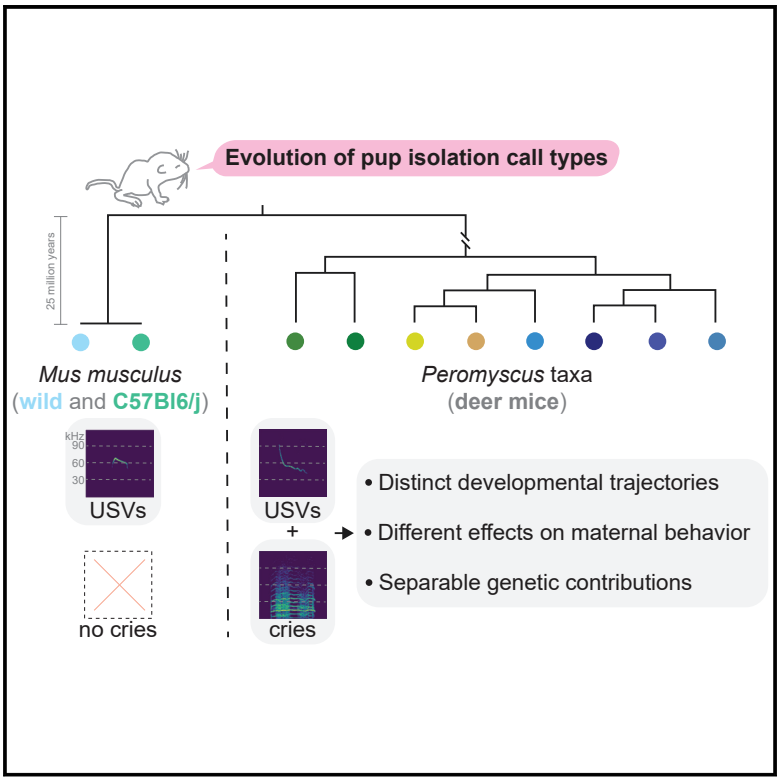
Article

Current Biology

Two pup vocalization types are genetically and functionally separable in deer mice

Graphical abstract



Authors

Nicholas Jourjine, Maya L. Woolfolk, Juan I. Sanguinetti-Scheck, John E. Sabatini, Sade McFadden, Anna K. Lindholm, Hopi E. Hoekstra

Correspondence

hoekstra@oeb.harvard.edu

In brief

Using unsupervised clustering algorithms, Jourjine et al. identify two isolation calls in neonatal deer mice (genus *Peromyscus*): ultrasonic vocalizations (USVs) and lower frequency cries. They characterize the evolution of these pup calls in eight closely related taxa and show that USVs and cries are both functionally and genetically distinct.

Highlights

- Deer mouse pups produce two types of isolation-induced calls: USVs and cries
- Wild and C57BL6/J *Mus musculus* pups produce isolation-induced USVs but not cries
- Deer mouse cries elicit significantly faster maternal approach than USVs
- Distinct genetic loci contribute to interspecific variation in USVs versus cries

Jourjine et al., 2023, Current Biology *33*, 1–12
April 10, 2023
https://doi.org/10.1016/j.cub.2023.02.045

CellPress

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

Current Biology

CellPress

Article

Two pup vocalization types are genetically and functionally separable in deer mice

Nicholas Jourjine,[1] Maya L. Woolfolk,[1] Juan I. Sanguinetti-Scheck,[1] John E. Sabatini,[1] Sade McFadden,[1] Anna K. Lindholm,[2] and Hopi E. Hoekstra[1,3,*]

[1]Department of Molecular & Cellular Biology, Department of Organismic & Evolutionary Biology, Center for Brain Science, Museum of Comparative Zoology, Harvard University and the Howard Hughes Medical Institute, 16 Divinity Avenue, Cambridge, MA 02138, USA

[2]Department of Evolutionary Biology & Environmental Studies, University of Zürich, Winterthurerstrasse, 190 8057 Zürich, Switzerland

[3]Lead contact

*Correspondence: hoekstra@oeb.harvard.edu

https://doi.org/10.1016/j.cub.2023.02.045

SUMMARY

Vocalization is a widespread social behavior in vertebrates that can affect fitness in the wild. Although many vocal behaviors are highly conserved, heritable features of specific vocalization types can vary both within and between species, raising the questions of why and how some vocal behaviors evolve. Here, using new computational tools to automatically detect and cluster vocalizations into distinct acoustic categories, we compare pup isolation calls across neonatal development in eight taxa of deer mice (genus *Peromyscus*) and compare them with laboratory mice (C57BL6/J strain) and free-living, wild house mice (*Mus musculus domesticus*). Whereas both *Peromyscus* and *Mus* pups produce ultrasonic vocalizations (USVs), *Peromyscus* pups also produce a second call type with acoustic features, temporal rhythms, and developmental trajectories that are distinct from those of USVs. In deer mice, these lower frequency "cries" are predominantly emitted in postnatal days one through nine, whereas USVs are primarily made after day 9. Using playback assays, we show that cries result in a more rapid approach by *Peromyscus* mothers than USVs, suggesting a role for cries in eliciting parental care early in neonatal development. Using a genetic cross between two sister species of deer mice exhibiting large, innate differences in the acoustic structure of cries and USVs, we find that variation in vocalization rate, duration, and pitch displays different degrees of genetic dominance and that cry and USV features can be uncoupled in second-generation hybrids. Taken together, this work shows that vocal behavior can evolve quickly between closely related rodent species in which vocalization types, likely serving distinct functions in communication, are controlled by distinct genetic loci.

INTRODUCTION

Vocal communication is fundamental to the social lives of vertebrates. Consistent with this critical function, vocalization is an ancient behavior, likely arising independently in multiple vertebrate lineages between 100 and 400 million years ago.[1,2] Since then, species have evolved differences in the acoustic features of their vocalizations and the social contexts in which those vocalizations have meaning for listeners. In vertebrates, studies in a few exceptionally vocal groups (e.g., birds and frogs) have shed light on the ecological and social factors contributing to the evolution of this variation.[3–5] However, less is known about the proximate mechanisms underlying the evolution of vocal behavior, particularly among closely related species.

Recently developed computational tools have allowed for rapid, unsupervised detection of vocalizations and characterization of vocal repertoires in diverse species.[6–10] These tools make it possible to measure vocal repertoires with few assumptions about what vocalizations should look like or how they should be categorized. As a result, it is now feasible to study groups of animals that, although well suited to answer questions about the evolution of vocal behavior, have received less attention in comparative studies.

One such group is rodents. Many rodent species are highly vocal, and they use vocalization in many of the same social contexts as other mammals.[11–15] Studies in laboratory mice (e.g., *Mus musculus* strain C57BL6/J) have focused on ultrasonic vocalizations (USVs) made by males during courtship with females[16–18] and by neonates ("pups") when isolated from their parents.[19] Pup isolation calls are of particular interest because they elicit search and retrieval behaviors from parents when pups become separated from the nest[20–23] and undergo a stereotyped postnatal development in their rate of production and in their acoustic features.[24] Recent studies have also begun to reveal genetic factors required for pup isolation calls in *Mus*.[25–28] However, relative to laboratory strains of *Mus*, we know less about the pup vocal behaviors of wild *Mus* (although adult vocalizations have been recorded in wild-derived *Mus*[29]) or other rodent species.

Deer mice (genus *Peromyscus*) diverged from *Mus* approximately 25–40 million years ago and have since undergone a radiation across North America, resulting in several closely related, but behaviorally diverse, species.[30] Differences have evolved

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

between species in the vocal repertoires of both adult and neonatal[31] *Peromyscus* mice. And while it has been hypothesized that at least some of these differences have resulted from adaptation to specific environmental or social factors,[31–36] the ultimate and proximate drivers of vocal behavior evolution in this genus remain poorly understood.

Here, we compare vocal behavior in eight *Peromyscus* taxa, the C57BL6/J strain of *Mus musculus*, and free-living, wild *Mus musculus*, focusing on the postnatal development of isolation calls, because they are relatively easy to elicit and record, can have a direct role in pup survival,[27] and because interspecific differences in isolation calls are likely to be heritable.[25,37] Using automated detection and unsupervised clustering of vocalizations made during pup isolation assays, we find that, although USVs are conserved across all these taxa, *Peromyscus* also produce lower frequency calls ("cries"). We then explore mechanisms driving variation both between call types and among species. We find that heritable vocal features have diverged quickly among *Peromyscus* taxa and that the distinct call types produced by *Peromyscus* pups likely serve different social functions and evolved via different genetic loci.

RESULTS

Unsupervised clustering identifies two types of pup isolation calls in *Peromyscus*

To characterize pup calls across species, we recorded isolation-induced vocalizations from 596 *Peromyscus* pups belonging to four species (eight subspecies) at seven postnatal ages spanning their first 2 weeks of life. We compared these recordings with isolation calls from pups of the same ages from laboratory *Mus musculus* (C57BL6/J; 116 pups) and free-living, wild *Mus musculus* (111 pups) (Figure 1A; Table S1). Using thresholding of spectrogram intensity values to automatically segment these recordings into vocalizations (Figure 1B), we first embedded spectrogram images of all vocalizations made by each taxa in two dimensions using uniform manifold approximation and projection (UMAP[10,38]; Figure 1C, top row). We found that all detected isolation calls from wild *Mus* resembled the high-frequency USVs that have been previously characterized in C57BL6/J: vocalizations from both wild and C57BL6/J *Mus* fell into a single cluster in UMAP space, consistent with recent descriptions of both adult[39] and pup[38] C57BL6/J vocalizations. By contrast, *Peromyscus* vocalizations separated into two distinct clusters, one of which contained short, high-frequency ultrasonic frequency sweeps, whereas the other contained longer, lower frequency vocalizations (Figure 1C, middle and bottom rows; Figure S1).

Although UMAP embeddings of spectrogram images recovered meaningful acoustic variation in vocalizations, applying non-linear dimensionality reduction algorithms to spectrograms can be difficult to interpret biologically compared with more conventional bioacoustics approaches.[6,38] We therefore calculated 26 acoustic features for each vocalization[40] and performed principal component analysis (PCA) to measure the extent to which these features explain variation between vocalizations in the full dataset of both *Peromyscus* and *Mus*. The first two principal components (PCs) explained 51% and 11% of the variation among vocalizations, respectively, with PC1 qualitatively separating the dataset into two clusters, one of which was occupied by all taxa, whereas the other was occupied exclusively by vocalizations from *Peromyscus* pups (Figure 1D). The top-loading acoustic features on PC1 were duration (ms) and average frequency (kHz) of vocalizations. Plotting each of these features by taxon revealed qualitatively bi-modal distributions for all *Peromyscus*, but unimodal distributions for wild and C57BL6/J *Mus* (Figure 1E), patterns consistent with the clustering observed in UMAP embedding of spectrogram images from these species.

We next performed hierarchical clustering of the same acoustic features calculated for each of the 50,000 vocalizations sampled randomly across all recorded species, labeling the leaves of the resulting dendrogram by species and by the UMAP cluster to which each vocalizations' spectrogram was embedded (from Figure 1B). Hierarchical clustering also splits the vocalizations into two groups that corresponded to spectrogram-image-based clustering: one containing short, high-frequency vocalizations and one containing longer, lower frequency vocalizations (Figure 1F). All *Peromyscus* taxa produced vocalizations in both categories, with vocalizations from both wild and lab *Mus musculus* clustering near each other and among the short, high-frequency *Peromyscus* vocalizations.

