Review

Peromyscus burrowing: A model system for behavioral evolution

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ABSTRACT

A major challenge to understanding the genetic basis of complex behavioral evolution is the quantification of complex behaviors themselves. Deer mice of the genus *Peromyscus* vary in their burrowing behavior, which leaves behind a physical trace that is easily preserved and measured. Moreover, natural burrowing behaviors are recapitulated in the lab, and there is a strong heritable component. Here we discuss potential mechanisms driving variation in burrows with an emphasis on two sister species: *P. maniculatus*, which digs a simple, short burrow, and *P. polionotus*, which digs a long burrow with a complex architecture. A forward-genetic cross between these two species identified several genomic regions associated with burrow traits, suggesting this complex behavior has evolved in a modular fashion. Because burrow differences are most likely due to differences in behavioral circuits, *Peromyscus* burrowing offers an exciting opportunity to link genetic variation between natural populations to evolutionary changes in neural circuits.

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Contents

1. Introduction

The North American continent is a patchwork of various habitats – ranging from rainforest to prairie to desert – and throughout these varied landscapes are deer mice of the genus *Peromyscus.* Since the early 1900's, natural historians have documented how *Peromyscus* mice have diversified to successfully inhabit almost all major habitat types across this continent. While there exists tremendous variation in many morphological and physiological traits, *Peromyscus* also show a striking diversity of behaviors. Yet, how complex behaviors are encoded and evolve is still largely unknown.

Multiple features of *Peromyscus* biology are conducive to addressing these longstanding questions. First, *Peromyscus* are tractable in the laboratory, which enables their use for the study of ecologically relevant behaviors in a controlled setting [1,2]. Examples of the behavioral diversity within and between many *Peromyscus* species that have been studied in the lab include predator response [3], social dominance hierarchy formation [4], aggression and parental behavior [5], nest site selection [6], and burrowing behavior. Second, many of these species-specific behaviors persist

across generations in lab-reared animals, suggesting that there is a measurable, heritable component to this variation in behavior. Finally, emerging genomic and transcriptomic resources in *Peromyscus* (e.g. [7–10]), combined with existing resources in closely related laboratory models (i.e. *Mus* and *Rattus*), as well as the ability to perform crosses between behaviorally divergent populations or species, empower the use of *Peromyscus* for behavioral-genetic studies. Complementary to identifying the genetic basis of behavior in traditionally inbred mouse models, the use of outbred *Peromyscus* populations can shed light onto the genetic architecture of complex behaviors and how they are shaped by natural selection.

One of the main challenges to dissecting the genetic and neurobiological basis of behavioral variation, in any organism, is the ability to measure the behavior of interest. In comparison to many morphological traits, behaviors are often highly variable and can be influenced strongly by internal stimuli (e.g. hormonal status) and the external environment (e.g. social, developmental and abiotic context) [11]. Furthermore, the measurement of behavior is often dependent on subjective decisions by human observers, which can be difficult to standardize [12]. Advances in high-throughput behavioral phenotyping in mice promise to alleviate some of the problem via automation (e.g. [13,14]); nonetheless it remains challenging to identify and quantify biologically relevant components of complex behaviors. One solution, however, is to take advantage of the idea of an "extended phenotype" [15]. Richard Dawkins described an extended phenotype as an animal artifact – that is, the morphological product of a heritable behavior – such as the nest of swallows, the dam of beavers, or a spider's web. Another example of an extended phenotype is the burrow produced by fossorial or semi-fossorial mammals, including *Peromyscus*, casts of which can be measured like a morphological trait but it is inherently a product of behavior.

Burrowing – the excavation of habitable space – is widespread across animals, and is a prominent behavior of some *Peromyscus* species. Burrows may play an important role in the ecosystem through movement of organic and abiotic substrates, shifting geochemical conditions, and providing shelter for commensal species [16–18]. Burrows are energetically costly to create [19], but can benefit animals by protecting against predation, buffering environmental fluctuations in temperature and humidity, and providing them a place to store resources [20]. Specializations for burrowing in mammals have been traced back to the Jurassic [21], and approximately half of extant terrestrial mammal genera have members that burrow [22]. This behavior has continued to diversify in extant groups. In particular, *Peromyscus* mice have undergone recent innovations in burrowing behavior resulting in a range of species-typical burrow sizes and shapes. This diversification of behavior between interfertile populations has enabled the investigation of the genetic architecture underlying burrow evolution (Fig. 1A-B) [23].

