The Genetic Basis of Behavior: Burrow Construction in Deer Mice (Genus Peromyscus)

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The genetic basis of behavior: burrow construction in deer mice (genus *Peromyscus*)

A dissertation presented

by

Hillery Claire Metz

to

The Department of Organismic and Evolutionary Biology

In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

in the subject of

Biology

Harvard University
Cambridge, Massachusetts

April 2015
The genetic basis of behavior: burrow construction in deer mice (genus *Peromyscus*)

**Abstract**

Understanding how complex, adaptive behavior evolves is a major goal of biological research. Phenotypic differences between closely-related species often arise due to evolution by natural selection and can be a powerful resource for understanding biological diversity and its mechanistic underpinnings. In this dissertation, I capitalize on striking behavioral differences between two interfertile sister species of *Peromyscus* rodents. I pursue the proximate mechanisms underlying this behavioral adaptation by investigating both the ontogeny and genetics of innate differences in burrow construction behavior in *Peromyscus polionotus* and *P. maniculatus*.

In Chapter 1, I compare the ontogeny of burrow construction behavior of *Peromyscus polionotus* and *P. maniculatus* across early development. I find that *P. polionotus* begins burrowing precociously (as early as 17 days of age) compared to *P. maniculatus* (27 days of age), despite *P. polionotus* being physically smaller and less active in a wheel running assay. Furthermore, juvenile *P. polionotus* constructed long burrows complete with species-specific escape tunnels. Interspecific cross-fostering did not alter the developmental trajectory of either species, indicating that these differences are innate. Moreover, F₁ hybrids followed the behavioral ontogeny of *P. polionotus*, indicating that precocious burrow construction segregates in a *P. polionotus*-
dominant manner. Finally, I show that a quantitative trait locus (QTL) associated with adult tunnel length in these species is predictive of precocious digging in recombinant F$_2$ hybrids, demonstrating that either a single pleiotropic locus or a group of tightly-linked genes control behavioral differences across life stages in *P. polionotus*.

In Chapter 2, I dissect the genetic architecture of this complex behavior in adult animals using an experimental cross. By introgressing the burrow architecture of *P. polionotus* into the genetic background of *P. maniculatus*, I analyze the underlying genetic architecture of differences in burrowing behavior, and show that escape tunnels are likely a threshold trait. Next, I use a novel image-based analysis to collect measurements of burrow shape and demonstrate the utility of a more rigorous measurement of extended phenotypes.

Finally, in Chapter 3, I combine two forward-genetics approaches—QTL mapping and transcriptome analysis—to nominate specific candidate genes for the differences in burrowing behavior between *P. polionotus* and *P. maniculatus*. Using a large advanced backcross mapping population (n=751), I detect five QTL contributing to differences in burrow architecture between these species: three loci for entrance tunnel length variation, and two loci for escape tunnel length. In the transcriptome study, I focus on gene expression in F$_1$ hybrids to detect allele-specific expression (ASE), as ASE in an F$_1$ hybrid indicates *cis*-regulatory differences between the parental lineages. I find widespread bias favoring expression from the *P. polionotus*-allele in F$_1$ hybrid brains, which may be a molecular reflection of *P. polionotus*-like burrowing behavior of hybrids. Finally, I use ASE to nominate candidate genes within the detected QTL regions, and find
genes related to behavioral disorders, circadian rhythms, and activity patterns; these genes represent promising candidates for future functional studies.
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"If you hold a cat by the tail, you learn things that cannot be learned in any other way."

--Mark Twain

Graduate school has been the most rewarding and transformative experience of my life so far. I recognize how incredibly lucky am to have spent the last seven years here—years spent on discovery and personal development while surrounded by such talented, lovely people. Is there anything better?

I would like to thank my Ph.D. committee members: Profs. Benjamin de Bivort, Catherine Dulac, Jonathan Losos, Bence Ölveczky and Joshua Sanes, for their generosity, availability, advice and mentorship over the years. Most especially, I would like to thank my dissertation advisor, Hopi Hoekstra, for being my guide through the rollercoaster of graduate school. Thank you for having confidence in me and for being the most generous of advisors. The Hoekstra lab is just the best, and you deserve credit for assembling such great team of people. In the larger community of biology at Harvard, I wish to thank Profs. Catherine Dulac, the late Farish Jenkins Jr., Charles Marshall, and Bence Ölveczky, for teaching memorable courses that were especially influential.

I wish to thank the organizations who have contributed to financing my education and research over the years, including the Department of Organismic and Evolutionary Biology, the Mind Brain and Behavior Initiative at Harvard, Chapman Funds for Vertebrate Locomotion, the National Science Foundation, and the American Association of University Women. Your support made this possible.

I thank the entire Hoekstra lab past and present, which would be quite a lengthy list, but I wish to thank a few people in particular. Marie Manceau—I admire you in so many ways. Thank you for being the first to believe in me, and for being an awesome rotation mentor. Emily Kay Delaney, thank you for bringing the fun! I learned so much from you and our trips to the field might have been the best part of grad school. Thanks to Heidi Fisher, Emily Jacobs-Palmer, Evan Kingsley, Celine Clabaut and Jean-Marc Lassance—for all the help over the years and being some of the best lab-mates one could hope for. I was so glad you have your company throughout grad school. Thanks to Zain Ali, Nicole Bedford, Brant Peterson and Jesse Weber for all the discussions on the brain, behavior, and evolution. I learned so much from our chats while we moved
endless piles of dirt around the burrowing room. Thanks to Brant Peterson, Jesse Weber, Emily Kay Delaney, Evan Kingsley, Emily Jacobs-Palmer and Andres Bendesky for training and assistance with experiments. I wish to thank undergraduate students Linda Pan, Rachelle Ludwick and Sarah Scalia for their help with experiments, and for showing me how rewarding it can be to mentor young scientists. Finally, I am just incredibly thankful for the help, humor and friendship of Kyle Turner, whose presence and assistance made everything better in the final year.

Beyond the Hoekstra lab, I am grateful to Talia Moore—thank you for introducing me to biomechanics and for sharing your enthusiasm and vast knowledge. Thanks to Judy Chupasko and Mark Omura from the MCZ Mammalogy department for years of assistance and training. I thank my collaborators, Joerg Fritz and Ryan York, who contributed substantially to this thesis. I send my gratitude to Joe Rocca for superb rodent care. Thanks to my friends from my graduate school cohort, especially Chris Baker, Emily Jacobs-Palmer, Martha Munoz and Jon Sanders—sharing graduate school with you was a joy and a privilege. To Aaron Levine—thank you for many fun adventures and bringing so much good to my life. I thank my friends, Nicole Bedford, Emily Kay Delaney, Stephanie Kinkel, Martha Munoz, and Fauna Samuel—I treasure your company, and thank you for making my life richer and happier. I adore you all.

I owe thanks to a few early influences—namely, Mr. Tim Cady, my teacher in grades 5 and 6. You were the first to put me on the track of being a “smart kid.” Thank you to my family: my mom, sister Hannah, and brothers Kalven and Talon.

To Farish, Charles, Hopi, Brant, and Emily—thank you for sharing your infectious excitement for science and nature.

And most of all, thanks to the mice that made this project possible.
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**List of Abbreviations**

- ASE — allele-specific expression
- cM — centimorgans
- ddRAD-Seq — double-digest restriction associated DNA sequencing
- DiffASE — differential allele-specific expression
- indel — insertion or deletion
- LOD — log of odds ratio
- Mb — megabases
- QTL — quantitative trait locus
- SNP — single nucleotide polymorphism
- TF — transcription factor
- BLAST — basic local alignment search tool
Introduction

“... the effort expended in discovering the genetics would be wasted. Better to allocate that effort to studying in evolutionary ways characters of evolutionary interest, and in a genetic way characters of genetic interest.”

-- Alan Grafen 1984

It is widely accepted that behavior is a discipline of biology. Thus, like other phenotypes behavior must have genetic, morphological and physiological foundations that have been shaped by evolutionary processes. But what are the proximate mechanisms underlying the evolution of behavior between lineages? Although evolutionary theory primarily addresses genetic processes, the study of behavior evolution has for decades largely ignored genes (Boake et al. 2002; Greenspan 2008; Wilson 1975). The reasons are several, but one leading reason is the embrace by behavioral ecologists of the “phenotypic gambit” (Grafen 1984), which assumes that natural selection is an optimizing process unconstrained by proximate mechanisms. Perhaps as a consequence, accounts of diversity in animal behavior and its evolution are plentiful (e.g. Bouchard and Lynch 1989; Price and Lanyon 2002; Wainwright and Turingan 1997; Winkler and Sheldon 1993) but little is known about the proximate mechanisms by which behaviors are constrained, or alternatively, change and evolve between lineages.
To understand behavioral evolution, it will be necessary to identify the genetic mechanisms that mediate ecologically-important behavioral variation. The details of genetic architecture (i.e. the manner in which genotypes produce phenotypes, including number and effect size of loci, epistasis, dominance, and pleiotropy) can influence and constrain the course of evolution in a variety of ways. To give a few examples: (1) the degree of pleiotropy affects the modularity and evolvability of traits (Gleason 2005; Kirkpatrick and Barton 2006); (2) the number and effect size of loci underlying a trait hints at whether evolution progresses in small, gradual steps or larger ones (Gleason 2005; Shaw and Wiley 2010); and (3) certain genetic architectures may be surprising for naturally-evolved traits. For example, a threshold trait implies that variant alleles at multiple loci must have increased in frequency before the phenotype appeared in the ancestral population (Pulido 2007). Thus, knowledge of genetic mechanisms is critical for the study of evolution.

Moreover, the mechanisms underlying natural differences in behavior may be fundamentally different from those discovered through traditional laboratory-based behavioral studies, which have typically focused on a handful of model organisms originally selected for their suitability for captive rearing, not for fitness-related behavioral differences. Moreover, such laboratory studies have relied heavily on artificial sources of variation, such as mutants, transgenics, and selected lines (e.g. Lynch 1980; Stowers et al. 2002; Fischbach and Heisenberg 1984). These “artificial phenotypes” often have large-scale and deleterious effects on organisms, and their relevance to behavioral changes in nature is not clear.
More specifically, the genetic basis of a behavior (i.e. all of the genes that give rise to the complete set of structures and molecular interactions that work together to produce a given behavior) may be fundamentally different from the genetic variation that gives rise to differences in behavior between natural populations or species (Shaw and Wiley 2010; Gleason 2005). When a behavior is altered by laboratory manipulation, any component part of the anatomy or physiology contributing to the behavior may be involved. Because each behavior involves neurons acting upon a musculoskeletal structure in the context of hormonal and neuromodulatory signals, and possibly modified by the environment and previous experience—there are many ways to artificially alter behavior. In contrast, when taking an evolutionary perspective, it is not necessary to catalog the full set of genes necessary for the expression of a behavior, but rather, to uncover the particular genetic differences that result in behavioral variations between lineages (Shaw and Wiley 2010).

With important exceptions (e.g. Rothenbuhler 1964; Scott and Fuller 1965), the divide between genetics and behavior largely stood for decades (Wilson 1975). But increasingly, it is feasible to study the genetic underpinnings of behavior in non-model organisms with ethologically-important differences (Boake et al. 2002; MacKay et al. 2009; Hofmann et al. 2014). Indeed, integrative approaches to the study of behavior have become widely and enthusiastically advocated in recent years (e.g. Ophir 2011; MacDougall-Shackleton 2011; Barrett et al. 2013; Bateson and Laland 2013; Hofmann et al. 2014; Rittschof and Robinson 2014), following a handful of successful integrative behavioral studies (e.g. Ben-Shahar et al. 2002; Krieger and Ross 2002; Sokolowski 1997;
Young and Wang 2004). While uniting all of Tinbergen’s questions (mechanism, ontogeny, phylogeny and function; 1963) in a single research project is a goal that is rarely attained, it is an ideal that has inspired my dissertation research.

**Study System**

In this dissertation, I bring an integrative approach to the study of burrowing behavior in *Peromyscus* rodents, a system that is ideal for uncovering the proximate mechanisms of an adaptive behavior with natural variation. Burrow construction is an ecologically important behavior, as burrows facilitate social interactions and provide shelter from predators and fluctuations in temperature (Hansell 2005; Jackson 2002). Indeed, burrowing has evolved myriad times among diverse animal taxa, from bivalves to carnivores (Hansell 2005). In *Peromyscus*, burrow architecture varies markedly between species. These differences are most pronounced between interfertile sister species *P. polionotus* (oldfield mouse) and *P. maniculatus* (deer mouse), and their respective burrow architectures are correlated with their habitat. *P. polionotus* inhabits fields and beaches with sparse vegetation, and excavates long burrows (~60 cm) of stereotyped structure comprising an entrance tunnel, nest chamber, and additional escape tunnel that radiates from the nest to near the soil surface, which is used for exiting the burrow following perturbation of the entrance (Sumner and Karol 1929; Hayne 1936). This burrow shape likely evolved in response to predation in open habitats (Blair 1951; Ivey 1949). In contrast, *P. maniculatus* dwells in forests and prairies with denser vegetation that provide natural shelter, and it digs shorter burrows (~20 cm)
with only a single tunnel (Dawson et al. 1988; Blair 1951; Ivey 1949). Furthermore, the evolutionary history of burrow architecture is well understood in *Peromyscus*: when viewed in a phylogenetic context, the long burrows and escape tunnels of *P. polionotus* are unique and derived traits (Weber and Hoekstra 2009). In sum, the burrowing adaptations of *P. polionotus* constitute an ecologically important, complex, and recently derived behavior.

Burrowing is also tractable for lab-based studies, as *P. polionotus* and *P. maniculatus* breed readily in captivity and each species reliably recapitulates their species specific burrow architectures in laboratory enclosures (Dawson et al. 1988; Weber and Hoekstra 2009). These burrows are a classic “extended phenotype” (Dawkins 1982) and their structure can be preserved by casting tunnels to capture a tangible, “morphological” representation of behavior (Felthauser and McInroy 1983). Importantly, these casts provide clear, discrete characters for measurement, circumventing some of the difficulties in quantifying behavior directly. Furthermore, the species-specific burrow differences are known to have a strong genetic component (Dawson et al. 1988). Because *P. polionotus* and *P. maniculatus* hybridize, genetic crosses are possible, and this enables a forward-genetics approach to uncover the underlying genetic basis of burrowing differences (Dawson et al. 1988; Weber et al. 2013). Together, these qualities make burrowing in *Peromyscus* an excellent system for the study of behavioral evolution at both the proximate and ultimate levels.
References


Chapter 1
Ontogeny of burrowing behavior: pleiotropic gene action across life stages

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In preparation for *Current Biology*

**Highlights**

- *Peromyscus polionotus* constructs burrows precociously compared to sister species, *P. maniculatus*
- Precocious burrow construction segregates dominantly in F₁ hybrids, and is correlated with adult traits in F₂ hybrids
- A QTL linked to adult tunnel length is predictive of precocious burrow construction in hybrids
- Causal variants appear to act pleiotropically—affecting both development (precocious digging) and adult behavior (burrow length)

**eTOC**

How does behavior evolve in the wild? Metz et al. find that *P. polionotus* mice exhibit precocious burrowing behavior compared to sister species *P. maniculatus*, a developmental difference that is unaltered by cross-fostering. In F₂ hybrids, precocious burrowing is co-inherited with adult traits. Furthermore, a QTL linked to adult tunnel length predicts precocious burrowing, suggesting that pleiotropy (or tight linkage) underlies behavioral differences across life stages.
Summary

Background. A central challenge in biology is to understand how behaviors diverge between closely related species. One way to elucidate how differences arise is to compare the timing and progression of development in species with distinct adult behaviors. Here, we capitalize on variation in burrow construction between two sister species, *Peromyscus maniculatus* and *P. polionotus*. As adults, these mice build dramatically different burrows: while *P. polionotus* excavates a long, multi-tunnel burrow of stereotyped form, the burrow of *P. maniculatus* is a short single tunnel.

Results. By measuring burrow construction in juveniles, we found striking ontological differences between species in both timing and development of burrow construction behavior. First, *P. polionotus* began excavating burrows 10 days earlier than *P. maniculatus*, and their burrows frequently included species-specific escape tunnels. In contrast, *P. maniculatus* did not build burrows early in development, despite their larger physical size. To test whether this behavior is influenced by early-life environment, pups were reciprocal cross-fostered, but this did not alter the characteristic burrowing behavior of either species. In backcross F$_2$ hybrids, we show that precocious burrowing in juveniles and adult tunnel length are genetically correlated, and that a QTL linked to adult tunnel length is also predictive of precocious burrowing.

Conclusions. In *Peromyscus*, developmental differences in behavior have a genetic basis. The coinheritance of developmental and adult traits indicates the same genetic region—either a single gene with pleiotropic effects or closely linked genes—acts on
behavior across life stages, and suggests that pleiotropy may be common in behavioral evolution.

**Introduction**

_Huxley likes to speak of ‘the three major problems of biology’: that of causation, that of survival value and that of evolution—to which I should like to add a fourth, that of ontogeny._

—Tinbergen (1963)

Striking phenotypic differences between closely-related species can be a powerful resource for understanding biological diversity and its mechanistic underpinnings—major goals of biological research. Teasing apart how such phenotypic differences arise is critical, as species-specific traits can result from a variety of causes, including genetic variation, differences in environment, or both. Indeed, an integrative research approach was advocated by Tinbergen (1963), who codified four distinct but mutually informative categories of inquiry for behavioral studies. These are now known as Tinbergen’s “Four Questions,” but Tinbergen’s chief contribution appears to be the addition of ontogeny to an existing list. What does ontogeny contribute to the study of behavior?