Taking these data together, automated segmentation and unsupervised clustering of 287,461 vocalizations from 10 rodent taxa suggest that isolation calls with a mean frequency of 66 kHz (min dominant freq: 50.4 ± 20 kHz; max dominant freq: 83.1 ± 18 kHz) are conserved between *Peromyscus* and *Mus*. We refer to this vocalization type as USVs as they have been referred to in previous studies of C57BL6/J mice. Finally, *Peromyscus*, but not *Mus*, produces a second type of isolation call consisting of lower frequency (min dominant freq: 16.6 ± 6.8 kHz; max dominant freq: 42.6 ± 20.8 kHz) and louder vocalizations (Figure S1). We refer to vocalizations in this category as cries, because they acoustically resemble the cry vocalizations produced by neonates in other mammalian species[41] (Video S1).

Interspecific variation in the cries and USVs of deer mice

We next sought to quantify interspecific variation in the acoustic features of *Peromyscus* cries and USVs. To perform analyses separately on these two vocalization types, we first annotated examples of each type for each species (Table S2). We then trained random forest classifiers to predict species identity from 14 predefined acoustic features for which we hypothesized that interspecific variation may be relevant for pup fitness (Figure 2A). Both cry and USV classifiers predicted species above chance (12.5%), indicating that features of both vocalization types carry species-specific information. To test the effect of sample size on performance, we trained a set of random forest classifiers on varying numbers of vocalizations per type, ranging from 20 to 1,400. Cry and USV classifiers performed above chance with as few as 20 training examples, although performance increased with sample size and differed between species. In addition, cry vocalizations were better predictors of species identity than USVs (Figure 2B). Together, these analyses indicate that acoustic features of both vocalization types contain information about species identity and suggest that the acoustic features of cries have diverged more among species than those of USVs.

To further quantify acoustic differences between cries and USVs across species, we used 3-component PCA models to

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

CellPress

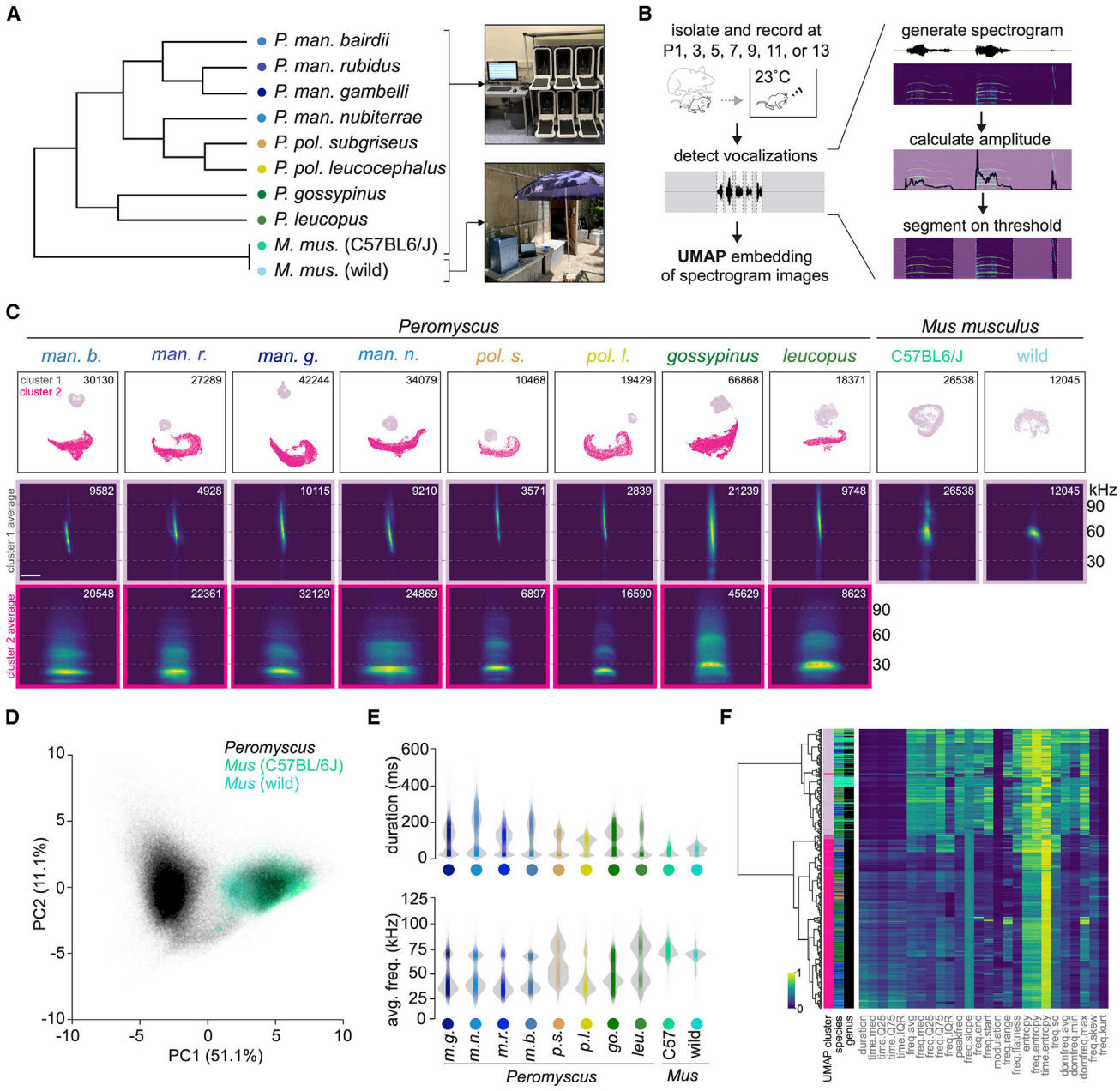


Figure 1. *Peromyscus* but not *Mus* produce cries when isolated

(A) Left: phylogenetic relationships of taxa recorded either under laboratory conditions (top, *Peromyscus* and *Mus* C57BL6/J) or at a field station in Zürich, Switzerland (bottom, wild *Mus musculus*).

(B) Recording paradigm. Left: pups were isolated and recorded at 1, 3, 5, 7, 9, 11, or 13 days after birth (day 0). Vocalizations were detected from each recording automatically using thresholding on spectrogram images.

(C) Top: UMAP embeddings for vocalizations of each taxon, colored by HDBSCAN clustering of UMAP coordinates. Total detected vocalizations for each taxon are given (upper right-hand corner of each image). Middle and bottom: average spectrogram image of all vocalizations belonging to each HDBSCAN cluster for each taxon. Number of detected vocalizations in each cluster is given (upper right-hand corner). Scale bar, 100 ms.

(D) Principal component analysis (PCA) on 26 acoustic features for each detected vocalization.

(E) Top-loading features from the PCA in (D) (duration and average frequency, respectively) for each taxon.

(F) Hierarchical clustering on acoustic features of 50,000 vocalizations sampled randomly from all vocalizations. Feature names: time/freq.med = median of the energy distribution in the time/frequency domain; time/freq.Q25 = first quartile of the energy distribution in the time/frequency domain; time/freq.Q75 = third quartile of the energy distribution in the time domain; time/freq.IQR = interquartile interval of the energy distribution in the time/frequency domain.

See also Figure S1, Table S1, and Video S1.

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

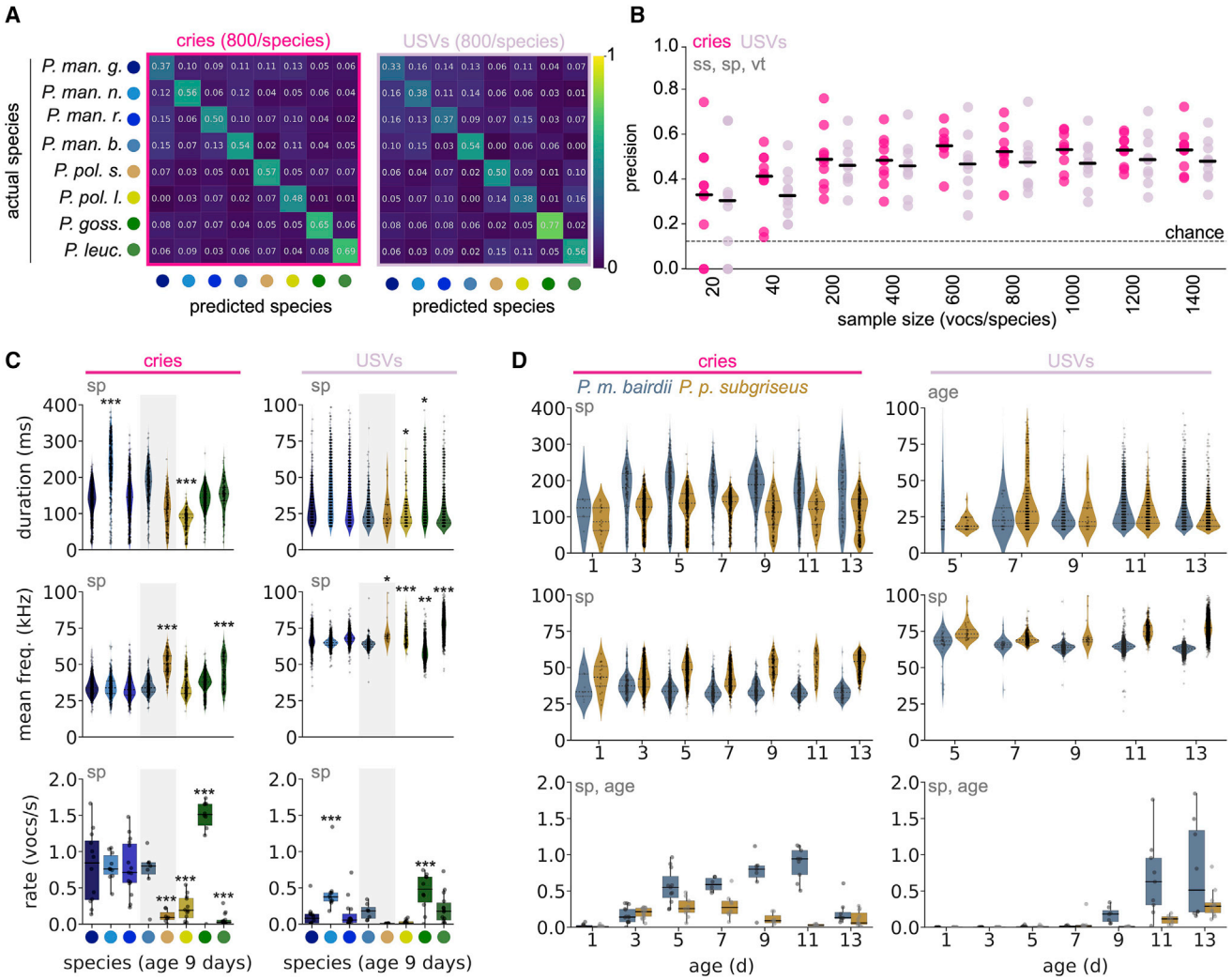


Figure 2. Natural variation in rate and acoustic features of *Peromyscus* pup isolation calls

(A) Confusion matrices showing precision of random forest classifiers (500 trees each, 80%/20% train test split) trained to predict taxon labels from 800 vocalizations per type (cry or USV) per taxon. Left: cries. Right: USVs.

(B) Relationship between sample size and model precision of random forest classifiers trained on between 20 and 1,400 vocalizations per type per taxon. Each dot represents one taxon, dashed line indicates chance classification. Black bars indicate medians. ss, effect of sample size ($p < 0.001$); sp, effect of species ($p < 0.001$); vt, effect of vocalization type (cry or USV; $p < 0.05$) by linear model of precision with sample size, species, and vocalization type as explanatory variables (see STAR Methods for details).

