Fitness Assays Reveal Incomplete Functional Redundancy of the HoxA1 and HoxB1 Paralogs of Mice

James S. Ruff,* Raed B. Saffarini,* Leda L. Ramoz,* Linda C. Morrison,* Shambralyn Baker,* Sean M. Laverty,† Petr Tvrdik,‡ and Wayne K. Potts*,[1]

*Department of Biology and ‡Department of Human Genetics, University of Utah, Salt Lake City, Utah 84112, and †Department of Mathematics and Statistics, University of Central Oklahoma, Edmond, Oklahoma 73034

ABSTRACT Gene targeting techniques have led to the phenotypic characterization of numerous genes; however, many genes show minimal to no phenotypic consequences when disrupted, despite many having highly conserved sequences. The standard explanation for these findings is functional redundancy. A competing hypothesis is that these genes have important ecological functions in natural environments that are not needed under laboratory settings. Here we discriminate between these hypotheses by competing mice (*Mus musculus*) whose *Hoxb1* gene has been replaced by *Hoxa1*, its highly conserved paralog, against matched wild-type controls in seminatural enclosures. This $Hoxb1^{A1}$ swap was reported as a genetic manipulation resulting in no discernible embryonic or physiological phenotype under standard laboratory tests. We observed a transient decline in first litter size for $Hoxb1^{A1}$ homozygous mice in breeding cages, but their fitness was consistently and more dramatically reduced when competing against controls within seminatural populations. Specifically, males homozygous for the $Hoxb1^{A1}$ swap acquired 10.6% fewer territories and the frequency of the $Hoxb1^{A1}$ allele decreased from 0.500 in population founders to 0.419 in their offspring. The decrease in $Hoxb1^{A1}$ frequency corresponded with a deficiency of both $Hoxb1^{A1}$ homozygous and heterozygous offspring. These data suggest that *Hoxb1* and *Hoxa1* are more phenotypically divergent than previously reported and support that sub- and/or neofunctionalization has occurred in these paralogous genes leading to a divergence of gene function and incomplete redundancy. Furthermore, this study highlights the importance of obtaining fitness measures of mutants in ecologically relevant conditions to better understand gene function and evolution.

KEYWORDS fitness assay; functional redundancy; *Hoxa1*; *Hoxb1*; intraspecific competition; subfunctionalization

GENE targeting techniques have led to the phenotypic characterization of thousands of genes across eukaryotes (for reviews see Thorneycroft *et al.* 2001; Capecchi 2005; Collins *et al.* 2007) and this characterization continues as this invaluable technology develops (*e.g.*, Meyer *et al.* 2012; Hsu *et al.* 2014). However, an estimated 10–15% of mouse genes show minimal to no phenotypic consequences when disrupted (mouse appears normal), despite many having highly conserved sequences (Barbaric *et al.* 2007). One explanation for these findings is functional redundancy—genes throughout the genome, typically paralogs of disrupted genes, code for the same, or at least overlapping, functions (Nowak *et al.* 1997; Kafri *et al.* 2009). A competing explanation for "no phenotype" gene disruptions is that these genes have important ecological functions in natural environments that are not needed, or are of minimal importance, within laboratory settings. Here we discriminate between these hypotheses by using mice that have experienced a manipulation previously reported to have no embryonic or physiological phenotype, wherein the coding sequence of the *Hoxb1* gene has been replaced by that of its paralog *Hoxa1* (Tvrdik and Capecchi 2006).

The traditional explanation for why redundant genes cannot be maintained over evolutionary time is because accumulation of degenerative mutations, leading to nonfunctionalization, will occur within the genome (Ohno 1970). However, incomplete or partial redundancy could result through several mechanisms: convergent evolution of unrelated genes, recent duplications that have not accumulated enough mutations to

doi: 10.1534/genetics.115.178079
Manuscript received May 19, 2015; accepted for publication July 28, 2015
Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.178079/-/DC1.
[1]Corresponding author: Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112. E-mail: Potts@biology.utah.edu

be completely nonfunctional, duplicates that have taken on new, but similar, functions through neofunctionalization, and through a process known as subfunctionalization (for reviews see Prince and Pickett 2002; Innan and Kondrashov 2010). Subfunctionalization mediated through the duplication–degeneration–complementation (DDC) model, which predicts that degenerative mutations in regulatory elements increase duplicate gene preservation by partitioning ancestral functions, has been invoked as the most likely explanation for the maintenance of paralogous *Hox* genes and experiments demonstrating functional redundancy between *Hox* mutants have been used to support the DDC model (Force *et al.* 1999). *Hox* genes encode proteins that act as transcription factors for cellular specification and have undergone two duplication events in tetrapods from the ancestral chordate state (Gehring and Hiromi 1986; Levine and Hoey 1988; Manley and Capecchi 1997, 1998; Chen *et al.* 1998; Chen and Capecchi 1999; Manzanares *et al.* 2000). *Hox* genes are phylogenetically conserved, especially regarding their collinear order and the DNA-binding homeodomains of their proteins (McGinnis and Krumlauf 1992; Lutz *et al.* 1996; Rijli and Chambon 1997).

Hoxa1 and *Hoxb1* are involved in the patterning of the brainstem. At the open neural tube stage, both genes display a similar expression pattern, encompassing the posterior and central hindbrain. At later stages [embryonic day 9.5 (E9.5) in the mouse], the expression of *Hoxa1* declines, while *Hoxb1* becomes strongly activated in the central segment of the developing hindbrain, the rhombomere 4 (Tvrdik and Capecchi 2006). This activation is dependent on an autoregulatory loop resulting from binding of the HoxB1 protein to its own unique enhancer (Popperl *et al.* 1995). *Hoxb1* expression in r4 persists until E13 and modulates neurogenesis in this segment. In the *Hoxa1* mutant mouse, expression of *Hoxb1* and other downstream genes are altered, the brainstem respiratory circuits are malformed, and *Hoxa1* newborn mutants die of apnea (del Toro *et al.* 2001; Tvrdik and Capecchi 2006). The *Hoxb1* mutant, on the other hand, is viable but displays facial paralysis due to the absence of the seventh cranial nerve originating from rhombomere 4 (Goddard *et al.* 1996). In humans, homozygous missense mutations in *HOXB1* cause bilateral facial palsy, hearing loss, and strabismus, correlating extensively with the mouse *Hoxb1* null phenotype (Webb *et al.* 2012). Homozygous *HOXA1* mutations, which have occurred in several human populations, are viable but cause either Bosley-Salih-Alorainy syndrome or Athabascan brainstem dysgenesis syndrome (Bosley *et al.* 2008; Bertrand *et al.* 2011). Surprisingly, homozygous $Hoxb1^{A1}$ swapped mice (*i.e.*, mice expressing HoxA1 protein from both *Hoxb1* alleles, with no expression of HoxB1) show no detectable phenotypic change relative to wild type under laboratory conditions despite a 15% amino acid sequence difference at the homeodomains and a mere 49% identity overall (Remacle *et al.* 2004; Tvrdik and Capecchi 2006). In hemizygous animals, which express only one $Hoxb1^{A1}$ swapped allele over *Hoxb1* null, fewer facial motor neurons are generated, resulting in hypomorphism of the seventh cranial nerve. However, the homozygous $Hoxb1^{A1/A1}$ swaps never displayed facial paralysis and the allele segregated normally in the laboratory population (Tvrdik and Capecchi 2006). Thus, the laboratory phenotypic assessment suggested that if expressed at sufficient levels, either protein could correctly execute the developmental program carried out by the other paralog.

To determine if mice homozygous for the $Hoxb1^{A1}$ swap suffer from cryptic negative phenotypes we utilized organismal performance assays (OPAs). Within OPAs, treatment and control mice compete directly for resources, territories, and mates under seminatural conditions. Mouse fitness is largely based upon intraspecific competition and can be measured directly in terms of reproductive success or indirectly through key fitness components such as survival and competitive ability. OPAs have previously been used to detect and quantify fitness costs of both cousin- and sibling-level inbreeding, the cost of bearing a selfish genetic element (*t complex*), and health consequences of added sugar consumption and pharmaceutical exposure (Meagher *et al.* 2000; Carroll *et al.* 2004; Ilmonen *et al.* 2008; Ruff *et al.* 2013, 2015; Gaukler *et al.* 2015). In all cases, OPAs revealed major fitness deficiencies that analyses with conventional, laboratory-based methods failed to detect.