Ontogeny is the study of how behavior changes across the life of an individual, and such inquiries into development can (1) reveal to what degree divergent behaviors arise from innate differences versus environmental factors, including learning, and thus
clarify the origins of complex behavior (e.g. Brainerd and Doupe 2002; Grimsley et al. 2011; Kikusui et al. 2011; Kroodsma and Canady 1985; Krützen et al. 2005; Tebbich et al. 2001); (2) when paired with phylogeny, uncover unexpected ancestral state reconstructions that were not discernible in studies of adults, resulting in novel hypotheses for the evolution of traits (e.g. Dial 2003; Moczek et al. 2006; Heers and Dial 2012); and (3) help expose underlying proximate mechanisms driving changes in behavior (Gardner et al. 2005; Moon-Fanelli 2011; Schneiderova 2013). In short, ontogeny informs and edifies answers to each of Tinbergen’s four questions.

Here, we focus on the burrow construction, an ecologically important behavior that varies dramatically between closely related sister species of North American Peromyscus rodents—*P. maniculatus* and *P. polionotus* (Figure 1.1; Dawson et al. 1988; Sumner and Karol 1929). The deer mouse, *P. maniculatus*, consistently excavates short (~20 cm) burrows, comprised of a single tunnel terminating at a small nest area. In contrast, the oldfield mouse, *P. polionotus*, builds an considerably longer burrow (~60 cm) composed of an elongated entrance tunnel, a nest chamber, and a secondary tunnel that extends upward from the nest toward the surface. This second tunnel does not penetrate the soil surface except during an emergency evacuation, and is thus often referred to as an “escape tunnel” (Figure 1.1b; Sumner and Karol 1929; Hayne 1936; Ivey 1949). These behavioral innovations in *P. polionotus* have been studied at multiple levels, including phylogenetic history (Weber and Hoekstra 2009), genetic mechanisms (Dawson et al. 1988; Weber et al. 2013), and speculations of adaptive function—namely that *P. polionotus* burrows may provide refuge from the elevated rates of predation that
occur with life in open, exposed habitats (Blair 1951; Jackson 2000). Yet Tinbergen’s fourth question, that of ontogeny, has remained unexamined until now.

More specifically, knowledge of development is important for understanding how the comparatively complex burrow architecture of *P. polionotus* evolved from a presumably simpler ancestral state. In particular, we want to know: When and how does species-specific burrow architecture emerge during development? Are juvenile burrows distinct from adult burrows in size or structure? If incomplete, are immature burrows truncated or miniaturized (Figure 1.1c)?

To address these questions, we compared the development of burrow construction in our two focal sister species. We first characterized the development of burrowing behavior in a time series of *P. maniculatus* and *P. polionotus* juveniles. Next, to disentangle the effects of genes and early-life environment, we assayed burrowing behavior in interspecific cross-fostered animals of both pure species. To examine the inheritance of developmental differences and their relationship with adult traits, we then evaluated burrowing in F$_1$ and recombinant F$_2$ hybrid mice. Finally, building upon previously-mapped QTL loci contributing to differences in adult burrow structure (Weber et al. 2013), we tested for statistical association between these QTLs and developmental traits in backcross F$_2$ hybrids, and tested if these juvenile and adult behaviors remained correlated. Our results demonstrate that striking species-specific differences in burrowing behavior appear early in ontogeny, are not measurably
affected by postnatal juvenile environment or maternal effects, and that developmental phenotypes share (in part) a genetic basis with adult traits.

Figure 1.1. (A) Timeline of general development for *P. polionotus* and *P. maniculatus*. (B) Adult oldfield mice (*P. polionotus*) dig complex burrows with a stereotyped architecture that is evolutionarily derived and dramatically different from the ancestral burrow seen in closely related species. Entrance tunnels are greatly elongated and a second “escape” tunnel is excavated beyond the nest area. (C) During ontogeny, we hypothesized that for a given length of excavated burrow, developing *P. polionotus* juveniles would either dig truncated versions of adult burrows (incomplete, long), thus compromising the architecture of the final product or produce smaller versions of adult burrows with escape tunnels (complete, miniature).
Results

Development of burrow construction

P. polionotus construct burrows precociously compared to P. maniculatus

We found striking interspecific differences in the developmental timing and progression of burrow construction (Figure 1.2). Notably, P. polionotus were precocious diggers relative to P. maniculatus, excavating complete burrows—defined as diggings with at least two components, an entrance tunnel plus a nest area—10 days earlier than their sister species. The first appearance of complete burrows was 17 days of age in P. polionotus (1 of 5 pups, Figure 1.2a), but not until 27 days of age in P. maniculatus (3 of 14 mice, Figure 1.2a), a considerable difference in developmental stage in these mice (see Figure S1 for timeline of development).

From 21 days of age on, nearly all P. polionotus individuals constructed burrows (41 of 47 mice), a frequency statistically indistinguishable from P. polionotus adults (Fisher’s exact test, $P = 0.575$). In contrast, no more than a third of sub-adult P. maniculatus (ages 29-31 days, Figure 1.2a) constructed burrows, a frequency that also did not differ from conspecific adults (Fisher’s exact test, $P = 0.640$). The absence of burrow construction in P. maniculatus before 27 days of age differs significantly from frequencies of burrow construction of both conspecific adults and conspecific juveniles ≥27 days of age (Figure 1.2a and Table 1.1; Fisher’s exact test, $P < 0.001$ and $P = 0.047$, respectively). In summary, P. polionotus burrowed at adult-like frequencies from 19
days of age, a developmental benchmark *P. maniculatus* did not reach until 27 days of age, a difference of 8 days.

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**Table 1.1.** Proportion of mice digging complete burrows varies between species and across life stages. Complete burrows are defined as diggings having at least two components—an entrance tunnel and a nest area. Table shows *P* values for 2 x 2 Fisher’s exact tests for comparisons shown. Mice are grouped according to developmental stage as follows: pre-weaning (ages 19-25 days), weaned juveniles (ages 27-31 days) and adults (60+ days of age). Significance codes are as follows: *P ≤ 0.05 = ‘*’, *P ≤ 0.01 = ‘**’, *P ≤ 0.001 = ‘***’.
In trials when mice did not create burrows, individuals of both species usually excavated small cup-shaped cavities or “divots” instead (see Figure 1.2e), suggesting the motor patterns for digging were at least partly (if not completely) developed. Only three of 97 mice (two 17-day-old *P. polionotus* and a 27-day-old *P. maniculatus*) failed to leave any signs of digging activity.

**JUVENILES CONSTRUCT BURROWS WITH MINIATURIZED ADULT ARCHITECTURE**

*Peromyscus polionotus* and *P. maniculatus* juveniles both produced burrows with architecture typical of adults of their respective species. From 19 days of age, nearly the earliest age tested, the burrows of *P. polionotus* included escape tunnels at a rate not significantly different from conspecific adults (Figure 1.2b; Fisher’s exact test, *P* = 0.523). Likewise, *P. maniculatus* juvenile burrows invariably featured only a single tunnel leading to the nest chamber, always lacking an escape tunnel (Figure 1.2b). Although complete with regard to architectural components, juvenile excavations were significantly shorter than those of adults (Figure 1.2c; Student’s *t*-test *P* < 0.0001 for both species), thus representing miniature versions of adult burrows (Figure 1.1c).

Whereas tunnel length increased with age in both species, reflecting a progression in burrowing ability with growth and development (Figure 1.2c; ANCOVA: dependent variable = excavation length, fixed effect = species, covariate = age in days, *P* < 0.0001), tunnel length varied considerably between species. *Peromyscus polionotus* constructed significantly longer burrows than *P. maniculatus* across ontogeny (Figure 1.2c; ANCOVA: dependent variable = excavation length, fixed effect = species, covariate
= age in days, \( P < 0.0001 \), consistent with the known differences in adult tunnel length (Dawson et al. 1988; Weber and Hoekstra 2009). Furthermore, the rate of increase in tunnel length with increasing age (i.e. the developmental rate) was higher in \( P. polionotus \) (Figure 1.2c; ANCOVA: dependent variable = excavation length, fixed effect = species, covariate = age in days, age x species interaction term, \( P = 0.030 \)).

To evaluate whether precocious burrow construction in \( P. polionotus \) might be due to advantages in physical development rather than behavioral development (e.g. Colonnese et al. 1996), we next examined general measures of morphological and motor development in both species. Two lines of evidence refute the hypothesis that \( P. polionotus \) are generally precocious compared to \( P. maniculatus \). First, \( P. polionotus \) juveniles travelled less distance in an ad libitum wheel-running assay than \( P. maniculatus \); we found significant effects of both age and species on distance travelled (Figure 1.2d; ANCOVA: dependent variable = distance travelled, fixed effect = species, covariate = age in days, age: \( F = 37.12, P < 0.001 \), species: \( F = 14.40, P < 0.001 \)). While total distance run increased with age at a comparable rate in both species (age x species interaction term, \( F = 0.278, P = 0.5993 \)), \( P. maniculatus \) ran significantly greater distances than age-matched \( P. polionotus \) across development. Second \( P. polionotus \) were smaller in both body size (ANCOVA: dependent variable = body mass, fixed effect = species, covariate = age in days, \( F = 136.49, P < 0.0001 \)) and had shorter hindfeet (ANCOVA: dependent variable = hindfoot length, fixed effect = species, covariate = age in days, \( F = 59.72, P < 0.0001 \)) than \( P. maniculatus \) (Supplemental Figure S1). Likewise, we did not observe heterochrony favoring \( P. polionotus \) with respect to developmental
milestones such as eye opening or tooth eruption, as *P. maniculatus* tended to develop earlier (Figure 1.1a; Supplemental Figure S1). Thus, early burrowing in *P. polionotus* juveniles likely reflects a true behavioral difference, not an advantage in motor or morphological development.

**Figure 1.2. Continued on following page.** The ontogeny of burrow construction differs dramatically between sister species *P. polionotus* and *P. maniculatus*. (A) *P. polionotus* begin constructing complete burrows (excavations comprised of ≥ 1 tunnel plus a nest area) 10 days earlier than *P. maniculatus*, and at the earliest age tested. The proportion of animals constructing burrows was adult-like from 21 days of age in *P. polionotus* juveniles (Fisher’s exact test, *P* = 0.5748). In contrast, *P. maniculatus* did not construct burrows until 27 days of age, from which age frequency of burrow construction was not significantly different from conspecific adults (Fisher’s exact test, *P* = 0.6404). Thus *P. polionotus* began constructing burrows precociously compared to *P. maniculatus*. The proportion of mice building burrows differed significantly between species (Table 1.1). (B) Juveniles constructed burrows with architecture typical of their species. Juvenile *P. polionotus* burrows were complete with escape tunnels as early as 19 days of age, while *P. maniculatus* never constructed escape tunnels. (C) Juvenile *P. polionotus* construct significantly longer excavations than age-matched *P. maniculatus* across ontogeny (ANCOVA, Species, *P* < 0.0001), and the lengths of their excavations increase at a greater rate with development (ANCOVA, Age x Species interaction, *P* = 0.013). Adult phenotypes are shown but not included in the ANCOVA model. (D) Despite digging shorter burrows, *P. maniculatus* ran further in a wheel running assay than *P. polionotus* (ANCOVA, Age: *F* = 37.12, *P* < 0.001, Species: *F* = 14.40, *P* < 0.001, Age x Species interaction, *P* = 0.5993). This suggests that the absence of burrows in *P. maniculatus* prior to 27 days of age is not due to a delay in motor ability or activity compared to *P. polionotus*. (E) Summary of data shown in A-C. With regard to burrow construction, *P. polionotus* juveniles are precocious relative to *P. maniculatus*, constructing burrows 10 days earlier and from the earliest ages tested. Moreover, juvenile mice prioritized burrow shape (architectural components) ahead of burrow size (length of tunnels), thus producing miniature versions of adult architecture. These miniaturized structures then extended toward adult proportions during development.
Figure 1.2. Continued.
Species-specific burrowing behavior unaltered by interspecific cross-fostering

To disentangle the effects of genetics from postnatal social environment, pups were reciprocally cross-fostered between the two species (Figure 1.3a). We reasoned that any resulting effects of environment on burrowing behavior were likely to be greatest during post-natal development. Because the burrowing performance of both singly and group cross-fostered animals did not differ (ANCOVA, total excavation length, \( P. polionotus \): Age \( F = 9.24 \ P = 0.0103 \), Foster Treatment \( F = 0.024 \ P = 0.8798 \), Age x Treatment interaction \( F = 0.183 \ P = 0.6767 \); \( P. maniculatus \): Age \( F = 10.678 \ P = 0.00561 \), Foster Treatment \( F = 0.062 \ P = 0.8066 \), Treatment x Age interaction \( F = 0.063 \ P = 0.853 \)), we grouped those data together for analysis (see below).

The behavior of fostered animals closely matched that of their non-fostered peers across all burrow characteristics examined. The developmental onset of burrow building did not differ significantly between cross-fostered and non-fostered \( P. maniculatus \). Prior to 27 days of age, \( P. maniculatus \) juveniles did not build complete burrows (Figure 1.3b) regardless of foster treatment. Following the onset of burrowing at 27 days of age, fostered animals constructed burrows no more frequently than pups raised by their biological parents (Figure 1.3b; Fisher’s exact test, one-tailed, \( P = 0.57 \)). Cross-fostered \( P. maniculatus \) did not excavate escape tunnels (Figure 1.3c), and the excavations of cross-fostered animals, whether burrows or divots, closely matched those of mice raised by their biological parents with regard to length (Figure 1.3d; ANCOVA, \( P = 0.63 \)).
Likewise, *P. polionotus* raised by heterospecific parents began burrowing at the earliest age tested (19 days of age; Figure 1.3e)—and from 21 days of age, nearly all cross-fostered *P. polionotus* excavated burrows (12 of 14 mice; Figure 1.3e), a frequency statistically indistinguishable from those of non-fostered juveniles or adult *P. polionotus* (Fisher’s exact test, one tailed, $P = 0.59$ and $P = 0.36$, respectively). Likewise, burrow structure did not change with cross-fostering treatment. Cross-fostered *P. polionotus* dug escape tunnels as early in ontogeny (from 19 days of age), and as frequently (50%, 8 of 16 mice) as non-fostered animals (41%, 22 of 53 mice; Figure 1.3f, Fisher’s exact test $P = 0.34$) and adults (67%, 6 of 9 mice; Fisher’s exact test $P = 0.58$). Finally, excavation lengths did not differ between cross-fostered animals and mice raised by their biological parents (ANCOVA, $P = 0.06$, Figure 1.3g). Indeed, in *P. polionotus*, burrows of fostered pups tended to be longer than those of non-fostered animals, although the difference was not significant. This is the opposite of the expected effect on excavation length if learning or parental care played an important role in the ontogeny of the species’ typical burrow construction. In summary, we found no differences between non-fostered *P. polionotus* and their peers who were fostered from birth by *P. maniculatus*. 
Figure 1.3. Reciprocal interspecific cross-fostering between *P. polionotus* and *P. maniculatus* does not alter ontogeny of species-specific burrowing behavior. (A) Schematic of cross-fostering design. (B-D) *P. maniculatus* and (E-G) *P. polionotus*. Cross-fostered individuals do not differ from pups raised by biological parents in frequency of burrow building in *P. maniculatus* (B; Fisher’s exact test, *P* = 0.57) or in *P. polionotus* (E; Fisher’s exact test, *P* = 0.59).
Figure 1.3. Continued. Likewise, species-specific escape tunnel construction was absent in cross-fostered *P. maniculatus* (C) and did not differ from species-typical frequency in cross-fostered *P. polionotus* (F; Fisher’s exact test, \( P = 0.34 \)). Sample sizes for each age and foster group shown. (D and G) Lengths of excavations do not significantly differ between juveniles raised by their biological parents and those fostered by heterospecific breeding pairs (*P. maniculatus* ANCOVA, \( P > 0.05 \); *P. polionotus* ANCOVA, \( P > 0.05 \)).

**Genetic basis of behavioral development**

Above, we report precocious burrow construction in *P. polionotus* compared to sister species *P. maniculatus*. Here, we test the hypothesis that this advanced developmental onset shares a common genetic basis with the well-characterized differences in adult burrow architecture (Dawson et al. 1988; Weber and Hoekstra 2009, Weber et al. 2013). To test this hypothesis, we first generated F\(_1\) hybrid mice to determine the dominance pattern of developmental traits including precocious burrow construction. Next, we generated a population of backcross F\(_2\) hybrids to test whether adult phenotypes (e.g. tunnel length, escape tunnel presence) segregate independently from developmental traits (e.g. precocious onset of behavior). These data are summarized in Figure 1.4.

**Ontogeny of burrow construction is *P. polionotus*-dominant**

The development of burrowing behavior in F\(_1\) hybrids closely matches their *P. polionotus* parent in each parameter examined, including frequency of burrow construction (Figure 1.4b; Fisher’s exact test, \( P = 0.3784 \)) frequency of escape tunnel digging (Figure 1.4b; Fisher’s exact test, \( P = 1.00 \)), and length of excavations (Figure 1.4c; ANCOVA, \( F = 3.164, P = 0.0858 \)).
Figure 1.4. Genetic dissection of precocious burrowing (a developmental trait) in *P. polionotus* X *P. maniculatus* *F₁* and *F₂* hybrids reveals precocious burrow construction is dominant and co-inherited with adult traits. (A) Schematic of breeding design and color legend. (B) *F₁* hybrids exhibit a developmental trajectory like that of their *P. polionotus* parent with respect to burrow construction. They construct burrows and escape tunnels as early and often as *P. polionotus* (Fisher’s exact test *P* = 0.3784), but more frequently than *P. maniculatus* (Fisher’s exact test, *P* < 0.0001). (C) Excavation lengths of *F₁* hybrids are likewise indistinguishable from *P. polionotus* (ANCOVA, *F* = 3.164, *P* = 0.0858), but significantly greater than *P. maniculatus* (ANCOVA, *F* = 46.62, *P* < 0.0001).
However, they differ significantly from *P. maniculatus* in all of these measures of burrowing behavior: frequency of burrow construction (Figure 1.4b; Fisher’s exact test, \( P < 0.0001 \)), frequency of escape tunnel digging (Figure 1.4b; Fisher’s exact test, \( P = 0.0188 \)), and length of excavations (Figure 1.4c; ANCOVA, \( F = 46.62, P < 0.0001 \)). This inheritance pattern indicates that the genetic underpinnings precocious burrowing, a developmental trait, are *P. polionotus*-dominant, consistent with the pattern of inheritance observed for adult burrowing behavior (e.g. Dawson et al. 1988; Weber et al. 2013).