(C) Duration (ms), mean frequency (kHz), and rate (vocalizations/second) of cries and USVs of P9 pups from each taxon. Dashed lines in violin plots represent quartiles; each dot is a vocalization. Box plots show quartiles with whiskers extending to 1.5 times the interquartile range; each dot a pup. sp, effect of species; s, effect of sex by linear mixed effects model with species and sex as main effects and pup identity and pup heat loss as random effects (see STAR Methods for details). Stars above taxa indicate difference from the reference taxon (*P. m. gambelli*, farthest left in each plot, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(D) Duration (ms), mean frequency (kHz), and rate (vocalizations/s) of cries and USVs of *P. m. bairdii* and *P. p. subgriseus* at all recorded ages. sp, effect of species; age, effect of age; s, effect of sex by linear mixed effects model with species, sex, and age as main effects and pup identity and pup heat loss as random effects (see STAR Methods for details).

See also Figures S2–S5 and Table S2.

reduce the dimensionality of 14 acoustic features for 500 annotated examples of each vocalization type per species (Figure S2). Mean frequency and duration were among the top-loading features of both PC1 and PC2 for both cries and USVs, suggesting that they are major features that distinguish vocalizations within each of these types. Because these are also features that may be important for eliciting parental care,[41] we asked how species differed in the frequency and duration of their cries and USVs. In addition, we considered the rate and the temporal rhythms with which each species produced each vocalization type, because these have also been suggested to be functionally meaningful aspects of isolation calls.[22,41,42]

To make these comparisons, we first automatically labeled all detected vocalizations for each species as cry or USV using a

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

random forest classifier, which allowed us to analyze cry and USV counts separately (Figure S3). We then asked how the vocalizations of *Peromyscus* taxa differed from one another across early postnatal development (Figure 2C, postnatal day 9 [P9] shown; see Figure S4 for all ages). We find a significant effect of species but not sex on the duration (Figure 2C, top), mean frequency (Figure 2C, middle), and rate (Figure 2C, bottom) of both cries and USVs. To compare temporal rhythms of each vocalization type, we considered the distributions of their inter-onset intervals, that is, the amount of time from the start of each vocalization to the start of the next[43,44] (Figure S5A). We found that the cries of all *Peromyscus* taxa had a similar bout-like rhythm and bi-modal distributions of inter-onset intervals (Figures S5B and S5C, left), whereas the rhythms of USVs from all species were less clearly structured in time (Figures S5B and S5C, right).

Two of the species we examined are of particular interest because they have previously been studied for their extreme differences in social system and parental care behavior: *P. maniculatus bairdii* and *P. polionotus subgriseus*. *P. m. bairdii* is highly promiscuous with uniparental (maternal) care of pups, whereas *P. p. subgriseus* is both genetically and socially monogamous, and pups receive biparental care.[45–47] Comparing these two species across development (P1–P13), we find significant effects of species but not sex on the duration (Figure 2D, top left), mean frequency (Figure 2D, middle left), and rate (Figure 2D, bottom left) of cries and on the mean frequency (Figure 2D, middle right) and rate (Figure 2D, bottom right) of USVs. Thus, these analyses identify examples of interspecific variation in pup isolation calls between two sister species of *Peromyscus*, diverged less than ~1 million years ago, demonstrating that these calls can evolve over short evolutionary timescales.

Cries and USVs differ in their ability to elicit maternal approach

As the largest differences we observe occur between call types within species (rather than species differences within call type), we next explored whether cries and USVs may serve different functions. We find that pups from different *Peromyscus* species produce cry and USV calls at different rates across neonatal development, with cries generally being the predominant vocalization type in pups younger than 9 days old and USVs predominating in older pups (Figure 2D, bottom; Figure S4). Because the amount of care pups require likely differs between these age categories, we hypothesized that cries and USVs may signal different levels of need and therefore would elicit responses from parents with different latency and/or speed. To test this hypothesis, we performed playback experiments in which a *P. m. bairdii* mother with a 9-day-old litter was presented with species-typical bouts of either cries or USVs, using recordings from 9-day-old *P. m. bairdii* pups modified to be matched in amplitude and temporal rhythm (Figures 3A and 3B). By automatically tracking the mothers' location, we extracted spatial and velocity data and aligned these to time points when cry or USV recordings began. We found both vocalization types could cause mothers to leave their pups (Figure 3C). However, mothers were more likely to leave their pups and approach the speaker after cries (Figures 3D and 3E), arrived at the speaker significantly sooner after the start of vocalization playback (Figure 3F, left, paired t test, $p < 0.01$, mean cry = 14.2 s, mean USV = 87.5), and reached a higher maximum velocity while moving toward the speaker (Figure 3F, right paired t test, $p < 0.05$, mean cry = 15.9 cm/s, mean USV = 7.4 cm/s). Thus, the sound of pup cries elicits more rapid behavioral responses from dams than the sound of USVs, consistent with the hypothesis that cries are vocal signals of urgent need.

Separable genetic contributions to interspecific variation in cries and USVs

In some rodents, pup isolation calls are heritable.[48] To identify features of deer mouse cries and USVs that have a heritable genetic contribution, we performed cross-fostering experiments between the two interfertile sister species (*P. m. bairdii* and *P. p. subgriseus*) in which we had identified differences in call rate, duration, and mean frequency (see Figure 2D). When litters from both species were born on the same day, we exchanged the entire litter between parents, then recorded the pups 9 days later and compared these recordings with those from litters that were not exchanged (Figure 4A). Specifically, for each pup, we compared the median value of call rate, duration, and mean frequency for all cries and USVs they produced. We found no effect of cross fostering on the rate, mean frequency, or duration of cries (Figure 4B, one-way ANOVA with Tukey post hoc test). We also performed PCA on 26 acoustic features calculated for the cries of each pup and found that cross-fostered pups largely fell into the same region of PCA space as predicted for their species, not the foster species (Figure 4C). We observed similar patterns for features of USVs with two exceptions (Figure 4D). First, we found no significant difference in USV duration between species, consistent with our previous observations (Figure 2D, top right). Second, the mean frequency of USVs in cross-fostered *P. p. subgriseus* were intermediate between that of control pups from each species (Figure 4D, one-way ANOVA with Tukey post hoc test), suggesting that some acoustic features of USVs may be sensitive to parental environment. However, interspecific differences in USV rate were not affected by cross fostering, and PCA of USV acoustic features calculated for each pup revealed that cross-fostered pups clustered largely as predicted for their species, although the separation between species was smaller for USVs than for cries (Figure 4E).

Encouraged by the large and plausibly heritable differences in vocal behavior between *P. m. bairdii* and *P. p. subgriseus*, we conducted an interspecific cross to identify genetic components that modulate vocal behavior in cries and USVs, separately. Specifically, we generated a population of F1 hybrids and then intercrossed these F1 hybrids to generate a large population of second-generation (F2) hybrids (n = 617 pups; Figure 5A). We found that the rate, mean frequency, and duration of cries made by F1 hybrids all resembled those of *P. p. subgriseus* (Figure 5B, one-way ANOVA with Tukey post hoc test). PCA on 26 acoustic features calculated for each pups' cries also revealed that F1 pups occupied the same region of PCA space as *P. p. subgriseus* but not *P. m. bairdii* (Figure 5C). We observed a different pattern of inheritance for USVs. Specifically, the mean frequency of F1 USVs more closely resembled that of *P. m. bairdii* than *P. p. subgriseus*, and the rate and duration of these USVs were each intermediate between the two species (Figure 5D, one-way ANOVA with Tukey post hoc test). PCA revealed that F1 pups occupied a region of PCA space intermediate between

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

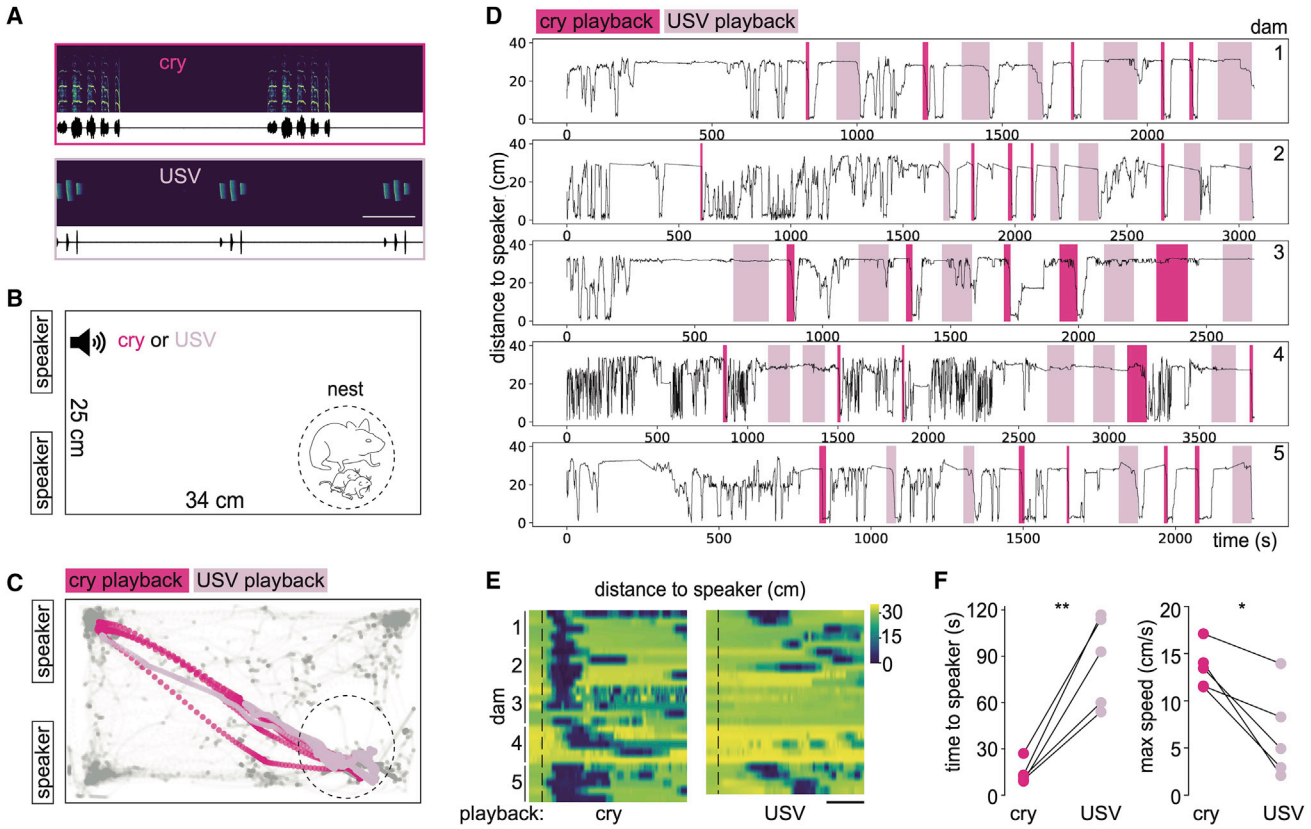


Figure 3. Cries elicit rapid behavioral responses from *Peromyscus* mothers

(A) Spectrograms (top) and wave forms (bottom) of exemplar cries and USVs used during playback experiment; scale bar, 1 sec.

(B) Experimental setup: *P. m. bairdii* mother with P9 pups was presented either the cry or USV recordings in (A), played repeatedly in a loop from one of two speakers until either the mother reached the speaker or 2 min elapsed, whichever came first (see STAR Methods for details). Dashed circle, nest location.

(C) Example of positional tracking (dam 5) during playback experiment. Dark pink dots, mother position in response to cries. Light pink dots, mother position in response to USVs. Gray dots, mother position when no sound was being played back. Dashed circle, nest location.