2. Burrow diversity in *Peromyscus*

Peromyscus range from northern Canada to Panama, occupying a wide diversity of habitats [24]. Early physiology experiments demonstrated that these small-bodied rodents must seek or create microenvironments that shelter against the temperature extremes of their habitats [25–27]. Consistent with this, *Peromyscus* nests have been found amongst vegetative or rocky cover, in trees, and in burrows (Table 1). The construction and location of nests is species-specific, suggesting the *Peromyscus* genus contains non-burrowing, facultative burrowing, and obligate burrowing species. This varying propensity to burrow in the wild is reflected in the frequency at which *Peromyscus* will burrow in the laboratory (Fig. 1A) [28].

The most conspicuous burrower of the *Peromyscus* genus is the oldfield mouse, *P. polionotus*. This species resides in the southeastern United States, specifically Alabama, Georgia, South Carolina and northern Florida and exclusively in open fields and sand dunes. In these sparsely vegetated habitats, mice are exposed to predators — primarily owls, herons and hawks, carnivores and snakes — and thus, burrows are an important retreat and escape [29], arguably more so than for mice living in heavily vegetated, heterogeneous environments. At a practical level, in these open habitats, the exposed entrances of active burrows — sometimes identifiable by a plume of freshly excavated of substrate — facilitated study by naturalists (Fig. 1C) [30–33].

The complex yet stereotyped architecture of the *P. polionotus* burrow was first described by Howell in 1921 as part of a faunal survey of Alabama [34]. Their burrow shape then was described more extensively by Sumner in 1929 [32], and later quantitative measurements of burrow dimensions were provided by Hayne [31] and Smith [35]. These naturalists described burrows from throughout *P. polionotus*' range — both mainland populations (e.g. *P. p. subgriseus*) and island populations (e.g. *P. p. leucocephalus*) — as consisting of an oft-plugged, downward sloping entrance tunnel, a nest chamber, and a second upward-sloping "escape tunnel" that approaches, but does not penetrate, the ground surface (Fig. 1A). These three burrow components are typically arranged along a plane, with a total length between 150 and 250 cm. And, when the burrow is disturbed, *P. polionotus* will erupt through the escape tunnel. This stereotyped burrow architecture and escape response have further facilitated capture and study by naturalists, such as Sumner, who colorfully described catching *P. polionotus* by inserting a "switch" into burrows and Rand and Host [36], who reported "By covering the ground with cheese cloth where the escape tunnel was expected to open, all the inhabitants of the burrow could be easily captured." When Weber and colleagues went to the field, nearly a century after Howell's first descriptions of the *P. polionotus* burrow, the burrow architecture, its dimensions, and the mouse's escape behavior were found intact throughout the species range [23].

Because most other *Peromyscus* species live in more vegetated environments, their wild burrows are more difficult to find and therefore, their morphology has not been well described. *P. polionotus* recapitulates its wild burrow structure in the laboratory setting [42,95], suggesting that a similar approach could be used to investigate the natural burrowing behaviors of its more elusive congeners. For example, the burrow architecture of its sister species, *P. maniculatus*, is relatively simple (Fig. 1A). *P. maniculatus* is the most widely-distributed *Peromyscus* species and is generally found in areas of dense vegetative cover such as forests (e.g. *P. m. nubiterrae*) and prairies (e.g. *P. m. bairdii*). The typical *P. maniculatus* burrow consists simply of an almost vertical entrance tunnel and a small nest chamber. Although the lengths of *P. maniculatus* burrows vary [37–39], these burrows are significantly shallower than *P. polionotus* burrows and lack escape tunnels. Because the species-specific burrowing behavior is shared across the respective subspecies, this suggests that the difference emerged approximately one million years ago around the divergence between the two species [40,41].

