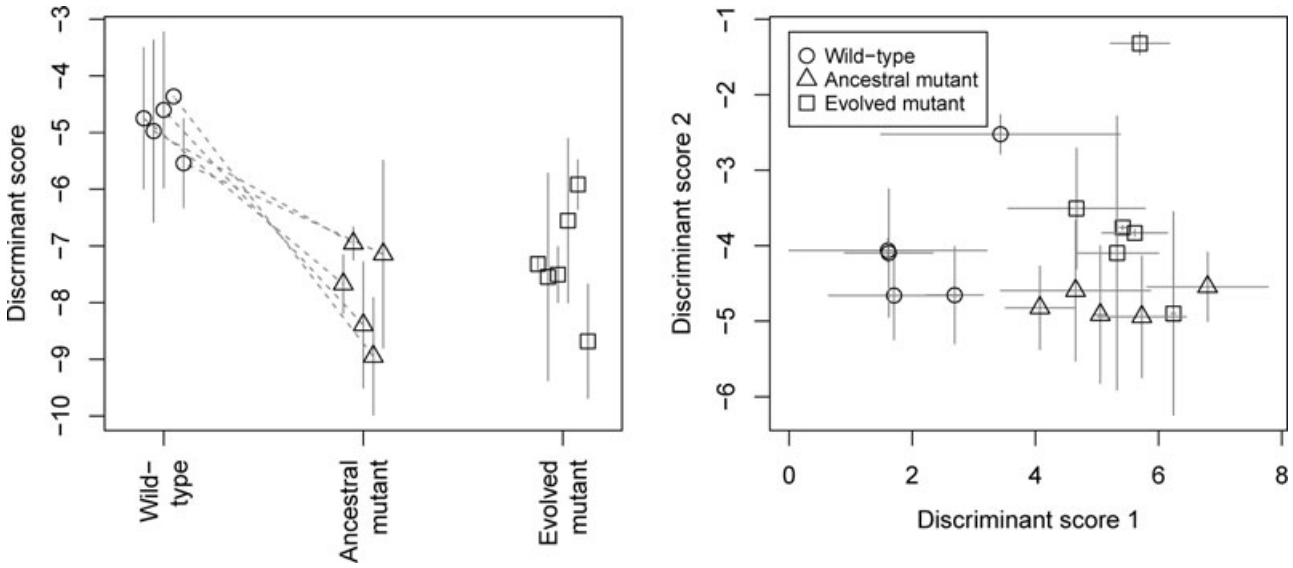

Figure 6. Results from discriminant function analyses of relative gene expression levels (ΔCT values) of sex-determining genes (*her-1*, *tra-2*, *tra-3*, *fem-3*, and *tra-1*) show that evolved mutant lines are qualitatively similar to ancestral mutant lines in overall gene expression profiles, even though their morphological phenotypes have become more like wild-type worms. (A) Loadings from a discriminant function analysis of just wild-type and ancestral mutant worm lines were applied to gene expression values of wild-type, ancestral, and evolved mutant worms to obtain discriminant scores. Dashed lines connect wild-type and ancestral mutant lines with the same genetic background. (B) Scores from a discriminant function analysis of all three treatment groups. Points represent line means ± two standard errors.

range of variation documented in the ancestral mutant lines and the unselected recombinant inbred lines from that study. The variability among the evolved lines, both in morphological phenotypes and in gene expression, is also notable. In other similar studies, compensatory adaptation is often repeatable among experimental replicates, including in the genes and pathways responsible for adaptation, even when they depend on parallel new mutations because they are initialized with isogenic populations (e.g., Stoebel et al. 2009; Charusanti et al. 2010; Denver et al. 2010; but see Estes et al. 2011). A few of our lines, in contrast, displayed a much stronger response than others, having almost completely flat reaction norms for tail morphology, and producing quite fertile hermaphrodites even at 24°C, which was never observed in any ancestral lines. (We are confident that this sharp change in a few lines is not due to contamination by wild-type worms or reversion mutations: we took great care to isolate experimental evolution lines from wild-type strains in our incubators and during transfers, and minor increases in tail intersexuality at the warmest temperatures confirm that the original mutations are still present. In addition, reversions in *xol-1* are extremely unlikely, because the *y9* allele is a deletion of the entire coding sequence.)

At present, we cannot be certain whether the observed changes are due to the sorting of preexisting alleles or to new mutations. The swift evolutionary response implies that standing variation in the ancestral populations likely played a role, and the existence of alleles capable of modifying these mutations' effects in ancestral populations is consistent with the results of Chandler (2010). Such preexisting, cryptic alleles may also represent examples of the multifunctional intermediate alleles necessary for the process of "pseudo-compensatory" evolution hypothesized by others (Haag and Molla 2005; Haag 2007). Note, however, that none of the five ancestral strains used to initialize the genetically variable experimental evolution populations displayed strongly rescued phenotypes. Therefore, if standing variation contributed to the compensatory response, this response must have relied on a synergistic interaction between preexisting alleles initially present in different genetic backgrounds, or at least transgressive segregation of additive alleles, of which evidence was also found by Chandler (2010).