(D) Distance of tested mothers (dam 1–5) to the active speaker. Shaded areas indicate the time periods during which cry (dark pink) or USV (light pink) stimuli were each played until the mother reached the speaker or 2 min elapsed.

(E) Distance to the playback speaker for each trial (rows, 4–5 for each mother) and mother aligned to the onset of cry (left panel) or USV (right panel). Dashed vertical line indicates start of first playback vocalization; scale bar, 30 s.

(F) Left: median time to the speaker for each mother (n = 5) following cry (dark pink) or USV (light pink) stimulus (paired t test, $^{**}p < 0.01$; mean cry = 14.2 s, mean USV = 87.5 s). Right: median of maximum speed reached for each mother following cry (dark pink) or USV (light pink) stimulus (paired t test, $^{*}p < 0.05$; mean cry = 15.9 cm/s, mean USV = 7.4 cm/s).

P. m. bairdii and *P. p. subgriseus* (Figure 5E). Thus, features of cries and USVs exhibit distinct modes of inheritance in F1 hybrids, raising the possibility that cries and USVs have distinct genetic contributions.

To determine if variation of cries and USVs are genetically separable, we examined 512 F2 hybrids, each with a unique combination of alleles from the two parental species. If the same loci contribute to variation in both cries and USVs, we expect variation in these call types to be correlated in this F2 population, but if different loci contribute to variation in each call type, we do not expect to find a correlation between cries and USVs (as recombination will have unlinked these loci from one another). First, we found a weak correlation in the rate at which pups produce cries and USVs in F2 hybrids (Figure 5F, Spearman's rho = 0.36, $p < 0.001$), suggesting that the genetic loci influencing variation in the rate at which pups produce cries and USVs are partially shared. In contrast, we found no correlation between cries and USVs in their mean frequency (Figure 5G, Spearman's rho = 0.08, $p > 0.1$) or duration (Figure 5H, Spearman's rho = 0.07, $p > 0.1$), arguing that the loci contributing to interspecific variation in these acoustic features are distinct for cries and USVs. Taken together, these data show that, although interspecific variation in the rate of cries and USVs may share some genetic contribution, variation in most features, in particular duration and frequency, appear to be independently controlled by distinct genetic loci.

DISCUSSION

Using automated detection and clustering of vocal syllables, we examine the evolution of pup isolation calls in *Peromyscus*. Unlike *Mus* pups that make only USVs in this social context, we find *Peromyscus* pups produce two acoustically distinct isolation call types: cries and USVs. These calls have distinct

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

CellPress

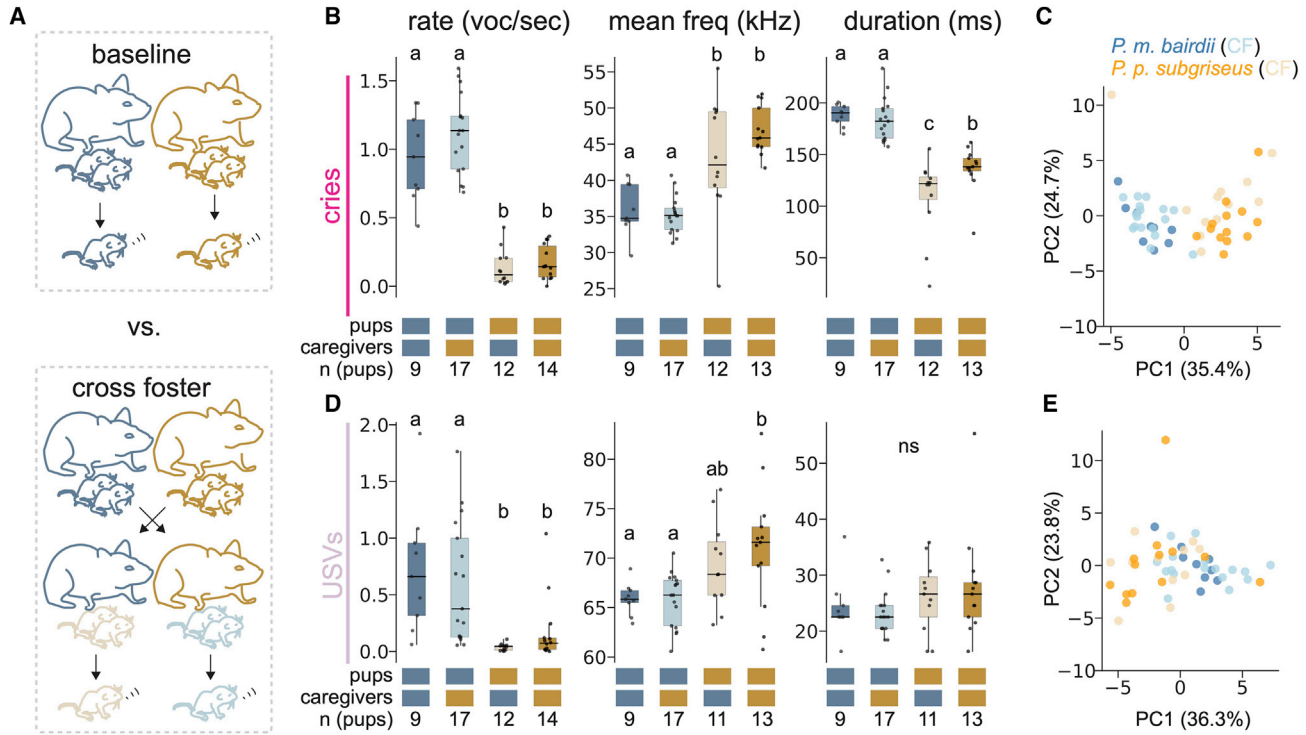


Figure 4. Effect of cross fostering on vocalization rate and acoustic features of cries and USVs

(A) Schematic of cross-fostering experimental design: pups from *P. m. bairdii* (blue) and *P. p. subgriseus* (gold) litters born on the same day were switched within 24 h of birth and recorded in isolation at P9.

(B) Effect of cross fostering on the rate (first column, $p < 0.001$), mean frequency (second column, median values for each pup, $p < 0.001$), and duration (third column, median values for each pup, $p < 0.001$) of cries. One-way ANOVA with Tukey post hoc test, letters indicate significantly different groups, baseline (dark colors) and cross-fostered (light) pups. Pups, species of the pups. Caregivers, species of the adults caring for the pups from postnatal day 1 until recording. Box plots show quartiles with whiskers extending to 1.5 times the interquartile range; each dot corresponds to one pup.

(C) Two-component PCA on acoustic features of cries aggregated by pup (median values for each pup), baseline (dark colors), and cross-fostered (light) pups.

(D) Effect of cross fostering on the rate (first column, $p < 0.001$), mean frequency (second column, median values for each pup, $p < 0.01$), and duration (third column, median values for each pup, $p > 0.05$) of USVs. One-way ANOVA with Tukey post hoc test, letters indicate significantly different groups, baseline (dark colors) and cross-fostered (light) pups. Box plots show quartiles with whiskers extending to 1.5 times the interquartile range; each dot corresponds to one pup.

(E) Two-component PCA on acoustic features of USVs aggregated by pup (median values for each pup), baseline (dark colors), and cross-fostered (light) pups.

developmental trajectories, as well as different effects on maternal behavior, with cries being produced by younger pups and triggering more rapid maternal approach behavior than USVs. By comparing pup isolation calls between two closely related species and their hybrids, we find variation in acoustic features of both cries and USVs that appear heritable, exhibit different patterns of dominance in F1 hybrids, and become largely uncoupled in F2 hybrids, suggesting that variation in vocal features can evolve rapidly via changes in distinct genetic loci.

Although we identify two call types in the vocal repertoires of *Peromyscus* pups, previous studies in which vocalization types were labeled by hand have reported a larger number[32] (e.g., "bark," "sustained vocalization," "simple sweep," and "complex sweep"), raising the question of how best to partition vocal repertoires into "types." Recent unsupervised clustering of adult courtship vocalizations in C57BL6/J *Mus*[38,39] also identified a smaller number of acoustic categories (i.e., one) than previous studies that relied on hand labeling by experts, a pattern we corroborate here for C57BL6/J pup isolation calls. Thus, some differences between vocalizations that appear discrete to human observers are, in fact, continuous in acoustic space. In *Peromyscus*, our analyses of >250,000 isolation calls suggest that this is the case for previously described bark and sustained vocalization types, and that both fall within the "cry" category recovered by our unsupervised clustering. The same is true for previously described simple sweeps and complex sweeps, which both fall within the USV type identified in this study. Importantly, the automated categorization of *Peromyscus* pup calls presented here and previously reported categorizations are each potentially informative. For example, vocalization types with acoustic differences that are salient to humans also have been shown to be behaviorally meaningful for mice during social interactions.[49–51] Future work combining unsupervised clustering of vocal behaviors with playback experiments will be important for better understanding how listeners partition the acoustic space of conspecific vocalizations into meaningful categories.[10]

Unlike *Peromyscus*, isolated *Mus* pups almost exclusively vocalize in the ultrasonic range, raising the question of whether the commonly studied laboratory strains of *Mus* have a reduced vocal repertoire, perhaps because of domestication. Using a unique experimental population of wild, free-living *Mus*

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

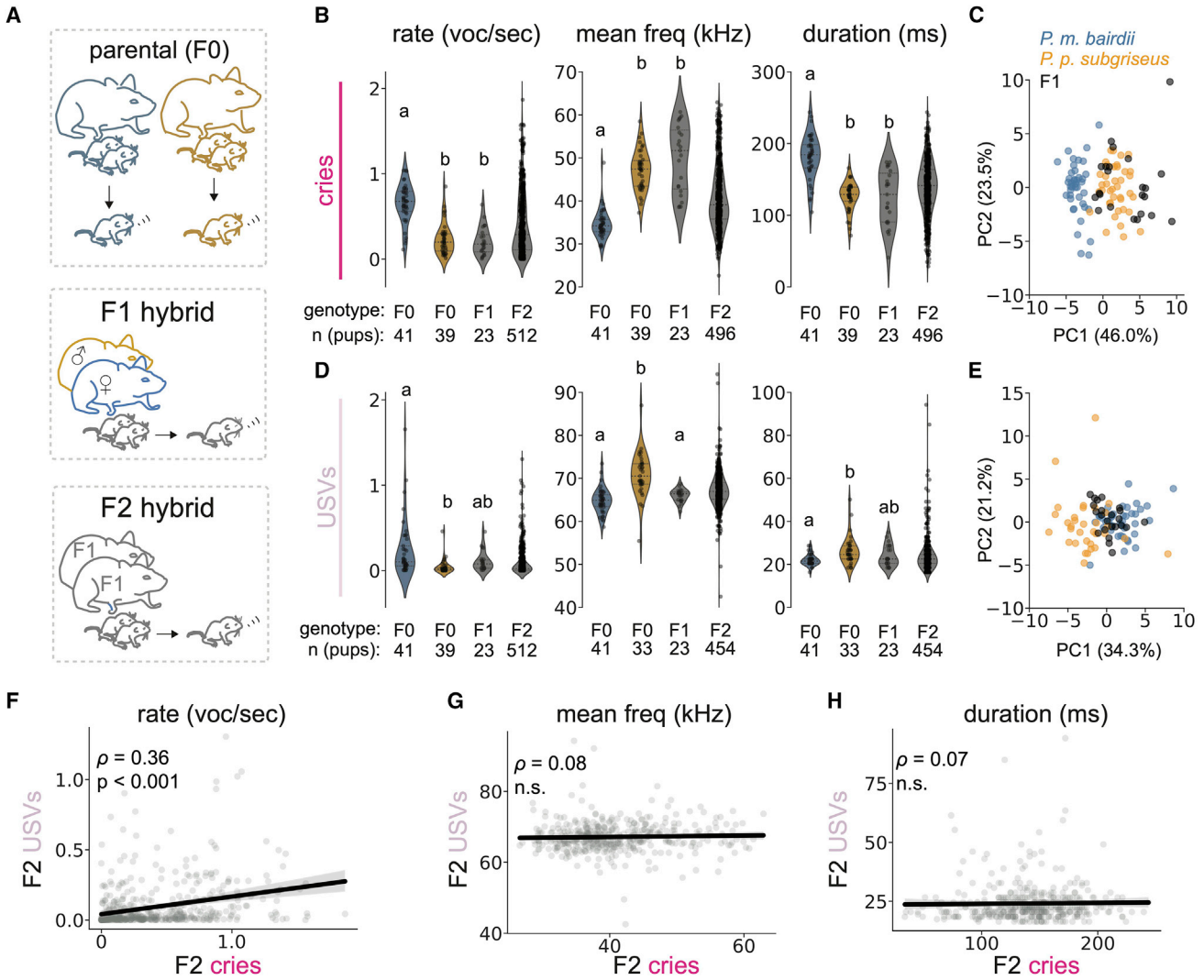


Figure 5. Separable genetic contributions to interspecific variation in cries and USVs

(A) Schematic of crossing scheme to generate first (F1) and second (F2) generation hybrids.