**Developmental and Adult Traits Share a Common Genetic Basis**

We next asked if the developmental traits described above (namely, precocious burrow construction) and adult traits (lengthened tunnels, escape tunnel digging) are associated in a *P. polionotus* x *P. maniculatus* experimental cross, in which alleles are recombined in F\(_2\) hybrids (Figure 1.4a). If traits have an independent genetic basis, they are expected to be uncoupled in the F\(_2\) generation; trait correlations in a recombinant population indicate shared or closely-linked genetic underpinnings for those traits. Thus, we produced 60 backcross F\(_2\) hybrids and assessed burrowing performance for each
individual at four time points: two juvenile (ages 21 and 24 days) and two adult (ages 61 and 64 days). 31 hybrid animals were scored as precocious burrowers, as they dug at least one juvenile burrow, and the remaining 29 animals were scored as delayed burrowers as they completed no burrows at ages 21 or 24 days. Consistent with there being a shared genetic basis for burrowing traits across life stages, we found that precocious burrowers went on to dig significantly longer burrows as adults (Figure 1.4d; Welch’s two sample t-test, $P = 0.0241$). Furthermore, juvenile excavation length was significantly associated with adult tunnel length (Figure 1.4e; least-squares linear regression, $R^2 = 0.1681$, $P = 0.0013$). Together, these data suggest that for burrowing behavior, developmental (precocious onset of burrowing) and adult (tunnel length) traits share a pleiotropic genetic basis, are closely linked, or both. Cross direction (i.e. the whether $F_1$ parent was the sire or dam) did not significantly impact juvenile or adult burrow length (Student’s t-tests, juveniles: $P = 0.3709$; adults: $P = 0.673$). Similarly, sex of $F_2$ hybrid had no significant relationship with burrow length (Student’s t-test, juveniles: $P = 0.6824$; adults: $P = 0.4311$).

To directly test the hypothesis that juvenile and adult traits share some genetic underpinnings, we next genotyped backcross $F_2$ mice at four unlinked markers, each representing a QTL peak linked to differences in adult burrow architecture in a previously published QTL-mapping study (Weber et al. 2013). Of these, we found that inheritance of a $P. polionotus$ allele was predictive of phenotype for only one of these four markers (Figure 1.5). Marker 2, on linkage group 2, is a QTL linked to entrance tunnel length variation in adult $F_2$ hybrids, and we found it is also predictive of multiple
juvenile phenotypes. Marker 2 genotype significantly predicted precocious burrow construction (Figure 1.5a; Fisher’s exact test, one-tailed, \( P = 0.0440 \)), juvenile excavation length (Figure 1.5a; Student’s t-test, \( P = 0.0136 \)), and juvenile burrow length (Student’s t-test, \( P = 0.0457 \), data not shown). In concordance with the study by Weber et al. (2013), marker 2 genotype predicted variation in adult tunnel length (Figure 1.5a; Student’s t-test \( P = 0.0329 \)). For each of the other markers examined, no significant relationships between genotype and phenotype were detected (Figure 1.5; Fisher’s exact tests and t-tests, \( P > 0.05 \)), possibly due to the small sample size examined in this study.

**Figure 1.5. Continued on following page.** Genotypes at QTLs previously linked to adult burrow architecture also predict juvenile behavior in recombinant hybrids. For each plot, 60 backcross \( F_2 \) hybrids (\( F_1 \times P. maniculatus \)) are split according to genotypes at one of four markers. Each marker represents the center of a QTL peak described in Weber et al. (2013): three QTLs were found to be linked to adult entrance tunnel length (markers 1-3), and one was linked to escape tunnel presence or absence (marker 4). Linkage groups (LG) from Weber et al. indicated for each marker. Genotypes are either MM (homozygous \( P. maniculatus \)) or PM (heterozygous). For each genotype group, the phenotypic mean is plotted, with error bars at +/- 1 SE of the mean. A-C: markers linked to adult tunnel length in Weber et al. (2013). (A) Relationship between noted genotype and Precociousness Score, where individuals received a score of 1 if they dug at least one discrete burrow at ages 21 or 24, and a score of 0 if not. Parental precociousness (a similar phenotype, but based on a single trial only, rather than the two trials for backcross animals plotted), at ages 21-24 are shown in cyan (\( P. polionotus \)) and yellow (\( P. maniculatus \)) for comparison. A \( P. polionotus \) allele at marker 2 is significantly predictive of precocious burrow construction (Fisher’s exact test, one-tailed, \( P = 0.0440 \)). Genotypes at other markers not significantly associated with this phenotype.
Figure 1.5. Continued. (B) Juvenile total excavation length split by marker genotypes. A *P. polionotus* allele at marker 2 is predictive of longer juvenile excavation length (Student’s t-test $P = 0.0136$). Markers 1 and 3 not found to be significant predictors of juvenile tunnel length in this population. (C) Adult total excavation length split according to genotype. In concordance with Weber et al. 2013, a *P. polionotus* allele at marker 2 is predictive of longer tunnel lengths in adults’ burrows (Student’s t-test, $P = 0.0329$). D-F: In this data set, genotype at marker 4 was not found to significantly predict frequency of escape tunnel construction in juveniles (D), juvenile escape length (E) or adult escape length (F) in the 60 backcross $F_2$ hybrids examined here.
Discussion

Burrow construction is a complex behavior driven by multiple motor patterns that results in the creation of a subterranean home comprised of tunnels and chambers (Hansell 2005). Here, we report on how the final product of digging behavior—the burrow—originates and progresses during the post-natal development of *Peromyscus maniculatus* and *P. polionotus*, two sister species with dramatically different adult burrow architectures. We find that the development of burrowing in juvenile mice mirrors the behavior of adults of their respective species (Weber and Hoekstra 2009): compared to *P. maniculatus*, *P. polionotus* juveniles dig longer tunnels and build burrows more frequently across ontogeny. Additionally, each species’ characteristic burrow architecture is intact in their juveniles, with *P. polionotus* frequently excavating escape tunnels from 19 days of age, and *P. maniculatus* never constructing escape tunnels. But strikingly, we find that *P. polionotus* are precocious with respect to burrow construction, compared to *P. maniculatus*, and begin building complete burrows at least 10 days earlier. We next report that all aspects of species-specific burrowing behavior is preserved in cross-fostered individuals of both species, demonstrating that burrowing behavior is likely to have a strong genetic basis, with post-natal environmental factors having little, if any, influence on the differences between species. Finally, we examined the genetic underpinnings of behavioral ontogeny in *P. polionotus* and *P. maniculatus* using a genetic cross. We found that a developmental trait (precocious onset of burrowing) and adult traits (lengthened tunnels of adult *P. polionotus* burrows) are co-inherited, both at the level of phenotypic co-variation and with respect to specific
genetic markers. These data point to a shared—likely pleiotropic—genetic basis influencing behavior across life stages.

While our cross-fostering results are consistent with a strong genetic basis for the striking burrowing differences between species—both in development and as adults—our study design does not eliminate environmental effects which may transpire prenatally (Francis et al. 2003). While embryo transfer experiments could be employed to test for prenatal effects, dysregulation of placental development due to genomic imprinting-related incompatibilities between species could be a limitation to these methods (Vrana et al. 2000).

Our findings have implications that extend to categories of explanations beyond ontogeny (i.e. of Tinbergen’s Four Questions). First, pleiotropy (or tight linkage of multiple causal mutations) can facilitate or inhibit evolution. On one hand, pleiotropy can produce effects that are not directly selected for, but secondarily “dragged along” by evolution (Stern 2013; Cooper et al. 2007). On the other hand, because changes in several traits are often involved during adaptation to a new environment (Fisher 1930; Orr 2000; Schluter 2000), co-inheritance of groups of phenotypes (e.g. by pleiotropy or linkage) can expedite adaptation (Kirkpatrick and Barton 2006; Hoffmann and Rieseberg 2008). Indeed, a common experimental outcome is to map multiple phenotypic traits to a shared genomic region (e.g. Mills et al. 2014; Hawthorne and Via 2001; Hall et al. 2006; Protas et al. 2008; Joron et al. 2011).
Here, one parsimonious explanation for the co-inheritance of precocious burrowing with lengthened adult tunnels is that evolution has shaped both behaviors by acting on innate internal states that persist across life history stages. Specifically, genetic variation may act to enhance motivation to engage in burrowing activity relative to alternative behavioral choices. A modification in drive to burrow between *P. maniculatus* and *P. polionotus* could pleiotropically affect both developmental onset of burrowing and length of tunnels in adults. Indeed, many behavior genetic studies suggest that evolution may be predisposed to act on neuromodulatory systems (Bendesky and Bargmann 2011). Moreover, shifts in behavioral drives and priorities can underlie variation in behavior across time within individuals (e.g. DeNardo and Sinervo 1994), and variation in behavior between species (e.g. Young and Wang 2004).

*P. polionotus* dug burrows at the earliest ages tested—17 days of age, and with adult-like frequencies by 19 days of age. We were unable to test mice younger than 17 days of age because they were too underdeveloped for prolonged separation from their dam. Thus, our data suggests that the limited physical ability (e.g. small size or dependence on their dam for nutrition and warmth) may be the primary constraint on burrowing in very young *P. polionotus*, rather than behavioral development. It is possible that this species would construct burrows at even earlier ages if provided adequate nutrition and warmth. In contrast, *P. maniculatus* did not construct burrows until 3 days post-weaning, at 27 days of age. This delay in developmental onset could be due to an innate timing difference in onset of motivation to construct burrows (relative to *P. polionotus*), or alternatively, could be due different mechanisms triggering the
onset of burrowing. For example, in *P. maniculatus*, burrowing may be a facultative response to separation from the mother (an environmental trigger), while in *P. polionotus*, onset of burrowing occurs at an early developmental stage regardless of environment. While beyond the scope of this study, the latter hypothesis could be tested by varying the age of weaning in *P. maniculatus*.

The precocious burrowing of *P. polionotus* may reflect natural selection for burrowing earlier in life, perhaps because (1) the open habitat of *P. polionotus* (Sumner and Karol 1929) exposes young mice to a higher rate of predation and thus increases the survival value of burrowing, or (2) juvenile burrow-building represents a form of “play” during a critical period of motor development (Byers 1998; Byers and Walker 1995; Burghardt 2005), in which the young animals practice a behavior that is ecologically-important to them as adults. Our results, which implicate a broadly-acting pleiotropic genetic mechanism, call into question which trait or traits have been selected—precocious juvenile burrowing, long adult burrows, or both.

The motivation hypothesis—that an increase in drive to dig burrows underlies some of the behavioral differences described between these species—is strengthened by our finding that juvenile *P. polionotus* excavate miniaturized adults burrows. If limited by physical ability to a given length of excavated tunnels, there are two distinct possibilities for *P. polionotus*: (1) truncate the complete architecture (but perhaps incrementally lengthen it over subsequent nights) or (2) miniaturize (Figure 1.1c). Our data clearly support the miniature hypothesis, which suggests that mice may “measure”
their burrowing efforts by physical exertion, rather than by true metrical distance. Miniaturization is a phenomenon that appears in other juvenile behaviors as well (e.g. Bleher 2000). This points toward a particular neurobiological mechanism—changes in internal (motivational) states or drives—as affecting the differences in burrowing behavior between these species.

**Experimental Procedures**

*Animal care and breeding*

Experiments were conducted using captive *Peromyscus* strains kept under controlled laboratory conditions. Strains of both species were originally acquired from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia SC, USA). These outbred lines, derived from wild-caught ancestors in 1948 (*P. maniculatus*, BW strain) and 1952 (*P. polionotus*, PO strain), have been laboratory-housed since capture, and have thus lived without access to soil for well over 100 generations. For each species, breeding pairs were formed by pairing unrelated adults that were checked daily for the presence of new pups. Only offspring of experienced parents (≥1 previous litter weaned) were used for experiments. Mice were housed at 21.1°C on a 16:8 h light:dark cycle and were provided high-fat breeding diet and water *ad libitum*. Animals were housed with access to cotton nesting material, corn cob bedding material, and 3-sided red-tinted plastic shelters (see Supplementary Table S1.2 for full details). We weaned juveniles from their parents 24 days after birth, and weanlings were subsequently housed in littermate groups until completion of experiments. Due to genomic imprinting
in these species, our cross design for production of F$_1$ hybrids was limited to \textit{P. polionotus} sires to \textit{P. maniculatus} dams (Vrana et al. 2000). Thus, this cross design excludes any \textit{P. polionotus} maternal effects acting in favor of \textit{P. polionotus}-like burrowing behavior. All procedures were approved by the Harvard University Institutional Animal Care and Use Committee.

\textit{Burrowing behavior trials: parental species and F$_1$ hybrids}

We tested the burrowing behavior in a total of 131 juvenile mice; 57 \textit{P. maniculatus} (including 18 cross-fostered individuals) and 74 \textit{P. polionotus} (16 cross-fostered) ranging in age from 17 to 31 days old, and 11 F$_1$ hybrids ranging in age from 19-24 days. For comparison, we also tested 17 adult \textit{P. maniculatus} and 9 adult \textit{P. polionotus} under similar conditions. Both males and females were tested, as no sex-based differences in burrowing behavior have been observed (Weber et al. 2013).

Burrowing trials were conducted in large, indoor sand-filled arenas as previously described (Weber and Hoekstra 2009), except duration was reduced from 48 hours to 14-17 hours (one 8-hour dark cycle followed by 6-9 hours of light) for juveniles because the youngest mice could not be separated from their dam for longer. Briefly, we released animals into large enclosures (1.2 × 1.5 × 1.1 m) filled with approximately 700 kg of moistened, hard-packed premium playground sand (Pharma-Serv Corp.). Mice were provided with nesting material, standard rodent food, and water during trials. Temperature and light:dark cycle were identical to housing conditions. For mice ≤ 24 days of age, we also provided Napa Nectar (Lenderking, MD, USA) and Nutrical (Tomlyn,
SC, USA), as is standard practice for weanling-age animals. We tested juveniles once, without previous experience, and thus our experiment targeted innate behavior and not learned ability. Thus, each mouse was tested once, singly. We tested mice of both species at postnatal ages 19, 21, 23, 24, 25, 27, 29 and 31 days. Because of the species’ early onset of burrowing behavior, we tested additional *P. polionotus* individuals at 17 days of age, the earliest possible age to separate a juvenile from its mother. We tested *F*$_1$ hybrids at 19, 21, and 24 days of age. Thus, we constructed a developmental time series for each species during key stages of motor and behavioral development.

*Burrowing behavior trials: backcross *F*$_2$ hybrids*

We generated 60 backcross *F*$_2$ hybrids by mating *F*$_1$ hybrids to *P. maniculatus* mates (Figure 1.4a). Both directions of the cross (reciprocal pairings) were used to generate hybrids. 22 animals were produced from *F*$_1$ dam x *P. maniculatus* sire and 38 from *P. maniculatus* dam X *F*$_1$ sire. We then characterized the juvenile and adult burrowing behavior of these mice, thus collecting developmental and adult phenotypes in the same individuals. We tested juvenile mice at 21 and 24 days, as the greatest differences in juvenile burrowing behavior between *P. maniculatus* and *P. polionotus* are observed during this time period (Figure 1.2a). We then tested these same mice as adults (61 and 64 days of age) using the same assay.

Behavioral trials for *F*$_2$ backcross hybrids were conducted as above with the following modifications. Each individual was tested four times in total, at juvenile ages 21 and 24 days, and adult ages 61 and 64 days. Furthermore, enclosure area was
reduced by half (i.e. to 0.6 × 1.5 × 1.1 m) for testing of both juvenile and adult backcross individuals to accommodate the large number of animals being tested.

Burrow Measurements

To quantify burrow construction, at the conclusion of each trial, we inspected enclosures for any excavations, which were qualitatively characterized as either burrows (comprised of ≥1 tunnel plus a discernable nest area), or divots (broad cup-shaped vertical diggings <10 cm); see Figure 1.2e. Burrows were cast by injecting them with polyurethane insulation foam (Hilti Corp., Schaan, Liechtenstein) as previously described (Weber and Hoekstra 2009), and the lengths of burrow components (entrance tunnel, nest chamber, and escape tunnel if present) were measured from these dried polyurethane casts. The length of divots was measured directly in the sand enclosures.

Cross-fostering

To disentangle the effects of genetics from social environment during postnatal development, pups were reciprocally cross-fostered between the two species. Age-matched (≤ 48 hrs age difference) *P. maniculatus* (N= 18) and *P. polionotus* (N=16) pups were traded between experienced (≥1 previous litter) breeding pairs 24-48 hours after birth, and were otherwise kept under constant environmental and housing conditions. To allow us to test for effects of parents versus siblings on the behavior of the test animal(s), two fostering paradigms were used: pups were fostered as either singles (one pup traded between litters, such that the fostered pup had heterospecific siblings and heterospecific parents) or as litters (entire litters traded between breeding pairs, such
that pups had heterospecific foster parents but conspecific siblings). Following weaning from their parents at 24 days, juveniles were housed with cage-mate siblings (biological or foster) until the beginning of behavioral trials.

