Title: Paternal aging causes atypical development of vocal communication in neonatal mice

Authors: Lingling Mai[1], Ryuichi Kimura[1, 2], Hitoshi Inada[1,3], Kouta Kanno[4], Takeru Matsuda[5,6], Ryosuke O. Tachibana[7], Kaichi Yoshizaki[8], Fumiyasu Komaki[5,6], Noriko Osumi[1]*

Affiliations:

[1] Department of Developmental Neuroscience, Tohoku University Graduate School of Medicine, Sendai, Japan, 980-8575.

[2] Department of Drug Discovery Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan.

[3] Laboratory of Health and Sports Sciences, Division of Biomedical Engineering for Health and Welfare Tohoku University Graduate School of Biomedical Engineering, Sendai, Japan.

[4] Faculty of Law, Economic and Humanities, Kagoshima University, Kagoshima, Japan.

[5] Department of Mathematical Informatics, Graduate School of Information Science and Technology, The University of Tokyo, Tokyo, Japan.

[6] Mathematical Informatics Collaboration Unit, RIKEN Center for Brain Science, Wako, Japan.

[7] Department of Life Science, Graduate School of Arts and Sciences, The University of Tokyo, Tokyo Japan.

[8] Department of Disease Model, Institute for Developmental Research, Aichi Developmental Disability Center, Aichi, Japan.

*Correspondence to: osumi@med.tohoku.ac.jp

Abstract:

The infant crying is an innate behavior for communication and impaired in some types of neurodevelopmental diseases. Since paternal aging has been reported to be a risk for neurodevelopmental diseases in offspring, we established a mouse model to examine its effect on development of vocal communication. Ultrasonic vocalization (USV) was recorded from pups separated from their mother, and qualitatively and quantitatively analyzed as sonograms. The pups derived from aged father emitted USV calls fewer in number and with less diversity. While the pups derived from young fathers showed a developmental convergence in vocal behavior, those from aged fathers exhibited more atypical patterns. Here we show for the first time a

significant influence of paternal aging on early vocal development from an individual perspective.

One Sentence Summary:

Paternal aging alters the early development of vocalization in offspring, inducing more individuals with atypical developmental pattern.

Main Text:

Human infant crying is an innate social communication (*1, 2*), which can attract attention from caregivers (*3*) and influence on adult cognitive control (*4*). It is reported that different crying patterns such as higher frequency and shorter duration are observed in infants with a high risk of neurodevelopmental disorders such as autism spectrum disorder (ASD) (*5, 6*), which is characterized by two core symptoms; i.e., social interaction impairment (including verbal and nonverbal communication deficits) and repetitive behavior (*7-9*). Although the etiology of ASD remains unclear, it is believed that genetic, environmental and epigenetic factors are involved (*10-12*). Recently, epidemiological studies repeatedly suggest a significant association between paternal aging and a risk of ASD in offspring (*13-16*). Thus, the influence of paternal aging on infant crying can be worth studying as one of the symptoms of an early developmental deficiency in ASD.

Vocal communication of animals is extensively studied in songbirds, whereas the ultrasonic vocalization (USV) of the rodent, especially of the mouse, has recently been paid attention in regard with neuropathology of communication deficits (*17, 18*). Interestingly, the separation of a pup from its mother and littermates induces the pup to emit ultrasonic vocalization (USV) calls, which triggers maternal approach and retrieval behavior (*19, 20*). Maternal separation-induced patterns of USV calls (i.e., here termed “syllables”) can thus be considered as a form of social communication and corresponding to the human infant crying (*21*). During normal postnatal development, pups’ USV gradually changes in acoustic features and syllable components (*22, 23*). In ASD model mice, many variations of USV parameters such as fewer calls, higher or lower frequency and shorter call duration are observed (*23-25*).

In this study, we have newly established semi-automatic and unsupervised modeling approaches, and comprehensively measured the maternal separation-induced USV of individual mouse pups during early postnatal development in the first two weeks. Not only comparing the developmental patterns of USV emitted from offspring’s groups derived from young and aged fathers, but also comprehensive analyses were performed by paying careful attention on individual variability in their syllable patterns. From the two different approaches, we conclude that paternal aging alters the trajectory of syllable development in both quantitative and qualitative aspects, and that paternal aging increases the individuals with atypical USV patterns during early infancy.

Paternal aging alters the acoustic features of syllables in offspring

Offspring were obtained by mating young wild type female mice together with either young (3 months) or aged (>12 months) wild type male mice (YFO and AFO, respectively, **Fig.1A**). We applied a semi-automatic procedure using USVSEG and an unsupervised modeling approach with variational autoencoder (VAE) to analyze the syllables. A large number of syllables were collected from YFO and AFO at postnatal day 3 (P3), P6, P9, and P12 (**Table S1**). The USVSEG showed that paternal aging altered the trajectories of overall syllables that emitted from AFO with a reduced number, shorter duration, and different patterns of the interval between two adjacent syllables (**Fig.1B**).

For deeper understanding, all syllables were first classified into twelve types (**Fig. 1C**) based on the shapes of spectrograms according to a previous report *(23, 26)*. The USV features of the twelve syllable types were showed in **Fig. S1**. In general, a total of nine types of syllables (upward, downward, short, chevron, wave, complex, one-jump, more-jumps and more-jumps + harmonics) showed significant alterations in the AFO. Among the altered syllables, six types of syllables (upward, chevron, wave, complex, more-jumps and more-jumps + harmonics) exhibited only the reduction in number; the alterations of two features were detected in the downward syllable (shorter duration and different development trajectory in intervals) and in the short syllable (less number and lower maximum frequency); meanwhile, one-jump syllable exhibited alterations in four features (less number, shorter duration, lower and different trajectory of maximum frequency, and different trajectory of intervals). Therefore, paternal aging impacted distinct types of syllables at different levels.

We further analyzed the composition of the twelve syllable types (**Fig. 1D** and **Table S2**). At P3, no statistical difference was detected. From P6 to P12, the AFO emitted USV calls with significantly different syllable components. Compared with the syllables of YFO on each postnatal day, AFO emitted higher percentages of “downward” syllable at P6, P9 and P12, along with “flat”, “short” and “harmonics” syllables at P9. By contrast, other minor syllables such as “chevron”, “wave”, “complex”, “one-jump”, “more-jumps”, “one-jump + harmonics” and “more-jumps + harmonics” showed lower percentage in the AFO during postnatal development. These results showed that paternal aging led to significantly different compositions of distinct syllables.