(B) Comparison of rate (first column), mean frequency (second column, median values for each pup), and duration (third column, median values for each pup) of cries made by *P. m. bairdii* and *P. p. subgriseus* and their F1 and F2 hybrids. Species and their F1 hybrids were compared by one-way ANOVA with Tukey post hoc test. Letters indicate significantly different groups ($p < 0.001$ for all comparisons). Dashed lines in violin plots represent quartiles; each dot corresponds to one pup.

(C) Two-component PCA on acoustic features of vocalizations aggregated by pup (median values for each pup).

(D) Comparison of rate (first column), mean frequency (second column, median values for each pup), and duration (third column, median values for each pup) of USVs made by *P. m. bairdii* and *P. p. subgriseus* and their F1 and F2 hybrids. Species and their F1 hybrids were compared by one-way ANOVA with Tukey post hoc test. Letters indicate significantly different groups ($p < 0.001$ for all comparisons). Dashed lines in violin plots represent quartiles; each dot corresponds to one pup.

(E) Two-component PCA on acoustic features of USVs aggregated by pup (median values for each pup).

(F) Cry call rate versus USV call rate in F2 hybrids; each dot corresponds to one pup. ρ: Spearman's rank correlation coefficient; black line: linear regression ± 95% confidence interval (gray shading).

(G) Cry mean frequency versus USV mean frequency in F2 hybrids using median values for each pup; each dot corresponds to one pup. ρ: Spearman's rank correlation coefficient; black line: linear regression ± 95% confidence interval (gray shading).

(H) Cry duration versus USV duration in F2 hybrids using median values for each pup; each dot corresponds to one pup. ρ: Spearman's rank correlation coefficient; black line: linear regression ± 95% confidence interval (gray shading).

musculus,[52] we find that the acoustic features of these vocalizations largely resemble those of C57BL6/J. Like C57BL6/J, but unlike *Peromyscus*, all wild *Mus* pup vocalizations are ultrasonic with mean frequency around 66 kHz, and clustered together in UMAP space. Thus, domestication appears to have not dramatically altered the isolation calls of *Mus* pups, and the presence of cry vocalizations in *Peromyscus*, but not C57BL6/J, is more likely the result of evolutionary divergence in wild populations rather

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

than an artifact of selection in the laboratory. Like *Mus*, isolation calls of pups from the closely related genus *Rattus* produce USVs with a mean frequency in the ultrasonic range (although, interestingly, adult USVs separate into two groups: aversive 25 kHz calls and affiliative 50 kHz calls[53]). Although the presence of isolation cries in other rodents (e.g., lemmings[54] and gerbils[55]) and mammals more generally[41] suggests that they were lost on the lineage leading to *Mus* and *Rattus* rather than gained in *Peromyscus*, without a more distantly related outgroup, our study cannot definitively say which of these states (presence or absence of cries) is ancestral. *Mus* pups do not appear to vocalize using lower frequency sounds in pup isolation assays, but they are capable of producing these sounds and, although less studied compared with USVs, they do so in other social contexts (e.g., "wriggling calls[56]"). Thus, the difference between *Peromyscus* and *Mus* we describe here does not reflect a difference in vocal ability, but rather a difference in the social contexts that elicit specific types of vocalizations.

Using playback assays, we find that cries elicit significantly faster behavioral responses from *P. maniculatus* dams than USVs. In all the *Peromyscus* species we examined, cries are primarily produced early in postnatal development, before eye opening, walking, and thermoregulation, whereas USVs are primarily made by older pups. In addition, we find that cries are louder and lower in frequency than USVs and therefore should degrade more slowly with distance during atmospheric transmission.[57,58] Thus, one hypothesis is that, although cries may be energetically more taxing to produce than USVs, cries may be used when pups are most vulnerable to exposure because they garner faster attention from caregivers. This leaves open the question of the communicative function of USVs. Early studies in *P. maniculatus* suggested that USVs function to suppress maternal aggression,[59] but, to the best of our knowledge, this hypothesis has not been tested. In *Mus*, USVs are thought to modulate maternal behavior, and the rate of USVs affects maternal responsiveness.[23,60,61] Because USVs in *Peromyscus* do elicit maternal response (albeit more slowly than cries), one hypothesis is that, although a less salient signal, USVs may be less detectable by predators (e.g., cats and foxes, which both have optimal hearing ranges below 10 kHz[62,63] and are common predators of deer mice[64]). Thus, as pups become more mature, the tradeoff between maximizing rapid parental response and minimizing detection by predators may change. Another question is the following: how do species-specific differences in pup vocalizations affect maternal (or paternal) responses? In at least some mammals, mothers do not distinguish between cries of their own young and those of other species,[65] but as we find that acoustic features of *Peromyscus* cries have diverged between closely related and/or sympatric taxa, it is possible that *Peromyscus* mothers might need to discriminate between isolation cries of different species in the wild. Given the robust response of *Peromyscus* mothers to pup vocalizations, future playback experiments can be used to measure parental responses to interspecific variation in vocalizations, as well as more fine-scale manipulation of specific acoustic features (e.g., rate, duration, and frequency) of both cries and USVs.

Although the function of cries versus USVs is likely distinct, the ultimate drivers of variation in these isolation calls remains unclear. One possible ecological driver is habitat. Indeed, some of the first studies of *Peromyscus* vocal behavior hypothesized that differences in vocalization rate result from different selection pressures to be heard by mothers in arboreal versus terrestrial habitats.[31] Although some of the species we examined are consistent with this hypothesis, this correlation breaks down as sympatric species, such as *P. maniculatus* and *P. leucopus*, vocalize at different rates, and the four *P. maniculatus* subspecies we tested occupy different habitats but vocalize at similar rates. Another possible evolutionary driver of interspecific differences is social system. In voles (genus *Microtus*), differences in pup isolation call rate have been attributed to social system complexity, with pups from monogamous, pair-bonding species vocalizing more than those from promiscuous species.[66] Four of the species we consider have differences in levels of parental care and the presence or absence of monogamy. However, in *Peromyscus*, we observe the opposite relationship: *P. p. subgriseus* is both socially and genetically monogamous; yet, pups from this species are less vocal than those of the highly promiscuous *P. m. bairdii*. Thus, the ultimate drivers of pup isolation call rate are likely multifaceted and may differ between species or genera in a way that belies simple correlations with habitat or social system.

We find that two pup vocalization types, which are acoustically and functionally distinct, have also diverged between closely related species, raising the question of how different aspects of a vocal repertoire (e.g., different call types or different acoustic features within a call type) may coevolve. For example, if different vocalization types share underlying genetic contributions, neural regulation, or production mechanisms, evolutionary changes in one type could result in (possibly deleterious) changes in others. On the other hand, if different vocalization types have separate underlying proximate mechanisms, changes in one call type would be less constrained, an evolutionary scenario that could facilitate functional specialization. In a comparison between two closely related species, we find that patterns of inheritance in first- and second-generation hybrids suggest that interspecific variation in vocal features of both call types are largely controlled by separate genetic loci. In comparisons of call types within species, we also find that cries and USVs are produced with different temporal rhythms, suggesting that at least partially distinct neural circuits pattern these behaviors.[67] Moreover, cries and USVs most likely arise from physically separate production mechanisms in the larynx, with the lower frequency features of cries being typical of sound produced by laryngeal vocal fold vibration and high-frequency sweeps of USVs likely produced by a separate mechanism.[68] Taking these observations together, distinct *Peromyscus* call types appear largely unconstrained by one another, which may contribute to their functional specialization in eliciting behavioral responses from parents.

Conclusions

Understanding the ultimate and proximate mechanisms driving the evolution of vocal behavior is a challenge, one that is currently being aided by rapid advances in computational tools to detect, label, and compare vocalizations across individuals and species. Using these tools in combination with playback experiments and genetic crosses, we have identified rapidly evolving features of a mammalian vocal repertoire in which interspecific variation in separable vocalization types is controlled by distinct genetic loci. Future work will identify those genetic loci

and their impact on neural circuits that both support social vocal communication and underlie its evolution in mammals.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - audio recording
  - playback
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - audio processing
  - data processing and statistical analyses

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.02.045.

ACKNOWLEDGMENTS

We would like to thank Barbara König and the members of the Hoekstra and Lindholm labs for their feedback and advice throughout the course of this research, Andri Manser for advice on statistical analyses, and the staff of the Harvard University Animal Facility for care of *Peromyscus* and C57BL6/J mouse colonies. We also thank Tim Sainburg, Gregg Castellucci, Kelsey Tyssowski, and two anonymous reviewers for their thoughtful comments on the manuscript. This work was supported by the Jane Coffin Childs Foundation (JCC Postdoctoral Fellowship to N.J.), National Science Foundation (NSF GRFP to M.L.W.), Howard Hughes Medical Institute (HHMI Gilliam Fellowship to M.L.W.), Human Frontiers Science Program (HFSP Postdoctoral Fellowship to J.I.S.-S.), the Harvard Museum of Comparative Zoology Grants-in-Aid of Undergraduate Research, and the Harvard Program for Research in Science and Engineering (to J.E.S.). H.E.H. is an Investigator of the Howard Hughes Medical Institute.

AUTHOR CONTRIBUTIONS

N.J. and H.E.H. conceived and planned the project. N.J., M.L.W., J.I.S.-S., J.E.S., and S.M. collected the data. N.J., M.L.W., J.I.S.-S., and J.E.S. analyzed the data. A.K.L. provided access to wild *Mus musculus* and input on interpreting their recordings. N.J. and H.E.H. wrote the manuscript with feedback from all co-authors.

DECLARATION OF INTERESTS

H.E.H. is a member of the Advisory Board for *Current Biology*.

INCLUSION AND DIVERSITY

We worked to ensure sex balance in the selection of non-human subjects. One or more of the authors of this paper self identifies as an underrepresented ethnic minority in their field of research or within their geographical location. One or more of the authors of this paper self-identifies as a gender minority in their field of research. One or more of the authors of this paper received support from a program designed to increase minority representation in their field of research.