Wheel-running Behavioral Trials

To compare the ontogeny of general activity levels between species, we performed a standardized wheel running assay. We tested juvenile *P. maniculatus* (N = 43) and *P. polionotus* (N = 40) between the ages of 17 and 31 days. Again, both males and females were tested. A 5-inch flying saucer exercise wheel (Ware Manufacturing Inc., Phoenix, AZ) was outfitted with a CC-COM10W wireless bike computer (Cateye Co. Ltd., Osaka, Japan) to record total distance run over 90 minutes. An exercise wheel was placed in the home cage 4 hours prior to testing to habituate the mouse to the novel object. The mouse was then placed in a new standard cage with a clean wheel and unlimited food and water. *Peromyscus* show strongly nocturnal patterns of wheel running (Dewsbury 1980) thus, all tests were performed between 16:00 and 22:00h during the dark cycle.

Statistical Analyses

To disentangle effects of age on burrowing behavior, we employed several statistical tests. We first tested for effects of age and species on burrowing behavior as well as for effects of sex, postnatal litter size, enclosure, and foster status at the intraspecific level using ANCOVA. Because no statistical differences were detected between treatments, singly cross-fostered individuals (having both heterospecific
parents and siblings) and litter-fostered animals (heterospecific parents but conspecific siblings) were pooled. We used Fisher’s exact test to evaluate the frequencies of burrow and escape tunnel digging between different genetic groups. Welch’s t-tests were used to compare means in F₂ hybrids. To evaluate phenotypic correlations in our F₂ cross, we used least squares linear regression. To detect associations between QTLs linked to adult behavior and precocious burrowing, we used Fisher’s exact test, and for continuous traits in juveniles (i.e. excavation length), Student’s t-tests. Two P. polionotus individuals that appeared in poor health (age >23 days) were excluded from all analyses. Two 17-day P. polionotus individuals were found shivering yet in otherwise good health and were included. All statistical analyses were performed using the R language.

Genotyping

We made use of restriction fragment length polymorphisms (RFLPs) to genotype the backcross F₂ population (N=60) at four regions corresponding to known QTL underlying adult burrowing behavior (Weber et al. 2013). All four assays were designed such that the P. polionotus allele contained a restriction enzyme cut site whereas the P. maniculatus allele did not. PCR was performed with a Taq DNA Polymerase kit (Qiagen) and custom primers (Integrated DNA Technologies; Table S1). We verified that the selected RFLPs were fixed between species by Sanger sequencing (3730XL DNA Analyser, Applied Biosystems) PCR amplicons in 4 unrelated individuals of each species, as well as the P. maniculatus and F₁ parents of the cross (BigDye® Terminator v3.1 Cycle
Sequencing Kit, Life Technologies). PCR products were digested with restriction enzymes (New England Biolabs, Ipswich MA) and separated by gel electrophoresis using a Quick-Load® 100bp DNA Ladder (New England Biolabs, Ipswich MA) as a size reference. Digested fragments were visualized under UV light. All backcross animals inherit at least one *P. maniculatus* allele; therefore, we interpreted the presence of a second smaller fragment (of appropriate size) as evidence of a *P. polionotus* allele.

**Author Contributions:** H.C.M. and H.E.H. conceived and designed the experiments. H.C.M., N.L.B. and L.P. performed the experiments, H.C.M. and N.L.B. analyzed the data. H.C.M. and H.E.H wrote the paper.

**Acknowledgements:** We thank S. Scalia, J. Mason and the Hoekstra Lab for assistance with behavioral assays and animal husbandry; A. Bendesky, B. de Bivort, C. Dulac, J. Losos, B. Ölvezczy, B. Peterson, and J. Weber for helpful discussions; Harvard’s Office of Animal Resources, particularly J. Rocca for animal husbandry; and S. Worthington for statistical consultation. We also thank H. Fisher and M. Munoz for comments on the manuscript. This research was funded by Chapman Funds for Vertebrate Locomotion, a National Science Foundation Graduate Research Fellowship, a Doctoral Dissertation Improvement Grant, and an American Fellowship from the American Association of University Women to H.C.M.; a Natural Sciences and Engineering Research Council Graduate Fellowship to N.L.B.; Harvard College Research Program award and a Harvard Museum of Comparative Zoology Grant for Undergraduate Research to L.P., and a Beckman Young Investigator Award and the Howard Hughes Medical Institute to H.E.H.

**References**


Chapter 2
Genetic architecture of an animal architecture: introgression of a complex behavioral phenotype in advanced backcross lines

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Abstract

What genetic architectures underlie differences in behavior between species? Here, we study sister species *Peromyscus polionotus* and *P. maniculatus*, which construct dramatically different burrow shapes, a behavior that is known to have a strong genetic basis. First, we dissect the genetic architecture of this complex behavior using an experimental cross. By introgressing the burrow architecture of *P. polionotus* into the genetic background of *P. maniculatus*, we show that the burrow architecture of *P. polionotus* can be maintained through at least a BC5 generation, suggesting a relatively simple genetic basis. However, the inheritance of *P. polionotus*-specific escape tunnels resembles a threshold trait mediated by multiple loci, rather than a Mendelian locus. Finally, we develop a novel image-based analysis to collect measurements of burrow shape from casts of burrow tunnels, demonstrating the utility of more rigorous measurement of extended phenotypes in the understanding of complex behavior.

Introduction

What is the genetic basis of behavior? This is a central question in not only in animal behavior and evolution, but in neurobiology and psychiatry. But despite years of research, less progress has been made in understanding the genetic underpinnings of behavior than for traits such as morphology and physiology (e.g. Abzhanov et al. 2004; Colosimo et al. 2005). One reason for this relative lack of progress is the inherent difficulty in quantifying behavior, perhaps because behavior is the output and raison d’être of highly complex nervous systems. An animal’s behavior is also less stable across
time than its morphology, making it more difficult to apply a measurement system reliably across a group of individuals. Even in a controlled environment, behavior is usually probabilistic rather than deterministic, making fair, consistent measurements difficult to obtain and interpret (Millinkski 1987; Bendesky and Bargmann 2011; Clutton-Brock and Harvey 1984). Although behavior measurement systems are continually being developed and improved (e.g. Dell et al. 2014), this problem is particularly prominent for complex natural behaviors, many of which are difficult if not impossible to compress into a laboratory setting.

Further difficulty lies in how to uncover the complete genetic underpinnings of natural behavioral differences. Forward genetic approaches such as QTL mapping can detect loci linked to traits of interest (e.g. Yalcin et al. 2004; MacKay et al. 2009), but not all quantitative trait loci (QTL) are equally likely to be detected. Mapping studies involve a finite number individuals and genetic markers and have little power to detect genetic factors of small to moderate effects (Boake 1994; MacKay et al. 2009). Indeed, QTL studies are known to underestimate the number of loci underlying traits, as well as to overestimate the effect sizes (i.e. proportion of segregation variance explained) of detected loci, a.k.a. the Beavis effect (Beavis 1994). Yet it is useful to accurately estimate some parameters of a trait’s genetic architecture before proceeding with follow-up experiments (Otto and Jones 2000). A basic question of genetic architecture is whether a trait is the product of a single or few genes with large effects (and perhaps modified by other genes of small influence) or by many genes each having minor individual effect on the trait. The number of genes producing segregating variation in a
trait influences both the tractability of a system for in-depth genetic analysis, as well as its evolutionary dynamics (Shaw and Wiley 2010; Albert et al. 2008).

Here, we capitalize on the intransience of animal architecture to uncover the genetic underpinnings of a natural, fitness-related behavior that varies dramatically in the wild. *Peromyscus* rodents, like many mammals, inhabit subterranean burrows that they construct through a series of behaviors (Hansell 2005). Burrow construction is an ecologically-important behavior, as burrows can facilitate social interactions and provide shelter from predators and temperature extremes (Hansell 2005; Jackson 2000). In *Peromyscus*, burrow architecture varies dramatically between species. These differences are most pronounced between interfertile sister species *P. polionotus* (oldfield mouse) and *P. maniculatus* (deer mouse), and are perhaps associated with differences in the species’ habitats (Figure 2.1). *Peromyscus polionotus* inhabits fallow fields and beaches with sparse vegetation in the Southeastern U.S.A., and excavates long burrows (~60 cm) with a stereotyped multi-component structure: an entrance tunnel, nest chamber, and additional escape tunnel that radiates from the nest to just below the soil surface, which is used for exiting the burrow following a disturbance. This burrow shape likely evolved in response to living in open habitats (Hayne 1939; Blair 1951; Ivey 1949; Sumner and Karol 1929). In contrast, *P. maniculatus* dwells in forests and prairies with denser vegetation that may provide natural shelter, and it digs shorter burrows (~20 cm) with only a single tunnel (Figure 2.1; Dawson et al. 1988).
In this chapter, to quantify complex behavioral variation, we evaluate burrow construction behavior as an “extended phenotype” (Dawkins 1982). Just as developmental processes give rise to body structures, behavioral steps can give rise to architectural structures whose building process has an analogous ontogeny (Hansell 2005). Thus, rather than examining and cataloging numerous ephemeral individual behaviors, the end-point of those behaviors can be directly measured. The final product—an extended phenotype—represents an integration of several behaviors across time.

**Figure 2.1.** Range, habitat, and burrowing behavior of *P. maniculatus* (ochre) and *P. polionotus* (cyan). Slanting lines point to locations where laboratory stocks were originally captured (Ann Arbor, MI and Ocala, FL). (A) While *P. maniculatus* ranges broadly across North America, *P. polionotus* inhabits a limited region of the Southeastern U.S.A. (Hall 1981). (B) Typical habitats of each species. *P. maniculatus* comprises several subspecies that live in diverse habitat types, but mainly prairies and forests. In contrast, *P. polionotus* is an open field specialist, living in fallow fields and on sandy beaches (Sumner and Karol 1929). (C) Typical burrow shapes reported for each species (Dawson et al. 1988; Weber and Hoekstra 2009; Weber et al. 2013).
In *The Origin of the Species*, Darwin (1859) argues that elaborate behaviors, including intricate animal architecture, are products of natural selection. And indeed these structures are known to evolve between lineages (e.g. Winkler & Sheldon 1993; Kusmierski et al. 1997; Collias and Collias 1964; Weber and Hoekstra 2009). Arguably, natural selection acts more directly on these products of behavior than on the individual component actions that give rise to the final structure. A comparable example from morphology would be selection acting most directly on limb length, rather than on the activity of each individual morphogen and tissue that patterns the limb during development. In the case of burrows, tunnels can be cast to create a tangible, “morphological” representation of behavior (Felthauer and McInroy 1983). These casts provide clear, discrete behavioral characters for measurement, circumventing some of the difficulties in quantifying behavior.

Here, we investigate the genetic architecture of the differences in burrow shape between *P. polionotus* and *P. maniculatus* using a genetic cross. Previously, these differences in burrow shape were reported to have a simple, modular genetic basis—with tunnel length influenced by just three loci and escape tunnel by perhaps a single Mendelian locus (Dawson et al. 1988; Weber et al. 2013). We dissect the genetic basis of this behavior further using an advanced backcross study, and find evidence that burrow architecture has a more complex genetic basis, with the escape tunnel appearing as a threshold trait. Next, we explore methods to measure many parameters of burrow architecture using automated image analysis, and find that comprehensive, detailed
measurement of burrow size and shape show promise for understanding this animal architecture.

Methods

Animal care and breeding

Experiments were conducted using captive *Peromyscus maniculatus*, *P. polionotus*, and their hybrid offspring, kept under controlled laboratory conditions. Strains of both species were originally acquired from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia SC, USA). These outbred lines, derived from wild-caught ancestors in 1948 (*P. maniculatus*, BW strain) and 1952 (*P. polionotus*, PO strain), have been laboratory-housed since capture, and have thus lived without access to soil for well over 100 generations. Mice were housed at 21.1°C on a 16:8 h light:dark cycle and were provided standard rodent diet and water *ad libitum* (see Supplementary Table S1.2 for details). Animals were housed with cotton nesting material, corn cob bedding material, and 3-sided red-tinted plastic shelters (see Supplementary Table S1.2). Juveniles were weaned from their parents 23-24 days after birth, and weanlings were subsequently housed in same-sex groups until behavioral testing. All procedures were approved by the Harvard University Institutional Animal Care and Use Committee, protocol number 27-09.

First generation hybrids were generated by crossing a single male *P. polionotus* to a *P. maniculatus* female. Genomic imprinting-related health issues prevent the reciprocal cross with these species (Vrana et al. 2000). 13 F₁ hybrids of both sexes were
subsequently paired with *P. maniculatus* mates to generate $F_2$ backcross hybrids, or BC1s (first backcross generation). From these recombinant hybrids (BC1 generation, as well as for subsequent generations BC2-BC5), a small subset of individuals were selected, based on phenotypic criteria, to be parents of an additional generation of backcross hybrids (Figure 2.2). These criteria were chosen based on findings of Weber et al. 2013: average entrance tunnel length $\geq 12$ cm and presence of at least one escape tunnel across three trials. Such individuals were each paired with a *P. maniculatus* mate of the opposite sex and housed as described above. Breeding continued through an $F_6$ (BC5) generation. For generations BC2-BC5, the number of selected parents giving rise to that generation were 14, 11, 7, and 1, respectively.

**Burrowing behavior trials**

We tested the burrowing behavior in a total of 892 mice ($n=339$ BC1, $n=163$ BC2, $n=287$ BC3, $n=97$ BC4, and $n=6$ BC5), ranging in age from 60 to 100 days old in large, indoor, sand-filled enclosures. Both males ($n=434$) and females ($n=458$) were included in all analyses, as no sex-based differences in burrowing behavior have been observed previously in hybrids (Weber et al. 2013), nor were any detected here (sex comparisons: average entrance tunnel length $t = 1.42, P = 0.156$; average escape length $t = 1.08, P = 0.279$; number of escape tunnels dug across 3 trials, $t = 0.724, P = 0.469$; Welch’s two sample $t$-tests).
Burrowing trials were conducted as previously described (Weber et al. 2013). Briefly, we released animals into large enclosures (1.2 × 1.5 × 1.1 m) filled with approximately 700 kg of moistened, hard-packed premium playground sand (Pharma-Serv Corp.). Mice were provided with nesting material, standard rodent food, and water during trials. Temperature and light:dark cycle were identical to housing conditions, as
behavior and housing shared a common room. We tested each mouse three times, consecutively, assigning mice to a new enclosure at random for each trial.

**Burrow measurements—experimental genetic cross**

To quantify burrow construction, at the conclusion of each trial, we inspected enclosures for any excavations, which were qualitatively characterized as either burrows (comprised of ≥1 tunnel plus a discernable nest area), or divots (broad cup-shaped vertical diggings <10 cm). Divots were measured directly from enclosures, while burrows were cast by injecting them with polyurethane insulation foam (Hilti Corp., Schaan, Liechtenstein) as previously described (Weber and Hoekstra 2009), and hand measurements of lengths of components (entrance tunnel length, nest chamber length, and escape tunnel length, which sum to give total burrow length) were taken directly from casts.

**Burrow measurements—automated analysis of animal architecture**

Burrows were cast by injecting them with polyurethane insulation foam (Hilti Corp., Schaan, Liechtenstein) as previously described (Weber and Hoekstra 2009), a process that leaves overflow matter on the soil surface (Figure 2.3a). During casting, in addition to identifying information, the following notations were made on each cast. (1) The escape tunnel (if present) was marked as such, and (2) lines indicating the burrow’s position with respect to gravity were drawn on the overflow material using a level tool, such that the original position of the burrow was recorded. Burrow casts were then individually photographed as follows: they were positioned such that their longest axis
was perpendicular to the imaging axis (i.e. burrows were positions parallel to the camera lens), against a dark background beside a regular grid (which provides a scale for each image). Where more than one tunnel reached the soil surface or radiated toward the surface, the escape tunnel overflow material was marked with a blue dot (Figure 2.3) either during photography (using blue tape on the overflow material) or in post processing (Adobe Photoshop CS6, where tunnel did not penetrate the surface). Burrow casts were photographed with a Nikon D90 with an AFS NIKKOR 18-55 mm lens at a distance of ~2m using ControlMyNikon software.

Custom scripts (MATLAB) were used to collect measurements from these photographs after automatic pre-processing with Adobe Photoshop CS5 to equalize brightness and saturation for all images. Burrow components (e.g. tunnels, nest chambers) were discerned using the following criteria: First the outline of the polyurethane foam was determined based on its brightness compared to the dark background. Second, the overflow material was distinguished from material in contact with tunnels by its brighter and glossier appearance (Figure 2.3a). Sand particles adhere to the material filling burrow tunnels, making these regions consistently more matte and dark compared to polyurethane that cures exposed to air. Next, each burrow component was measured using the following criteria (values were measured at the pixel level, but rescaled to the actual physical dimension with a scale that is taken with each image); (1) burrow area: total area in pixels of isolated burrow; (2) burrow length: length of central line of burrow, computed as local maxima in pixel distance to the border of the burrow area; (3) tunnel thickness: average length of lines drawn
perpendicular to central line of burrow at unit pixel intervals; (4) escape tunnels (number, length, area): for casts where a second tunnel reached the surface or radiated toward the surface (marked with a blue dot during photography) the escape tunnels were identified as those where the first derivative of the central line was positive for ≥4 cm (about 1/2 mouse body length), length and area are measured just as for the main burrow (5) nest chamber (length/area/depth/angle): identified by a sharp increase (≥20% within 1cm) in tunnel thickness for ≥4 cm and/or an angular change of ≥30 degrees in orientation and/or as the longest segment of above average thickness, esp. if near terminus of tunnel or lowest depth of burrow, burrow angle is measured relative to the direction of gravity; (6) curviness: average curvature of a spline through the points that define the burrow length; (7) orientation: the angle (with respect to gravity) for a minimum enclosing ellipsoid of the burrow area; (8) entry angle: the angle, with respect to the local surface) that the burrow forms at the entry to the main tunnel (9) work: a mouse’s investment in constructing a burrow of given length is not expected to increase linearly with increasing tunnel length. This is because burrow excavation required two primary steps, substrate loosening, followed by removal of soil from burrow. As the tunnel extends, the cost of removing soil to the surface for each additional increment of length is expected to be more costly than the previous increment. Thus, we estimated the work invested in burrow construction using two formulas that attempt to quantify these detailed interactions. The first is

\[
W_1 = NCOT_b \cdot (l + d) + \left( NCOT_p \frac{\int \rho \pi t^2 dt}{M_a} + \frac{M_agl}{d} \right) \cdot \frac{\int \rho \pi t^2 dt}{M_a}(l + d)
\]
utilizing leading order terms of the theory described by White et al. (2006). Here $NCOT_b$ is the net cost of transport by burrowing and $NCOT_p$ is the net cost of pedestrian transport, experimental values for both can be found in the reference. The burrow length $l$, depth $d$, and thickness $t$ follow directly from our measurements. $M_a$ is the average animal mass, $g$ the gravitational constant, and $\rho$ is the soil/sand density.