Here we use OPAs to test if mice homozygous for $Hoxb1^{A1}$ express adverse phenotypes relative to matched, wild-type controls. OPA endpoint measures include survival, male competitive ability, and reproductive success (measured in terms of both allelic frequencies and genotypic counts of offspring born within OPA enclosures). Corresponding laboratory measures of reproductive success are assessed by comparing litter sizes from $Hoxb1^{A1}$ homozygotes and heterozygotes to genetically matched wild-type controls and by analyzing genotypic frequencies of offspring produced in $Hoxb1^{A1}$ heterozygous breedering cages for anomalies. If no differences are observed between $Hoxb1^{A1}$ and control mice then near-complete functional redundancy at both the proximate and the ultimate level will be supported. However, if OPAs reveal differential gene function at the ultimate (*i.e.*, fitness) level, then previous measures of functional redundancy based on proximate measures will have been overestimated, highlighting the importance of a naturalistic environment when quantifying differential performance between mutants and controls.

Materials and Methods

Animals

Many laboratory strains of mice do not possess the natural and functional behaviors required for OPA assessment (Manning *et al.* 1992; Nelson *et al.* 2013); therefore, suitable mice with the $Hoxb1^{A1}$ swap and an appropriate control had to be generated (Figure 1). Specifically, a $Hoxb1^{A1}$ treatment lineage was bred starting with 16 $Hoxb1^{A1(g)/A1(g)}$-harboring 129 × C57BL/6 hybrid mice, generated by homologous recombination in 129 R1 ES cells (see reference Tvrdik and Capecchi 2006 for a detailed description), were bred to genetically diverse wild-derived mice and the resulting $Hoxb1^{A1(g)/+}$ heterozygotes (F_1) were crossed ($n = 93$) to establish the next

(F_2) generation (Figure 1A). Progeny were genetically screened and only *Hoxb1*$^{A1(g)/A1(g)}$ individuals were selected as OPA founders and are hereon referred to as Hoxb1^{A1} founders. Three OPA populations were established with these F_2 animals and three more were established with F_3 animals produced from F_2 *Hoxb1*$^{A1(g)/A1(g)}$ homozygous breeding pairs ($n = 16$). A control lineage of animals was bred to rule out potential confounding effects due to differential genetics surrounding the swapped region. To achieve this, 12 *Hoxb1*$^{+(g)/+(g)}$ 129 × C57BL/6 hybrid mice were crossed with the same wild stock used in the Hoxb1^{A1} treatment lineage. *Hoxb1*$^{+(g)/+(g)}$ 129 × C57BL/6 hybrid mice were generated in the same manner as the *Hoxb1*^{A1} swaps and tagged with the same internal ribosome entry site (IRES)–τ-GFP marker, but expressing the normal HoxB1 protein from the *Hoxb1* locus. The resulting F_1 *Hoxb1*$^{+(g)/+}$ generation ($n = 55$) were then crossed to produce the F_2 generation (Figure 1B). Only *Hoxb1*$^{+(g)/+(g)}$ mice were selected as control OPA founders and are hereafter referred to as controls. Three OPA populations were established with these F_2 animals and three more were established with F_3 animals produced from F_2 *Hoxb1*$^{+(g)/+(g)}$ homozygous breeders ($n = 12$). Therefore, except for the *Hox* gene region of interest, both Hoxb1^{A1} and control founders had the same background genetics on average, since other parts of the genome were expected to segregate randomly. Wild-derived animals were from the eighth generation of the colony originally described by Meagher *et al.* (2000). All P_0, F_1 animals as well F_2 and F_3 animals (prior to OPA release) were housed according to standard protocols under a 12:12 h light:dark cycle with food and water available *ad libitum*. All protocols were approved by the animal care guidelines of the Institutional Animal Care and Use Committee at the University of Utah.

Genotyping

Hoxb1 genotype was determined using a three primer PCR amplification system where a 3′ common primer (5′-AAA TAT CTG CTG ACT TGA ACC C) anneals between exons 1 and 2 within the bridging intron and specific 5′ primers, which anneal within exon 1—for *Hoxb1*$^{+/+}$ (5′-GAG TGT GAT CAC GAT CGT GAA AC) and for *Hoxb1*$^{A1/A1}$ (5′-AAT AAC TCC TTA TCC CCT CTC C)—yield a 157-bp and 258-bp fragment, respectively. These amplicons were visualized on 5% polyacrylamide gels (Figure 1D). Likewise, to distinguish between τ-GFP-tagged wild-type and true wild-type individuals a similar genotyping system was used. A 3′ common primer (5′-CCA TCA ATC ATC CCT CCA CC) and a 5′-specific primer for *Hoxb1*$^{+(g)/+(g)}$ (5′-ACA ACC ACT ACC TGA GCA CC) located within the τ-GFP site and for *Hoxb1*$^{+/+}$ (5′-TCC ATC ACC TCT TGA ATT GAA C), located 5′ of where the τ-GFP insertion within animals possessing it, yield a 366-bp and 204-bp fragment, respectively, which were visualized on 5% polyacrylamide gels (Figure 1D).

A combination of both genotyping systems was used to genotype all F_2 progeny, F_2 and F_3 founders, and all pups from OPA enclosures. A total of 1145 genotypes were obtained for 1155 F_2 offspring for a success rate of 99.1%. Similarly, all F_2 and F_3 OPA founders' genotypes were confirmed before release. Regarding OPA pups, 1145 genotypes of the 1194 individuals were determined representing a success rate of 95.9%.

OPA enclosures

OPA enclosures are 30 m^2 and are subdivided into six subsections by wire mesh to create environmental complexity and promote territory formation. Subsections have food and water sources provided *ad libitum* that are associated with a set of nest boxes in either one of the four "optimal" territories (with enclosed nest boxes) or two "suboptimal" territories (with exposed nest boxes). Photographs of OPA enclosures and detailed descriptions may be found elsewhere (Ruff *et al.* 2013, 2015; Gaukler *et al.* 2015). OPAs are designed to promote natural mouse mating behavior wherein males compete for territories, a limited resource, which attracts high-quality females; these competitive interactions structure the base unit of house mouse biology—demes (for reviews see Sage 1981; Berdoy and Drickamer 2007).

Six independent OPA enclosures were founded by populations of 28–30 individuals, 8–10 males and 18–20 females for a total of 176 individuals (58 male and 118 female). Populations were created in two sets with three being founded with F_2 animals and the remaining three with F_3 founders. Equal numbers of Hoxb1^{A1} and control founders were represented in each sex within all populations. To prevent confounding behaviors associated with relatedness, no male individual was related at the cousin level or above to any other individual within a given population. Relatedness between female founders was also avoided, though sister pairs were included in the second series of populations, which is common in nature. When this was the case, sister pairs were balanced across treatments. Mean age of F_2 founders was 37.3 ± 1.2 (M ± SD) weeks for females and 37.5 ± 0.6 weeks for males at the time of release and for F_3 founders, females were 33.1 ± 5.8 weeks old and males were 31.4 ± 6.1. To prevent incidental breeding before the establishment of male territories, unmanipulated females were released with the male Hoxb1^{A1} founders at the onset of each population to allow male territory formation prior to release of female Hoxb1^{A1} treatment and control founders. After 1 week, the unmanipulated females were removed and the female Hoxb1^{A1} treatment and control founders released, marking the start (week 1) of the study. OPA populations were maintained for 25 weeks.