Moreover, we focused on the diversity of the syllables. Our results showed that YFO developed to emit more types of syllables along with postnatal days, while the AFO produced significantly fewer syllable types from P3 to P12 (**Fig. 1E**). To evaluate the different diversity of the syllable types between the two groups, we calculated the entropy scores as an indicator of production uniformity across the syllable types in individual offspring (*22*) (**Fig. 1F**). As we expected, a continuous rise of the entropy scores was observed in all the offspring during development, although the AFO always showed lower entropy scores compared with those from YFO across all postnatal stages. The data reflected that the AFO exhibited fewer types and narrower diversity of syllables, meaning that they had a poorer repertoire in vocalization.

For capturing important USV variation missed by USVSEG and preserving as much information as possible from an objective perspective, we introduced an unsupervised modeling approach VAE to characterize and quantify the subtle change in behavioral variability. A series of points were mapped in the inferred latent space and visualized (**Fig. 1G**). The number of syllables was detected by VAE demonstrated again that the AFO emitted a smaller number of syllables ($p = 0.028$, ANOVA) (**Table S1**). Moreover, a unique syllable distribution in each developmental stage across YFO and AFO confirmed a dynamic process indeed existed in the USV early development. Interestingly, the patterns of syllable distribution were similar between YFO and AFO at P3, while the differences became apparent between the groups from P6 to P12, especially at P12. Although quantitative and comprehensive analyses were performed based on the data in the section below (**Fig. 3**), the major difference shown in **Fig. 1G** is that an aggregated distribution pattern was obvious in YFO, but less in AFO. The missing part of aggregated syllables might include the important syllables that might reflect developmental milestones and indicate the deficiency of vocal development in AFO. Our two different approaches reached the same results that paternal aging indeed altered the syllable development and led to deficiencies of composition and diversity.

Paternal aging increases individuals with atypical patterns in syllable development

We then addressed the "individual" differences among pups belonging to YFO and AFO groups because ASD children often exhibit "atypical" development. In order to understand the longitudinal syllable development in individuals, based on the USVSEG data, clustering analyses with Gaussian mixture models were applied first. The Akaike Information Criterion (AIC) was applied to determine the cluster number objectively.

Because the number and duration of the overall syllables showed the positive correlation (Pearson correlation coefficient 0.519, $p < 0.001$: YFO; 0.511, $p < 0.001$: AFO), and the syllables emitted from AFO significantly decreased their number and duration, we first separated the offspring into different clusters based on the syllable number and duration (**Fig. 2A**). AIC implied that the choice of five clusters is optimal. The developmental patterns of the syllable number and duration in YFO were distributed to five clusters and concentrated on the fourth cluster, whereas the patterns in AFO only dispersed among four clusters and focused on the third cluster. Chi-square independence test revealed that the clustering patterns, i.e., the proportion of individuals in each cluster, were significantly different between the YFO and AFO ($p = 0.02$). These results exhibited that the dominant developmental patterns of the syllable number and duration were different between YFO and AFO.

We next classified the syllable types into four clusters based on AIC (**Fig. 2B**). Interestingly, the syllables emitted from YFO were classified into three clusters, while those from AFO were into four clusters. Most of the syllable emitted from the YFO belonged to the second cluster, whereas most of those from the AFO to the first cluster. Interestingly, the third cluster only included the individuals of AFO. The cluster distribution was significantly different between the two groups ($p = 0.003$,

chi-squared test). Furthermore, we clustered the normalized entropy to clarify individual development in syllable diversity (**Fig. 2C**). The number of clusters was selected to five based on AIC. The cluster distribution was significantly different between the YFO and AFO (chi-squared test, $p < 0.001$). YFO occupied four clusters and dominated in the fourth cluster, while the AFO did five clusters and dominated in the first cluster. Again, the second cluster only included the individuals of AFO. Data shown here indicated the diversity in developmental patterns of syllables in YFO, which differs from those of AFO. Therefore, the longitudinal development patterns of offspring were found to be significantly influenced by paternal aging and some patterns were unique only for the individuals of AFO.

To trace the developmental patterns among individuals, we further applied PCA analyses using USVSEG data to summarize the syllable features of each individual (**Fig. 3A**). At P3, the circles including 90% of the offspring's data were large, indicating that each pup exhibits a wide variety of individual difference in vocal communication regardless of the paternal age. However, the minor difference between YFO and AFO was already significant ($p = 0.024$, MANOVA). The difference became much clearer from P6 to P12 (P6, $p = 0.007$; P9, $p = 0.004$; P12, $p < 0.0001$, MANOVA); the YFO showed a developmental convergence in vocal communication patterns, while the variability regions of AFO were kept wider. In other words, the variability of individuals in the AFO was extremely clear. We also applied t-SNE using VAE data to analyze the USV repertoire in individual pup (**Fig. 3B**). Corresponding to the syllable map (**Fig. 1G**), the syllable development also displayed as a dot for each individual. Again, the vocal repertoires of YFO demonstrated the developmental convergence even though their trajectory differed, whereas those of AFO scattered here and there during development. The most significant difference was also observed at P12 ($p < 0.0001$, MANOVA), consistent with the PCA result. We then summarized the individual trace and highlighted the initial stage (P3) and the terminal stage (P12) (**Fig. 3C**). In both PCA and t-SNE, it was clear that at P3, the inferred spatial positions of YFO and AFO were similar, while spatial distributions of YFO and AFO were significantly different in later stages. Finally, the two different approaches verified the same finding that paternal aging led to misregulation of the vocal development inducing more atypical individuals.

Discussion

In the study, we reported for the first time in the mouse model that paternal aging affects developmental trajectory at early postnatal stages using sophisticated analyses with two different approaches. Considering epidemiological observation in human studies suggesting paternal aging as one of the risk factors for ASD in offspring (*13-16, 27*), our study in mice indeed suggests that the age of the father actually gives a significant effect on the alterations of vocal communicative behavior in infant mice.

Previous studies have shown that pup's USV can be analogous to human baby's cry and thus used as one of a few tools to understand behavioral development during the early postnatal period (*28, 29*). In our study, YFO emitted the diverse types of syllables during the postnatal periods, which is consistent with a previous study (*22*).