The second approach follows Vleck (1979). Here the work can be expressed as

$$W_2 = (K_s + K_p \cdot l)\int \rho \pi t^2 \, dl$$

where $K_s$ and $K_p$ are experimental constants that depend on soil density and body mass of the animal.

**Statistical Analysis**

We used several statistical tests to evaluate the inheritance of burrow traits in backcross mice. All tests were performed using the R statistical package. First, we calculated pairwise Spearman correlations and principal components for hand-measured traits. To compare trait values between the two sexes, we used a Welch’s $t$-test. To compare length traits across backcross generations, we employed ANOVA, and to compare proportions of mice digging escape tunnels between generations, we used Fisher exact tests.
Figure 2.3. Automated image analysis of burrow architecture (i.e. size and shape) from polyurethane casts. (A) Each polyurethane burrow cast was photographed against a dark background with a regular grid (ruler) in frame. If an escape tunnel was present, it was marked with blue laboratory tape. (B) The analysis determines the scale of the image using inter-node distances from the grid and distinguishes sandy tunnels from overflow material to determine the burrow outline, main axis, and nest area (see full methods for details). (C) Four examples of casts analyzed by our image-based measurement system.
Results

**CORRELATIONS AMONG BURROW MEASUREMENTS**

Phenotypes having independent genetic underpinnings (i.e. in linkage equilibrium and without pleiotropic effects) should segregate independently in recombinant generations of an experimental cross, and thus show only weak correlations. However, if they share a genetic basis (due to linkage or pleiotropy), correlations between phenotypes should be stronger. Thus, we examined the correlations among all hand measurements taken from burrow casts of the BC1 generation (Figure 2.4), which unlike further generations, is free of phenotypic selection effects. The degree of correlation varied, and often reflected associations that are numerically inevitable (e.g. when one measurement is a component of another, as entrance tunnel length is a component of the total burrow length). However, measurements of the nest length and entrance tunnel length were only weakly correlated with measurements of the escape tunnel (number of escape tunnels, average escape tunnel length; Figure 2.4). This correlation structure is recapitulated in the results of a principal components analysis. The first principal component loads tunnel length measurements including average best burrow length, best burrow length, and best entrance tunnel length and explains a large proportion (0.74) of the variance in the data. The second principal component loads average escape tunnel length and number of escape tunnels, and explains a smaller proportion of the variance (0.14). Together,
these data are consistent with the previously-reported modular genetic architecture for burrow shape (Weber et al. 2013).

**Figure 2.4.** Spearman correlations between all pairs of burrow measurements in *P. polionotus* X *P. maniculatus* backcross hybrid mice (BC1 generation, n=339). Measurements of descending tunnel lengths (i.e. entrance tunnel and nest compartment tunnel) are strongly correlated (large red box), and separately, measures of escape tunnels (small red box) are strongly correlated. However, entrance and nest tunnel lengths are only weakly correlated to escape tunnel measurements. Measurements of total burrow length are correlated with entrance, nest, and escape tunnel measurements, as expected due to numerical association. (No. = number, entr. = entrance, len. = length, esc. = escape).
INHERITANCE OF *P. polionotus* PHENOTYPES ACROSS BACKCROSS GENERATIONS

We found that a key characteristic of the *P. polionotus* burrow phenotype—long entrance tunnels—became more *P. maniculatus*-like (i.e. shorter) with advancing backcross generations, even under our strict phenotype introgression regime where only individuals exhibiting complete *P. polionotus*-like burrowing behavior were selected as parents of the next generation (Figure 2.5a; ANOVA, $F = 43.35, P < 0.0001$). However, this decline in entrance tunnel length stabilized across the BC3-BC5 generations, and no significant differences in entrance tunnel length among generations BC3, BC4, and BC5 were detected (Figure 2.5a; ANOVA, $F = 2.15, P = 0.115$). Furthermore, in each generation, a proportion of individuals recapitulated the entrance tunnel phenotype of the *P. polionotus* parental strain (Figure 2.5a; Table 2.1). Additional burrow component tunnel lengths showed a similar pattern of decreasing length across generations (Supplemental Figure S2).

Similarly, the proportion of backcrossed mice digging an escape tunnel declined across generations, but became stable between the BC3-BC4 generations (Figure 2.5b; Table 2.1). When comparing the proportion of escape-digging mice (≥1 escape tunnel across 3 trials) between all pairs of generations from BC1-BC4 (BC5 was excluded for small sample size), all 2x2 comparisons were significantly different (Fisher exact tests, $P < 0.001$) except for the comparison of BC3 to BC4 (Fisher exact test, $P = 1.0$).
Figure 2.5. (A) Inheritance of entrance tunnel length (average of 3 trials) across five sequential generations of *Peromyscus polionotus* X *P. maniculatus* backcross hybrids. Violin plots show kernel densities for entrance tunnel length phenotype. Black dots represent the median value for each generation. Parental medians (solid lines) and quartiles are shown in the background (ochre for *P. man*, and cyan for *P. pol*). Data for parental species and 272 BC1 hybrids adapted from Weber et al. (2013). (B) Inheritance of escape tunnel digging across five backcross generations of *Peromyscus* hybrids. Data for parental species and 272 BC1 hybrids adapted from Weber et al. 2013. Error bars show the standard error of the mean. The proportion of mice digging escapes did not differ between BC3 and BC4 generations, but were significantly different in each other pairwise comparison of BC1-BC4 generations (Fisher exact tests, *P*=1.0 and *P* < 0.001, respectively).
AUTOMATED MEASUREMENT OF BURROW CASTS

Our novel, automated analysis collected several measurements from photographs of burrow casts. Many of these measurements are not obtainable using hand measurements, including burrow area and estimations of work. This set of measurements does not immediately appear to fall into two clear phenotypic modules when pairwise Spearman correlations of measurements (within each cast) are considered, but there is a moderately strong correlation between curvature and escape tunnel length (Figure 2.6; Spearman correlation = 0.473), as well as among measurements of length, area, and work (Figure 2.6; e.g. Spearman correlations: work1 and area = 0.674; work1 and main length = 0.361; area and main length = 0.743).

Table 2.1. Proportions of mice exhibiting *P. polionotus*-like burrowing phenotypes across five sequential backcross generations of *Peromyscus polionotus* X *P. maniculatus* backcross hybrids. Traits examined include “long entrances” (criteria: average length >12 cm across 3 trials), and escape tunnel digging (criteria: at least one escape tunnel dug across 3 trials). Values given as proportions, with the standard error of the mean in parentheses. Data for 272 BC1 hybrids adapted from Weber et al. (2013).

<table>
<thead>
<tr>
<th>Generation</th>
<th>n</th>
<th>Proportion digging long entrances</th>
<th>Proportion digging escape tunnels</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1</td>
<td>339</td>
<td>0.233 (0.023)</td>
<td>0.478 (0.027)</td>
</tr>
<tr>
<td>BC2</td>
<td>163</td>
<td>0.135 (0.027)</td>
<td>0.288 (0.036)</td>
</tr>
<tr>
<td>BC3</td>
<td>287</td>
<td>0.112 (0.019)</td>
<td>0.112 (0.019)</td>
</tr>
<tr>
<td>BC4</td>
<td>97</td>
<td>0.103 (0.031)</td>
<td>0.052 (0.022)</td>
</tr>
<tr>
<td>BC5</td>
<td>6</td>
<td>0.167 (0.167)</td>
<td>0.167 (0.167)</td>
</tr>
</tbody>
</table>
Figure 2.6. Spearman correlations between all pairs of measurements collected from images of burrow casts using a novel, automated image-based analysis. Burrow casts were made from the tunnels of backcross hybrid mice (generations BC1-BC5, n=1129 casts) and represent a wide diversity of shapes and sizes.
Discussion

Here, we examine the inheritance of complex *P. polionotus* burrow architecture as it is introgressed by phenotypic selection into the genetic background of its sister-species *P. maniculatus*. Our goal is to uncover the underlying genetic architecture of burrow construction behavior. We find that *P. polionotus* phenotypes—both long entrance tunnels and escape tunnels—are preserved through at least a BC5 generation, suggesting that a fairly large proportion of the genetic variation influencing these traits must be under the control of a small number of loci. Thus, this is a tractable genetic architecture for follow-up studies such as QTL mapping.

To estimate genetic architecture, we also attempted to compute the Castle-Wright estimator (Castle 1921; Wright 1952; Lande 1981; Zheng et al. 1990, 1999). However, for each phenotype examined, the variance in the F<sub>1</sub> generation was greater than in the segregating generation (F<sub>2</sub>)—leading to negative segregation variance (data not shown) and making the Castle-Wright estimations unworkable with these data. The high variance observed in all measures of burrowing behavior in F<sub>1</sub> hybrids is unexpected, as F<sub>1</sub> hybrids are heterozygous but genetically uniform as a population, with the exception of any polymorphisms due to heterozygosity in the parental strains. However, the F<sub>2</sub> as well as the F<sub>1</sub>s would inherit such polymorphisms, so this is not a potential explanation here. One explanation for this high variance could be genetic incompatibilities in hybrids between these species (e.g. Vrana et al. 2000). Moreover,
the Castle-Wright estimator requires that several assumptions are met, and often does not perform well with real data (Zheng et al. 1990).

Moreover, although the proportion of mice exhibiting long entrance tunnels and escape tunnels declined in the advanced backcross generations, the proportions for both phenotypes stabilized around 0.10 in the BC3, BC4 and BC5 generations. A precise estimation of genetic architecture would require intensive, large-scale genetic studies to disentangle the potential effects of locus number, effect size, epistasis, and penetrance (Boake 1994; MacKay 2001), which is beyond the scope of this study. However, the proportion of 1 in 10 progeny expressing a trait is suggestive of a genetic architecture involving approximately 3-4 genes (i.e. $1/2^n$, where $n$ is the number of alleles controlling the trait), and this number is congruent with QTL mapping results from multiple studies (Weber et al. 2013; Chapter 3).

Classic Mendelian inheritance of a dominant “escape” locus should produce 50% escape-tunnel-digging-progeny in each backcross generation. Instead, we observed a decline in the number of animals constructing an escape tunnel across backcross generations. If the escape tunnel is considered a binary trait, this result suggests the underlying architecture is quantitative and controlled as a threshold trait (e.g. Wright 1934; Pulido 2007), where multiple loci each increase the liability of the organism to express the trait, yet the trait only appears if liability exceeds some threshold. From an evolutionary perspective, this is an interesting genetic architecture, as it implies that multiple alleles would have had to arise and increase in frequency in the ancestral
population before the trait was expressed and visible to selection (Pulido 2007).

However, as a partial (i.e. incomplete) escape tunnel likely does not allow for evasion of predators, an all-or-nothing threshold genetic architecture for escape tunnel digging may be adaptive, ensuring that mice either invest in the complete structure or not at all.

Acknowledgements. We thank R Ludwick, L Pan, S Scalia, JN Weber, and Z Ali for help with behavior experiments. Thanks to A Bendesky, B de Bivort, C Dulac, B Ölveczky and J Sanes and the Hoekstra Lab for helpful discussions. We thank Harvard’s Office of Animal Resources, particularly J Rocca for excellent animal husbandry. This research was funded by the Mind Brain Behavior Initiative at Harvard, the Chapman Funds for Vertebrate Locomotion, a National Science Foundation Graduate Research Fellowship, a Doctoral Dissertation Improvement Grant, and an American Fellowship from the American Association of University Women to HCM; and a Beckman Young Investigator Award and the Howard Hughes Medical Institute to HEH.

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Chapter 3
Pinpointing molecular mechanisms underlying natural behavioral variation:

intersection of fine-scale QTL mapping and transcriptome analysis

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Abstract

What genes underlie changes in behavior between species, and how do genetic mutations act in the nervous system (or elsewhere) to alter behavior? These are fundamental questions in animal behavior, yet they remain inadequately understood. Here, we combine two forward-genetics approaches—QTL mapping and transcriptome analysis—to nominate specific candidate genes for the dramatic differences in burrowing behavior between *P. polionotus* and *P. maniculatus*. Using a large advanced backcross mapping population (n=751), we detect five QTL contributing to differences in burrow architecture between these species: three loci associated with entrance tunnel length variation, and two loci associated with escape tunnel length. In a complementary brain transcriptome study, we focus on gene expression in F$_1$ hybrids to detect allele-specific expression (ASE), as ASE in an F$_1$ hybrid indicates *cis*-regulatory differences between the parental lineages. We find bias favoring expression from the *P. polionotus*-allele in F$_1$ hybrid brains, which may be a molecular reflection of *P. polionotus*-like burrowing behavior of hybrids. Finally, we use ASE to nominate candidate genes within the detected QTL regions, and find genes related to behavioral disorders, circadian rhythms, and activity patterns; these genes represent promising candidates for future functional studies.
Introduction

Evolution by natural selection has produced a diverse array of complex behaviors, but perhaps none so elaborate or measurable (Chapter 2) as the construction behaviors that give rise to animal architecture. These structures—such as webs, nests, and burrows—though diverse in form, often share common properties such as construction through unlearned sequences of motor patterns, stereotyped species-specific forms, and clear fitness-related functions, such as protection from predators and temperature extremes (Hansell 2005; Jackson 2000). How genes encode the blueprints for construction behavior into neural circuits remains unknown. However, construction behavior involves the integration of multiple underlying behavioral components including internal (motivational) states, activity patterns, and spatial orientation (Hansell 2005). Thus, studying natural variation in animal architectures may inform our understanding of brain circuits underlying these core components of behavior.

Moreover, the findings produced by studies based on natural differences in behavior may be more relevant to understanding variation in human behavior than traditional laboratory studies, which have historically focused on artificial behavioral variation such as manufactured mutants and transgenics (e.g. Stowers et al. 2002; Zhou and Palmiter 1995). Such man-made manipulations tend to have outsized or harmful effects, while behavioral adaptations from nature are by definition beneficial to the organisms that have evolved them. Additionally, most of the known examples of genetic mechanisms governing evolutionary changes in behavior have been limited to reverse
genetics—that is, through manipulation and investigation of *a priori* candidate genes (e.g. Robinson et al. 2008; Lim et al. 2004; Ben-Shahar et al. 2002; Davis and Fernald 1990; Fitzpatrick et al. 2005).

Here, we use forward genetics approaches to examine the molecular underpinnings of dramatic differences in burrowing behavior between two species of wild mice, *Peromyscus polionotus* and *P. maniculatus*. *P. polionotus* digs stereotyped burrows with long entrance tunnels and an escape tunnel that radiates up towards the soil surface but doesn’t penetrate the surface (Figure 3.1). This complex burrow shape is likely important for mouse survival (especially from snake and weasel predators) in its exposed habitats (beaches and fallow fields of the Southeast United States; Sumner and Karol 1929; Hayne 1936). In contrast, *P. maniculatus* digs a burrow shape that is ancestral in this genus (Weber and Hoekstra 2009), consisting of a single short tunnel terminating at a nest area (Figure 3.1). These species-specific architectures are strongly influenced by genetics, as interspecific cross-fostering does not alter burrow shape (Chapter 1), and burrow differences have previously been mapped to QTL loci (Weber et

![Figure 3.1](image-url)

**Figure 3.1.** The species-specific burrow architectures of *P. maniculatus* and *P. polionotus*. Burrows show a stereotyped structure within species and are constructed by captive mice held in laboratory conditions (Dawson et al. 1988; Weber et al. 2013). The outline colors (cyan, ochre) will be used throughout the chapter to identify species.
In summary, the burrows built by *P. polionotus* represent a derived innovation in a complex natural behavior, and moreover, one that is tractable for genetic analysis.

To uncover the genetic basis of this behavior, we used a two-pronged approach. First, we used an advanced-backcross strategy to fine-scale map the quantitative trait loci (QTL) that underlie differences in burrow construction behavior between the two species. Second, we employed a transcriptomic approach to identify lineage-specific cis-regulatory variation that has diverged between *P. polionotus* and *P. maniculatus*. These approaches are complementary. QTL mapping is a valuable tool for unraveling the genetic architecture of phenotypic traits, and provides the advantages of identifying those loci that underlie segregating differences in a particular, defined behavior. Yet QTL mapping is limited to detecting loci (i.e. regions of chromosomes), and typically does not pinpoint individual genes (Bendesky and Bargmann 2011; Boake et al. 2002; Flint et al. 2005; MacKay et al. 2009). On the other hand, a transcriptomic approach allows for the identification and measurement of expression differences at the level of genes, and furthermore can be targeted to the tissue of interest (Bendesky and Bargmann 2011; MacKay et al. 2009). However, whether the discovered differences in gene expression are causally linked to a given behavior (rather than just correlated) is more difficult to ascertain. Here, we combine the strengths of both methods and move closer to understanding the molecular basis of a complex mammalian behavior.
Methods

Overview. Our genetic mapping strategy builds upon previous work in this genus, in which the differences in burrowing behavior were demonstrated to be \textit{P. polionotus}-dominant in F\textsubscript{1} hybrids (Dawson et al. 1988) and primarily influenced by a handful of genetic regions, mapped to broad, poorly-resolved loci (Weber et al. 2013). This allowed us to further dissect the genetic basis of burrowing using an advanced backcross introgression strategy (Chapter 2; Figure 2.2): phenotypic selection for \textit{P. polionotus}-like burrows at each generation paired with repeated backcrossing to \textit{P. maniculatus}. This cross design acts to isolate the loci responsible for \textit{P. polionotus}-like burrows in the genetic background of \textit{P. maniculatus}. Thus, it improves mapping precision by repeated rounds of recombination, which reduces linkage disequilibrium between causative nucleotides and neighboring variants, as the number of recombination events increases proportionately with the number of generations (Darvasi et al. 1995; MacKay 2001).