Although a few populations displayed nearly complete phenotypic recovery, the majority exhibited more modest gains. The evolutionary response in the latter may have been limited by the amount of initial genetic variation, because populations were seeded with only five genetic backgrounds. However, natural variation in the *C. elegans* sex determination genes surveyed to date has been found to be extremely low (Graustein et al. 2002, Haag and Ackerman 2005), so starting with a larger pool of wild-type genetic backgrounds may not alter outcomes substantially. New mutations, on the other hand, may have been important in the lines showing extreme phenotypic rescue. Whole-genome resequencing of ancestral and evolved lines could help identify any such mutations.

GENE EXPRESSION

Compared to wild-type worms, the mutant lines used to establish the experimental evolution populations initially displayed altered expression patterns of these sex-determining genes, as a whole and for individual genes (Tables 1 and 2, Figs. 5 and 6). For example, *tra-1* transcript levels were increased in mutant worms, even though *tra-1* transcript abundance is similar between the sexes in wild-type worms and its activity is normally regulated in other ways (Zarkower and Hodgkin 1992; Starostina et al. 2007). Moreover, there was strong evidence that a background-by-genotype interaction influenced transcript levels in *her-1* and *fem-3*, suggesting that there is segregating variation capable of modifying these mutations' downstream effects on transcript levels of other genes. In addition, there is heritable variation for gene expression in *C. elegans*, both genome-wide (Li et al. 2006; Rockman et al. 2010), and for at least some of these sex-determining genes (Table 1 and Fig. 5). Thus, it was plausible that changes in transcript levels could account for the observed phenotypic changes, especially given that coding sequence polymorphism in *C. elegans* is relatively low (Graustein et al. 2002; Denver et al. 2003; Cutter 2006). For example, if these mutations alter sex determination at least in part by disrupting the transcription of other genes, then restoring transcripts to wild-type levels might ameliorate sexual phenotypes. In addition, *tra-2(ar221)* is a putative temperature-sensitive hypomorph; thus, we also hypothesized that evolved lines might partially compensate for the reduced activity of this *tra-2* allele by upregulating it or its cofactors, or by downregulating its antagonists.

However, none of these hypotheses were supported. First, transcript levels of the targeted sex-determining genes were not always altered by the mutations in the expected direction, meaning that evolutionarily downregulating male-promoting genes or upregulating female-promoting genes might not rescue the mutations' jointly masculinizing effects. For example, the ancestral mutant worms displayed lower levels of *her-1*, which is involved in male somatic development, and higher levels of *tra-1*, involved in female somatic development, than wild-type worms, in spite of their masculinized phenotypes.

Second, we found only limited evidence for evolutionary changes in the expression of just a single gene, *tra-2*, suggesting a slight increase from the ancestral levels, as predicted (Table 2). However, even this result may be driven partially by one evolved line displaying extreme divergence in *tra-2* expression (Fig. 5). In addition, outcomes were variable across evolved lines, and there was no apparent association between sexual phenotypes and gene expression levels.

Looking at the expression patterns of these five genes as a whole, wild-type and ancestral mutant worms are readily distinguishable by discriminant function analysis, but evolved lines are clearly more similar to the ancestral mutant lines than to wild-types (Fig. 6). Thus, the presence of these mutations in *tra-2* and *xol-1* clearly alters overall expression profiles of the targeted sex-determining genes, but expression profiles have not been restored to wild-type states in the evolved lines. Instead, wild-type like phenotypes can be produced even in spite of mutant-like gene expression patterns. In fact, this independence between gene expression and somatic phenotype may explain some of the among-strain variation in transcript levels in wild-type worms, if it allows expression levels to evolve neutrally, at least within a certain range. Combined, all these results imply that transcript levels of these sex-determining genes cannot explain the restoration of more wild-type phenotypes, with the caveat, of course, that we only assayed expression at one time point in whole worms. Several alternative hypotheses may explain our findings. First, sex determination in *C. elegans* involves regulation at many levels (reviewed in Wolff and Zarkower 2008), so changes in translation rates or protein stability, trafficking, or modification, for example, might also account for the compensatory response. In addition, changes in other genes might be partly responsible. Indeed, Chandler (2010) found that at least some quantitative trait loci (QTLs) responsible for variation in these mutations' effects in the N2 and CB4856 backgrounds mapped to loci without any known

Table 2. DIC scores of models testing for differences in relative expression levels (ΔCT) of each gene between evolved mutant, ancestral mutant, and wild-type worms, to test whether there has been an evolutionary change in expression levels, and if so, whether the evolved levels are also different from wild-type levels; and 95% highest posterior density confidence intervals for parameter estimates from full models assuming fixed effects of treatment (wild-type, ancestral mutant, or evolved mutant).