Received: November 26, 2022
Revised: February 11, 2023
Accepted: February 14, 2023
Published: March 8, 2023

REFERENCES

1. Chen, Z., and Wiens, J.J. (2020). The origins of acoustic communication in vertebrates. Nat. Commun. *11*, 369.
2. Jorgewich-Cohen, G., Townsend, S.W., Padovese, L.R., Klein, N., Praschag, P., Ferrara, C.R., Ettmar, S., Menezes, S., Varani, A.P., Serano, J., et al. (2022). Common evolutionary origin of acoustic communication in choanate vertebrates. Nat. Commun. *13*, 6089.
3. Escalona Sulbarán, M.D., Ivo Simões, P., Gonzalez-Voyer, A., and Castroviejo-Fisher, S. (2019). Neotropical frogs and mating songs: the evolution of advertisement calls in glassfrogs. J. Evol. Biol. *32*, 163–176.
4. Hennelly, L., Habib, B., Root-Gutteridge, H., Palacios, V., and Passilongo, D. (2017). Howl variation across Himalayan, North African, Indian, and Holarctic wolf clades: tracing divergence in the world's oldest wolf lineages using acoustics. Curr. Zool. *63*, 341–348.
5. Miles, M.C., Schuppe, E.R., and Fuxjager, M.J. (2020). Selection for rhythm as a trigger for recursive evolution in the elaborate display system of woodpeckers. Am. Nat. *195*, 772–787.
6. Odom, K.J., Araya-Salas, M., Morano, J.L., Ligon, R.A., Leighton, G.M., Taff, C.C., Dalziell, A.H., Billings, A.C., Germain, R.R., Pardo, M., et al. (2021). Comparative bioacoustics: a roadmap for quantifying and comparing animal sounds across diverse taxa. Biol. Rev. Camb. Philos. Soc. *96*, 1135–1159.
7. Steinfath, E., Palacios-Muñoz, A., Rottschäfer, J.R., Yuezak, D., and Clemens, J. (2021). Fast and accurate annotation of acoustic signals with deep neural networks. eLife *10*, e68837.
8. Cohen, Y., Nicholson, D.A., Sanchioni, A., Mallaber, E.K., Skidanova, V., and Gardner, T.J. (2022). Automated annotation of birdsong with a neural network that segments spectrograms. eLife *11*, e63853.
9. Fonseca, A.H., Santana, G.M., Bosque Ortiz, G.M., Bampi, S., and Dietrich, M.O. (2021). Analysis of ultrasonic vocalizations from mice using computer vision and machine learning. eLife *10*, e59161.
10. Sainburg, T., and Gentner, T.Q. (2021). Toward a computational neuroethology of vocal communication: from bioacoustics to neurophysiology, emerging tools and future directions. Front. Behav. Neurosci. *15*, 811737.
11. Banerjee, A., Phelps, S.M., and Long, M.A. (2019). Singing mice. Curr. Biol. *29*, R190–R191.
12. Okanoya, K., and Screven, L.A. (2018). Rodent vocalizations: adaptations to physical, social, and sexual factors. In Springer Handbook of Auditory Research (Springer), pp. 13–41.
13. Pasch, B., Bolker, B.M., and Phelps, S.M. (2013). Interspecific dominance via vocal interactions mediates altitudinal zonation in Neotropical singing mice. Am. Nat. *182*, E161–E173.
14. Rieger, N.S., and Marler, C.A. (2018). The function of ultrasonic vocalizations during territorial defence by pair-bonded male and female California mice. Anim. Behav. *135*, 97–108.
15. Fernández-Vargas, M., Riede, T., and Pasch, B. (2022). Mechanisms and constraints underlying acoustic variation in rodents. Anim. Behav. *184*, 135–147.
16. Holy, T.E., and Guo, Z. (2005). Ultrasonic songs of male mice. PLoS Biol. *3*, e386.
17. Chabout, J., Sarkar, A., Dunson, D.B., and Jarvis, E.D. (2015). Male mice song syntax depends on social contexts and influences female preferences. Front. Behav. Neurosci. *9*, 76.
18. Egnor, S.R., and Seagraves, K.M. (2016). The contribution of ultrasonic vocalizations to mouse courtship. Curr. Opin. Neurobiol. *38*, 1–5.
19. Zimmer, M.R., Fonseca, A.H.O., Iyilikci, O., Pra, R.D., and Dietrich, M.O. (2019). Functional ontogeny of hypothalamic Agrp neurons in neonatal mouse behaviors. Cell *178*, 44–59.e7.

20. Ehret, G. (2005). Infant rodent ultrasounds: A gate to the understanding of sound communication. Behav. Genet. *35*, 19–29.

21. Ehret, G., and Haack, B. (1982). Ultrasound recognition in house mice: key-Stimulus configuration and recognition mechanism. J. Comp. Physiol. *148*, 245–251.

22. Schiavo, J.K., Valtcheva, S., Bair-Marshall, C.J., Song, S.C., Martin, K.A., and Froemke, R.C. (2020). Innate and plastic mechanisms for maternal behaviour in auditory cortex. Nature *587*, 426–431.

23. Uematsu, A., Kikusui, T., Kihara, T., Harada, T., Kato, M., Nakano, K., Murakami, O., Koshida, N., Takeuchi, Y., and Mori, Y. (2007). Maternal approaches to pup ultrasonic vocalizations produced by a nanocrystalline silicon thermo-acoustic emitter. Brain Res. *1163*, 91–99.

24. Castellucci, G.A., Calbick, D., and McCormick, D. (2018). The temporal organization of mouse ultrasonic vocalizations. PLoS One *13*, e0199929.

25. Ashbrook, D.G., Roy, S., Clifford, B.G., Riede, T., Scattoni, M.L., Heck, D.H., Lu, L., and Williams, R.W. (2018). Born to Cry: A genetic dissection of infant vocalization. Front. Behav. Neurosci. *12*, 250.

26. Barnes, T.D., Wozniak, D.F., Gutierrez, J., Han, T.U., Drayna, D., and Holy, T.E. (2016). A mutation associated with stuttering alters mouse pup ultrasonic vocalizations. Curr. Biol.. S0960-9822(16)30179-8.

27. Hernandez-Miranda, L.R., Ruffault, P.L., Bouvier, J.C., Murray, A.J., Morin-Surun, M.P., Zampieri, N., Cholewa-Waclaw, J.B., Ey, E., Brunet, J.F., Champagnat, J., et al. (2017). Genetic identification of a hindbrain nucleus essential for innate vocalization. Proc. Natl. Acad. Sci. USA *114*, 8095–8100.

28. Castellucci, G.A., McGinley, M.J., and McCormick, D.A. (2016). Knockout of Foxp2 disrupts vocal development in mice. Sci. Rep. *6*, 23305.

29. Nicolakis, D., Marconi, M.A., Zala, S.M., and Penn, D.J. (2020). Ultrasonic vocalizations in house mice depend upon genetic relatedness of mating partners and correlate with subsequent reproductive success. Front. Zool. *17*, 10.

30. Bedford, N.L., and Hoekstra, H.E. (2015). *Peromyscus* mice as a model for studying natural variation. eLife *4*, e06813.

31. Hart, F.M., and King, J.A. (1966). Distress Vocalizations of young in two subspecies of *Peromyscus maniculatus*. J. Mammal. *47*, 287–293.

32. Kalcounis-Rueppell, M.C., Pultorak, J.D., Blake, B.H., and Marler, C.A. (2018). Ultrasonic vocalizations of young mice in the genus *Peromyscus*. In Handbook of Ultrasonic Vocalizations (Elsevier Academic Press), pp. 149–156.

33. Kalcounis-Rueppell, M.C., Pultorak, J.D., and Marler, C.A. (2018). Ultrasonic vocalizations of mice in the genus *Peromyscus*. Handb. Behav. Neurosci. *25*, 227–235.

34. Kobrina, A., Letowt, M.E., and Pasch, B. (2022). The influence of social context on pinyon mouse (*Peromyscus truei*) vocalizations. J. Mammal. *103*, 275–286.

35. Miller, J.R., and Engstrom, M.D. (2007). Vocal stereotypy and singing behavior in *Baiomyine* mice. J. Mammal. *88*, 1447–1465.

36. Miller, J.R., and Engstrom, M.D. (2012). Vocal stereotypy in the rodent genera *Peromyscus* and *Onychomys* (Neotominae): taxonomic signature and call design. Bioacoustics *21*, 193–213.

37. Lesch, R., Schwaha, T., Orozco, A., Shilling, M., Brunelli, S., Hofer, M., Bowling, D.L., Zimmerberg, B., and Fitch, W.T. (2021). Selection on vocal output affects laryngeal morphology in rats. J. Anat. *238*, 1179–1190.

38. Sainburg, T., Thielk, M., and Gentner, T.Q. (2020). Finding, visualizing, and quantifying latent structure across diverse animal vocal repertoires. PLoS Comp. Biol. *16*, e1008228.

39. Goffinet, J., Brudner, S., Mooney, R., and Pearson, J. (2021). Low-dimensional learned feature spaces quantify individual and group differences in vocal repertoires. eLife *10*, e67855.

40. Araya-Salas, M., and Smith-Vidaurre, G. (2017). warbleR: an R package to streamline analysis of animal acoustic signals. Methods Ecol. Evol. *8*, 184–191.

41. Lingle, S., Wyman, M.T., Kotrba, R., Teichroeb, L.J., and Romanow, C.A. (2012). What makes a cry a cry? A review of infant distress vocalizations. Curr. Zool. *58*, 698–726.

42. Gaub, S., and Ehret, G. (2005). Grouping in auditory temporal perception and vocal production is mutually adapted: the case of wriggling calls of mice. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *191*, 1131–1135.

43. Ravignani, A., Dalla Bella, S., Falk, S., Kello, C.T., Noriega, F., and Kotz, S.A. (2019). Rhythm in speech and animal vocalizations: a cross-species perspective. Ann. N. Y. Acad. Sci. *1453*, 79–98.

44. Burchardt, L.S., and Knörnschild, M. (2020). Comparison of methods for rhythm analysis of complex animals' acoustic signals. PLoS Comp. Biol. *16*, e1007755.

45. Bendesky, A., Kwon, Y.-M., Lassance, J.-M., Lewarch, C.L., Yao, S., Peterson, B.K., He, M.X., Dulac, C., and Hoekstra, H.E. (2017). The genetic basis of parental care evolution in monogamous mice. Nature *544*, 434–439.

46. Birdsall, D.A., and Nash, D. (1973). Occurence of succesful multiple insemination of females in natural populations of deer mice (*Peromyscus maniculatus*). Evolution *27*, 106–110.

47. Foltz, D.W. (1981). Genetic evidence for long-term monogamy in a small rodent, *Peromyscus polionotus*. Am. Nat. *117*, 665–675.

48. Brunelli, S.A. (2005). Selective breeding for an infant phenotype: rat pup ultrasonic vocalization (USV). Behav. Genet. *35*, 53–65.

49. Pultorak, J.D., Alger, S.J., Loria, S.O., Johnson, A.M., and Marler, C.A. (2018). Changes in behavior and ultrasonic vocalizations during pair bonding and in response to an infidelity challenge in monogamous California mice. Front. Ecol. Evol. *6*, 125.

50. Rieger, N.S., Monari, P.K., Hartfield, K., Schefelker, J., and Marler, C.A. (2021). Pair-bonding leads to convergence in approach behavior to conspecific vocalizations in California mice (*Peromyscus californicus*). PLoS One *16*, e0255295.

51. Screven, L.A., and Dent, M.L. (2019). Perception of ultrasonic vocalizations by socially housed and isolated Mice. eNeuro *6*. ENEURO.0049-19.2019.

52. König, B., Lindholm, A.K., Lopes, P.C., Dobay, A., Steinert, S., and Buschmann, F.J.-U. (2015). A system for automatic recording of social behavior in a free-living wild house mouse population. Anim. Biotelem. *3*, 39.

53. Portfors, C.V. (2007). Types and functions of ultrasonic vocalizations in laboratory rats and mice. J. Am. Assoc. Lab. Anim. Sci. *46*, 28–34.