Examination of additional *Peromyscus* species suggests that the *P. polionotus* burrow architecture is indeed unique. While other species dig long burrows, such as *P. aztecus*, whose burrow lengths in lab enclosures rival that of some *P. polionotus*, no other *Peromyscus* species tested thus far produces a recognizable escape tunnel [28]. When examined in a phylogenetic context, the *P. polionotus* escape tunnel is a derived trait [28], highlighting a gain in behavioral complexity along this lineage. This diversity suggests that if these behaviors are heritable, there may be separate genes responsible for the length of the burrow and the presence or absence of the escape tunnel (see "Genetic Architecture" below).
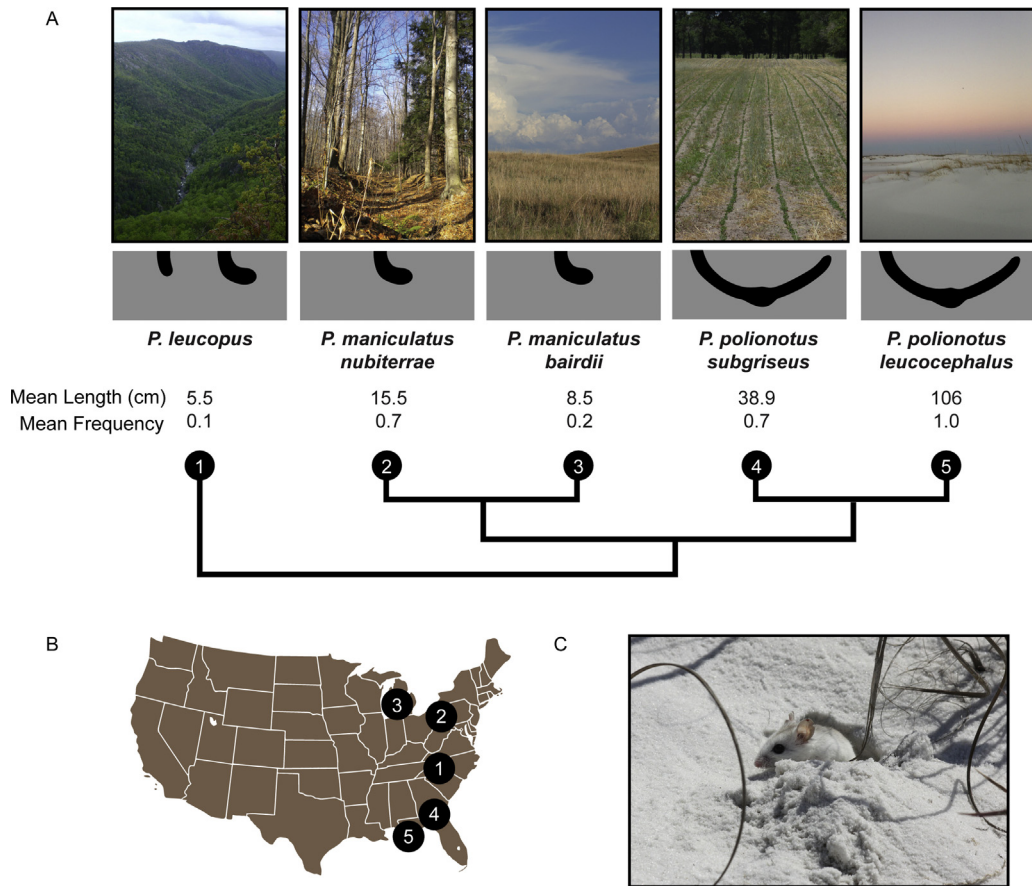
Fig. 1. *Peromyscus* burrow and habitat diversity. (A) *Peromyscus* species-typical burrow shapes and habitats. Burrow measurements were acquired in the laboratory with laboratory-reared animals. Burrow lengths (in cm) are the mean of the longest burrow length from three test trials, and burrow frequencies are the mean of the ratio of trials animals burrowed out of three total trials. The mean length and frequency are provided for each species. Phylogeny, burrow shapes, and burrow metrics adapted from [28], with the exception of *P. maniculatus nubiterrae* and *P. polionotus leucocephalus* (Hu and Bedford, unpublished). Burrow shape diagrams are representative of overall burrow structure and are not to scale. (B) Locations from which laboratory population founders were originally collected [24]. (C) *P. polionotus leucocephalus* at its burrow entrance. Note the plume of freshly excavated sand.