OPA measures

Survival: Survivorship of population founders was determined by periodic checks in each enclosure. Dead founders were identified by passive integrated transponder (PIT) tags and personalized ear markings. Date of death was estimated based on three factors: date of last check, the last date an animal was recorded feeding, and corpse condition. To avoid altering territorial dynamics and influencing infanticide, researchers entered OPAs only to rotate PIT-tag readers

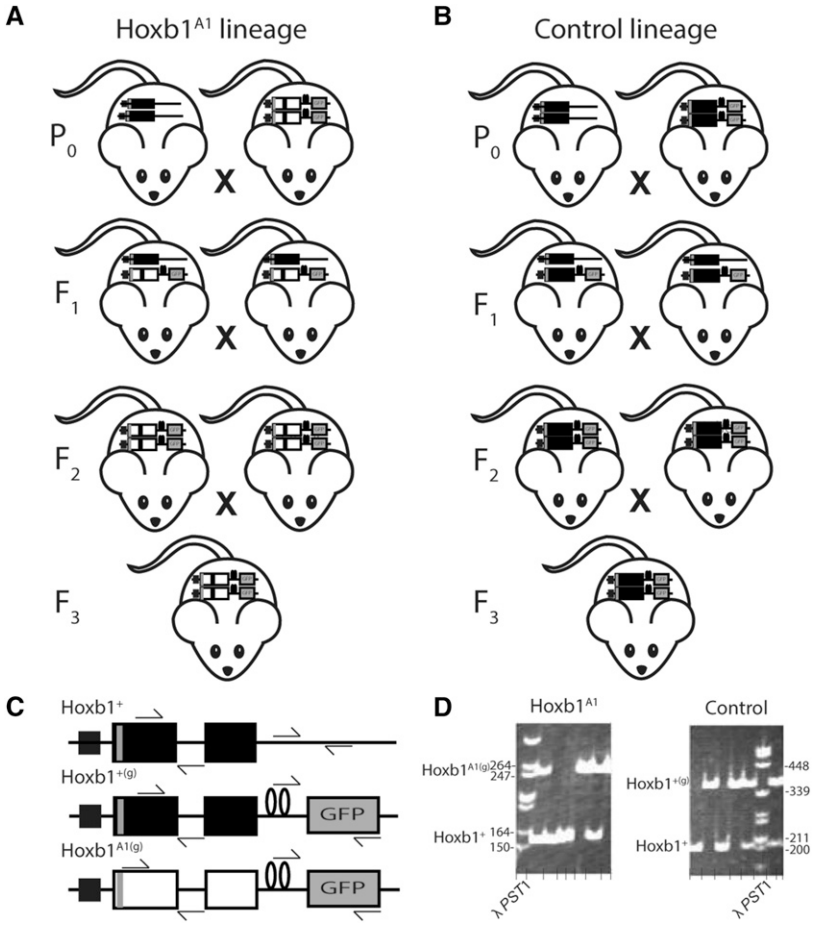

Figure 1 Breeding design for production of Hoxb1^{A1} and control founders. (A) To produce animals bearing *Hoxb1^{A1}*swaps that also possess the functional behaviors needed for OPAs, *Hoxb1$^{A1(g)/A1(g)}$* 129 × C57BL/6 mice were bred to outbred, wild-derived mice. The resulting *Hoxb1$^{A1(g)/+}$* heterozygotes were crossed to establish the next (F_2) generation. Progeny were genetically screened and only *Hoxb1$^{A1(g)/A1(g)}$* individuals were selected as OPA founders. Three OPA populations were established with these F_2 animals and three more were established with F_3 animals produced from F_2 *Hoxb1$^{A1(g)/A1(g)}$* homozygous breeding pairs. (B) To control for potential confounding effects due to differential genetics surrounding the swap control animals were bred by crossing *Hoxb1$^{+(g)/+(g)}$* 129 × C57BL/6 mice with the same wild stock used in the Hoxb1^{A1} treatment lineage. The *Hoxb1$^{+(g)/+}$* were then crossed to produce the F_2 generation. Only *Hoxb1$^{+(g)/+(g)}$* mice were selected as OPA founders. Three OPA populations were established with these F_2 animals and three more were established with F_3 animals produced from F_2 *Hoxb1$^{+(g)/+(g)}$* homozygous breeders. (C) Illustrations of wild type (*Hoxb1^{+}*), wild type with the IRES–τ-GFP tag (*Hoxb1$^{+(g)}$*), and *Hoxb1^{A1}* swap with the IRES–τ-GFP tag (*Hoxb1$^{A1(g)}$*) are provided. Large rectangles represent exons 1 and 2 of Hoxb1 (black) and Hoxa1 (white). The *Hoxb1* promoter is conserved across all genotypes and solid squares represent the *Hoxb1* autoregulatory enhancer. Loops separating the τ-GFP tag from the second exon depict the IRES. Arrows approximate primer binding sites. Illustrations are not to scale. (D) Image of polyacrylamide gel discrimination between *Hoxb1$^{A1(g)}$* and *Hoxb1^{+}* (left) and between *Hoxb1$^{+(g)}$* and *Hoxb1^{+}*(right) alleles.

between pens, refresh food and water, and conduct pup sweeps (described in paragraph below). Corpses were therefore collected in a variety of conditions that precluded necropsies.

Reproductive success: To determine founder reproductive success, tissue samples were gathered during "pup sweeps" in which pups born during the previous cycle were removed from the population. Sweeps occurred every 5 weeks to prevent offspring born in enclosures from breeding. In all six populations, five pup sweeps occurred. A total of 1194 individual samples were collected with 199.7 ± 70.6 (M ± SD) per population. Population level reproductive success was determined for Hoxb1^{A1} and control founders, using the genotyping technique described above.

Male competitive ability: One week prior to entrance, founders of both sexes were implanted with unique PIT tags (TX1400ST, BioMark, Boise ID). A set of PIT antennae and readers (FS2001F-ISO, BioMark) were rotated through the populations throughout the study and placed at each of the feeders; data were streamed to a computer equipped with data-logging software (Minimon, Culver City, CA). Readers were rotated between populations as only two sets of readers were available, and more than two populations were running concurrently. As dominant males do not tolerate competitors within their territories, dominance was assigned when a male had >80% of all male PIT-tag reads at a single location over the course of a multiday (minimum of 3 day) reader session. Thus, paired measurements of the number of territories controlled by Hoxb1^{A1} and control males were gathered for each population multiple times throughout the 25-week study. Female behavioral data were also acquired, but are not presented here.

Statistical methods

Breeding cage measures: To assess for genotype frequency differences in *Hoxb1^{A1}* treatment and control lineage heterozygote breeding cages, comparisons were made between the specific homozygotes and between the summed homozygote *vs.* heterozygote counts. As reproduction data are discrete counts, we modeled offspring counts within the first litter using a generalized linear mixed model (GLMM) with a Poisson distribution and a logarithmic link. Genotype was modeled as a fixed effect while individual breeding cage was modeled as a random effect as two measures were taken from a single breeding cage. Both models for Hoxb1^{A1} treatment lineage breeding cages were based on 186 observations from 93 breeding cages, while models for control lineage breeding cages were based on 110 observations from 55 cages. This method of analysis was selected as individual pups can be

grouped by breeding cage and it is appropriate for the Poisson distribution of litter count data.

As only one measure per breeding cage was used to compare litter sizes between Hoxb1^{A1} treatment and control lineage heterozygous breeding cages, a Mann–Whitney *U*-test was conducted. To compare litter sizes between Hoxb1^{A1} treatment and control lineage homozygous breeders, multiple litters (up to three) from individual breeding cages were used, therefore a GLMM assuming a Poisson distribution and using logarithmic link was based on 75 observations from 28 breeding cages. The model predicts litter size with the main effects of genotype, litter parity, and their interaction on litter size. Breeding cage was modeled as a random effect with a random slope generated for each.

OPA survival: Survivorship of the 176 OPA founders was analyzed by Cox proportional hazard models. Week one was defined as when Hoxb1^{A1} and control female founders entered OPA enclosures. A multivariate model was used to assess the effects of genotype, population, and their interaction on survival. Individuals that survived the duration of the trial or that were removed from the study were censored. There were 30 mortality events and 146 censorings.

Male competitive ability: To assess the effects of genotype, time, and time-by-genotype interaction on male competitive ability, we used a GLMM to predict the probability of territory ownership. As a territory can only be defended or not, we used a binomial distribution with a logit link. Territorial control within populations by each genotype was assessed multiple times throughout the study for a total of 124 observations. The intercept of the model was set at the grand mean (week 13.44). Time, genotype, and their interaction were treated as fixed effects and population was modeled as a random effect with a random intercept calculated for each.