However, compared with YFO, the AFO emitted a narrower spectrum of the syllable types, which reminds us a poorer repertoire in language ability in some of ASD patients. The restricted syllable diversity has also been described in genetic ASD models, i.e., *Cd157* KO mice (*30*) and *Tbx1*-deficient heterozygous mice (*31*). Additionally, we observed the different composition of the syllables in AFO, which has previously been reported in other genetic ASD mouse models such as *Reelin* mutant (*32*), *fmr1* knockout (*33*) and *ScSn-Dmd*^{mdx}*/J* mutant (*34*). It is thus reasonable to assume that paternal aging leads to ASD-like impairments in syllable development and diversity.

We highlight here that our study successfully revealed the "atypical" USV development in individual mice. Compared with behavioral development of children with typical development (so called "neurotypical"), ASD children show the variety of "atypical" behavioral phenotypes (*7, 35, 36*); they do not uniformly exhibit impaired scores in various criteria. Moreover, these atypical behaviors grow distinct with age. Some ASD-specific behaviors (e.g. impairment in social cognition, eye contact, language abilities) are not obvious under 6 months, but become gradually clear from the latter part of the first year and second years (*37-39*). Correspondingly, in our mouse model, all of the cluster analyses, PCA and tSNE expressed unique patterns in each individual mouse pup in the YFO, whereas more "atypical" patterns were observed in the AFO. We also found that YFO exhibited a typical development, which shows gradual convergence during development; while AFO showed greater variabilities, which became most significant at the latest postnatal day we examined (P12). It is worth mentioning that the data-driven approach of VAE was first applied in our analyses of USV development. The dynamic mapping of syllable development in both group and individual levels clearly showed common and individually diverse features during USV development. We would emphasize that the two different approaches yielded a common conclusion that paternal aging indeed leads to a deficiency development of vocal communication in atypical individuals. For early intervening ASD, our study spotlights again the significance of identifying the "atypical", or "neuro-diverse" behaviors and elucidating the underlying neural basis at the early infancy.

Materials and Methods

Animals

All experimental procedures were approved by the Ethics Committee for Animal Experiments of Tohoku University Graduate School of Medicine (#2014-112) and animals were treated according to the National Institutes of Health guidance of the care and use of laboratory animals. Three-month-old (young) or >12-month-old (aged) male C57BL/6J mice were crossed with 10-week-old (young) virgin female C57BL/6J mice. After mating, each female mouse was separated from male mouse and isolated alone to minimize possible confounding factors against the behavior of offspring. In this study, 32 offspring were obtained from 5 young fathers and 29 offspring from 5 aged fathers. Offspring that died during experiment periods were excluded from analyses (mortality ratio, 5.7%: YFO vs 12.1%: AFO). At postnatal

day 3 (P3), each offspring was tattooed with an Aramis Animal Microtattoo System (Natsume Co., Ltd., Tokyo, Japan) for individual recognition after the USV test (described below). The average litter size and number of pups were not significantly different between YFO and AFO; i.e., 7.00 ± 0.35 (n = 32) and 6.6 ± 0.97 (n = 29) in YFO and AFO, respectively (p = 0.68, two-tailed t-test). Therefore, we assume that the factor by litter size would be neutral to evaluation of paternal aging effects on the offspring's vocal communication. All animals were housed in standard cages in a temperature and humidity-controlled room with a 12-hour light/dark cycle (light on at 8:00) and had free access to standard lab chow and tap water.

USV collection

According to previously described protocols (*26*, *40-41*), each pup separated from its mother and littermates one by one and placed on a transparent plastic dish with wood chip bedding, and accessed within the sound-attenuating chamber for USV test on P3, P6, P9 and P12. An ultrasound microphone (Avisoft-Bioacoustics CM16/CMPA) was placed through a hole in the middle of the cover of the chamber, about 10 cm above the offspring in its dish to record their vocalizations. The recorded vocalizations were transferred to the UltraSound Gate 416H detector set (Avisoft Bioacoustics, Germany) at 25-125 kHz. After a 5-min recording session, pups were measured their body weight and returned to the nest. This procedure was repeated in sequence until all pups had completed the recording phase. Both male and female pups were analyzed. Room temperature was maintained at 22˚C.

Syllable segmentation and classification

Acoustic waveforms were processed using a GUI-based MATLAB script ("USVSEG") originally developed for segmenting rodents' ultrasonic vocalizations (*42*). Briefly, the script computed the spectrograms from each waveform (60 seconds/block), put a threshold to eliminate the noise component of the signal and detected syllables within a frequency range of 60-120 kHz. A criterion of 10-ms minimum gap was used to separate two syllables and 2-ms as the minimum duration of a syllable. The duration, inter-syllable interval, maximum frequency (peak frequency at maximum amplitude) and maximum amplitude of each syllable were calculated automatically by the program script. The syllable intervals of distinct types were identified as the intervals between the specific type of syllable and the following syllable. If the inter-syllable interval is wider than 250 ms, this interval will be identified as a silence gap. Segmented syllables were manually classified into twelve categories of syllable types by visual inspection of enhanced spectrograms which were generated by the MATLAB program script. Ten of the syllable types (#1-10 below) were similar to previous descriptions (*23,26*). Noise sounds that were mistakenly segmented by the program (e.g. scratching noise) were manually identified and eliminated from further analyses. Definitions of syllable categories are below (see also Fig 1C):

1) Upward syllables were upwardly modulated with a terminal frequency change ≥ 6.25 kHz than the beginning of the syllable.

2) Downward syllables were downwardly modulated with a terminal frequency change ≥ 6.25 kHz than the beginning of the syllable.
3) Flat syllables were continuous with a frequency modification ≤ 3 kHz.
4) Short syllables were displayed as a dot and shorter than or equal to 5 ms.
5) Chevron syllables were formed like a U or a reversed U.
6) Wave syllables were regulated with two directional changes in frequency > 6.25 kHz.
7) Complex syllables were regulated with three or more directional changes in frequency > 6.25 kHz.
8) One-jump syllables contained two components, in which the second component was changed ≥10 kHz frequency than the first component and there was no time interval between the two components.
9) More-jumps syllables contained three or more than three components, in which the second component was changed ≥10 kHz frequency than the first and third components respectively. There was no time interval between adjacent components.
10) Harmonics syllables were displayed as one main component stacking with other harmonically components of different frequencies.
11) One-jump + harmonics syllables were contained one jump syllable and harmonics syllable together and there was no time interval between each other.
12) More-jumps + harmonics syllables were contained more jump syllable and harmonics syllable together and there was no time interval between each other.