Photograph credits: *P. leucopus* habitat from Wikimedia Commons, under CC0 license, *P. maniculatus nubiterrae* habitat by Evan P. Kingsley, *P. maniculatus bairdii* habitat and *P. polionotus leucocephalus* at its burrow by Nicole L. Bedford, *P. polionotus subgriseus* habitat by United States Department of Agriculture, and *P. polionotus leucocephalus* habitat by Caroline K. Hu.

Table 1

Field and laboratory observations of *Peromyscus* digging behavior and burrows. Only species with data for at least one of the four categories were included. Not applicable (NA) indicates species do not generate burrows. Dash (−) indicates there is no data for that species. Asterisk (*) denotes species that do not burrow in the wild and also did not generate burrows when tested in the laboratory.

Species	Field nest site location	Field burrows measured	Laboratory digging observed	Laboratory burrows measured
P. alstoni	Burrows, pocket gopher burrows [77]	Yes [77]	–	–
P. attwateri	Trees, rock crevices [78]	NA	–	–
P. aztecus	Rock crevices, burrows [79]	–	–	Yes [28]
P. boylii	Rock and log crevices, burrows [80,81]	–	–	–
P. californicus	Ground level under logs, rock cavities, woodrat dens [82]	NA	Yes [55]	Yes* [28]
P. crinitus	Rock cavities [77]	NA	Yes [55]	–
P. eremicus	Rock cavities, burrows [83]	–	–	Yes* [28]
P. gossypinus	Log cavities, ground level under vegetation, tortoise burrows, burrows [33,84]	Yes [84]	Yes [44,57]	–
P. leucopus	Trees, log and rock cavities, burrows [85,86–88]	–	Yes [44,55,57,89]	Yes [28]
P. maniculatus	Burrows (*P.m. bairdii*), trees (*P.m. nubiterrae*) [37–39,85,88]	Yes [37–39]	Yes [55,57,58,89]	Yes [23,28,42]
P. melanocarpus	Tree root system crevices [90]	NA	–	–
P. melanophrys	–	–	–	Yes [28]
P. melanotus	Burrows [91]	Yes [92]	–	–
P. mexicanus	Burrows, ground level under vegetation, tree root cavities [93]	–	–	–
P. pectoralis	Ground level under vegetation, rock crevices [78]	NA	–	–
P. perfulvus	Trees, ground level under vegetation [94]	NA	–	–
P. polionotus	Burrows [30–33]	Yes [23,30–33,36]	Yes [36,44,55,57,58,95]	Yes [23,28,36,42,95]
P. truei	Trees, rock and log crevices, ground level under vegetation, burrows [96,97]	–	–	–

Peromyscus polionotus

Video 1.

3. Mechanisms underlying burrowing variation

Several factors may contribute to the diversity in the size and shape of burrows observed among *Peromyscus* species, including soil composition, mouse morphology or physiology, and differences in burrowing behavior. Below, we discuss each of these factors in turn and focus primarily on the differences between *P. polionotus* and *P. maniculatus*, which are the most well characterized species.

3.1. Substrate

Although *P. polionotus* and *P. maniculatus* have non-overlapping ranges with contrasting soil composition, differences in soil composition likely explain very little of the species differences in burrow architecture. Within *P. maniculatus*, the length and volume of burrows decrease with increased silt percentage [38]. These study populations were in southeastern Idaho, where silt is on average 50% of soil. By contrast, a broad sampling of the *P. polionotus* range, in which silt is <15%, found no relationship between *P. polionotus* burrow length with silt percentage. *P. polionotus* burrows were, however, shallower with increased silt percentage [23]. Both of these intraspecies burrow effects are consistent with siltier soil being more energetically demanding to excavate. However, when both species were presented with the same soil in a laboratory setting, burrow differences between these sister species in the field were recapitulated by lab-reared animals [42]. This finding suggested that heritable differences between *P. polionotus* and *P. maniculatus*, rather than differences in the abiotic environment, are largely responsible for species differences in burrow shape.