OPA allele frequencies: A linear mixed-effects model (LMM) was used to assess the frequencies of *Hoxb1*$^{A1(g)}$ and *Hoxb1*$^{+(g)}$ in the offspring of OPA founders across the five pup sweeps. The model predicted the main effects of allele [*Hoxb1*$^{A1(g)}$ *vs. Hoxb1*$^{+(g)}$], time, and their interaction on allele frequency across the six populations. Paired gene frequencies were predicted five times at 5-week intervals for a total of 60 observations. Time, allele, and the interaction, were modeled as fixed effects and population was modeled as a random effect, to control for repeated measures with a random intercept and slope calculated for each. The intercept was set at the grand mean (week 15).

OPA genotypic counts of offspring: As reproduction data are discrete counts we modeled offspring counts over time in a GLMM with a Poisson distribution and a logarithmic link. We predicted population-level fitness across the six populations by modeling the main effects of genotype [*Hoxb1*$^{A1(g)/A1(g)}$ *vs.* *Hoxb1*$^{+(g)/+(g)}$], time, and their interaction. Offspring genotypes were measured five times at 5-week intervals for a total of 60 observations. Time, genotype, and the interaction were modeled as fixed effects and population was modeled as a random effect with a random intercept. The intercept was set at the grand mean (week 15).

To test for a deficiency (or excess) of heterozygotes within OPAs, a GLMM with the same intercept and distribution as in the specific homozygote comparison was used. However, four distinct genotype groups were assessed: observed heterozygotes, summed homozygotes, and the expected (2×) count of heterozygotes based upon each of the homozygote counts; 30 observations were available for each. Time, genotype, and their interaction were modeled as fixed effects and population was modeled as a random effect with a random intercept and slope.

All mixed-effects models were fit in R using the *lmer* or *glmer* function of the "lme4" library (Bates *et al.* 2014; R Development Core Team 2015). For all mixed-effects models several candidate models for the random effects terms were generated, including models estimating both intercept and/or slope for random effects. In all cases the model that explained at least some of the variance with random effects and had the lowest Akaike's information criterion score was selected. Degrees of freedom and resulting *P* values for LMMs were determined with a Satterthwaite approximation using the lmerTest library (Kuznetsova *et al.* 2014). Estimating degrees of freedom in LMMs remains controversial, but all effects deemed statistically significant on the basis of *P* values also possess a *t* value $>|2|$. This conservative criterion for significance is recommended by the library's authors. All α-values were 0.05 and tests were two tailed.

Data availability

All pertinent data from breeding cage and OPA studies presented in this manuscript are available in File S1. This Includes litter size and pup genotypes from *Hoxb1*^{A1} treatment and control lineage breeding cages, male competitive ability within OPAs, genotypic counts of offspring born within OPAs, and survival data for OPA founders.

Results

No deficiencies of homozygous or heterozygous F_2 offspring were observed from F_1 heterozygous Hoxb1^{A1} treatment or control lineage breeding cages; however, litter sizes were larger in Hoxb1^{A1} treatment breeding cages (Table 1). In F_1 Hoxb1^{A1} treatment lineage heterozygous [*Hoxb1*$^{A1(g)/+}$] breeding cages (n = 93), no deficiency of *Hoxb1*$^{A1(g)/A1(g)}$ homozygotes was observed relative to the count of *Hoxb1*$^{+/+}$ offspring (GLMM; Z = 1.12, P = 0.262) and no deficiency of *Hoxb1*$^{A1(g)/+}$ heterozygotes was seen relative to the number of summed homozygotes (GLMM; Z = 0.44, P = 0.663). In *Hoxb1*$^{+(g)/+}$ control lineage breeding cages (n = 55) no deficiency of *Hoxb1*$^{+(g)/+(g)}$ homozygotes was observed relative to *Hoxb1*$^{+/+}$ (GLMM; $Z = -1.64$, $P = 0.101$) and no deficiency of heterozygotes was observed (GLMM; $Z = -0.36$, P = 0.723). However, litter sizes from the Hoxb1^{A1} treatment lineage

Table 1 Summary of genotypic counts and litter sizes of Hoxb1^{A1} treatment and control lineage breeding cages

Lineage	Design (*N*)	Mutant homozygotes	Wild-type homozygotes	Heterozygotes	Total[a]
F_1 heterozygous breeding cages					
Hoxb1^{A1} treatment	*Hoxb1*$^{A1(g)/+}$ × *Hoxb1*$^{A1(g)/+}$ (55)	2.0 ± 0.1[b]	2.2 ± 0.2	4.0 ± 0 2	8.2 ± 0.2^{A}
Control	*Hoxb1*$^{+(g)/+}$ × *Hoxb1*$^{+(g)/+}$ (93)	1.9 ± 0.2	1.5 ± 0.2	3.6 ± 0 3	7.1 ± 0.4^{B}
F_2 homozygous breeding cages					
Hoxb1^{A1} treatment	*Hoxb1*$^{A1(g)/A1(g)}$ × *Hoxb1*$^{A1(g)/A1(g)}$ (16)	4.7 ± 0.5			4.7 ± 0.5^{A}
Control	*Hoxb1*$^{+(g)/+(g)}$ × *Hoxb1*$^{+(g)/+(g)}$ (12)	7.7 ± 0.1			7.7 ± 0.1^{B}

[a] Totals followed by different capital letters significantly differ. For more detailed statistical summaries see Table S1 and Table S2.
[b] Values are means ± SE for first litters.

heterozygous breeding cages were larger (8.20 ± 0.20; M ± SEM) than from control lineage breeding cages (7.13 ± 0.35) (Mann–Whitney; $U = 1851$, $P = 0.005$). For mixed model results see Supporting Information, Table S1.

Hoxb1^{A1} treatment lineage F_2 homozygous breeders produced fewer F_3 offspring, at least initially, than did homozygous control lineage breeders (Table 1). In first litters (the model intercept) *Hoxb1*$^{A1(g)/A1(g)}$ breeders produced 4.66 (+0.52, –0.46; M ± SEM) offspring per breeding cage, while *Hoxb1*$^{+(g)/+(g)}$ homozygous control breeders produced 7.66 (+1.18, –1.02) offspring per breeding cage (GLMM; $Z = 3.46$, $P = 0.001$); asymmetric SEMs are reported as they are back transformed from logarithmic values. However, it was observed that control lineage breeders had decreased rates of reproduction in progressing litters (GLMM; $Z = -3.01$, $P = 0.003$) and with *post hoc* Mann–Whitney tests it was confirmed that there was only a significant ($P < 0.05$) difference between breeding groups during the first litter. For mixed model results see Table S2.

Within OPA enclosures no difference in survival was observed between Hoxb1^{A1} and control founders [proportional hazards (PH); $\chi^2 = 0.002$, $P = 0.964$; Figure S1). Furthermore, survival did not differ among OPA populations (PH; $\chi^2 = 4.21$, $P = 0.519$), nor did the effect of genotype differ by population (PH; $\chi^2 = 7.76$, $P = 0.170$).

The probability of territorial ownership was lower for male Hoxb1^{A1} founders than for control founders (Figure 2). At the model intercept (week 13.44), the probability that a territory was dominated by a male Hoxb1^{A1} founder was 44.7%, while for controls it was 55.3% (GLMM; $Z = 2.56$, $P = 0.010$). Neither time (GLMM; $Z = 0.318$, $P = 0.751$), nor genotype by time affected territorial acquisition (GLMM; $Z = -0.45$, $P = 0.653$), indicating that the competitive disadvantage of male Hoxb1^{A1} founders persisted over the course of the study. For mixed model results see Table S3.

The *Hoxb1*$^{A1(g)}$ allele was selected against within OPA enclosures. The initial frequency of *Hoxb1*$^{A1(g)}$ was 0.500 in population founders; however, in offspring born within OPAs, this frequency was reduced to 0.419 ± 0.037 (M ± SEM), resulting in a selection coefficient (s) of 0.162 (Figure 3A). This difference was found to be statistically significant (LMM; $t = 3.102$, $P = 0.003$). For mixed model results see Table S4.