54. Volodin, I.A., Yurlova, D.D., Ilchenko, O.G., and Volodina, E.V. (2021). Ontogeny of audible squeaks in yellow steppe lemming *Eolagurus luteus*: trend towards shorter and low-frequency calls is reminiscent of those in ultrasonic vocalization. BMC Zool. *6*, 27.

55. Zaytseva, A.S., Volodin, I.A., Ilchenko, O.G., and Volodina, E.V. (2020). Audible calls and their ontogenetic relationship with ultrasonic vocalization in a rodent with a wide vocal range, the fat-tailed gerbil (*Pachyuromys duprasi*). Behav. Processes *180*, 104241.

56. Ehret, G., and Bernecker, C. (1986). Low-frequency sound communication by mouse pups (*Mus musculus*): wriggling calls release maternal behaviour. Anim. Behav. *34*, 821–830.

57. Marten, K., and Marler, P. (1977). Sound transmission and its significance for animal vocalization: I. Temperate habitats. Behav. Ecol. Sociobiol. *2*, 271–290.

58. Wiley, R.H., and Richards, D.G. (1978). Physical constraints on acoustic communication in the atmosphere: implications for the evolution of animal vocalizations. Behav. Ecol. Sociobiol. *3*, 69–94.

59. Smith, J.C. (1972). Sound production by infant *Peromyscus maniculatus* (Rodentia: Myomorpha). J. Zool. *168*, 369–379.

60. Bowers, J.M., Perez-Pouchoulen, M., Edwards, N.S., and McCarthy, M.M. (2013). Foxp2 mediates sex differences in ultrasonic vocalization by rat pups and directs order of maternal retrieval. J. Neurosci. *33*, 3276–3283.

61. D'Amato, F.R., Scalera, E., Sarli, C., and Moles, A. (2005). Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? Behav. Genet. *35*, 103–112.

62. Heffner, R.S., and Heffner, H.E. (1985). Hearing range of the domestic cat. Hear. Res. *19*, 85–88.

63. Malkemper, E.P., Topinka, V., and Burda, H. (2015). A behavioral audiogram of the red fox (*Vulpes vulpes*). Hear. Res. *320*, 30–37.

64. King, J.A. (1968). Biology of *Peromyscus* (Rodentia) (American Society of Mammalogists, Lawrence, Kansas).

65. Lingle, S., and Riede, T. (2014). Deer mothers are sensitive to infant distress vocalizations of diverse mammalian species. Am. Nat. *184*, 510–522.

66. Blake, B.H. (2012). Ultrasonic calling in 2 species of voles, *Microtus pinetorum* and *M. pennsylvanicus*, with different social systems. J. Mammal. *93*, 1051–1060.

67. Zhang, Y.S., and Ghazanfar, A.A. (2020). A hierarchy of autonomous systems for vocal production. Trends Neurosci. *43*, 115–126.

68. Riede, T., Kobrina, A., Bone, L., Darwaiz, T., and Pasch, B. (2022). Mechanisms of sound production in deer mice (*Peromyscus* spp.). J. Exp. Biol. *225*, jeb243695.

69. Kingsley, E.P., Kozak, K.M., Pfeifer, S.P., Yang, D.-S., and Hoekstra, H.E. (2017). The ultimate and proximate mechanisms driving the evolution of long tails in forest deer mice. Evolution *71*, 261–273.

70. Bedford, N.L., Gable, J.T., Hu, C.K., Wooldridge, T.B., Sokolov, N.A., Lassance, J.-M., and Hoekstra, H.E. (2021). Automated tracking reveals the social network of beach mice and their burrows. Preorint at bioRxiv. https://doi.org/10.1101/2021.08.07.455531.

71. Delaney, E.K., and Hoekstra, H.E. (2018). Sexual imprinting and speciation between two *Peromyscus* species. Evolution *72*, 274–287.

72. Hager, E.R., Harringmeyer, O.S., Wooldridge, T.B., Theingi, S., Gable, J.T., McFadden, S., Neugeboren, B., Turner, K.M., Jensen, J.D., and Hoekstra, H.E. (2022). A chromosomal inversion contributes to divergence in multiple traits between deer mouse ecotypes. Science *377*, 399–405.

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

CellPress

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Peromyscus maniculatus bairdii	*Peromyscus* Stock Center (Univ of South Carolina)	https://sc.edu/study/colleges_schools/pharmacy/centers/peromyscus_genetic_stock_center/
Peromyscus maniculatus gambelli	*Peromyscus* Stock Center (Univ of South Carolina)	https://sc.edu/study/colleges_schools/pharmacy/centers/peromyscus_genetic_stock_center/
Peromyscus maniculatus rubidus	wild caught (Harvard)	N/A
Peromyscus maniculatus nubiterrae	wild caught (Harvard)	N/A
Peromyscus polionotus subgriseus	*Peromyscus* Stock Center (Univ of South Carolina)	https://sc.edu/study/colleges_schools/pharmacy/centers/peromyscus_genetic_stock_center/
Peromyscus polionotus leucocephalus	wild caught (Harvard)	N/A
Peromyscus leucopus spp.	*Peromyscus* Stock Center (Univ of South Carolina)	https://sc.edu/study/colleges_schools/pharmacy/centers/peromyscus_genetic_stock_center/
Peromyscus gossypinus spp.	wild caught (Harvard)	N/A
Mus musculus domesticus (C57BL6/J strain)	Jackson Labs (Bar Harbor, ME)	IMSR_JAX:000664
Mus musculus domesticus (wild, free living)	N/A	N/A
Software and algorithms		
Custom Analysis Code	Nicholas Jourjine	https://zenodo.org/badge/latestdoi/585089651
Scikit-learn	N/A	https://scikit-learn.org/stable/
SciPy	N/A	https://scipy.org/
Audicity	N/A	https://www.audacityteam.org/
Deposited data		
Peromyscus Pup Vocal Evolution Data Set	custom	https://doi.org/10.5061/dryad.g79cnp5ts

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hopi Hoekstra (hoekstra@oeb.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Audio recordings have been deposited on Dryad. Accession numbers are listed in the key resources table.

All original code has been deposited at https://github.com/nickjourjine/peromyscus-pup-vocal-evolution. DOIs are listed in the key resources table.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We focused on eight *Peromyscus* taxa, representing four species (*P. maniculatus*, *P. polionotus*, *P. leucopus*, *P. gossypinus*). We established colonies of *P. m. bairdii*, *P. m. gambelli*, *P. p. subgriseus* and *P. leucopus* from animals originally obtained from the Peromyscus Stock Center at the University of South Carolina and established *P. m. nubiterrae*,[69] *P. p. leucocephalus*,[70] *P. gossypinus*,[71] and *P. m. rubidus*[72] colonies from wild caught animals. We focused on two strains of *Mus musculus domesticus*: the C57BL6/J strain obtained from Jackson Labs (Bar Harbor, ME) and a wild, free-living population of approximately 200 mice living in a barn in Zürich, Switzerland.[52]

We housed all *Peromyscus* and C57BL6/J animals in barrier, specific-pathogen-free conditions with 16 h light: 8 h dark at 22° C in individually ventilated cages 18.6 cm x 29.8 cm x 12.8 cm height; Allentown, New Jersey) with quarter-inch Bed-o-cob bedding (The

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

Andersons, Maumee, Ohio). Breeding animals and their litters were fed irradiated PicoLab Mouse Diet 20 5058 (LabDiet, St. Louis, Missouri) *ad libitum* and had free access to water. We weaned animals at 23 days of age into same strain and sex cages. After weaning, we fed animals irradiated LabDiet Prolab Isopro RMH 3000 5P75 (LabDiet) *ad libitum* with free access to water and provided them with nesting material (Nestlet, Ancare, Bellmore, New York) and a polycarbonate translucent red hut. Free-living *Mus musculus domesticus* had access to *ad libitum* food (1:1 mix of "Hafer Flockiert", UFA AG, 3360 Herzogenbuchsee, Switzerland and "Meerschwienchen und Hamster Futter", Landi Schweiz AG 3293 Dotzigen, Switzerland) and water.

All experiments on *Peromyscus* and *Mus* (C57BL6/J) were approved by the Harvard University Faculty of Arts and Sciences Institutional Animal Care and Use Committee. All recordings of wild *Mus musculus* were approved under permit ZH076/2022 granted by the Veterinary Office of Canton, Zürich, Switzerland.

METHOD DETAILS

audio recording

We recorded pups in three separate paradigms: (1) a developmental time course of *Peromyscus* and *Mus* (lab and wild) pups; (2) *P. maniculatus bairdii* and *P. polionotus subgriseus* pups that were cross-fostered; and (3) first- (F1) and second-generation (F2) hybrid pups generated from an intercross between *P. m. bairdii* and *P. p. subgriseus*. Pups were recorded in sound-attenuating chambers (i.e., a styrofoam box for *Wild Mus* recordings and an Igloo "wheelie cool" cooler lined with acoustic foam for all other recordings). We collected audio data using either a single channel recording system (Avisoft Ultrasoundgate 116Hb) for wild *Mus* recordings or a multichannel recording system (Avisoft 816hb Ultrasoundgate, http://www.avisoft.com/ultrasoundgate/816h/) for all other recordings. We used Avisoft CM16/CMPA microphones (http://www.avisoft.com/ultrasound-microphones/cm16-cmpa/) with a 250 kHz sampling rate and 16 bit encoding with a Windows 10 operating system.

developmental time course recordings

In the laboratory, we recorded isolation calls from pups of eight *Peromyscus* taxa, as well as an inbred strain of *Mus* (C57BL6/J). We established pup age by daily nest checks, using the convention that pups are 1 day old on the day of litter discovery (day of birth is day 0). We removed breeding cages containing pups that were either 1, 3, 5, 7, 9, 11, or 13 days old from their colony room to a designated recording room and left them undisturbed for 10 minutes. We then removed all pups from their nest, placed them individually into clean, empty mouse cages (18.6 cm x 29.8 cm x 12.8 cm height; Allentown, Allentown, New Jersey), and immediately recorded their temperatures using an infrared thermal camera (FLIR, model C5) directed at the back of their neck from a distance of approximately 3 inches. We then placed each pup into its own recording chamber. Then, audio recording for all pups commenced simultaneously and lasted 10 minutes, at which point we removed pups and measured their temperatures again, as described above. Pups were then weighed, sexed using anogenital distance, examined for the presence of a milk spot (an indicator of recent feeding), and returned to the nest. Each litter/pup was recorded only once. Recordings were made in white light (day) conditions between 5 and 2 hours prior to the transition to red light (night).

We recorded wild *Mus musculus domesticus* pups taken from a free-living population in a barn near Zürich, Switzerland that is continuously monitored as part of an ongoing, long-term study.[52] We placed pups in a clean, empty plastic container (P1 - P11) or a clean mason jar (P13, to prevent escape) and recorded each individually for 5 minutes. After audio recording, we recorded weight, sex, and age. Four wild *Mus* pups (one litter) were recorded at postnatal day four (P4). All handling necessary for the long-term study was done following pup recording to minimize handling effects on vocal behavior. We used unique tattoo markings on pups to avoid duplicate recordings (pups were recorded once). Recordings were made between 10am and 3pm between July 1 and 21, 2022.

cross-foster recordings

On days when litters were discovered (<24h old) simultaneously from *P. m. bairdii* and *P. p. subgriseus* breeding pairs, we exchanged the litters from these pairs and then returned cages to their racks until the pups were 9 days old. We found no evidence for rejection of the pups. We then removed pups from their nest into a clean Allentown cage and recorded each pup individually, as described above, for 3 minutes. Following recording, we sexed and weighed each pup and returned them to their foster parents.