3.2. Morphology

Many mammals exhibit morphological and physiological adaptations for digging and subsisting underground [43]. *Peromyscus* dig primarily using repetitive bouts of forelimb strokes to loosen substrate and push it towards the hindlimbs, which then kick the substrate backwards (Video 1) [44]. A comparison of seven species in the laboratory found no relationship between body mass and burrow length [28]. Strikingly, *P. polionotus* is the smallest of North American *Peromyscus* [45], yet it can propel substrate twice as far as the larger-bodied *P. gossypinus* [44]. How the musculoskeletal system of the small-bodied *P. polionotus* has adapted to become powerful at digging is still largely unknown. Within *P. polionotus*, the beach-dwelling subspecies have longer hindfeet relative to body length, which may be a local adaptation for locomoting on and digging in sand [45,46]. Compared to its sister species *P. maniculatus*, *P. polionotus* also has a shorter tail relative to its body length, which is a common feature of semi-fossorial and fossorial species [45,47]. In sum, gross differences in body size do not account for

digging variation in *Peromyscus*, but the possibility of more subtle morphological differences contributing to digging ability warrants more careful study.

3.3. Physiology

While burrows can stabilize temperature and humidity, these underground environments can bring the novel challenges of low oxygen (hypoxia) and high carbon dioxide levels (hypercapnia). Oxygen decreases, and carbon dioxide increases, with increased burrow depth [48]. *P. polionotus* is able to withstand low oxygen levels (<5%) that are lethal to the semi-arboreal *P. leucopus*, which digs similar burrows to *P. maniculatus* [35]. Both *P. polionotus* and *P. maniculatus* are active at night and undergo daily reductions in body temperature or metabolism during the light phase when held at constant ambient temperatures in the laboratory [49,50]. However, it has been predicted that *P. polionotus* must be capable of even lower metabolic rates in the wild, especially when inhabiting burrows with litters [49]. Differences in hypoxia resistance have been found in other sister species of rodents in which one species is semi-fossorial and the other surface-dwelling [51], or in which both species dig burrows that differ in air exchange [52]. In this latter example, genetic differences were found in genes such as transferrin and hemoglobin, which are involved in oxygen transport [52]. High-altitude populations of *P. maniculatus*, which endure chronic hypoxia, also possess hemoglobin polymorphisms that increase fitness in these habitats [53]. Variation in *P. polionotus* serum proteins has been described [54], but it is yet unknown whether *P. polionotus* with deeper burrows have adapted to lower oxygen levels through changes in oxygen transport proteins or other means. While data support *P. polionotus* having undergone physiological changes permissive to dwelling deeper below ground than its sister species, there is a lack of evidence suggesting that differences in physiology drive differences in burrow construction between these two species.

3.4. Digging behavior

Perhaps the most striking differences among *Peromyscus* species in relation to burrowing are in the intensity of digging behavior. King and colleagues assayed the digging of six *Peromyscus* species in the laboratory using the amount of sand removed from a sand-filled tube as a proxy for digging behavior [55]. The percentage of animals that removed >1 kg of sand in the 15 min test period varied widely by species, from 4 to 70%. When five of these species were later tested in large sand enclosures [28], performance in the acute digging assay corresponded well with the burrow lengths excavated: the two lowest percentage species (*P. californicus* and *P. eremicus*) did not burrow, the two mid-range (*P. maniculatus* and *P. leucopus*) dug short burrows, and the highest percentage species (*P. polionotus*) dug the longest burrows. It is important to note that digging is also a component of food caching, which is performed by non-burrowing species such as *P. californicus* [56], nonetheless the amount of substrate excavated in a short sampling period does appear reflective of species burrow length.