Vocalization characterization

The variational autoencoder (VAE) (*43*), which is an unsupervised learning method and does not rely on syllable boundaries, was introduced to apply the task of characterizing vocalization. The VAE enable reduce the dimensions of raw data and quantify subtle changes in behavioral variability on tens-of-milliseconds timescales in order to preserve as much information as possible. We implemented the VAE in PyTorch (v1.1.0) and trained to maximize the standard evidence lower bound. The latent dimension was fixed to 32 because previous study found 32 is sufficient for all training runs. The USV syllables were detected and segmented by USVSEG with the following parameters: frequency range = 60-120 kHz; minimum gap = 10-ms; minimum duration = 2-ms. After training, the false positive syllables were removed as noise syllables. Note that we excluded several pups (i.e., 2 of YFO and 4 of AFO at P3; 2 of AFO at P6; 2 of AFO at P12) from this analysis because the VAE did not detect syllables in these pups. The VAE was trained to map single-syllable spectrogram images which were visualized in **Fig. 1G**. To visualize the individual variation across individuals, a t-distributed Stochastic Neighbor Embedding (t-SNE) was computed for each pup and showed in **Fig. 3B-3C**. Distances between points represent the similarity in vocal repertoires.

Statistical analysis

For better visualization, the acoustic features of syllables are presented as Z score (Z score = (score – Mean) / Standard Deviation). A two-way analysis of variance (ANOVA) with False Discovery Rate (FDR) correction (0.05) was used to investigate the statistical significance of syllables data which includes Z score and original data of number, duration, maximum frequency, maximum amplitude and interval of overall and distinct syllables. Two main effects (i.e., father's age and postnatal day effect) and the interaction (i.e., father's age × postnatal day effect) were examined by ANOVA. A multivariate analysis of variance (MANOVA) was performed to detect the difference of syllable percentage component, principle components, and t-SNEs between the YFO and AFO in each postnatal day with the independent variables of father's age. *Post-hoc* comparisons were performed using two-tailed *t*-test with Benjamini-Hochberg correction (BH correction) to detect the difference between two groups in each postnatal day when ANOVA revealed the significant interaction (paternal aging × postnatal day effect). The correlation between body weight and USV parameters was detected by the Pearson correlation coefficient.

To detect the diversity of syllable types, we used the information entropy as a measure of uniformity in production rates across syllable types for each offspring. The entropy score was ranged between 0 and 1. The score gets close to 1 when the animal produced all the syllable types evenly (or diversely), while it becomes closer to 0 if the animal preferred to produce fewer specific syllables types (less diversely). We obtained this entropy score by the following calculation:

$$\text{entropy} = \frac{-\sum_{t=1}^{T} p_t \log_2 p_t}{\log_2 T}$$

where, T indicates the number of syllable types, and p_t means the production rate of a specific syllable type t. Note that we excluded several offspring (i.e., 2 of YFO and 4 of AFO at P3; 2 of AFO at P6; 2 of AFO at P9; 2 of AFO at P12) from this analysis since the total number of syllables in 5 minutes were insufficient (less than 10) to analyze their entropies. The entropy scores were compared between the YFO and AFO across different postnatal days by using two-way ANOVA. To understand the individual development, clustering analysis with Gaussian mixture models (GMMs) was applied, where the data dimension is eight corresponding to the number and duration of syllables at four time points. We fit GMMs with diagonal covariance Gaussian components by the MATLAB function *fitgmdist*. The number of clusters was selected by minimizing the Akaike Information Criterion (AIC) (*44-45*). Based on the fitted GMM, we classified each individual mouse pup into the cluster with maximum posterior probability. Then the chi-square independence test was applied to determine whether the cluster distribution was significantly different between the two groups. Principal Component Analysis (PCA) was performed to objectively characterize the typical syllable patterns of individual offspring. In the present study, the syllable data including syllable number, number of types, duration, maximum frequency and maximum amplitude were normalized by Z score, then inputted for the PCA to generate principal components. For all comparisons, significance was set at $p = 0.05$. JMP13 Pro software (SAS Institute, Cary, NC, USA) was used for statistical

analyses. Values are shown as mean ± standard error of the mean (S.E.M.) for each group. Scatter plots shown in Fig. 3A and 3B, or 3C were drawn by JMP13 Pro software or R software, respectively (*46*).

References and Notes

1. C. Acebo, E. B. Thoman, Crying as social behavior. *Infant Mental Health Journal* **13**, 67-82 (1992).
2. C. Acebo, E. B. Thoman, Role of infant crying in the early mother-infant dialogue. *Physiology and Behavior* **57**, 541-547 (1995).
3. J. Soltis, The signal functions of early infant crying. *Behav Brain Sci.* **27**, 443-490 (2004).
4. J. Dudek, A. Faress, M. H. Bornstein, D. W. Haley, Infant cries rattle adult cognition. *PLoS One* **11**, e0154283 (2016).
5. S. J. Sheinkopf, J. M. Iverson, M. L. Rinaldi, B. M. Lester, Atypical cry acoustics in 6-month-old infants at risk for autism spectrum disorder. *Autism Res* **5**, 331-339 (2012).
6. L. M. Unwin *et al.*, Acoustic properties of cries in 12-Month old infants at high-risk of autism spectrum disorder. *J Autism Dev Disord* **47**, 2108-2119 (2017).
7. American Psychiatric Association, *Diagnostic and statistical manual of mental disorders: DSM-5 (5th ed.).* (American Psychiatric Association, Washington, DC, 2013).
8. S. E. Folstein, J. Piven, Etiology of autism: genetic influences. *Pediatrics* **87**, 767-773 (1991).
9. L. J. Lotspeich, R. D. Ciaranello, The neurobiology and genetics of infantile autism. *Int Rev Neurobiol* **35**, 87-129 (1993).
10. G. B. Schaefer, N. J. Mendelsohn, Genetics evaluation for the etiologic diagnosis of autism spectrum disorders. *Genet Med* **10**, 4-12 (2008).
11. S. Tordjman *et al.*, Gene x environment interactions in autism spectrum disorders: role of epigenetic mechanisms. *Frontiers in Psychiatry* **5**, (2014).
12. S. Nardone, D. S. Sams, A. Zito, E. Reuveni, E. Elliott, Dysregulation of cortical neuron DNA methylation profile in autism spectrum disorder. *Cereb Cortex* **27**, 5739-5754 (2017).
13. A. Reichenberg *et al.*, Advancing paternal age and autism. *Arch Gen Psychiatry* **63(9)**, 1026-1032 (2006).
14. C. M. Hultman, S. Sandin, S. Z. Levine, P. Lichtenstein, A. Reichenberg, Advancing paternal age and risk of autism: new evidence from a population-based study and a meta-analysis of epidemiological studies. *Mol Psychiatry* **16**, 1203-1212 (2011).
15. S. Lundstrom *et al.*, Trajectories leading to autism spectrum disorders are affected by paternal age: findings from two nationally representative twin studies. *J Child Psychol Psychiatry* **51**, 850-856 (2010).
16. K. M. Lampi *et al.*, Parental age and risk of autism spectrum disorders in a Finnish national birth cohort. *J Autism Dev Disord* **43**, 2526-2535 (2013).