F2 hybrid recordings

To generate a F2 hybrid population, we first mated two *P. m. bairdii* females to two *P. p. subgriseus* males. These founders were chosen because they had species-typical weights, heat loss, and vocalization rates when measured at P7 and P9. We then paired 54 F1 hybrid siblings, when they were between 40 and 90 days old, to produce F2 hybrids. We recorded from 25 F1 and 617 F2 mice, following the protocol described above for the developmental time course recordings. F1 and F2 hybrids were recorded twice, once at P7 and once at P9, and compared to *P. m. bairdii* and *P. p. subgriseus* pups that were also recorded twice at the same ages and under identical conditions (data from P9 pups shown).

playback

Breeding pairs of *P. m. bairdii* were checked daily for pups, which were aged as described above. When pups from a given breeding pair were 8 days old, we relocated the mother, her nest, and her litter to the cage in which we performed the playback experiments (25 cm x 34 cm x 19 cm height; Allentown, Allentown, New Jersey). We modified the cage to have two grids of holes, on either end of one wall, for audio playback. After 24 hours, we moved the cage containing the mother and her litter to a separate room and left it on a table-top for 10 minutes. Once the mother had remained in her nest for an additional 1 minute, we played pre-recorded pup vocalizations from one of the two ultrasonic speakers (Avisoft, http://www.avisoft.com/playback/vifa/) until the mother touched the cage

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

wall immediately in front of the speaker, or for 2 minutes, whichever came first. We then stopped playing the audio recording until the mother returned to her nest and remained there for 1 minute, at which point we recommenced playback, using the python package random to determine whether we played cries or USVs. This regime continued for 10 rounds. We used an ultrasonic microphone placed above the nest to confirm that both vocalization types were detectable at the location of the mother during playback.

We used the software package bonsai to track the mother's position during playback and align position measurements to the active speaker (https://open-ephys.org/bonsai). All playback experiments were performed under red light conditions. All audio was recorded using the hardware and recording specifications described above. To produce the audio for playback, we first chose a species-typical bout of *P. m. bairdii* cries from a recording of a P9 pup. We then concatenated copies of this bout with alternating periods of silence using the software package Audacity (https://www.audacityteam.org/). The length of this silent period was chosen to match a typical silent period between groups of cries, using data in Figure S5. A species-typical bout of USVs was chosen in the same manner and concatenated with periods of silence that were matched to those of the cries. To account for the large difference in amplitude between cries and USVs and to minimize differences in background (non-vocal) silence in each recording, cry and USV recordings were matched in amplitude (USV increased to match cry) and background using the Amplify and Noise Reduction effects in Audacity.

QUANTIFICATION AND STATISTICAL ANALYSIS

audio processing

We processed raw audio by first segmenting it into vocalizations, then calculating acoustic features from those segments. For developmental time course recordings, we also embedded spectrogram images of vocalizations in two dimensions using UMAP, trained random forest classifiers to predict vocalization type (cry or USV) and species identity from acoustic features of vocalizations, and calculated inter-onset intervals for cries and USVs. All code needed to carry out these processing and analysis steps can be found at: https://github.com/nickjourjine/peromyscus-pup-vocal-evolution.

audio segmenting

To segment the raw audio recordings into vocalizations, we used the get_onsets_offsets function from the python package AVA (https://github.com/pearsonlab/autoencoded-vocal-analysis) using the following parameters for *Peromyscus* and C57BL6/J *Mus*: Minimum frequency: 20 kHz, Maximum frequency: 125 kHz, nperseg: 1024, noverlap: 512, minimum log-spectrogram value: 0.8, maximum log-spectrogram value: 6, segmenting threshold 1:.03, segmenting threshold 2:.3, segmenting threshold 3: 0.35, minimum duration in seconds: 0.015, maximum duration in seconds: 1, smoothing timescale 0.00025, softmax: False. Because we recorded wild *Mus* in the field, background sound levels were higher than in laboratory recordings. We therefore segmented wild *Mus* recordings using the same parameters as above, but with the minimum log-spectrogram value of 2. For all recordings, detected vocalizations separated by less than 0.004 seconds were merged into a single vocalization.

unsupervised clustering of spectrogram images

To cluster segmented vocalizations from the developmental time course recordings, we generated a spectrogram from audio clips of each detected vocalization using the following specifications: Minimum frequency: 5 kHz, Maximum Frequency 125 kHz, nperseg: 512, noverlap: 128, maximum spectrogram value: 10. To account for slight differences in background noise between recordings, we also chose an example audio clip consisting of background noise from each recording and confirmed by visual examination that it did not contain any vocalizations. For each vocalization spectrogram, we then set the minimum pixel value as 2-standard deviations above the median spectrogram value of this corresponding background noise clip. To reduce the size of spectrogram images while preserving image features, we used the python package AVA[39] to pad spectrograms to the length of the longest vocalization made by each species, then sampled them at 128 time points spaced evenly between the start and end of the signal (time) and 128 frequency points sampled evenly between the minimum and maximum frequency (frequency), linearly interpolating between time and frequency points using the interp2d function from the python package SciPy. This resulted in a 128x128 pixel image for each segmented vocalization. For each species, we then linearized, z-scored, and embedded these images in 2 dimensions using the default settings of the python package umap-learn (https://umap-learn.readthedocs.io/en/latest/). To label clusters of spectrogram images in these embeddings, we used the python package hdbscan (https://github.com/scikit-learn-contrib/hdbscan/blob/master/docs/index.rst) to perform unsupervised clustering of umap coordinates with the min_cluster_size set to 100, allow_single_cluster set to True, and all other parameters left as default.

acoustic feature calculation

To calculate acoustic features and sound pressure levels from each vocalization detected using the above parameters, we used the specan and sound_pressure_level functions of the R package warbleR[40] (version 1.1.27, https://github.com/maRce10/warbleR), with "harmonicity" set to False and "Fast" set to True. Sound pressure level measurements were made relative to a reference of 20 uPa (warbleR default). To estimate the lower and upper frequencies of each vocalization, we used the minimum dominant frequency ('mindom') and maximum dominant frequency ('maxdom') features calculated by the specan function of warbleR, which are the minimum and maximum frequency with the highest energy measured across a spectrogram (https://cran.r-project.org/web/packages/warbleR/warbleR.pdf)

training classifiers on acoustic features

To train random forest classifiers to label "cry" and "USV" in amplitude thresholded clips, we randomly sampled between 2000 and 6000 spectrograms from each hdbscan labeled umap cluster from each taxa using the pandas.sample method (https://pandas.pydata.org/docs/index.html). We then visually inspected each vocalization and labeled them as "cry", "USV", or "nonvocal sound"

Please cite this article in press as: Jourjine et al., Two pup vocalization types are genetically and functionally separable in deer mice, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.02.045

and calculated acoustic features from each of these annotated vocalizations as described above using the R package warbleR. The features used to described each clip were (using warbleR naming conventions) 'duration', 'time.median', 'time.Q25', 'time.Q75', 'time.IQR', 'meanfreq', 'freq.median', 'freq.Q25', 'freq.Q75', 'freq.IQR', 'meanpeakf', 'dfslope', 'enddom', 'startdom', 'modindx', 'dfrange', 'sfm', 'entropy', 'sp.ent', 'time.ent', 'sd', 'meandom', 'mindom', 'maxdom', 'skew', and 'kurt'. We then used these features and corresponding labels for each sampled vocalization to train a 10,000 tree Random Forest Classifier using the RandomForestClassifier class from the python package scikit-learn (https://scikit-learn.org/stable/) and an 80%/20% train/test split. Information gain was used as the optimization criterion (the 'criterion' parameter was set as 'entropy'). To assess the species-specificity of cry and USV acoustic features, we trained 500 tree random forest classifiers on of human-validated cries or USVs consisting of either 20, 40, 200, 400, 600, 800, 1000, 1200, or 1400 vocalizations per call type, sampled using the pandas.sample method, as above. The features used to describe each vocalization were (using warbleR naming conventions) 'duration', 'dfslope', 'time.median', 'time.IQR', 'time.Q25', 'time.Q75', 'meanfreq', 'meandom', 'freq.IQR', 'freq.Q25', 'freq.Q75', 'freq.median', 'sp.ent', and 'time.ent'. For each sample size and vocalization type (cry or USV), we then used the RandomForestClassifier class from the python package sklearn and an 80%/20% train/test split to predict species identity as described above.

calculating inter-onset intervals

For each developmental time course recording, we calculated inter-onset intervals for cries and USVs, respectively, using the diff method from the python package pandas to calculate the difference in seconds between the start time of each consecutive vocalization of each type. We generated recurrence plots[44] by log transforming the interonset intervals for each type, taking the difference of every log transformed interval with every other interval, then sorting the differences from smallest to largest and plotting the resulting matrix as a heatmap (Figure S5).

data processing and statistical analyses

All statistical analyses were performed using R version 4.2.2.

UMAP embedding

During preliminary data exploration, we discovered 85 detected C57BL6/J vocalizations that were artifacts resulting from a recording hardware problem. We excluded these from UMAP embedding and analyses in Figure 1, but otherwise did not filter vocalizations.

Developmental time course analyses

To compare species at a single postnatal day, we used linear models (one for each feature: duration, mean frequency, or rate), with the feature as the response variable and species and sex as fixed effects. Pup identity and pup temperature loss (defined as the difference in °C between the pup's temperature measured immediately after and immediately before recordings) were included as a random effects for duration and mean frequency models. As there is only one vocalization rate measurement per pup, only pup temperature loss was included as a random effect when modeling cry and USV vocalization rates. To compare pups across postnatal development, we used linear models (one for each feature: duration, mean frequency, or rate), with the feature as the response variable and species, sex, and age as fixed effects. Pup identity and pup temperature loss were included as a random effects for duration and mean frequency models; pup heat loss was the only random effect for vocalization rate models. Linear models were fit with the lmer function of the R package lme4. As clipping (i.e., saturation or near-saturation of the recording microphone by loud sounds) can introduce frequency artifacts, clipped vocalizations were excluded from analysis of spectral acoustic features. Clipped vocalizations were defined as those that reached 95% of the maximum sound level for 16-bit encoded wav files at any point. Suckling pups that had to be removed from their dam by the experimenter prior to recording were excluded from all analyses. Recordings from 10 pups were found to contain artifacts resulting from a recording hardware problem and were excluded from all analyses.

cry and USV playback

To analyze the behavioral responses of *P. maniculatus bairdii* dams to playback of cry or USV vocalizations, we calculated the median time it took each dam to reach the speaker for each of 5 cry and 5 USV playback exposures, and the median of the maximum speed reached during each of 5 cry and 5 USV playback exposures. We tested the effect of vocalization type on median time to reach the speaker and median of maximum speed using paired t-tests implemented with the t.test function in R with "paired" set to True.

cross-foster and F2 hybrid recordings

We tested the effect of cross fostering condition and genotype with ANOVA and Tukey posthoc tests using the aov and TukeyHSD functions in R, respectively, using median values of acoustic features for each pup. Only pups for which a feature could be calculated for all vocalizations of a given type (cry or USV) made by the pup were included for analyses of that feature. To test for correlations between features of cries and USVs we used Spearman's rank correlation coefficient using the cor.test function in R with method set to 'spearman'. In addition, we excluded four *P. maniculatus bairdii* pups from the cross-foster dataset (because they produced atypical, extremely short vocalizations that made them poor representatives for testing whether cross fostering affected species typical vocal behavior), and two cross fostered *P. polionotus subgriseus* pups (one litter) because they exhibited evidence of excessive grooming by their foster dam. Clipped vocalizations were excluded from calculations of spectral acoustic features and suckling pups that had to be removed from their dam by the experimenter prior to recording were excluded from all analyses.