Next, we used a transcriptomic approach to identify regulatory divergence between \textit{P. polionotus} and \textit{P. maniculatus}. Whole brain transcriptome (i.e. RNA-seq) data from F\textsubscript{1} hybrids were used to identify cases of allele-specific expression (ASE) where, within a given individual, one allele of a gene is overexpressed relative to the alternate (thus providing a built-in control for species differences in trans-regulation and environmental effects). We determined species-of-origin for transcripts by first identifying SNPs that were fixed and different between \textit{P. polionotus} and \textit{P. maniculatus}, and used species-specific transcript counts in F\textsubscript{1} hybrids to assess ASE. ASE
in an F₁ hybrid implies the presence of a cis-acting polymorphism affecting expression levels, as there is a 1:1 balance between species at the level of the genome (Figure 3.2a). Importantly, the causative nucleotides underlying cis-regulatory variation are by definition closely linked to the differentially-expressed gene. Specifically, cis-regulatory variants often occur in regions upstream of genes such as promoters and enhancers and often affect transcription-factor (TF) binding (Figure 3.2a). In contrast, trans-acting regulatory variation is mediated by a diffusible intermediate and thus, the causative nucleotides may be encoded anywhere in the genome (Figure 3.2b). ASE analysis in an F₁ hybrid can therefore be used to detect lineage-specific selection on gene expression levels, to infer the direction (i.e. which species’ allele is overexpressed) and magnitude (i.e. fold difference) of detected differences (Ronald et al. 2005).

To link differences in gene expression to burrowing behavior, we additionally searched for cases of differential ASE (DiffASE). DiffASE analysis compares ASE across multiple conditions, which allows for insights into regulatory differences that are specifically linked to that condition. More specifically, environmental conditions may induce sensory- or activity-dependent transcription factor (TF) expression, these TFs would go on to bind cis-regulatory regions, and expression differences resulting from cis-regulatory variation may only then be expressed. In the absence of such stimulus- or activity-specific TFs, cis-regulatory variation would remain invisible in transcriptome data. Here, we computed DiffASE across behavioral conditions of (1) burrowing: construction of a burrow, and (2) control: displacement to a new home cage (Figure 3.2c). This control treatment involves both a novel environment and the olfactory
experience of sand, while precluding burrow construction (Figure 3.2c). We then searched for cases of ASE unique to burrowing animals—that is (1) displaying ASE in the burrowing treatment, and (2) lacking ASE, or showing ASE biased in the opposite direction, in the control animals. This allowed us to link ASE to our behavior of interest, although it represents only one genetic mechanism by which genetic variation could influence this behavior. In summary, ASE represents more constitutive cases of expression differences (persisting across behavioral conditions), while DiffASE represents those that are specific to the context of a burrowing behavioral assay.

Using these two independent but complementary forward-genetics methods (Bendesky and Bargmann 2011), our aim is to uncover the molecular mechanisms of behavioral differences between these species, and through them, to eventually understand the brain pathways that shape natural variation in behavior.
Figure 3.2. Analysis of allele-specific expression (ASE) in F₁ hybrid mice.
Animal care and breeding

Experiments were conducted using captive *Peromyscus maniculatus* x *P. polionotus* hybrids kept under controlled laboratory conditions. Both species were obtained as outbred, captive strains from the *Peromyscus* Genetic Stock Center (University of South Carolina, Columbia SC, USA). Strains were derived from wild-caught ancestors in 1948 (*P. maniculatus*, BW strain) and 1952 (*P. polionotus*, PO strain), have been laboratory-housed since capture, and have thus lived without access to soil for well over 100 generations. Mice were housed at ~21.1°C on a 16:8 h light:dark cycle and were provided standard rodent diet and water *ad libitum*. Animals were housed with cotton nesting material, corn cob bedding material, and 3-sided red-tinted plastic shelters. Juveniles were weaned from their parents 23-24 days after birth, and
weanlings were subsequently housed in same-sex groups until the time of behavioral testing. All procedures were approved by the Harvard University Institutional Animal Care and Use Committee.

First generation hybrids were generated by crossing a single male *P. polionotus* to a *P. maniculatus* female. Genomic imprinting-related issues prevent the reciprocal cross between these species (Vrana et al. 2000). Thirteen F₁ hybrids of both sexes were subsequently paired with *P. maniculatus* mates to generate F₂ backcross hybrids, or BC1s (first backcross generation). From these recombinant hybrids (as well as for subsequent generations BC2-BC4), a subset of individuals were selected based on phenotypic criteria (average entrance tunnel length ≥ 14 cm and presence of at least one escape tunnel across three trials), to be parents of the following generation of backcross hybrids. Selected individuals were each paired with a *P. maniculatus* mate and housed as described above. Breeding continued through production of an F₅ (BC4) generation (Chapter 2; Figure 2.2; e.g. Luo et al. 2002, Bendesky and Bargmann 2011).

We tested the burrowing behavior in a total of 751 mice (n = 287 BC1, n = 97 BC2, n = 292 BC3, and n = 75 BC4) in large, indoor, sand-filled enclosures. Mice were tested as young adults (ages 60-100 days of age), and included both males and females as no sex-based differences in burrowing behavior have been detected (Weber et al. 2013; Chapter 2).

Burrowing trials were conducted as previously described (Weber et al. 2013). Briefly, we released animals into large enclosures (1.2 × 1.5 × 1.1 m) filled with
approximately 700 kg of moistened, hard-packed premium playground sand (Pharma-Serv Corp.) for ~46 hours per trial. Mice were provided with nesting material, standard rodent food, and water during trials. Temperature and light:dark cycle were identical to housing conditions, as behavior and housing shared a common room. We tested each mouse three times, consecutively (i.e. across 6 days), with each animal assigned to a new enclosure at random for each trial.

**Burrow measurements**

After completion of each burrowing trial, mice were hand-captured and housed singly in standard rodent cages while the burrowing enclosures were evaluated and prepared again. Enclosures were inspected for the presence of discrete burrows, and each burrow was injected with polyurethane insulation foam (Hilti, Schaan, Lichtenstein), which expands and then hardens to form a permanent cast of the burrow components. Small diggings < 2 cm were measured directly from the enclosures. Burrow components (entrance tunnel length, escape tunnel length) were directly measured from the dried casts.

**Genotyping**

DNA was extracted from tail, ear or liver tissue using a high-throughput, automated phenol-chloroform platform (Autogen, Holliston MA). Libraries of restriction cut-site-specific, genome-wide fragments were prepared for each individual using ddRAD-Seq (Peterson et al. 2012). In short, DNA was digested with two restriction enzymes, EcoRI and MspI (New England Biolabs, Ipswich MA), custom adapters
(Integrated DNA Technologies, Coralville, IA) were annealed to cut ends with T4 ligase (New England Biolabs, Ipswich, MA), libraries were size-selected using a Pippin Prep (Sage Science, Beverly, MA), and amplified and indexed using PCR (Phusion, New England Biolabs, Ipswich, MA). At each stage of preparation, fragments were purified using Ampure XP beads (Beckman Coulter Genomics, Danvers MA). Library quality was assessed prior to sequencing using a Bioanalyzer (Agilent Technologies, Santa Clara, CA) and sequenced 2x150 on the Illumina HiSeq (San Diego, CA) platform. Using Stampy (Lunter and Goodson, 2011), Illumina sequences were aligned to an existing set of 1,127 marker sequences containing species-diagnostic SNPs and ordered onto a *P. polionotus* x *P. maniculatus* linkage map with a total map length of 1835.5 cM (Weber et al. 2013).

**QTL mapping and genome alignment**

To search for QTLs while controlling for unequal relatedness in our mapping population, we employed QTLRel (Cheng et al. 2011), a package for the R language that implements Haley-Knott method (Haley and Knott 1992). We computed condensed identity coefficients (CICs, a measure of relatedness) from a population pedigree, imputed missing genotypes using R/qtl (Broman et al. 2009), and used permutation tests (n=1,000) to determine genome-wide thresholds at an α=0.05 significance level. Sequences of markers within the resulting 1.5-LOD confidence intervals of detected QTLs were then aligned to *P. maniculatus* genome scaffolds using NCBI blastn. These scaffolds were subsequently searched against NCBI lab mouse (*Mus*) nucleotide records.
and then matched to the Mus reference genome to determine syntenic intervals and physical bounds of QTL intervals (Supplementary Table S3.2).

Transcriptome Study

Behavioral assays and sample preparation

We analyzed gene expression levels (whole brain) of six F₁ hybrids (Figure 3.2; three burrowing treatment and three control treatment), and additionally for two mice from each parental species in order to identify species-diagnostic SNPs for the analysis of ASE in hybrids. All mice were male and aged 61-65 at time of death, and were experienced burrowers prior to tissue collection, having completed two burrowing trials according to the following schedule. Days 1 and 4: overnight burrowing trial, enclosure prepared as described previously (Weber et al. 2013), with Days 2, 3, 5 and 6 as rest days in the animal’s own home cage. Day 7: 90 minutes prior to tissue collection, each mouse was released into a behavioral arena: for the burrowing treatment, a burrowing enclosure—a 1.2 × 1.5 × 1.1 m arena filled with approximately 700 kg of moistened, hard-packed premium playground sand); for the control, a new home cage (0.197 x 0.305 x 0.165 m ) to which ~0.14 kg sand from a burrowing enclosure had been added to control for presence of substrate and olfactory experience, while precluding the possibility of burrow construction. After 90 minutes, whole brains were rapidly dissected in chilled RNAse-free PBS, flash-frozen in liquid nitrogen, and stored at -80 °C. Tissues were later homogenized using a TissueLyser (Qiagen, Venlo Netherlands) in Trizol (Life Technologies, Cambridge MA), and total RNA was extracted according to the
manufacturer’s protocol using PhaseLock Gel Tubes (5Prime, Gaithersburg, MD), followed by clean-up over RNeasy columns (Qiagen, Venlo, Netherlands). Libraries were prepared with a TruSeq Stranded mRNA Library Prep Kit following manufacturer’s directions (Illumina, San Diego CA).

**P. maniculatus x P. polionotus variant calling**

To identify fixed single nucleotide polymorphisms between *P. maniculatus* and *P. polionotus*, we combined multiple sets of RNA-seq data derived from each of the parental strains as well as interspecific F₁ hybrids. Adapters and overlapping segments between mate-pairs were removed using SeqPrep (https://github.com/jstjohn/SeqPrep) and reads were mapped to the *P. maniculatus bairdii* genome assembly Pman_1.0 (NCBI genome assembly accession GCF_000500345.1) using Tophat 2 version 2.0.9 (Kim et al. 2013) in --very-sensitive mode and trimming the first 6 nucleotides off of the 5’ ends of reads. Duplicate reads were removed using the PICARD tools (http://broadinstitute.github.io/picard) then all mapped reads were pooled into three groups: *P. maniculatus* parental, *P. polionotus* parental or F₁ hybrid. Indels were realigned within each pool using the Genome Analysis Toolkit version 3.3 (McKenna et al. 2010), followed by variant calling relative to the reference sequence via the UnifiedGenotyper with the following arguments: -nt 12 -ploidy 2 -glm BOTH -stand_call_conf 30 -stand_emit_conf 10.

After removing all low confidence SNP calls, we excluded all SNPs that were within 125 bp (the read length) of an indel, as improper alignment of indels can lead to
reference allelic bias. We retained remaining SNPs for ASE analysis if neither parent showed evidence of polymorphism at the SNP site, and parental base calls at a site supported the alleles observed in the hybrids. In cases where *P. polionotus* parental coverage was insufficient to confirm a SNP detected in the hybrids, we retained heterozygous sites in the hybrid that contained the *P. maniculatus* reference allele and one other.

*ASE analysis in F₁ hybrids*

We generated a reference *P. maniculatus* genome with all potential SNPs, including those of low confidence, masked by ‘N’. Reads from each sample and replicate were mapped to this reference using STAR version 2.4.0i (Dobin et al. 2013), with the following options: --outFilterMultimapNmax 1 --outSAMtype BAM SortedByCoordinate -outSAMattributes MD --clip3pNbases 6. Duplicates were removed from the resulting mapped files using the PICARD tools, and allelic counts were calculated for each individual SNP using custom PERL and Python scripts. Each read and its paired-end mate contributed a count only to a single SNP, and in cases where a read/mate overlapped multiple SNPs, its allelic count was randomly assigned to a single SNP. We further excluded any SNPs that (1) lacked any counts in all hybrid samples, (2) lacked counts in either of the two parental alleles in all hybrid samples, or (3) had >= to 90% of counts favoring one parental allele in all samples with more than 10 counts. Our final SNP set comprised 186,984 SNPs, allowing for estimation of ASE in 21,245 of genes in the annotation (80%).
Gene-level counts were then computed by summing across individual SNPs within the longest isoform. In cases where SNPs overlapped multiple annotated features, they were not used for counting. Data were generated using a stranded library preparation method, therefore ASE counts were only calculated from reads mapping to the appropriate strand. ASE in F1 hybrids was computed by taking the $\log_2$ ratio of the *P. maniculatus* allele counts over those for *P. polionotus* at each gene, which produces values centered at 0. Positive values reflect allelic bias toward *P. maniculatus* and negative values reflect bias toward *P. polionotous*. We calculated a binomial p-value for each gene, which tests for deviation from the expected allelic ratio. The expected ratio was computed empirically and was found to be $0.49 \ P. polionotus / 0.51 \ P. maniculatus$ for each sample. Thus, there is no evidence of reference bias, as the idealized ratio is 0.5/0.5. Because genes with low overall expression can lead to unreliable ASE estimations, we limited our analysis to genes with over 20 counts. Genes showing differential ASE (DiffASE) between burrowing and control animals were ranked by t-test p-values comparing log2(ASE) ratios for each of the 3 burrowing animals and the 3 control animals. Systematic bias toward either parental species for genes with a t-test p-value $< 0.05$ (328 genes) was assayed using the fisher.test function in the R language. P-values were calculated by comparing the genes that showed the most DiffASE to the distribution of parental bias for all other genes in which ASE was calculated. We eliminated from immediate consideration all genes known to be imprinted in *Mus* (Tables S3.3 and S3.4).
Results

**ASE and DiffASE show *P. polionotus*-biased expression**

In our whole transcriptomes, we detected expression of 12,287 genes with > 20 transcripts, and found widespread ASE in F₁ hybrids—with 2,629 genes showing ASE before correcting for multiple comparisons (Figure 3.3a, binomial test, \( P < 0.002 \) in each of 3 burrowing replicates) after eliminating known imprinted genes (Tables S3.3 and S3.4). In contrast, DiffASE was limited to 328 genes before controlling for multiple comparisons (Figure 3.3a, Student’s t-test, \( P < 0.05 \) in each of 3 replicates).

Both ASE and DiffASE showed significant bias favoring expression from the *P. polionotus* allele. Of the genes showing ASE in F₁ hybrids (binomial test, \( P < 0.002 \) in each of three burrowing replicates), 56.5 % had negative ASE ratios, a difference from the null hypothesis of 50% (Figure 3.3b; two sample test for equality of proportions, \( X^2 = 22.2, P < 0.001 \)). This over-representation of *P. polionotus*-biased genes in whole brain remains consistent while varying the significance threshold (Table S3.5), and is thus not sensitive to choice of threshold.

DiffASE genes (comparing conditions of burrowing and control; Fig 3.3c), while biased in favor of expression from *P. polionotus* (two sample test for equality of proportions, \( X^2 = 42, P < 0.0001 \)), were not significantly more biased than ASE across the genome (Figure 3.3c; Fisher’s exact test, \( P = 0.2521 \)). However, DiffASE genes have more negative ASE ratios than brain-expressed genes as a whole (Figure 3.3e; Kruskal-Wallis test \( P = 0.0089 \)), yet there was no significant difference in ASE ratios between burrowing
and control groups (Figure 3.3d; Kruskal-Wallis test \( P = 0.8988 \)). Moreover, when DiffASE genes are ranked by p-value, 19 out of the top 20, and 43 of the top 50 most significant DiffASE genes are biased toward expression from the \( P. polionotus \) allele (Figure 3.3g); both of these proportions are more biased toward negative values than all transcripts considered in ASE analysis (Figure 3.3f-h; Fisher’s exact test, \( P = 0.0082 \) and \( P < 0.0001 \), respectively). Thus, there is even more extreme bias favoring expression from the \( P. polionotus \) allele in the most significant DiffASE genes than the bias toward \( P. polionotus \) expression already observed across all ASE genes.