17. E. D. Jarvis, Evolution of vocal learning and spoken language. *Science* **366**, 50-54 (2019).
18. R. Mooney, The neurobiology of innate and learned vocalizations in rodents and songbirds. *Current opinion in neurobiology* **64**, 24-31 (2020).
19. F. D'Amato, E. Scalera, C. Sarli, A. Moles, Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? . *Behav Genet.* **35 (1)**, 103-112 (2005 Jan).
20. M. E. Hahn, M. J. Lavooy, A Review of the methods of studies on infant ultrasound production and maternal retrieval in small rodents. *Behav Genet.* **35 (1)**, 31-52 (2005).
21. G. Esposito, N. Hiroi, M. L. Scattoni, Cry, baby, cry: expression of distress as a biomarker and modulator in autism spectrum disorder. *International Journal of Neuropsychopharmacology* **20**, 498-503 (2017).
22. J. M. Grimsley, J. J. Monaghan, J. J. Wenstrup, Development of social vocalizations in mice. *PLoS One* **6**, e17460 (2011).
23. M. L. Scattoni, S. U. Gandhy, L. Ricceri, J. N. Crawley, Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. *PLoS One* **3**, e3067 (2008).
24. M. Wohr, Ultrasonic vocalizations in Shank mouse models for autism spectrum disorders: detailed spectrographic analyses and developmental profiles. *Neurosci Biobehav Rev* **43**, 199-212 (2014).
25. J. D. Dougherty *et al.*, The disruption of Celf6, a gene identified by translational profiling of serotonergic neurons, results in autism-related behaviors. *J Neurosci* **33**, 2732-2753 (2013).
26. T. Kikusui *et al.*, Cross fostering experiments suggest that mice songs are innate. *PLoS ONE* **6 (3)**, e17721 (2011).
27. B. E. Itzchak, E. Lahat, D. A. Zachor, Advanced parental ages and low birth weight in autism spectrum disorders--rates and effect on functioning. *Res Dev Disabil* **32**, 1776-1781 (2011).
28. I. Branchi, D. Santucci, E. Alleva, Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. *Behav Brain Res* **125**, 49-56 (2001).
29. M. L. Scattoni, J. Crawley, L. Ricceri, Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. *Neurosci Biobehav Rev* **33**, 508-515 (2009).
30. O. L. Lopatina, K. Furuhara, K. Ishihara, A. B. Salmina, H. Higashida, Communication Impairment in Ultrasonic Vocal Repertoire during the Suckling Period of Cd157 Knockout Mice: Transient Improvement by Oxytocin. *Frontiers in Neuroscience* **11**, (2017).
31. T. Takahashi *et al.*, Structure and function of neonatal social communication in a genetic mouse model of autism. *Mol Psychiatry* **21**, 1208-1214 (2016).
32. E. Romano, C. Michetti, A. Caruso, G. Laviola, M. L. Scattoni, Characterization of neonatal vocal and motor repertoire of reelin mutant mice. *PLoS One* **8**, e64407 (2013).

33. J. K. Lai *et al.*, Temporal and spectral differences in the ultrasonic vocalizations of fragile X knock out mice during postnatal development. *Behav Brain Res* **259**, 119-130 (2014).
34. R. Miranda *et al.*, Altered social behavior and ultrasonic communication in the dystrophin-deficient mdx mouse model of Duchenne muscular dystrophy. *Mol Autism* **6**, 60 (2015).
35. R. Grzadzinski, M. Huerta, C. Lord, DSM-5 and autism spectrum disorders (ASDs): an opportunity for identifying ASD subtypes. *Mol Autism* **4**, (2013).
36. R. H. Wozniak, N. B. Leezenbaum, J. B. Northrup, K. L. West, J. M. Iverson, The development of autism spectrum disorders: variability and causal complexity. *Wiley Interdiscip Rev Cogn Sci* **8**, e1426 (2017).
37. L. Zwaigenbaum *et al.*, Behavioral manifestations of autism in the first year of life. *Int J Dev Neurosci* **23**, 143-152 (2005).
38. J. Piven, J. T. Elison, M. J. Zylka, Toward a conceptual framework for early brain and behavior development in autism. *Mol Psychiatry* **22**, 1385-1394 (2017).
39. S. Ozonoff *et al.*, A Prospective study of the emergence of early behavioral signs of autism. *J Am Acad Child Adolesc Psychiary* **49**, 256-266 (2010).
40. K. Yoshizaki et al., Paternal Aging Affects Behavior in Pax6 Mutant Mice: A Gene/Environment Interaction in Understanding Neurodevelopmental Disorders. *PLoS ONE* 11, e0166665 (2016).
41. K. Yoshizaki, K. Koike, R. Kimura, N. Osumi, Early postnatal vocalizations predict sociability and spatial memory in C57BL/6J mice: Individual differences in behavioral traits emerge early in development. *PLoS ONE* 12, e0186798 (2017).
42. R. O. Tachibana, K. Kanno, S. Okabe, K. I. Kobayasi, K. Okanoya, USVSEG: A robust method for segmentation of ultrasonic vocalization in rodents. *PLoS ONE*, 15, e0228907 (2020).
43. J. Goffinet, R. Mooney, J. Pearson, Inferring low-dimensional latent descriptions of animal vocalizations. *bioRxiv*, (2019).
44. Konishi S, Kitagawa G. Information Criteria and Statistical Modeling. Springer-Verlag New York Press; 2008.
45. Mclachlan G, Peel D. Finite Mixture Models. 1st ed. Wiley-Interscience Press; 2000.
46. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/ (2016)