Does *P. polionotus'* digging prowess derive from increased digging efficiency, frequency, or duration? Direct observation of *P. polionotus*, *P. gossypinus*, and *P. leucopus* digging in substrate-filled enclosures found that *Peromyscus* dig in "bouts", first using a series forelimb strokes to loosen and gather substrate beneath the body, then kicking away the substrate with the hind limbs (Video 1) [36,44]. Observation suggests species-specific differences in limb movement sequence: *P. polionotus* had the fastest and most numerous forelimb strokes per bout and greater alternation between forelimb and hindlimb movements of the three species

[44]. Nonetheless, it is still unclear whether these motor patterns are more efficient at substrate removal because substrate displaced has yet to be measured in parallel with time spent digging. It has been, however, repeatedly shown that *P. polionotus* will perform more frequent digging bouts and spend more time digging than other tested *Peromyscus* species [44,57,58]. A comparison of twelve rodent species (including *P. polionotus*, *P. maniculatus*, *P. leucopus*, and *P. gossypinus*) found that although total time spent digging in an enclosure did correlate with bout duration, bout duration did not differ between any tested species [57]. Increased overall digging bout initiation, but not duration, therefore likely underlies the increased length of *P. polionotus* burrows, but does not explain the unusual shape of the *P. polionotus* burrow, especially its escape tunnel. The motor behavior that produces this tunnel has not been well characterized because it does not commence until the animal is hidden below ground. This challenge can be overcome, however, by using a thin sand enclosure that constricts the animal to digging against a transparent surface (see Video 1 in [24]). Collectively, observations of *Peromyscus* burrowing behavior suggest that a major target of natural selection in *Peromyscus* has been the neural circuitry regulating digging motor behaviors and the initiation of digging behavior.

4. Neurobiological basis of burrowing behavior

The neural basis of *Peromyscus* burrowing is currently under investigation. To date, the few insights into the neural basis of burrowing in mammals come from only a handful studies in the inbred laboratory mouse, *Mus musculus*. Inbred *Mus* strains, like their wild counterparts, dig multi-tunnel burrows that are less stereotypical in size and shape than *Peromyscus* burrows [59–61]. Deacon and colleagues designed an abbreviated burrowing assay that uses substrate displaced from an artificial burrow as a proxy for burrowing [62]. Prion injection or cytotoxic lesion of the hippocampus greatly reduced performance in this assay [63,64], and lesion of the medial prefrontal cortex produced a milder deficit [65]. Both of these regions are components of a circuit for representation of goal-directed trajectories in familiar environments [66]. Whether burrow excavation is similarly a goal-directed trajectory, but into the novel space within substrate, is unknown. In addition, one study finds that burrowing may also be regulated by the serotonergic system. Serotonin transporter overexpression or knockout *M. musculus* displace more substrate more or less substrate, respectively, than controls [67]. These findings suggest serotonin availability may negatively regulate burrowing in *Mus* and is a candidate for variation in *Peromyscus* burrowing behavior. Serotonin has been strongly implicated in the initiation of aggressive and impulsive behaviors [68,69]. Thus, studying burrowing behavior in *Peromyscus* may shed light on how natural selection can act on a major neurotransmitter system to affect a natural mammalian behavior.

5. Genetic architecture of burrow evolution

Several features of *Peromyscus* burrowing behavior make it a well-suited system for tackling how genetic change leads to complex behavioral change. Many behaviors are difficult to quantify because of experience and environmental-dependent variability as well as the transience of the behavior. As mentioned previously, a burrow is a behavioral product ("extended phenotype") that can be measured like a morphological trait, for which forward-genetic approaches combined with high-throughput genotyping in *Peromyscus* have been successful in identifying causal alleles [70,71]. Second, *Peromyscus* burrowing behavior is largely innate—lab-reared animals will excavate burrows similar to their

wild counterparts [28,42]. Finally, burrowing behavior has diverged significantly between two interfertile sister species, *P. maniculatus* and *P. polionotus*. Thus, this represents an exciting system in which to connect behavioral variation to its underlying genetic causes through a classical forward-genetic cross.