Gene	DIC_{Full}	DIC_{Null}	Intercept (evolved mutant mean) HPD	Ancestral mutant effect HPD	Wild-type effect HPD
her-1	61.3	61.5	−6.59 to −5.93***	−1.13 to 0.575	−0.665 to 2.22
tra-2	35.1	36.7	−2.42 to −1.45***	−0.979 to −0.041†	−0.766 to 0.407
tra-3	9.90	6.75	−2.91 to −2.64***	−0.275 to 0.122	−0.226 to 0.173
fem-3	60.5	59.2	−2.88 to −1.67***	−1.45 to 0.164	−2.70 to 0.172†
tra-1	−11.6	−11.9	−0.501 to −0.272***	−0.067 to 0.254	−0.430 to −0.066*

***$P<0.001$; **$P<0.01$; *$P<0.05$; †$P<0.1$.

sex-determining genes. Perhaps changes at other such loci have initiated the evolution of a novel sex determination state, rendering careful control over the expression of our targeted sex-determining genes less critical to achieving successful hermaphrodite development. Nevertheless, the observation that most lines at least partly recovered phenotypes in this relatively short time frame highlights the microevolutionary flexibility of SDMs, consistent with results of directed genetic manipulations in them (Hodgkin 2002).

Conclusions

It is tempting to speculate that temperature-sensitive mutations, such as *tra-2(ar221)*, might facilitate an evolutionary transition to temperature-dependent sex determination. Indeed, environmental sex determination is known in some nematode groups (Blackmore and Charnov 1989; Schouten 1994). At face value, the reduction in fitness accompanied by these mutations (Chandler et al. 2009) suggests that they would be purged quickly from natural populations, but our results show that those fitness effects could be ameliorated relatively swiftly. However, in this case, compensatory evolution actually led to a dampening, and in some cases, a nearly complete loss, of this temperature sensitivity, in favor of universally more hermaphrodite-like phenotypes. This result suggests that hermaphrodites could only "improve" at the expense of male somatic function (male germline function is still necessary for hermaphrodites to produce sperm), indeed supporting the idea of a genomic conflict between the sexes. Moreover, selection to maintain outcrossing and males (Anderson et al. 2010) was, in this case, probably weaker than selection to recover from these mutations, thus leading to the resolution of this conflict in favor of hermaphrodites. It would be interesting to test whether similar outcomes would be observed in an obligate outcrossing species, constrained to preserve both female and male somatic functions.

Regardless, our *C. elegans* lines demonstrate that organisms can accommodate deleterious developmental mutations on relatively short time scales, at least once those mutations are fixed, supporting a potential role for compensatory adaptation in the evolution of sex determination mechanisms. In addition, the genetic and genomic mechanisms underlying these changes may not be as predictable or repeatable as one might expect. Future research to identify the causal loci and determine the relative contributions of preexisting variants and new mutations, as well as examine the rate of evolutionary change, will help elucidate how compensatory adaptation influences the microevolution of sex determination pathways. In turn, such mechanistic work might inform our understanding of the diverse phylogenetic patterns and extensive macroevolutionary dynamism of SDMs.

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Supporting Information

The following supporting information is available for this article:

Table S1. Primer sequences for RT-PCR reactions.
Table S2. Parameter estimates of binary models testing for differences between ancestral and evolved lines in sex ratios.
Table S3. Parameter estimates of binary models testing for differences between ancestral and evolved lines in the frequencies of hermaphrodites carrying visible eggs/oocytes.
Table S4. Parameter estimates of ordinal models testing for differences between ancestral and evolved lines in tail phenotypes.
Table S5. Parameter estimates (mean and 95% highest posterior density confidence interval) and *P*-values from the best-fitting model predicting relative expression levels of *her-1* in wild-type and ancestral mutant lines (CT = Intercept + Genotype + Background + Interaction + *e*).
Table S6. Parameter estimates (mean and 95% highest posterior density confidence interval) and *P*-values from the best-fitting model predicting relative expression levels of *tra-2* in wild-type and ancestral mutant lines (CT = Intercept + Genotype + *e*).
Table S7. Parameter estimates (mean and 95% highest posterior density confidence interval) and *P*-values from the best-fitting model predicting relative expression levels of *tra-3* in wild-type and ancestral mutant lines (CT = Intercept + *e*).
Table S8. Parameter estimates (mean and 95% highest posterior density confidence interval) and *P*-values from the best-fitting model predicting relative expression levels of *fem-3* in wild-type and ancestral mutant lines (CT = Intercept + Genotype + Background + Interaction + *e*).
Table S9. Parameter estimates (mean and 95% highest posterior density confidence interval) and *P*-values from the best-fitting model predicting relative expression levels of *tra-1* in wild-type and ancestral mutant lines (CT = Intercept + Genotype + Background + *e*).

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