Acknowledgments:
The authors thank Ms. Sayaka Makino for animal care. The authors also appreciate all members of their laboratory for contributive discussions. **Funding**: This work was supported by KAKENHI in the Innovative Areas (Grant Number 16H06530) from MEXT. **Author contributions**: L.M. and N.O. conceived and designed experiments; L.M. collected all the USV data; L.M. performed the USVSEG analyses and the

statistical analyses; R.K. performed the VAE analyses; K.K. provided technical support of USVSEG analyses; R.K., H.I., T.M., R.T. and F.K. provided technical support of statistical analyses; All authors discussed and interpreted results; L.M., R.K., H.I. and N.O. wrote the manuscript with input from all authors. **Competing interests**: Authors declare no competing interests. **Data and materials availability**: All data are available in the manuscript or in the supplementary materials.

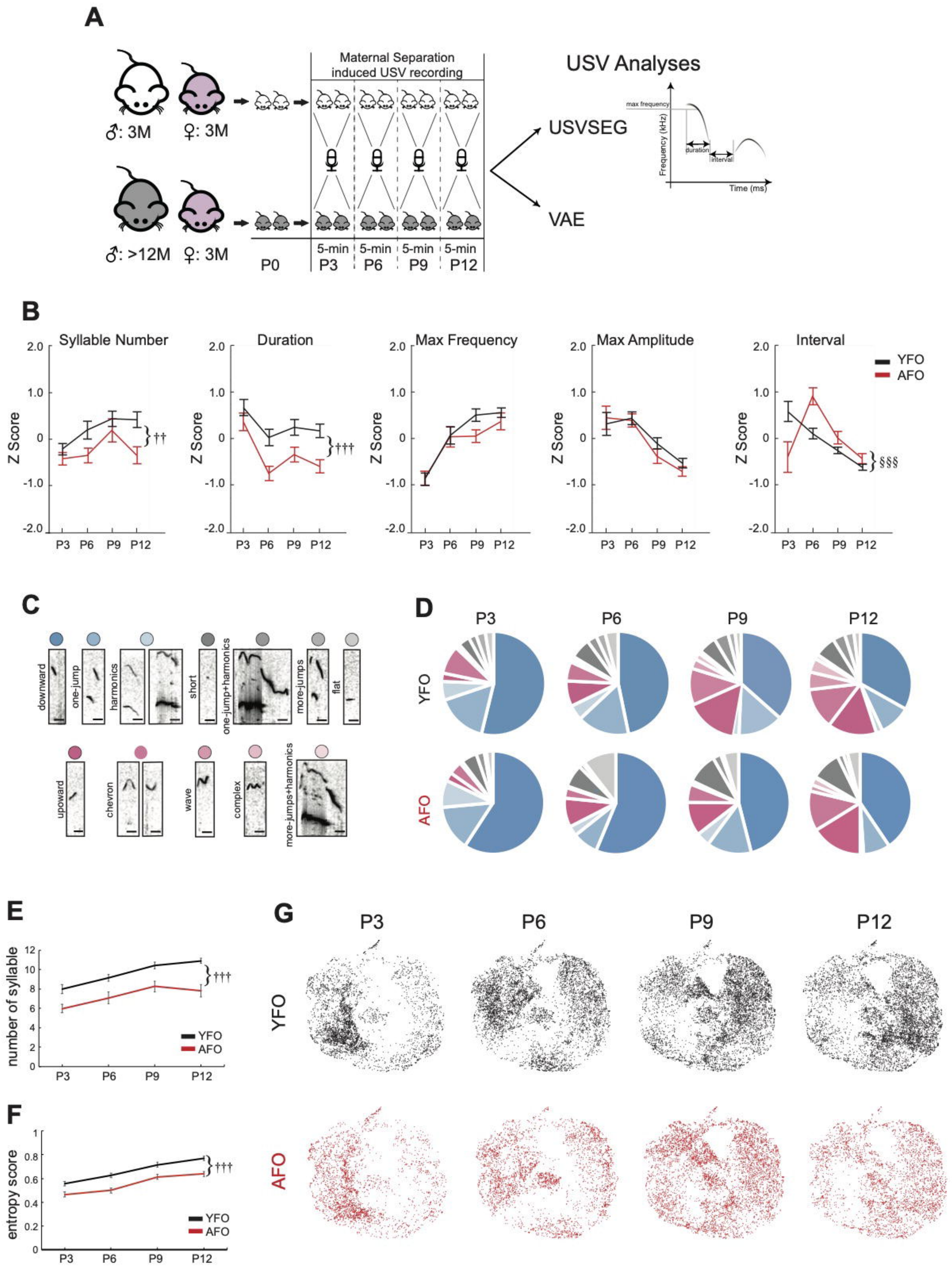
Legend

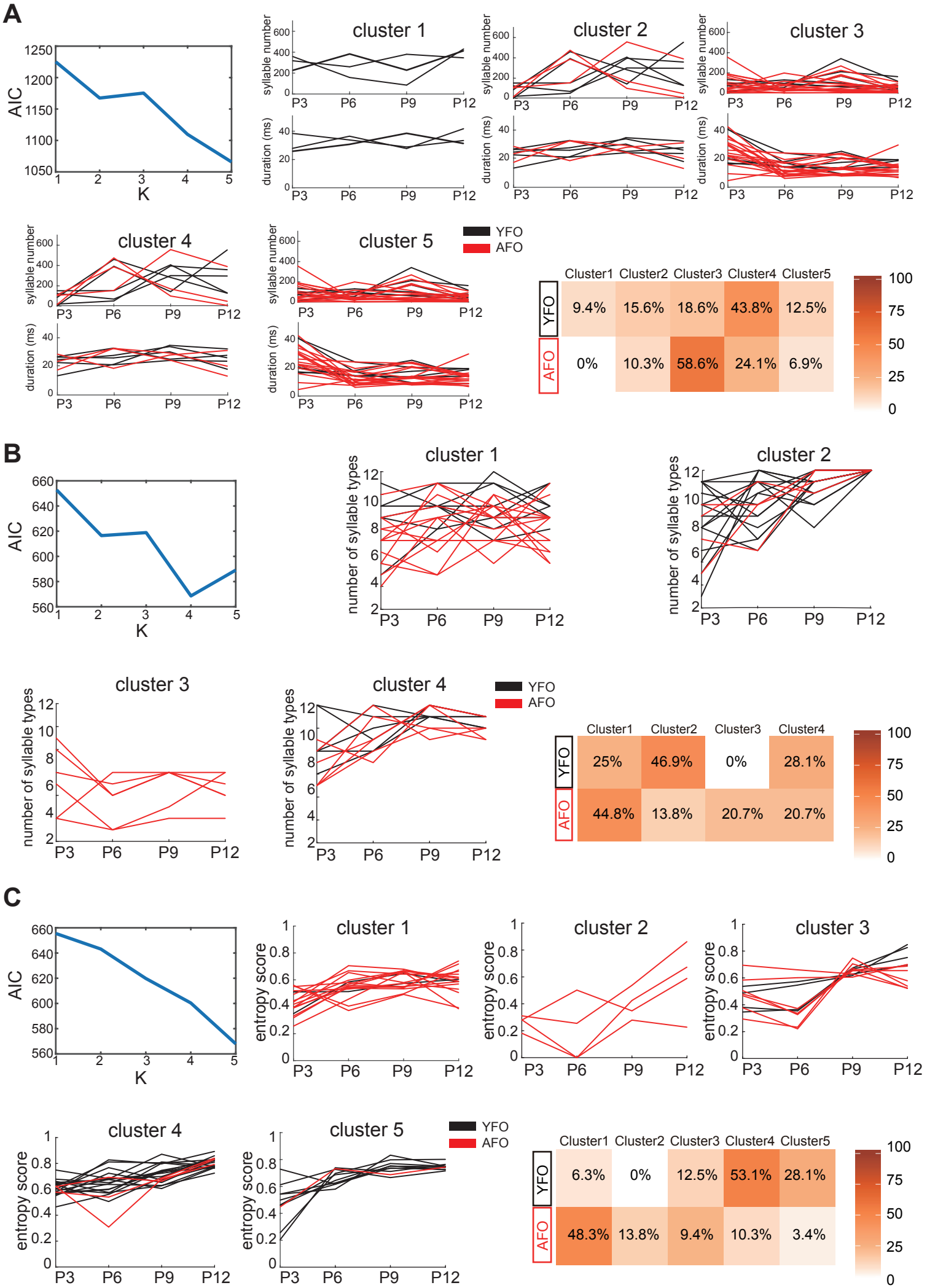
Fig.1. Paternal aging alters the acoustic features of syllables in offspring. (**A**) Experimental design. (**B**) Z scores of syllable number, duration, maximum frequency, maximum amplitude and interval were obtained from YFO and AFO. $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ indicates a significant main effect of father's age, $^{\S\S\S}p < 0.001$ indicates a significant interaction of father's age × day effect (Two-way ANOVA). (**C**) Typical sonograms of twelve types of syllables obtained from USVSEG. Scale bar = 60 kHz, 20 ms. (**D**) Pie graphs exhibit the percentage composition of twelve types of syllables from P3 to P12 in YFO and AFO. The color indicates the syllable type that corresponds to (C). The colors of magenta, blue and grey spectra indicate the syllable with increased, decreased and non-specifically changed percentages in YFO, respectively. (**E**) The developmental trajectories of syllable types in YFO and AFO. $^{\dagger\dagger\dagger}p < 0.001$ indicates a significant main effect of the father's age (Two-way ANOVA). (**F**) Normalized entropy scores of syllables from YFO and AFO. $^{\dagger\dagger\dagger}p < 0.001$ indicates a significant main effect of the father's age (Two-way ANOVA). (**G**) The VAE takes spectrograms from YFO and AFO as input, maps and visualizes them to a vector of latent dimensions from P3 to P12. Each point in this latent space represents a single spectrogram of syllable. Each point in the figure represents a single syllable spectrogram. The distance between points is indicated as a neighbor similarity, with closer points more similar.