Wallace Dawson, the founder of the *Peromyscus* Stock Center, first explored the inheritance of burrow shape using a small interspecific cross between *P. maniculatus* and *P. polionotus* [42]. Dawson focused on lactating females, which field observations and preliminary laboratory testing suggested may be the most proficient burrowers. By permitting animals to dig freely in large sand enclosures, he observed that *P. maniculatus* x *P. polionotus* F1 hybrids dug *P. polionotus*-like burrows, suggesting dominance of *P. polionotus* allele(s). When F1 hybrids were bred with *P. maniculatus*, the twelve backcross offspring dug burrows whose lengths were distributed across the range between the pure species (i.e., from short to long), but importantly, several mice still built *polionotus*-like burrows. Moreover, roughly half of animals produced escape tunnels. These results, although based on an extremely small sample size, hinted that the genetic basis of burrow shape may, in fact, be tractable [42].

To both follow up this earlier result and localize the causal regions in the genome, hybrid animals from a much larger cross were assayed for burrowing behavior and then genotyped. The experiment was expedited by testing sexually inexperienced animals of both sexes, which still exhibit species differences in burrow shape [28]. Furthermore, rather than excavating a burrow to measure it, which effectively destroys the burrow, burrows were cast with polyurethane foam, enabling more sophisticated and repeatable measurements. Specifically, Weber and colleagues measured the burrow phenotypes of 272 back cross animals, and the same animals were then genotyped using a high-throughput genotype-by-sequencing approach (Fig. 2) [23]. This larger set of back cross animals first corroborated the inheritance pattern observed by Dawson. Approximately one eighth of back cross animals dug *P. polionotus*-like long entrance tunnels and one half dug escape tunnels, indicating just a few loci may be responsible for the derived *P. polionotus* burrow. There was only weak correlation between tunnel size and tunnel shape, suggesting that these two burrow traits were under separate genetic control.

As a next step, these same simple phenotypic measures – burrow entrance length and the presence/absence of an escape tunnel – were used as the focal traits for quantitative trait locus (QTL) mapping to localize the genetic regions responsible for differences in burrowing behavior. Three genomic regions were identified that each contribute to burrow entrance tunnel length and a single region for the presence (or absence) of an escape tunnel. Together, the entrance tunnel length QTL accounted for 15% of the variance of this trait, which is over half of the estimated heritability, and the single escape tunnel QTL explained 6% of variance in the frequency of escape tunnel [28]. As suggested by the inheritance pattern, the regions associated with burrow length and presence of escape tunnel did not overlap in the genome. Importantly, this finding confirmed that different genes are involved and that the underlying genetic architecture is modular. In all cases, the *P. polionotus* alleles increased burrow size or complexity, suggesting the evolutionary history of the *P. polionotus* burrow may have been one of rapid fixation of a few ecologically favored, dominant alleles. The localization of multiple behavioral loci, as opposed to a single locus, is consistent with other behavioral genetic studies using inbred laboratory populations [72], as well as those investigating behaviors in outbred populations [73,74]. The identification of genomic regions associated with burrow size and shape is an exciting result because it represents the first step in the identification of the causal genes responsible for variation in a naturally evolved, complex behavior.
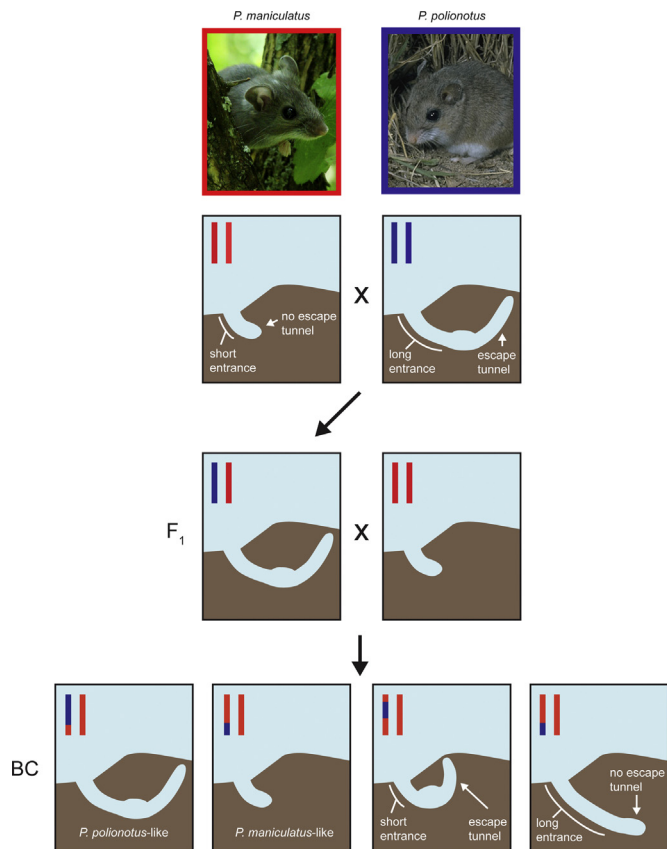
Fig. 2. Cross design for identifying genetic architecture of burrowing differences between *P. maniculatus* (red) and *P. polionotus* (blue). The two species are crossed to generate F1 hybrids, which have a chromosome from each of the parents (red and blue). F1 hybrids, which produce *P. polionotus*-like burrows, are then backcrossed to *P. maniculatus*. The resultant backcross generation (BC) shows a range of burrow architectures: burrows that resemble the parents (short entrance tunnel, without an escape tunnel and long entrance tunnel, with an escape tunnel) as well as new architectures (short entrance tunnel, with an escape tunnel and long entrance tunnel, without an escape). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Photograph credit: *P. maniculatus* and *P. polionotus* by United States Fish and Wildlife Service.