Hoxb1^{A1} founders contributed only 64.4% of the reproduction enjoyed by controls as measured by homozygous offspring (Figure 3B). At the model intercept (week 15), 7.82 (+0.90, –0.80) *Hoxb1*$^{A1(g)/A1(g)}$ offspring per population were produced in OPAs, while 12.18 (+0.81, –1.20) *Hoxb1*$^{+(g)/+(g)}$ offspring per population were produced (GLMM; $Z = 5.03$, $P < 0.001$). Both groups of founders increased reproductive output over time (GLMM; $Z = 2.84$, $P = 0.005$), and as there was no interaction between time and genotype (GLMM; $Z = 0.951$, $P = 0.342$) the decreased reproduction of Hoxb1^{A1} founders at the intercept was maintained throughout the study. For mixed model results see Table S4.

A 16.2% deficiency of *Hoxb1*$^{A1(g)/+(g)}$ heterozygotes was also observed in OPAs compared to the observed number of summed homozygotes (Figure 3B; GLMM; $Z = 2.66$, $P = 0.008$). At the model intercept (week 15), 16.74 (+1.70, –1.55) *Hoxb1*$^{A1(g)/+(g)}$ offspring per population were produced, while 19.65 (+1.22, –1.15) homozygous offspring were produced. As expected from the homozygote comparisons, both the number of heterozygotes and homozygotes produced increased over time (GLMM; $Z = 2.40$, $P = 0.016$). There was no time-by-genotype interaction (GLMM; $Z = 1.48$, $P = 0.139$), indicating that the decreased production of heterozygotes present at the intercept lasted throughout the study. The observed numbers of heterozygotes produced in OPAs are lower than those expected based on the counts of *Hoxb1*$^{+(g)/+(g)}$ offspring (GLMM; $Z = 6.01$, $P < 0.001$), but do not differ from expected levels predicted by observed *Hoxb1*$^{A1(g)/A1(g)}$ homozygotes (GLMM; $Z = -1.16$, $P = 0.246$). For mixed model results see Table S4.

Discussion

Within breeding cages, no deleterious effects of possessing a single *Hoxb1*$^{A1(g)}$ allele (*i.e.*, being heterozygous) were observable, but being homozygous did decrease the size of first litters in the Hoxb1^{A1} treatment lineage. In Hoxb1^{A1} treatment and control lineage heterozygous breeding cages no deficiency of either *Hoxb1*$^{A1(g)/A1(g)}$ or *Hoxb1*$^{+(g)/+(g)}$ homozygotes was observed relative to the wild type (*Hoxb1*$^{+/+}$), and no deficiency of heterozygotes was detected in relation to the homozygous offspring. This indicates that neither the IRES–τ-GFP-tagged control nor the IRES–τ-GFP-tagged *Hoxb1*^{A1} swap contributed to embryonic mortality. However, litters from Hoxb1^{A1} treatment lineage breeding cages with *Hoxb1*$^{A1(g)/+}$ heterozygous pairs were larger than those from control lineage breeding cages (*Hoxb1*$^{+(g)/+}$), suggesting that animals heterozygous for the *Hoxb1*$^{A1(g)}$ swap were more fit,

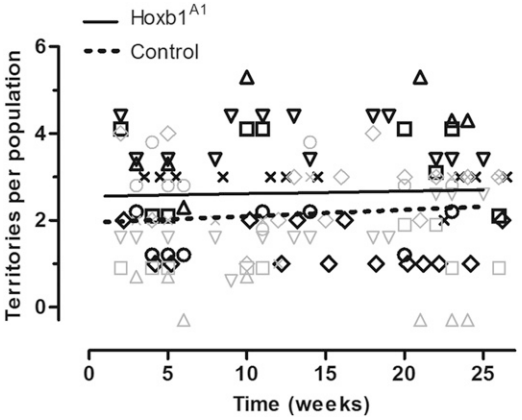

Figure 2 Competitive ability of Hoxb1^{A1} and control founders in OPAs. A 10.6% reduction in the probability of territorial ownership of Hoxb1^{A1} founders was observed relative to control founders (GLMM; $Z = 2.56$, $P = 0.010$). Competitive ability of both groups was assessed at multiple time points across populations ($n = 6$) for a total of 124 observations. Provided lines are simple linear regressions based upon raw data to help illustrate overall trends. Observations from each population are demarcated by shape and paired at each time point (solid outline for control and shaded outline for Hoxb1^{A1} founders) and cannot sum to more than six, as this is the maximum number of territories per population. If more than one population was assessed at a given time, then data points from multiple populations appear in a column; vertical scatter was added to prevent overlap and aid in visualization.

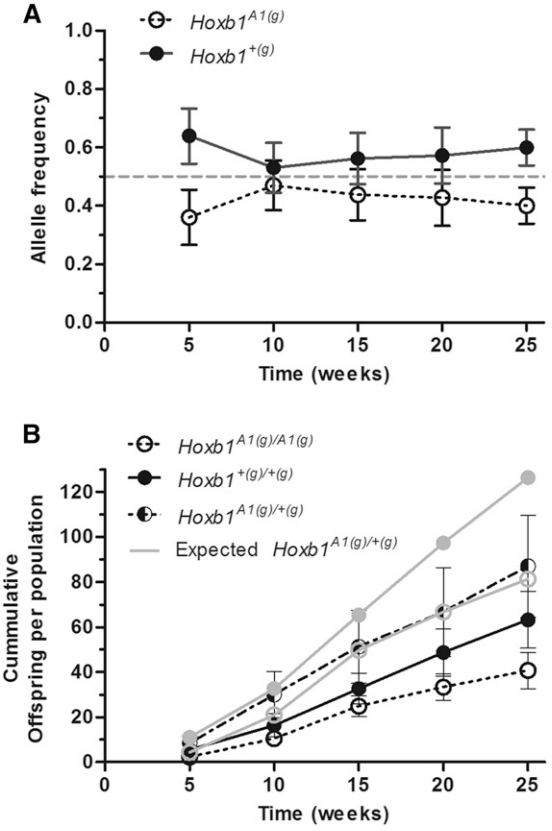

Figure 3 Allele frequencies (A) and genotypes of offspring born within OPAs (B). (A) The *Hoxb1*$^{A1(g)}$ allele was selected against ($s = 0.162$) within OPAs as the frequency of the mutant allele decreased in offspring born to population founders (LMM; $t = 3.10$, $P = 0.003$). Founders possessed the mutant allele at a frequency of 0.500 (signified by the shaded dashed line). (B) Hoxb1^{A1} founders had 64.4% of the reproduction achieved by controls as measured by homozygous offspring within OPA enclosures (GLMM; $Z = 3.52$, $P < 0.001$). Likewise, a 16.2% deficiency of *Hoxb1*$^{A1(g)/+(g)}$ offspring was observed as compared to summed homozygotes (GLMM; $Z = 2.66$, $P = 0.008$). The observed number of heterozygotes was lower than expected levels based on *Hoxb1*$^{+(g)/+(g)}$ homozygote counts (GLMM; $Z = 6.01$, $P < 0.001$; shaded line with closed circles), but did not differ from those predicted by counts of *Hoxb1*$^{A1(g)/A1(g)}$ pups (shaded line with open circles). All genotypes were assessed at multiple times across populations ($n = 6$) for a total of 30 observations. Black lines connect population means and error bars represent standard error.

at least in the breeding cage environment, than those bearing the control allele. Conversely, Hoxb1^{A1} treatment lineage homozygous *Hoxb1*$^{A1(g)/A1(g)}$ pairings produced fewer offspring than did mice in *Hoxb1*$^{+(g)/+(g)}$ control lineage breeding cages; this effect was only observed in first litters and disappeared in subsequent litters. The findings from heterozygous breeders and from all but the first litter of homozygous breeders are in accordance with previous investigations of this transgenic line that support a near-complete degree of functional redundancy between these paralogous genes (Tvrdik and Capecchi 2006); however, the decreased size of first litters experienced by *Hoxb1*$^{A1(g)/A1(g)}$ breeders argues for subtle reproductive impairment in these mice.