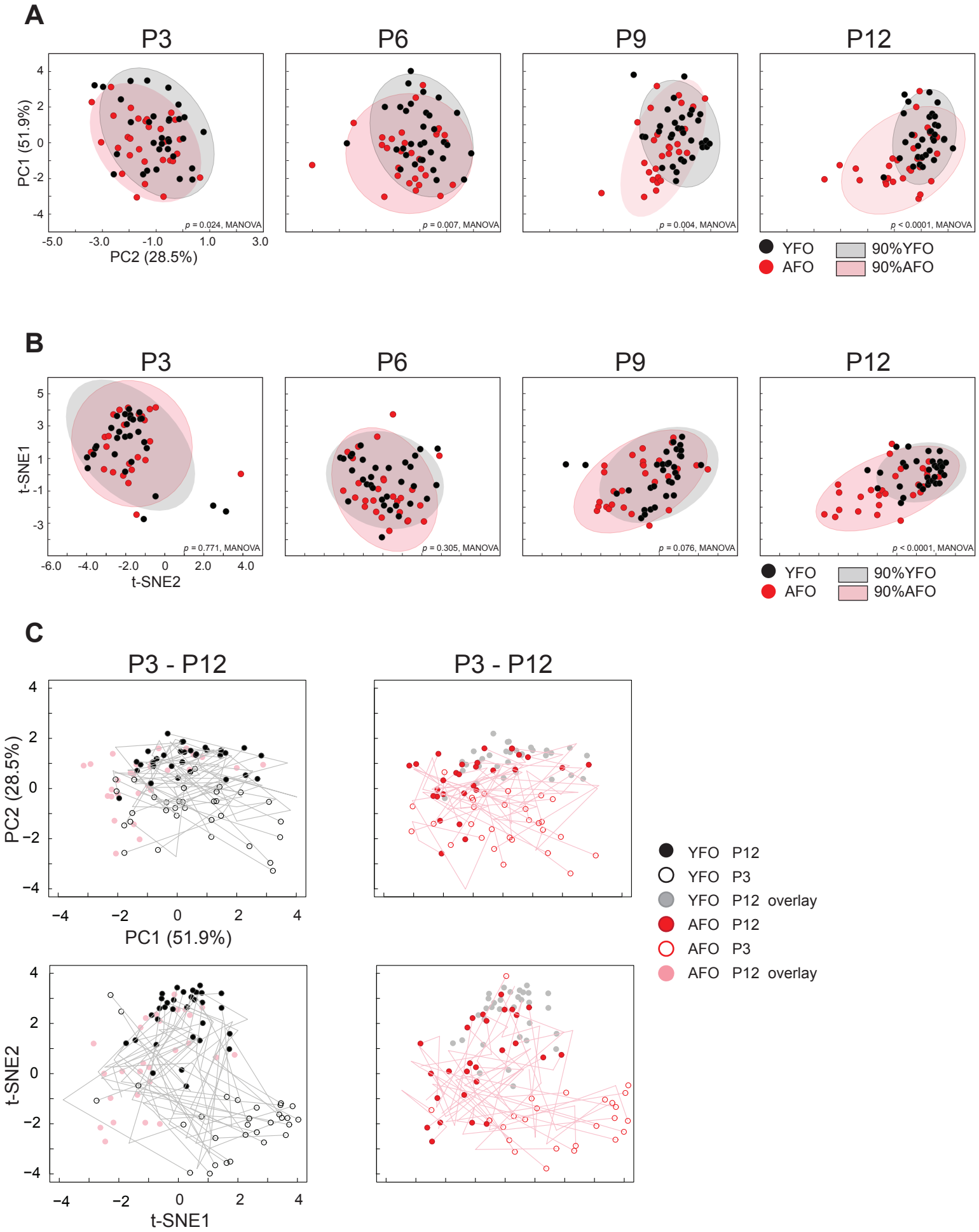
Fig. 2. Clustering analyses reveals the individuals' differences between YFO and AFO. (**A**) Separation of individual patterns according to the syllable number and duration. The cluster number was chosen as five by using AIC as the minimum number (shown on X-axis) with AIC values (Y-axis). Clustering analyses separated the individual offspring into five clusters according to the syllable number and duration. The heat map summarized the percentages of YFO and AFO that were distributed to each cluster. (**B**) Separation of individual patterns according to the syllable types. The cluster number was chosen as four by using AIC as the minimum number (shown on X-axis) with AIC values (Y-axis). Clustering analyses separated the individual offspring into four clusters according to the syllable types. The heat map summarized the percentages of YFO and AFO that were distributed to each cluster. (**C**) Separation of individual patterns according to the normalized entropy. The cluster number was chosen as five by using AIC as the minimum number (shown on X-axis) with AIC values (Y-axis). Clustering analyses separated the individual offspring into five clusters according to the normalized entropy. The heat map summarized the percentages of YFO and AFO that were distributed to each cluster.

Fig. 3. Paternal aging increases individuals with atypical development pattern. (**A**) Based on USVSEG, PCA summarized individual pattern of YFO and AFO from P3 to P12. Each point represents one pup's syllable features. Closer distances between points represent more similar in syllable features. The circles in gray or red included a 90% population of YFO or AFO. (**B**) Based on VAE, t-SNE summarized individual pattern of YFO and AFO from P3 to P12. Each point represents one pup's syllable features. Closer distances between points represent more similar in vocal repertoires. The circles in gray or red included a 90% population of YFO or AFO. (**C**) a summary and highlight of the individual trace at P3 and P12 based on (**A**) and (**B**),. Each point represents one pup's syllable features.

Fig. S1. Paternal aging alters the developmental trajectories of distinct types of syllables. The syllable number, duration, maximum frequency, maximum amplitude and interval of distinct types of syllables from YFO and AFO. (**A**)-(**L**) show the developmental trajectories of five syllable features in twelve types of syllables. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ indicates a significant main effect of father's age. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ indicates a significant interaction of father's age × day effect (two-way ANOVA).


A
♂: 3M
♀: 3M
♂: >12M
♀: 3M
P0
Maternal Separation induced USV recording
5-min P3
5-min P6
5-min P9
5-min P12
USV Analyses
USVSEG
VAE
max frequency
Frequency (kHz)
duration
interval
Time (ms)
B
Syllable Number
Duration
Max Frequency
Max Amplitude
Interval
Z Score
YFO
AFO
P3 P6 P9 P12
C
downward
one-jump
harmonics
short
one-jump+harmonics
more-jumps
flat
upoward
chevron
wave
complex
more-jumps+harmonics
D
P3
P6
P9
P12
YFO
AFO
E
number of syllable
YFO
AFO
F
entropy score
YFO
AFO
G
P3
P6
P9
P12
YFO
AFO


A
AIC
K
cluster 1
cluster 2
cluster 3
cluster 4
cluster 5
syllable number
duration (ms)
P3
P6
P9
P12
YFO
AFO
Cluster1 Cluster2 Cluster3 Cluster4 Cluster5
YFO 9.4% 15.6% 18.6% 43.8% 12.5%
AFO 0% 10.3% 58.6% 24.1% 6.9%
B
number of syllable types
Cluster1 Cluster2 Cluster3 Cluster4
YFO 25% 46.9% 0% 28.1%
AFO 44.8% 13.8% 20.7% 20.7%
C
entropy score
Cluster1 Cluster2 Cluster3 Cluster4 Cluster5
YFO 6.3% 0% 12.5% 53.1% 28.1%
AFO 48.3% 13.8% 9.4% 10.3% 3.4%


A
P3
P6
P9
P12
PC1 (51.9%)
PC2 (28.5%)
p = 0.024, MANOVA
p = 0.007, MANOVA
p = 0.004, MANOVA
p < 0.0001, MANOVA
YFO
AFO
90%YFO
90%AFO
B
t-SNE1
t-SNE2
p = 0.771, MANOVA
p = 0.305, MANOVA
p = 0.076, MANOVA
p < 0.0001, MANOVA
C
P3 - P12
PC2 (28.5%)
PC1 (51.9%)
t-SNE2
t-SNE1
YFO P12
YFO P3
YFO P12 overlay
AFO P12
AFO P3
AFO P12 overlay