6. Conclusions

The extended phenotype of *Peromyscus* – the burrow – has undergone recent innovations during the diversification of this genus [28]. Understanding the mechanistic basis of complex behavior is currently a major frontier of both genetic and neuroscience research, and this easily measured behavioral product has proven to be a productive study system for linking genes to behavior [23]. Because *Peromyscus* burrowing is a naturally evolved behavior, there are ample future opportunities to explore and connect both the ultimate and proximate mechanisms of behavioral evolution.

First, much is still unknown about the fitness benefits of burrow traits and the selection pressures on *Peromyscus* burrowing behavior. While early naturalists were able to document *P. polionotus* burrows and their occupants, they were impeded from observing burrow use in, for example, predator evasion or social interaction by the nocturnal and subterranean activity pattern of *Peromyscus*. We can overcome this challenge largely with the use of modern camera and tracking technologies in the field, which, when combined with mark-recapture data, can begin to address how burrows (and variation in burrow architecture) may contribute to survival and reproductive success in *P. polionotus*. Moreover, further characterization of other *Peromyscus* species may provide insight into the ecological drivers (e.g. habitat correlates such as vegetative cover,

temperature, predators) promoting burrowing and differences in burrow architecture. Thus far, evidence of burrowing or the lack of burrow use has been documented for less than half of *Peromyscus* species (Table 1). Given the diverse habitats *Peromyscus* occupy, there is potential to identify examples of convergent burrowing evolution or novel burrow architectures. The molecular substrate of this convergent or novel burrowing evolution can once again be explored with forward-genetics or through comparative genomics.

Second, the association of genomic regions with burrowing behavior provides a remarkable first step towards connecting genetic changes to changes in the neural circuits underlying behavior. Examples of causal alleles underlying behavior variation in natural populations are rare, and those that determine the mechanism of gene action are rarer still [75,76]. Thus, to mechanistically bridge genes, brain, and burrowing behavior in *Peromyscus*, immediate challenges include furthering our understanding of the neural basis of burrowing (e.g. identifying which brain regions are involved in burrowing and later, the underlying circuit organization), and the identification of candidate genes within burrow-associated genomic regions. Together, these findings can then be combined to design and perform functional tests of the effects that allelic differences have on circuit function, burrowing behavior, and, finally, burrow architecture. Together, this work will illuminate both the ultimate and proximate mechanisms driving the evolution of this complex animal behavior.

Acknowledgements

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