Within OPAs, Hoxb1^{A1} founders were outcompeted by control founders as measured by competitive ability and fitness. Specifically, male Hoxb1^{A1} founders were less likely to acquire a territory than control founders and the frequency of the *Hoxb1*$^{A1(g)}$ allele decreased from 0.500 in population founders to 0.419 in their offspring. The declining allelic frequency of *Hoxb1*$^{A1(g)}$ is driven by decreased reproduction of Hoxb1^{A1} founders who only produced 64% as many offspring as control founders, as measured by homozygous offspring. It is likely that the decreased competitive ability of Hoxb1^{A1} male founders contributed to the marked decrease in reproduction. It has been shown that dominant males sire the majority (~80%) of pups within OPA enclosures (Carroll *et al.* 2004); however, as the discrepancy in territorial acquisition was small (~10%), decreased competitive ability is insufficient to explain the observed differences in reproduction. Furthermore, the measures of decreased performance of Hoxb1^{A1} founders should be considered robust, as the high level of genetic diversity in wild-derived mice should make the influence of the *Hoxb1*^{A1} swap more difficult to detect.

A possible mechanism for the decreased reproductive success of Hoxb1^{A1} founders within OPAs is embryonic lethality, especially as *Hox* genes are of critical developmental importance. However, lethality is not supported by breeding cage data, which indicate equal frequencies of *Hoxb1*$^{A1(g)/A1(g)}$ and wild-type homozygotes. Litter sizes were reduced from Hoxb1^{A1} treatment lineage homozygous [*Hoxb1*$^{A1(g)/A1(g)}$] breeding cages relative to homozygous [*Hoxb1*$^{+(g)/+(g)}$] control lineage breeding cages similar to the degree observed in OPAs. However, as this effect was only present in first litters, it cannot explain the decreased reproductive success of

Hoxb1^{A1} founders across the 25-week study. It is possible that the natural stressors present within OPAs could exacerbate embryonic or early neonatal death or reproductive impairment, as has been seen with caloric restriction, territorial instability, and increased exposure to pathogens, all of which are elevated within OPAs relative to standard breeding cages (Bruce 1959; Rivers and Crawford 1974; Ilmonen *et al.* 2008).

Nonrandom mating has been observed in OPAs previously and could also explain the reproductive deficit of Hoxb1^{A1} founders (Potts *et al.* 1991). Specifically, the deficiency of *Hoxb1*$^{A1(g)/A1(g)}$ homozygotes and *Hoxb1*$^{A1(g)/+(g)}$ heterozygous offspring could be explained by control founders mating preferentially with each other, leaving Hoxb1^{A1} founders to mate randomly with remaining partners. This assertion is supported by the finding that the number of heterozygous pups observed within OPAs match expected values based on the number of *Hoxb1*$^{A1(g)/A1(g)}$ homozygotes, but not the levels expected based on *Hoxb1*$^{+(g)/+(g)}$ homozygotes. Additional mechanisms leading to reproductive decline are likely at work in Hoxb1^{A1} founders, and though they have yet to be determined, the characterization of the organismal phenotype should hasten their discovery.

Though it has been argued that many *Hox* paralog swaps, including *Hoxb1*^{A1}, are functionally redundant, data presented here indicate that the degree of functional redundancy has been overestimated by proximate assessments. Though many proximate defects associated with *Hoxb1*$^{-/-}$ complete knockouts may be masked by the *Hoxb1*$^{A1(g)}$ swap, the animals fail to achieve equal levels of Darwinian fitness under seminatural conditions and exhibit hints of fitness declines in laboratory cages. This fitness inequality could explain the extreme conservation seen in *Hox* genes across taxa, including *Hoxb1* and *Hoxa1*, as purifying selection will remove allelic variants from populations when their fitness is lower; variants do not need to possess gross alteration in morphological or behavioral traits to be selected against, but only need to be less fit than alternatives.

Paralogous *Hox* genes have provided a case study to further understand how duplicated genes can avoid nonfunctionalization and be maintained across evolutionary time, with the leading explanation being subfunctionalization via the DDC model (Prince and Pickett 2002). Examples of functional redundancy between paralogous genes have been cited as evidence for this model, though if subfunctionalization has occurred, one would expect incomplete redundancy. In this case, the level of redundancy is inversely related to the level of subfunctionalization that has occurred. Therefore, our findings, that the degree of redundancy between *Hoxb1* and *Hoxa1* is lower than previously acknowledged, does not argue against the DDC model, but suggests that there is less overlap in gene function between the paralogs tested than previously thought. Likewise, illustrating decreased redundancy does not necessarily help distinguish between candidate explanations for the maintenance of duplicated genes, such as neofunctionalization or subfunctionalization (either via the DDC model or the escape from adaptive conflict model) (Des Marais and Rausher 2008), but it does argue that for these paralogs, the degree to which these processes have altered gene function is higher than initially conceived.

With OPAs we are able to characterize inequalities between mice homozygous for a *Hoxb1*^{A1} swap and control mice that are missed by more traditional proximate investigations. This is likely due to the competitive nature of house mice, which vigorously compete with one another over resources. Differences in physiological performance that are too cryptic, diffuse, or subtle to cause gross defects may nonetheless lower fitness in a competitive environment and this concept has driven the use of fitness assays in *Drosophila*, RNA virus, and yeast communities (*e.g.*, Shabalina *et al.* 1997; Thatcher *et al.* 1998; Lauring *et al.* 2012); unfortunately, similar approaches have not been adopted by those working with vertebrate model systems (with the notable exception of genes involved in sperm function or competition, *e.g.*, Sutton *et al.* 2008). In addition to the *Hoxb1*^{A1} phenotypes herein, OPAs have revealed adversities associated with three other genetic treatments, including cousin- and sibling-level inbreeding and bearing the selfish genetic element known as the *t complex*, which had escaped detection for decades (Meagher *et al.* 2000; Carroll *et al.* 2004; Ilmonen *et al.* 2008). Examples such as these give weight to the argument that fitness assays are necessary for functional genomics, especially when confronted with no-phenotype knockouts, swaps, enhancers, deletions, and other mutants (Carroll and Potts 2006).

Acknowledgments

We thank M. Capecchi for graciously providing transgenic mice; D. Cornwall for help in figure design; and C. Davis, J. Timm, R. Ussing, and S. Wright for data collection. We also acknowledge two anonymous reviewers whose comments greatly improved this manuscript. This work was supported by National Institutes of Health (NIH) grant R01-GM039578 and was partially conducted while W.K.P was supported by National Science Foundation (NSF) grant DEB 09-18969 and NIH grant R01-GM109500. L.L.R. was supported by the NSF-funded Western Alliance to Expand Student Opportunities.

Literature Cited

Bates, D., M. Maechler, B. M., Bolker and S. Walker, 2014 Lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1–7. Available at: http://CRAN.R-project.org/package=lme4.

Barbaric, I., G. Miller, and T. N. Dear, 2007 Appearances can be deceiving: phenotypes of knockout mice. Brief. Funct. Genomic Proteomic. 6: 91–103.

Bertrand, N., M. Roux, L. Ryckebusch, K. Niederreither, P. Dolle *et al.*, 2011 Hox genes define distinct progenitor subdomains within the second heart field. Dev. Biol. 353: 266–274.