Figure 3.3 Continued on following page. Allele-specific expression (ASE) in \( P. polionotus \) \( \times \) \( P. maniculatus \) \( F_1 \) hybrids. (A) Raw ASE numbers. We identified 12,287 genes expressed in whole brain with > 20 transcripts. Of these, 2,651 showed significant ASE (binomial test \( P < 0.001 \) across all replicates), and 328 showed significant DiffASE (Student’s t-test, \( P < 0.05 \) across all replicates). (B) Of all genes showing ASE, 56.4% (1,496) were biased toward expression from the \( P. polionotus \) allele, a significant deviation from the null expectation of no species bias (two-sample test for equality of proportions, \( \chi^2 = 87.7, P < 0.001 \)). (C) In the 328 genes showing DiffASE between burrowing and control treatments, there was not a more pronounced species-bias than the genome as a whole (Fisher’s exact test, \( P = 0.2521 \)). (D) ASE ratios are not significantly different between the burrowing (mean ASE ratio -0.1535) and control (mean ASE ratio -0.1518) groups (Kruskal-Wallis rank sum test, \( P = 0.8988 \)). (E) However, DiffASE genes have significantly lower (that is, more \( P. polionotus \)-biased) ASE ratios than the genome as a whole (Kruskal-Wallis rank sum test, \( P = 0.0089 \)). (F) A volcano plot reveals highly \( P. polionotus \)-biased gene expression among genes with increasingly significant DiffASE between behavioral conditions. (G) When ranked according to p-value, of the 50 most significant DiffASE genes, 43 are biased toward expression from the \( P. polionotus \) allele, a significant deviation from the empirically-determined genome ratio (Fisher’s exact test, \( P < 0.0001 \)). Among the top 20 most-significant genes, 19 are \( P. polionotus \)-biased (data not shown, Fisher’s exact test, \( P < 0.0082 \)). (H) Heatmap comparing ASE between burrowing and control conditions, with genes ranked from left to right in order of increasing p-value (and thus decreasing statistical significance).
Figure 3.3 Continued. Allele-specific expression (ASE) in *P. polionotus* X *P. maniculatus* F₁ hybrids.
Furthermore, we detected ASE in 24 validated imprinted genes from *Mus* (Table S3.3), and detected expression of a further 18 genes that did not meet the significance threshold (Table S3.3; binomial test, P < 0.002 across three burrowing replicates). Importantly, not one of these 42 genes showed DiffASE between burrowing and control conditions, consistent with a mechanism of allele-silencing that is more permanent, such as methylation, which often underlies imprinted gene expression differences (Reik et al. 1987). These observations suggest that our threshold is sensitive enough to detect known allele-specific expression differences (assuming conservation of genomic imprinting patterns across rodents), perhaps at a cost of some false negatives.

**Fine-scale QTL mapping detects five loci for burrow components**

Components of burrow architecture (entrance tunnel length and escape tunnel length) mapped to five genomic loci on a *Peromyscus* linkage map (Weber et al. 2013). In the present study, QTL for entrance tunnel length mapped to three linkage groups (LG 2, 9 and 14) and escape tunnel length mapped to two linkage groups (LG 1 and 9) (Figure 3.4), partially overlapping with QTL detected on LG 1 and 2 in a previous study (Weber et al. 2013). The two QTLs on linkage group 9—one for entrance tunnel length and one for escape tunnel length—had broad, overlapping confidence intervals (Figure 3.4c). With our present data, we cannot distinguish whether the underlying genetic architecture on LG 9 is a single pleiotropic QTL or separate, linked QTLs.

The confidence intervals for detected QTLs ranged from 8.5 cM (LG 2) to 75.6 cM (LG 9), and each QTL interval was found to be largely syntenic to a physical interval from
the *Mus* genome (Table S3.2; ), consistent with expectations (Ramsdell et al. 2008). In particular, our mapping of LG 1 to *Mus* chromosome 7 concurs with the results of Ramsdell et al. (2008). The other syntenic regions we found to harbor QTL are not expected to have experienced genomic rearrangements (Ramsdell et al. 2008). Thus, we were able to infer which genes are likely present in each QTL interval, with the assumption of synteny across these rodent genera. For genes present in these QTL intervals, we next searched our F$_1$ transcriptome data to determine whether they (1) are expressed in brain, (2) show ASE in F$_1$ hybrids, or (3) show DiffASE between behavioral conditions (Figure 3.4). Our rationale is that genes that meet these three criteria—in increasing order as listed—are more likely to affect differences in burrowing behavior between lineages, and can be given priority for follow-up studies. Finally, we expect causative genes to be biased toward negative ASE ratios, as hybrid burrowing behavior closely matches the *P. polionotus* parent (Dawson et al. 1988; Weber et al. 2013).

We next turned our attention to identifying candidate genes from our transcriptomic data. Since the number of genes showing DiffASE were limited to 328, we considered DiffASE genes within all detected QTLs (Figure 3.4). While the QTLs on LG 1 and LG 9 are broad, those on LG 2 and LG 14 are limited in size, containing relatively few genes expressed in brain (Figure 3.4b and 3.4d). In particular, LG 2 (syntenic to *Mus* chromosome 2) is narrowed to only 7.6 Mb (Figure 3.5), and LG 14 spans a gene-sparse 33.3 Mb of the *Mus* genome (Figure 3.6). Thus, we examined genes within these intervals for likely candidates based on ASE ratios, DiffASE ratios, biological information
and reported gene function. Candidate genes are shown in Figures 3.5 (LG 2), 3.6 (LG 14), 3.7 (LG 1 and LG 9), and in Table 3.1.

**Figure 3.4.** (A-D) Linkage groups 2, 9 and 14 contain QTLs for entrance tunnel length (black) and escape tunnel length (purple) QTLs are found on linkage groups 1 and 9. Each point represents a genetic RAD marker. Log-of-odds (LOD) significance thresholds (genome wide, $\alpha = 0.05$) determined empirically by permutation tests are shown in orange. 1.5 LOD confidence intervals are indicated in Mb or cM as noted. The counts of brain expressed (“Exp”; >20 transcripts per gene), ASE and DiffASE genes harbored in each QTL confidence interval are indicated below each QTL peak.
Figure 3.5. Candidate genes within the QTL on LG 2. (A) The 1.5 LOD confidence interval spans just 7.6 Mb of the syntenic Mus chromosome 2 (Table S3.2), and we evaluated candidate genes in a wider 13 Mb interval. Approximate positions of all genes (grey rectangle) and candidates (text and triangles) are indicated. (B) ASE and DiffASE ratios for indicated genes. Negative values reflect P. polionotus-biased expression, while positive values indicate P. maniculatus-bias.
Figure 3.6. Candidate genes within QTL on LG 14. (A) The 1.5 LOD confidence interval spans 33.3 Mb of syntenic *Mus* chromosome 14 (Table S3.2), yet this region is notably gene-sparse. Approximate positions of all genes (grey rectangle) and candidates (text and triangles) are indicated. (B) ASE and DiffASE ratios for indicated candidate genes. Negative values reflect *P. polionotus*-biased expression, while positive values indicate *P. maniculatus*-bias.
**Discussion**

Here, two forward genetics approaches—QTL mapping and transcriptome analysis—are used in concert to identify candidate genes that may account for the striking differences in burrow construction behavior between *P. polionotus* and *P. maniculatus*. The intersection of the two techniques produced a short list of candidate genes for further study (Bendesky and Bargmann 2011), and our findings have implications for how evolution acts on genes to bring about adaptive behavioral change in divergent lineages.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Name</th>
<th>Behavior or Neural Phenotype(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duox1</td>
<td>2</td>
<td>Dual oxidase 1</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Gpr176</td>
<td>2</td>
<td>G-protein receptor 176</td>
<td>Feeding behavior (cattle liver)</td>
<td>Chen et al.</td>
</tr>
<tr>
<td>Klj5</td>
<td>14</td>
<td>Kruppel-like factor 5</td>
<td>Schizophrenia</td>
<td>Yanagi et al.</td>
</tr>
<tr>
<td>Slc30a4</td>
<td>2</td>
<td>Solute carrier family 30 member 4</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Tbc1d4</td>
<td>14</td>
<td>TBC1 domain family, member 4</td>
<td>Glucose homeostasis</td>
<td>Dash et al.</td>
</tr>
<tr>
<td>Tmem87a</td>
<td>2</td>
<td>Transmembrane protein 87A</td>
<td>Fear learning</td>
<td>Ponder et al.</td>
</tr>
<tr>
<td>Vwa8</td>
<td>14</td>
<td>Von Willebrand factor A domain containing 8</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Acer3</td>
<td>7</td>
<td>Alkaline ceramidase 3</td>
<td>Alcohol preference</td>
<td>Hoffman et al.</td>
</tr>
<tr>
<td>Appl1</td>
<td>7</td>
<td>Amyloid beta (A4) precursor-like protein 1</td>
<td>Reduced body weight, neurogenesis</td>
<td>Lazarov et al.</td>
</tr>
<tr>
<td>Arhgap42</td>
<td>9</td>
<td>Rho GTPase activating protein 4</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Edc3</td>
<td>9</td>
<td>Enhancer of mRNA decapping 3 homolog</td>
<td>Intellectual disability, neurodevelopment</td>
<td>Ahmed et al.</td>
</tr>
<tr>
<td>Fah</td>
<td>7</td>
<td>Fumarylacetoacetate hydrolase</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Fam118b</td>
<td>9</td>
<td>Family with sequence similarity 118, member B</td>
<td>Bipolar disorder GWAS</td>
<td>Johnson et al.</td>
</tr>
<tr>
<td>Knc1</td>
<td>7</td>
<td>Potassium voltage gated channel, Shaw-related</td>
<td>Social disinhibitions GWAS, KO: reduced body weight, sleep loss, motor impairments, hyperactivity</td>
<td>Archer et al.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>subfamily, member 1</td>
<td>Circadian rhythms</td>
<td>Kudo et al.</td>
</tr>
<tr>
<td>Lctl</td>
<td>9</td>
<td>Lactase-like</td>
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<td>NA</td>
</tr>
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<td>Lrp3</td>
<td>7</td>
<td>Low density lipoprotein receptor-related protein 3</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Lrrc28</td>
<td>7</td>
<td>Leucine rich repeat containing 28</td>
<td>None found</td>
<td>NA</td>
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<tr>
<td>Lrrc49</td>
<td>9</td>
<td>Leucine rich repeat containing 49</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Map3k10</td>
<td>7</td>
<td>Mitogen-activated protein kinase kinase kinase 10</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Polr3e</td>
<td>7</td>
<td>Polymerase (RNA) III (DNA directed) polypeptide E</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Prkd2</td>
<td>7</td>
<td>Protein kinase D2</td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td>Rsf1</td>
<td>7</td>
<td>Remodeling and spacing factor 1</td>
<td>Behavioral maturation honey bee (putative)</td>
<td>Zayed et al.</td>
</tr>
<tr>
<td>Snap91</td>
<td>9</td>
<td>Synaptosomal-associated protein 91</td>
<td>Role in synapses and their development</td>
<td>NA</td>
</tr>
<tr>
<td>Sphk2</td>
<td>7</td>
<td>Sphingosine kinase 2</td>
<td>Neuronal survival, Snpc</td>
<td>Sivasubramanian et al.</td>
</tr>
<tr>
<td>St5</td>
<td>7</td>
<td>Suppression of tuorigenicity 5</td>
<td>Mental retardation</td>
<td>Gohring et al.</td>
</tr>
<tr>
<td>Tbc1d17</td>
<td>17</td>
<td>TBC1 domain family, member 17</td>
<td>Alzheimer Disease</td>
<td>Kohli et al.</td>
</tr>
<tr>
<td>Tle3</td>
<td>9</td>
<td>Transducin-like enhancer of split 3</td>
<td>Target of FOXP2 in IFC (human), Glucocorticoid response</td>
<td>Spiteri et al.</td>
</tr>
</tbody>
</table>

**Table 3.1. Candidate genes for burrowing behavior.** DiffASE (first section) and ASE (second section) candidate genes for differences in burrowing behavior between *P. maniculatus* and *P. polionotus*.  

101
<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr</th>
<th>Name</th>
<th>Behavior or Neural Phenotype(s)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><em>Akap11</em></td>
<td>14</td>
<td>Akap11</td>
<td>A kinase (PRKA) anchor protein 11</td>
<td>Kelly &amp; Qiu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hormonal regulation arcuate nucleus</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Autism spectrum disorders</td>
<td>Poelmans et al.</td>
</tr>
<tr>
<td><em>Bahd1</em></td>
<td>2</td>
<td>Bahd1</td>
<td>Bromo adjacent homology domain containing 1</td>
<td>None found</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td><em>Casc4</em></td>
<td>2</td>
<td>Casc4</td>
<td>Cancer susceptibility candidate 4</td>
<td>None found</td>
</tr>
<tr>
<td><em>Cog3</em></td>
<td>14</td>
<td>Cog3</td>
<td>Component of oligomeric golgi complex 3</td>
<td>None found</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td><em>Commd6</em></td>
<td>14</td>
<td>Commd6</td>
<td>COMM domain containing 6</td>
<td>None found</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>None found</td>
<td>NA</td>
</tr>
<tr>
<td><em>Dgkh</em></td>
<td>14</td>
<td>Dgkh</td>
<td>Diacylglycerol kinase, et al</td>
<td>Bipolar disorder GWAS</td>
</tr>
<tr>
<td><em>Itm2b</em></td>
<td>14</td>
<td>Itm2b</td>
<td>Integral membrane protein 2B</td>
<td>Neurodegeneration, memory</td>
</tr>
<tr>
<td><em>Ivd</em></td>
<td>2</td>
<td>Ivd</td>
<td>Isovaleryl coenzyme A dehydrogenase</td>
<td>None found</td>
</tr>
<tr>
<td><em>Lcp1</em></td>
<td>14</td>
<td>Lcp1</td>
<td>Lymphocyte cytosolic protein 1</td>
<td>None found</td>
</tr>
<tr>
<td><em>Lpcat4</em></td>
<td>2</td>
<td>Lpcat4</td>
<td>Lyso phosphatidylycholine acetyltransferase 4</td>
<td>Brain development and aging</td>
</tr>
<tr>
<td><em>Map10a</em></td>
<td>2</td>
<td>Map10a</td>
<td>Microtubule-associated protein 1 A</td>
<td>Development of neurites</td>
</tr>
<tr>
<td><em>Med4</em></td>
<td>14</td>
<td>Med4</td>
<td>Mediator complex subunit 4</td>
<td>None found</td>
</tr>
<tr>
<td><em>Mzt1</em></td>
<td>14</td>
<td>Mzt1</td>
<td>Mitotic spindle organizing protein 1</td>
<td>None found</td>
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<tr>
<td><em>Nop10</em></td>
<td>2</td>
<td>Nop10</td>
<td>NOP10 ribonucleoprotein</td>
<td>None found</td>
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<tr>
<td><em>Pcdh17</em></td>
<td>14</td>
<td>Pcdh17</td>
<td>Protocadherin 17</td>
<td>Role in development,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>regionalization and functional differentiation of brain</td>
<td>NA</td>
</tr>
<tr>
<td><em>Pcdh9</em></td>
<td>14</td>
<td>Pcdh9</td>
<td>Protocadherin 9</td>
<td>Autism spectrum disorders</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>social recognition and sensorimotor traits (mice)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>boldness behavior in dogs</td>
<td></td>
</tr>
<tr>
<td><em>Pdlim2</em></td>
<td>14</td>
<td>Pdlim2</td>
<td>PDZ and LIM domain 2</td>
<td>None found</td>
</tr>
<tr>
<td><em>Phyhip</em></td>
<td>14</td>
<td>Phyhip</td>
<td>Phytanoyl-CoA hydroxylase interacting protein</td>
<td>None found</td>
</tr>
<tr>
<td><em>Pibf1</em></td>
<td>14</td>
<td>Pibf1</td>
<td>Progesterone immunomodulatory binding factor 1</td>
<td>None found</td>
</tr>
<tr>
<td><em>Ppip5k1</em></td>
<td>2</td>
<td>Ppip5k1</td>
<td>Diphosphoinositol pentakisphosphate kinase 1</td>
<td>None found</td>
</tr>
<tr>
<td><em>Serf2</em></td>
<td>2</td>
<td>Serf2</td>
<td>Small EDRK-rich factor 2</td>
<td>Mouse grooming behavior,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>open field test</td>
<td></td>
</tr>
<tr>
<td><em>Serinc4</em></td>
<td>2</td>
<td>Serinc4</td>
<td>Serine incorporator 4</td>
<td>None found</td>
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<tr>
<td><em>Shf</em></td>
<td>2</td>
<td>Shf</td>
<td>Src homology 2 domain containing F</td>
<td>None found</td>
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<tr>
<td><em>Spred1</em></td>
<td>2</td>
<td>Spred1</td>
<td>Sprouty protein with EVH-1 domain 1, related sequence</td>
<td>Synaptic plasticity,</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Learning</td>
<td>Legius syndrome</td>
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<tr>
<td><em>Srpr14</em></td>
<td>2</td>
<td>Srpr14</td>
<td>Signal recognition particle 14</td>
<td>None found</td>
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<tr>
<td><em>Sugt1</em></td>
<td>14</td>
<td>Sugt1</td>
<td>SGT1, suppressor of G2 allele of SKP1</td>
<td>None found</td>
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<tr>
<td><em>Ttbk2</em></td>
<td>2</td>
<td>Ttbk2</td>
<td>Tau tubulin kinase 2</td>
<td>Spinocerebellar ataxia type 11</td>
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<tr>
<td><em>UchI3</em></td>
<td>14</td>
<td>UchI3</td>
<td>Ubiquitin carboxyl-terminal esterase L3</td>
<td>Spatial learning and working memory</td>
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<td><em>Vps39</em></td>
<td>2</td>
<td>Vps39</td>
<td>Vacuolar protein sorting 39</td>
<td>None found</td>
</tr>
</tbody>
</table>

**Table 3.1.** Continued
First, our results suggest widespread *cis*-regulatory changes have acted on brain-expressed genes between these sister species. We found 21.5% of genes expressed in adult brain showed evidence of ASE in F$_1$ hybrids. This proportion is not easily comparable across studies, as it relies on coding polymorphisms between parental species (to produce SNPs for determination of parent-of-origin), methodological details (e.g. sequencing depth, tissue choice), and the chosen significance thresholds, but it is not dissimilar from rates reported in other organisms (e.g. McManus et al. 2010; Keane et al. 2011). This result is consistent with a growing body of work showing the importance of regulatory regions in evolution (MacKay 2001; Hoekstra and Coyne 2007; Wray 2007; Prud’homme et al. 2006).