Berdoy, M., and L. C. Drickamer, 2007 Comparative social organization and life history of *Rattus* and *Mus*, pp. 380–392 in *Rodent Societies: An Ecological and Evolutionary Perspective*,

edited by J. O. Wolff, and P. W. Sherman. University of Chicago Press, Chicago.
Bosley, T. M., I. A. Alorainy, M. A. Salih, H. M. Aldhalaan, K. K. Abu-Amero *et al.*, 2008 The clinical spectrum of homozygous hoxa1 mutations. Am. J. Med. Genet. A. 146A: 1235–1240.
Bruce, H. M., 1959 An exteroceptive block to pregnancy in the mouse. Nature 164: 105.
Capecchi, M. R., 2005 Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat. Rev. Genet. 6: 507–512.
Carroll, L. S., and W. K. Potts, 2006 Functional genomics requires ecology. Adv. Stud. Behav. 36: 173–215.
Carroll, L. S., S. Meagher, L. Morrison, D. J. Penn, and W. K. Potts, 2004 Fitness effects of a selfish gene are revealed in an ecological context. Evolution 58: 1318–1328.
Chen, F., and M. R. Capecchi, 1999 Paralogous mouse hox genes, hoxa9, hoxb9, and hoxd9, function together to control development of the mammary gland in response to pregnancy. Proc. Natl. Acad. Sci. USA 96: 541–546.
Chen, F., J. Greer, and M. R. Capecchi, 1998 Analysis of hoxa7/hoxb7 mutants suggests periodicity in the generation of the different sets of vertebrae. Mech. Dev. 77: 49–57.
Collins, F. S., J. Rossant, and W. Wurst, 2007 A mouse for all reasons. Cell 128: 9–13.
del Toro, E. D., V. Borday, M. Davenne, R. Neun, F. M. Rijli *et al.*, 2001 Generation of a novel functional neuronal circuit in hoxa1 mutant mice. J. Neurosci. 21: 5637–5642.
Des Marais, D. L., and M. D. Rausher, 2008 Escape from adaptive conflict after duplication in an anthocyanin pathway gene. Nature 454: 762–765.
Force, A., M. Lynch, F. B. Pickett, A. Amores, Y. L. Yan *et al.*, 1999 Preservation of duplicate genes by complementary, degenerative mutations. Genet. 151: 1531–1545.
Gaukler, S. M., J. S. Ruff, T. Galland, K. A. Kandaris, T. K. Underwood *et al.*, 2015 Low-dose paroxetine exposure causes lifetime declines in male mouse body weight, reproduction and competitive ability as measured by the novel organismal performance assay. Neurotoxicol. Teratol. 47: 46–53.
Gehring, W. J., and Y. Hiromi, 1986 Homeotic genes and the homeobox. Annu. Rev. Genet. 20: 147–173.
Goddard, J. M., M. Rossel, N. R. Manley, and M. R. Capecchi, 1996 Mice with targeted disruption of Hoxb-1 fail to form the motor nucleus of the VIIth nerve. Development 122: 3217–3228.
Hsu, P. D., E. S. Lander, and F. Zhang, 2014 Development and applications of crispr-cas9 for genome engineering. Cell 157: 1262–1278.
Ilmonen, P., D. J. Penn, K. Damjanovich, J. Clarke, D. Lamborn *et al.*, 2008 Experimental infection magnifies inbreeding depression in house mice. J. Evol. Biol. 21: 834–841.
Innan, H., and F. Kondrashov, 2010 The evolution of gene duplications: classifying and distinguishing between models. Nat. Rev. Genet. 11: 97–108.
Kafri, R., M. Springer, and Y. Pilpel, 2009 Genetic redundancy: new tricks for old genes. Cell 136: 389–392.
Kuznetsova, A., P. B. Brockhoff and R. H. B. Christensen, 2014 lmerTest: tests in linear mixed effects models. R package version 2.0–20. Available at: http://CRAN.R-project.org/package=lmerTest.
Lauring, A. S., A. Acevedo, S. B. Cooper, and R. Andino, 2012 Codon usage determines the mutational robustness, evolutionary capacity, and virulence of an rna virus. Cell Host Microbe 12: 623–632.
Levine, M., and T. Hoey, 1988 Homeobox proteins as sequence-specific transcription factors. Cell 55: 537–540.
Lutz, B., H. C. Lu, G. Eichele, D. Miller, and T. C. Kaufman, 1996 Rescue of Drosophila labial null mutant by the chicken ortholog hoxb-1 demonstrates that the function of hox genes is phylogenetically conserved. Genes Dev. 10: 176–184.
Manley, N. R., and M. R. Capecchi, 1997 Hox group 3 paralogous genes act synergistically in the formation of somitic and neural crest-derived structures. Dev. Biol. 192: 274–288.
Manley, N. R., and M. R. Capecchi, 1998 Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands. Dev. Biol. 195: 1–15.
Manning, C. J., W. K. Potts, E. K. Wakeland, and D. A. Dewsbury, 1992 What's wrong with MHC mate choice experiments? pp. 229–235 in *Chemical Signals in Vertebrates*, edited by R. L. Doty, and D. Müller-Schwarze. Plenum, New York.
Manzanares, M., H. Wada, N. Itasaki, P. A. Trainor, R. Krumlauf *et al.*, 2000 Conservation and elaboration of hox gene regulation during evolution of the vertebrate head. Nature 408: 854–857.
McGinnis, W., and R. Krumlauf, 1992 Homeobox genes and axial patterning. Cell 68: 283–302.
Meagher, S., D. J. Penn, and W. K. Potts, 2000 Male-male competition magnifies inbreeding depression in wild house mice. Proc. Natl. Acad. Sci. USA 97: 3324–3329.
Meyer, M., O. Ortiz, M. Hrabe De Angelis, W. Wurst, and R. Kuhn, 2012 Modeling disease mutations by gene targeting in one-cell mouse embryos. Proc. Natl. Acad. Sci. USA 109: 9354–9359.
Nelson, A. C., J. W. Cauceglia, S. D. Merkley, N. A. Youngson, A. J. Oler *et al.*, 2013 Reintroducing domesticated wild mice to sociality induces adaptive transgenerational effects on mup expression. Proc. Natl. Acad. Sci. USA 110: 19848–19853.
Nowak, M. A., M. C. Boerlijst, J. Cooke, and J. M. Smith, 1997 Evolution of genetic redundancy. Nature 388: 167–171.
Ohno, S., 1970 *Evolution by Gene Duplication*, Springer-Verlag, New York.
Potts, W. K., C. J. Manning, and E. K. Wakeland, 1991 Mating patterns in seminatural populations of mice influenced by mhc genotype. Nature 352: 619–621.
Prince, V. E., and F. B. Pickett, 2002 Splitting pairs: the diverging fates of duplicated genes. Nat. Rev. Genet. 3: 827–837.
Popperl, H., M. Bienz, M. Studer, S. K. Chan, S. Aparicio *et al.*, 1995 Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. Cell 81: 1031–1042.
R Development Core Team, 2015 R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/.
Remacle, S., L. Abbas, O. De Backer, N. Pacico, A. Gavalas *et al.*, 2004 Loss of function but no gain of function caused by amino acid substitutions in the hexapeptide of hoxa1 in vivo. Mol. Cell. Biol. 24: 8567–8575.
Rijli, F. M., and P. Chambon, 1997 Genetic interactions of hox genes in limb development: learning from compound mutants. Curr. Opin. Genet. Dev. 7: 481–487.
Rivers, J. P., and M. A. Crawford, 1974 Maternal nutrition and the sex ratio at birth. Nature 252: 297–298.
Ruff, J. S., A. K. Suchy, S. A. Hugentobler, M. M. Sosa, B. L. Schwartz *et al.*, 2013 Human-relevant levels of added sugar consumption increase female mortality and lower male fitness in mice. Nat. Commun. 4: 2245.
Ruff, J. S., S. A. Hugentobler, A. K. Suchy, M. M. Sosa, R. E. Tanner *et al.*, 2015 Compared to sucrose, previous consumption of fructose and glucose monosaccharides reduces survival and fitness of female mice. J. Nutr. 145: 434–441.
Sage, R. D., 1981 Wild mice, pp. 40–90 in *The Mouse in Biomedical Research*, edited by H. L. Foster, J. D. Small, and J. G. Fox. Academic Press, New York.

Shabalina, S. A., L. Yampolsky, and A. S. Kondrashov, 1997 Rapid decline of fitness in panmictic populations of Drosophila melanogaster maintained under relaxed natural selection. Proc. Natl. Acad. Sci. USA 94: 13034–13039.

Sutton, K. A., M. K. Jungnickel, and H. M. Florman, 2008 A polycystin-1 controls postcopulatory reproductive selection in mice. Proc. Natl. Acad. Sci. USA 105: 8661–8666.

Thatcher, J. W., J. M. Shaw, and W. J. Dickinson, 1998 Marginal fitness contributions of nonessential genes in yeast. Proc. Natl. Acad. Sci. USA 95: 253–257.