Notably, a majority of these biased genes we show a bias favoring expression from *P. polionotus* alleles, in both ASE and DiffASE comparisons. This may represent a molecular reflection of *P. polionotus*-dominant burrowing behavior of F$_1$ hybrids (Dawson et al. 1988; Weber et al. 2013). Indeed, such a result may be expected given that the burrowing behavior of F$_1$ hybrids closely matches the *P. polionotus* parental strain. However, in the examined F$_1$ transcriptomes, species-of-origin (i.e. *P. polionotus* or *P. maniculatus*) is perfectly confounded with sex-of-parent--all sires were *P. polionotus* and all dams *P. maniculatus*, as known imprinting effects preclude the reciprocal cross (Vrana et al. 2000). While we eliminated from our analyses all validated imprinted genes known from *Mus* (Tables S3.3 and S3.4), it is possible that some of the observed *P. polionotus*-bias in ASE is due to genomic imprinting. This is especially true
for genes involved in phenotypes such as metabolism and feeding behavior, as these traits are common targets for the effects of imprinting.

Our fine-scale QTL mapping successfully narrows in on a handful of genomic loci that segregate in hybrid animals. In particular, the QTLs on LG2 and LG14 are narrowed to regions containing relatively few genes. In total, 28 of 328 DiffASE genes identified in our analysis were localized within detected QTL peaks. The remaining 300 genes may indeed be involved in burrow construction behavior, but do not appear to be related to segregating differences between species.

If genes exhibiting DiffASE in a burrowing-specific manner are eventually shown to be causally related to segregating differences between *P. polionotus* and *P. maniculatus*, this may indicate that the gene expression changes are context-specific, rather than a constitutive aspect of physiology. That is, expression differences only arise following sensory- or activity-induced transcription factor expression. Similarly, sex-specific gene expression differences in mice have been shown to be reliant on hormone signals, and capable of irregularity across time (Xu et al. 2012), and Chan et al. have demonstrated that *cis*-regulatory variation can evolve to act in a tissue-specific manner (2010). These studies, and (if verified) our finding of behavior-specific DiffASE in *Peromyscus* brains, may illustrate an argued strength of evolution via *cis*-regulatory variation—*cis*-regulation can act with great spatiotemporal specificity (Hoekstra and Coyne 2007), be it a given hormonal environment, a defined tissue, or a particular behavioral condition.
Although ASE and DiffASE are useful tools for a genome-wide search for candidate genes driving differences between species, it is important to note the several limitations of our transcriptomic approach. First, our transcriptome analysis was limited to whole brain tissue, which will likely only detect large-scale and wide-spread changes in gene expression. Smaller differences in expression level would likely not be detected with use of stringent significance thresholds. Furthermore, multiple sub-region-specific expression differences of different directionality (i.e. increased and decreased expression in different brain areas) could balance out across whole brain, rendering them invisible in whole-brain approaches. Furthermore, our tissue sampling was limited to one age group, and would not detect expression differences acting in development. Last, read-mapping bias may prove to be particularly challenging in data sets comparing divergent species, such as ours (Degner et al. 2009). Moreover, there are many other ways in which genetic variation could shape behavior which we have not yet examined, including trans-acting regulatory differences, differences in development, actions in other tissues, and coding polymorphisms. Follow-up experiments to this study could focus on these other potential mechanisms of evolutionary change.

Our analysis points to a handful of candidates genes that are associated by QTL mapping and transcriptome analysis to species-specific differences in burrowing behavior. Moving from QTLs to specific genes is difficult (MacKay 2001; MacKay et al. 2009, Flint et al. 2005), and functional studies to link these genes definitively to burrowing behavior will be essential follow-up experiments. Of particular interest are genes known to be involved in brain development (e.g. Edc3, Snap91, Pcdh17, Map1a),
and behavior, including activity patterns (e.g. *Serf2, Kcnc1*), social behavior and its disorders (e.g. *Pcdh9, Kcnc1, Dgkh, Tbc1d17*), reward/addiction pathways (e.g. *Acer3, Gpr176*), and fear/anxiety (e.g. *Tmem87a*). While these candidates await functional studies to demonstrate their role in burrowing behavior, if confirmed, they would represent new examples of the molecular units underlying behavior evolution.

**Author Contributions.** QTL study-- HCM and HEH conceived and designed the experiments. HCM performed the experiments and analyzed the data. Transcriptome study—HCM, NLB, HF and HEH conceived and designed the experiments. NLB conducted the behavioral trials. HCM and NLB prepared the RNA-Seq libraries. RAY analyzed the gene expression data. HCM and HEH wrote the paper.

**Acknowledgements.** We thank Z Ali, R Ludwick, L Pan, S Scalia, and JN Weber for help with behavior experiments, BK Peterson and JM Lassance for assistance with ddRAD analyses, CL Lewarch for assistance with dissections, K Turner for assistance with library preparation, and the Hokestra Lab, particularly A Bendesky, C Hu, HS Fisher and E Jacobs-Palmer for helpful discussions. We thank B de Bivort, C Dulac, B Ölveczky and J Sanes for helpful discussions. We thank EHK Delaney for comments on the manuscript, and N. Rubinstein for assistance with considerations of genomic imprinting. Harvard’s Office of Animal Resources, particularly J Rocca for excellent animal husbandry. This research was funded by the Mind Brain Behavior Initiative at Harvard, the Chapman Funds for Vertebrate Locomotion, a National Science Foundation Graduate Research Fellowship, a Doctoral Dissertation Improvement Grant, and an American Fellowship from the American Association of University Women to HCM; a Natural Sciences and Engineering Research Council Graduate Fellowship to NLB; and a Beckman Young Investigator Award and the Howard Hughes Medical Institute to HEH.

**References**


Kohli, M et al. 2014. The dissection of high-penetration variants in extended late-onset Alzheimer Disease families by whole exome sequencing. *Neurology* 82(10):


Discussion

In this dissertation, I make progress in understanding the genetic basis of differences in a complex, natural behavior that evolved in the wild. My study of behavior ontogeny (Chapter 1) shows that pleiotropic genetic changes that act on pathways modulating burrowing-specific motivation likely play a role in the behavioral differences between these species, which are seen across life stages. More generally, evolution can shape behavior by linking performance of evolutionarily-advantageous behaviors with feelings of satisfaction or pleasure, and performance of disadvantageous behaviors with feelings of disappointment or pain. These percepts of quality (or valence) guide animals’ behavioral choices (e.g. Janak and Tye 2015, Namburi et al. 2015). Thus, evolution may alter behavioral priorities between species by adjusting levels of neuromodulatory molecules that act in a behavior-specific manner. For example, *Peromyscus polionotus* (relative to *P. maniculatus*) may inherently experience greater pleasure from the construction of burrows. Alternatively, *P. polionotus* (again relative to *P. maniculatus*) may experience reduced satisfaction from the performance of other behaviors, leading *P. maniculatus* but not *P. polionotus* to attend to those alternative behaviors and ultimately producing the measureable differences in burrowing between species. With the help of molecular, neurobiological, and genetic tools for non-model mammals, future studies could examine these two species for neural evidence of differential reward signaling linked to burrowing behavior.

From the candidate genes nominated in Chapter 3, some are thought to possibly affect motivational pathways, including *Gpr176*, *Acer3*, and *Sphk2*. Beyond the genes
detected in our ASE and DiffASE analyses, additional genes in our QTL peaks have known roles in regulating motivational pathways, notably \textit{Chrm5} on \textit{Peromyscus} LG 2/Mus Chromosome 2. Functional experiments to demonstrate a causal role for candidates in burrowing behavior could be accomplished either by bringing genetic tools to \textit{Peromyscus} (e.g. viral manipulation, CRISPR), or perhaps by studying burrowing behavior in \textit{Mus}, in which a powerful array of genetic tools already exist.

Interestingly, genes related to certain human neurological disorders appear repeatedly in our ASE and DiffASE candidate gene lists, including autism spectrum disorder (\textit{Akap11, Pcdh9}), Alzheimer Disease (\textit{Aplp1, Tbc1d17}), and mental disorders including bipolar disorder and schizophrenia (\textit{Klf5, Fam118b, Dgkh}). Whether these genes are related to species-specific differences in burrowing behavior or not, these expression level differences are interesting and highlight how studies of diverse animals can inform knowledge of human disease.

The next experiments in studying the genetic mechanisms underlying differences in burrowing behavior between these species should include examination of brain sub-regions. While it is not clear which brain regions could underlie innate differences in tunneling behavior in a mammal, a transcriptome study of regions such as hypothalamus, amygdala, hippocampus, and striatum (i.e. regions known to be involved in reward, motor control, and navigation) could provide improved differential expression results and avoid many limitations of a whole brain strategy—albeit with the risk of selecting irrelevant brain regions, or selecting overly-large brain regions.
However, immediate early gene studies could guide selection of brain regions for such studies, reducing these risks.

Although my QTL mapping results show greater mapping resolution than previous studies (Weber et al. 2013), they could be further improved by taking advantage of the new genomic resources for *Peromyscus*. Some genome scaffolds were recently made public, and additional *Peromyscus* genomes are being sequenced by the Broad Institute. With these genomic tools, multiplexed shotgun genotyping (Andolfotto et al. 2011), for example, could be implemented. Additionally, the *Peromyscus* genome scaffolds and the *P. polionotus* and *P. maniculatus* brain transcriptomes (Chapter 3) could be used to search for changes in regulatory elements upstream of QTL, ASE and DiffASE genes, and to search for coding changes, respectively, thus broadening the scope of the considered candidate genes.

In Chapter 2, I demonstrate the potential for applying an automated image-based analysis to the measurement of animal architectures, including burrowing. This method allows for a much richer set of phenotypes to be gathered from burrow casts. Based on the promising early results, this method shows great potential for being applied to the study of trait inheritance across generations (Chapter 2) and QTL mapping (Chapter 3), perhaps after principal component analysis to reduce the dimensionality of the data. Of particular interest to me is the correlation of escape tunnel digging with curvature in the analyzed casts (Figure 2.6). If some mice have the potential to dig escape tunnels yet lack sufficient motivation to dig them, perhaps their tendency to dig
escapes is revealed in the curviness of their tunnels. Thus, by measuring curvature, we may be able to study behavioral tendencies that were previously unaccounted for in our analysis methods.

*Peromyscus* rodents are a powerful system for the study of behavior, as they show a great deal of fitness-related behavioral variation and are tractable for intensive laboratory study. Of particular interest to me is the relationship between social behavior and burrowing behavior. *P. polionotus* is a genetically and socially monogamous species (Foltz, 1981) while *P. maniculatus*, like most mammals, is promiscuous (Dewsbury, 1981). These two behaviors (mating system and burrowing behavior) may be related: whereas *P. maniculatus* presumably must regularly invest a great deal of time into finding mates, a behavior that involves traveling through space, *P. polionotus* attaches to a single mate and together they invest in the construction of a complex burrow, which must exist in a home range (JN Weber and W Tong, unpublished data). Thus, a shift in mating system (a social behavior) may allow for the evolution of greater investment in home ranges and housing, including burrows, or vice versa (e.g. Nowak et al. 2010). Members of the Hoekstra research group, including Nicole Bedford, Andrés Bendesky, and Brant Peterson are already investigating the genetic basis of burrow construction and mating system in a single F$_2$ intercross between *P. polionotus* and *P. maniculatus*, which should reveal whether these two behaviors share a genetic basis. Whether these traits (mating system, burrow construction) have shared or independent genetic mechanisms, the behavioral adaptations of *P. polionotus* hold much promise for uncovering the mechanisms of behavior evolution in a wild mammal.
References


Supplementary Figures

Figure S1. Morphological and motor development in two species of Peromyscus rodents. *P. polionotus* and *P. maniculatus* have similar developmental trajectories, but *P. maniculatus* is larger and runs further in a voluntary wheel-running assay. Indicated data adapted from Kirkland, GL and JN Layne. 1989. *Advances in the study of Peromyscus (Rodentia).* Texas Tech University Press, Lubbock, TX.
Figure S2. Inheritance of tunnel length (average of 3 trials) across five sequential generations of *Peromyscus polionotus* X *P. maniculatus* backcross hybrids. Violin plots show kernel densities for each phenotype. Black dots represent the median value for each generation. Data for 272 BC1 hybrids adapted from Weber et al. 2013.
## Supplementary Tables

### Standard housing and husbandry information

| Caging          | Individually ventilated Allentown caging made of polysulfone (Allentown, Allentown, NJ)  
|                 | Positive pressure ventilation  
|                 | Air changes/hour: 60  
|                 | Dimensions: 7.75" wide x 12" long x 6.5" high (or 75 in$^2$ or 484 cm$^2$)  
| Bedding        | For standard bedding: Anderson’s Bed o cob, ¼ inch (The Andersons, Inc., Maumee, OH)  
| Cage changes   | At least every 14 days, more often if cage condition requires  
| Light cycle    | 16 hours light, 8 hours dark  
| Temperature    | 21°C ± 1  
| Humidity       | 30-70%  
| Disinfectants  | Chlorine dioxide (hoods, forceps, hands); Clidox (Pharmacal, Naugatuck, CT)  
|                | Quaternary ammonium compound (floors, cage wash); Quatricide PV (Pharmacal, Naugatuck, CT)  
|                | Cage wash detergent; Alka-Det (Pharmacal, Naugatuck, CT)  
| Feed           | Standard: irradiated LabDiet Prolab Isopro RMH 3000 SP75 (LabDiet, St. Louis, MO)  
|                | Breeder: irradiated PicoLab Mouse Diet 20 5058 (LabDiet, St. Louis, MO)  
| Water          | Reverse osmosis deionized water is chlorinated at 2 ppm and typically provided via automatic watering system.  
| Enrichment     | Nestlet (2 x 2” compressed cotton square, Ancare, Bellmore, NY)  
|                | Enviro-Dri (8-10 g added at cage change; strips of folded brown paper, Shepherd Specialty Papers, Watertown, TN)  
|                | Mouse Huts (certified polycarbonate; 3 3/4" wide x 1 7/8" tall x 3" long, BioServ, Flemington, NJ)  

The facility is AAALAC, Intl. accredited since 1990, and also holds a current USDA Research Registration (number available on request from the IACUC).

Table S1.2. Housing conditions for *Peromyscus* mice at Harvard University.
## Table S3.2: Summary of sequence alignments for hypothesized synteny between the *P. polionotus* x *P. manculatus* linkage map (Weber et al. 2013) and *Mus* physical genome within the listed QTL intervals. “Rep. genes” are representative genes (not a complete list) of *Mus* nucleotide records that aligned to the indicated *P. manculatus* genome scaffolds. Grey shading indicates that a marker did not align to any scaffold.

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<td>NW_006501607.1</td>
<td>-0.172</td>
<td>-</td>
</tr>
<tr>
<td>Qpct</td>
<td>NW_006501403.1</td>
<td>-0.549</td>
<td>yes</td>
</tr>
<tr>
<td>Rasgrf1</td>
<td>NW_006501564.1</td>
<td>-0.036</td>
<td>yes</td>
</tr>
<tr>
<td>Scyl2</td>
<td>NW_006501165.1</td>
<td>-0.173</td>
<td>-</td>
</tr>
<tr>
<td>Stx16</td>
<td>NW_006501107.1</td>
<td>0.037</td>
<td>-</td>
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<tr>
<td>Stx6</td>
<td>NW_006501298.1</td>
<td>-0.047</td>
<td>-</td>
</tr>
<tr>
<td>Tpx2</td>
<td>NW_006501294.1</td>
<td>-1.844</td>
<td>yes</td>
</tr>
<tr>
<td>Trappc9</td>
<td>NW_006501447.1</td>
<td>2.277</td>
<td>yes</td>
</tr>
<tr>
<td>Ube3a</td>
<td>NW_006501596.1</td>
<td>1.447</td>
<td>yes</td>
</tr>
<tr>
<td>Wars</td>
<td>NW_006501050.1</td>
<td>0.050</td>
<td>-</td>
</tr>
<tr>
<td>Zdbf2</td>
<td>NW_006501089.1</td>
<td>-4.609</td>
<td>yes</td>
</tr>
</tbody>
</table>

**Table S3.3:** Detected ASE for validated imprinted genes. Binomial significance indicated when $P < 0.002$ across all three burrowing F1 replicates.
<table>
<thead>
<tr>
<th>Validated imprinted genes, expression (&gt;20 transcripts) not detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF365933</td>
</tr>
<tr>
<td>Ago2</td>
</tr>
<tr>
<td>Airn</td>
</tr>
<tr>
<td>AK014392</td>
</tr>
<tr>
<td>AK029869</td>
</tr>
<tr>
<td>AK039108</td>
</tr>
<tr>
<td>AK141557</td>
</tr>
<tr>
<td>Ascl2</td>
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<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

**Table S3.4**: List of validated imprinted genes found at <20 reads in transcriptomic data.
<table>
<thead>
<tr>
<th>Threshold</th>
<th>No. of ASE genes</th>
<th>No. <em>P. pol</em>-biased</th>
<th>Proportion <em>P. pol</em>-biased</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>7786</td>
<td>4528</td>
<td>0.582</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>0.002</td>
<td>2629</td>
<td>1485</td>
<td>0.565</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>0.001</td>
<td>2321</td>
<td>1315</td>
<td>0.567</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>0.0001</td>
<td>1677</td>
<td>959</td>
<td>0.572</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Top 100</td>
<td>100</td>
<td>60</td>
<td>0.600</td>
<td>0.1552</td>
</tr>
<tr>
<td>Top 50</td>
<td>50</td>
<td>32</td>
<td>0.640</td>
<td>0.1574</td>
</tr>
</tbody>
</table>

**Table S3.5:** ASE species-bias proportions while varying significance threshold. In each case, threshold must have been met independently in each of three biological replicates (burrowing animals; see Figure 3.2). *P*-values given for two sample test for equality of proportions.