Thorneycroft, D., S. M. Sherson, and S. M. Smith, 2001 Using gene knockouts to investigate plant metabolism. J. Exp. Bot. 52: 1593–1601.

Tvrdik, P., and M. R. Capecchi, 2006 Reversal of hox1 gene subfunctionalization in the mouse. Dev. Cell 11: 239–250.

Webb, B. D., S. Shaaban, H. Gaspar, L. F. Cunha, C. R. Schubert *et al.*, 2012 Hoxb1 founder mutation in humans recapitulates the phenotype of hoxb1−/− mice. Am. J. Hum. Genet. 91: 171–179.

Communicating editor: B. A. Payseur

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.178079/-/DC1

Fitness Assays Reveal Incomplete Functional Redundancy of the HoxA1 and HoxB1 Paralogs of Mice

James S. Ruff, Raed B. Saffarini, Leda L. Ramoz, Linda C. Morrison, Shambralyn Baker, Sean M. Laverty, Petr Tvrdik, and Wayne K. Potts

DOI: 10.1534/genetics.115.178079

Table S1 Summary of mixed model results for genotype frequencies in Hoxb1^{A1} treatment and control lineage heterozygous F_1 breeding cages

***Hoxb1$^{A1(g)/+}$* X *Hoxb1$^{A1(g)/+}$* Homozygote Comparison**				
GLMM with Poisson distribution and logarithmic link (186 observations, 93 groups)				
Random effects	*Variance*	*Std. Deviation*		
Breeding cage (Intercept)	0.000	0.000		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	0.666	0.074	8.96	<0.0001***
Genotype *(Hoxb1$^{+/+}$)*	0.115	0.102	1.12	0.262
***Hoxb1$^{A1(g)/+}$* X *Hoxb1$^{A1(g)/+}$* Summed Homozygotes *vs.* Heterozygotes**				
GLMM with Poisson distribution and logarithmic link (186 observations, 93 groups)				
Random effects	*Variance*	*Std. Deviation*		
Breeding cage (Intercept)	0.066	0.256		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	1.386	0.052	26.74	<0.0001***
Genotype (Homozygote)	0.032	0.073	0.44	0.663
***Hoxb1$^{+(g)/+}$* X *Hoxb1$^{+(g)/+}$* Homozygote Comparison**				
GLMM with Poisson distribution and logarithmic link (110 observations, 55 groups)				
Random effects	*Variance*	*Std. Deviation*		
Breeding cage (Intercept)	0.068	0.262		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	0.632	0.104	6.07	<0.0001***
Genotype *(Hoxb1$^{+/+}$)*	-0.242	0.147	-1.64	0.101
***Hoxb1$^{+(g)/+}$* X *Hoxb1$^{+(g)/+}$* Summed Homozygotes *vs.* Heterozygotes**				
GLMM with Poisson distribution and logarithmic link (110 observations, 55 groups)				
Random effects	*Variance*	*Std. Deviation*		
Breeding cage (Intercept)	0.000	0.000		
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|z|)*
Intercept	1.281	0.071	18.02	<0.0001***
Genotype (Homozygote)	-0.036	0.101	-0.36	0.723

***Indicates a p value < 0.001.

Table S2 Summary of mixed model results for litter size in Hoxb1^{A1} treatment and control lineage homozygous F_2 breeding cages

Litter Size in Homozygous Breeding Cages				
GLMM with Poisson distribution and logarithmic link (Intercept at litter 1, 75 obs, 28 groups)				
Random effects	*Variance*	*Std. Deviation*		
Breeding cage (Slope)	0.001	0.034		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	1.540	0.105	14.67	<0.0001***
Genotype *(Hoxb1$^{+(g)/+(g)}$)*	0.497	0.144	3.46	0.001***
Litter Parity	0.155	0.082	1.90	0.058
Genotype *(Hoxb1$^{+(g)/+(g)}$)* X Litter Parity	-0.372	0.124	-3.01	0.003**

Indicates a p value <0.01, * < 0.001

Table S3 Summary of mixed model results for founder competitive ability within OPAs

Male Competitive Ability				
GLMM with binomial distribution and logit link (Intercept at week 13.44, 124 obs, 6 groups)				
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.000	0.000		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	-0.212	0.117	-1.81	0.070
Genotype *(Hoxb1$^{+(g)/+(g)}$)*	0.425	0.166	2.56	0.010*
Time	0.005	0.015	0.32	0.751
Genotype *(Hoxb1$^{+(g)/+(g)}$)* X Time	-0.009	0.021	-0.45	0.653

*Indicates a p value < 0.05

Table S4 Summary of mixed model results for allele frequencies and genotypic counts of offspring born within OPAs

Allele Frequencies				
LMM (Intercept at week 15, 60 obs, 6 groups)				
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.000	0.000		
Population (Slope)	0.000	0.000		
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Pr(>|t|)*
Intercept	0.419	0.037	11.38	<0.0001***
Allele *(Hoxb1$^{+(g)}$)*	0.162	0.052	3.10	0.003**
Time	0.001	0.005	0.14	0.886
Allele *(Hoxb1$^{+(g)}$)* X Time	-0.002	0.007	-0.20	0.840
Reproduction Homozygote Comparison				
GLMM with Poisson distribution and logarithmic link (Intercept at week 15, 60 obs, 6 groups)				
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.045	0.213		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	2.057	0.109	18.89	<0.0001***
Genotype *(Hoxb1$^{+(g)/+(g)}$)*	0.423	0.084	5.03	<0.0001***
Time	0.026	0.009	2.84	0.005**
Genotype *(Hoxb1$^{+(g)/+(g)}$)* X Time	0.011	0.011	0.95	0.342
Reproduction Heterozygote Comparison				
GLMM with Poisson distribution and logarithmic link (Intercept at week 15, 120 obs, 6 groups)				
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.045	0.211		
Population (Slope)	0.000	0.015		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept (Observed Heterozygotes)	2.818	0.0970	29.08	<0.0001***
Observed Summed Homozygotes	0.160	0.060	2.66	0.008**
Expected Based on *Hoxb1$^{A1(g)/A1(g)}$*	-0.074	0.064	-1.16	0.246
Expected Based on *Hoxb1$^{+(g)/+(g)}$*	0.349	0.058	6.01	<0.0001***
Time	0.021	0.009	2.40	0.016*
Observed Summed Homozygotes X Time	0.013	0.009	1.48	0.138
Expected Based on *Hoxb1$^{A1(g)/A1(g)}$* X Time	0.006	0.009	0.65	0.518
Expected Based on *Hoxb1$^{+(g)/+(g)}$*) X Time	0.017	0.008	2.09	0.037*

*Indicates a p value < 0.05,** < 0.01, *** < 0.001

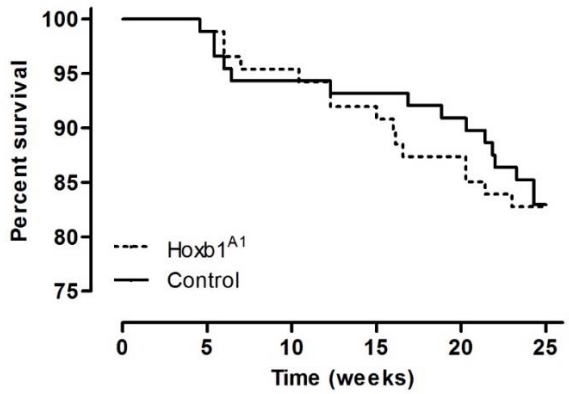

Figure S1 Survival of Hoxb1^{A1} and control founders within OPAs. Differential survival was not observed between Hoxb1^{A1} and control founders (Proportional Hazards; χ^2= 0.002, n = 176, p = 0.964).

File S1

Manuscript data file

File S1 is available for download as an Excel file at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.178079/-/DC1

Sheet 1 concerns litter size and pup genotypes from Hoxb1^{A1} treatment and control lineage breeding cages, Sheet 2 contains data for male competitive ability within OPAs, Sheet 3 provides the genotypic counts of offspring born within OPAs, and Sheet 4 has survival data for OPA founders.