Paternal aging causes atypical development of vocal communication in neonatal mice

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

[1]Department of Developmental Neuroscience, Graduate School of Medicine, Tohoku University, Sendai, Japan

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

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

[4]Mathematical Informatics Collaboration Unit, RIKEN Center for Brian Science, Wako, Japan

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

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

Abstract

Autism Spectrum Disorder (ASD) is one of the neurodevelopmental disorders and characterized with persistent impairments in social communication (including verbal and nonverbal communication) and repetitive behavior together with various comorbid symptoms. Epidemiological studies suggest a significant association between advanced paternal age and incidence of ASD in offspring, which has been modeled in rodents. However, how paternal aging makes an impact on the offspring's early communicative behavior, especially at the individual level, has not been addressed. Here we systematically analyzed postnatal development of vocal communication of pups by measuring ultrasonic vocalization (USV) induced by maternal separation that is considered to correspond to baby's cry, an earliest verbal communication in human. Maternal separation-induced USV of each offspring derived from young (3 months) or aged (>12 months) father was individually measured for 5 minutes at postnatal day 3 (P3), P6, P9, and P12. We classified USV syllables into twelve types according to Scattoni's classification with minor modifications, and analyzed duration, maximum frequency, maximum amplitude and interval of each syllable. Compared between the two groups, the offspring derived from aged fathers emitted the syllables with less number, shorter duration, different syllable components and less diversity. From an individual perspective, offspring derived from young father showed a developmental convergence in vocal communication, while those from aged fathers exhibited atypical developmental patterns. Taken together, we showed for the first time a significant influence of paternal aging on early vocal development with qualitative and quantitative aspects in individual mice.

Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by two core symptoms; i.e., social interaction impairment (including verbal and nonverbal communication deficits) and repetitive behavior[1-3]. In most cases, the exact etiology of ASD remains unclear, although researchers believe that genetic, environmental and epigenetic factors are involved in the neuropathology of ASD[4-6]. In recent years, epidemiological studies repeatedly suggest a significant association between paternal aging and a risk of ASD in offspring. Compared with the children of young fathers, the children were more likely to be diagnosed ASD if their fathers were older[7-10]. Thus, paternal aging can be worth to be focused as one of the non-genetic mechanisms leading to ASD.

In the new criteria of DSM-5, the communication and social interaction parts are combined into one, i.e., “Social/Communication Deficits”. This modification emphasizes the importance of the social communication domain in ASD in early infancy. Human infant crying is an innate social communication[11, 12], which has a natural peak in frequency of approximately 2.5 hours of crying per day at around 6-8 weeks of age[13]. The infant crying can attract attention from caregivers[14] and influences adult cognitive control[15]. It is reported that different crying patterns such as higher frequency and shorter duration are observed in infants with a high risk of ASD[16, 17]. Interpretation of autistic infant crying is, however, may have limitations in separating possible etiological factors as well as in the sample size.

Rodents have been serving as suitable models to better understand genetical and environmental factors of ASD[18, 19]. To elucidate mechanisms underlying communication impairment in early postnatal stages, ultrasonic vocalization (USV) of pups induced by maternal separation has recently been paid more attention. The separation of a pup from its mother and littermates induces the pup to emit USV calls, which triggers maternal approach and retrieval behavior[20, 21]. The patterns of USV calls (i.e., here termed "syllables") can be seen as a form of social communication and corresponding to the infant crying[22]. During normal postnatal development, murine pups' USV gradually changes in acoustic features and syllable components[23, 24]. In mouse models for ASD, researchers observe many variations of USV parameters such as fewer calls, higher or lower frequency and shorter call duration [24-26]. However, how paternal aging impacts early developmental patterns of detailed USV, especially from an individual level, has not been addressed yet.

Here we measured the maternal separation-induced USV of individual mouse pups during postnatal development at four stages. Not only comparing the developmental patterns of USV emitted from offspring derived from young and aged father, but also comprehensive analyses were performed by paying careful attention on individual diversity in their syllables. We found that paternal aging influenced the trajectory of syllable development in both quantitative and qualitative aspects, inducing more atypical individuals derived from aged fathers.

Results

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). The average litter size and sample size 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 (*t*-test for litter size, $p = 0.68$). Therefore, we assume that our experimental setup was suitable to evaluate paternal aging effects on the offspring's vocal communication.

1. USV measurement

To examine the effects of paternal aging on pup's USV, a large number of syllables were collected from YFO and AFO at postnatal day 3 (P3), P6, P9, and P12 (Table 1).

To highlight differences between the groups of YFO and AFO clearly, the acoustic features of USV were transformed to Z score (i.e., calculated by (Score – Mean) / Standard Deviation), and shown in Fig 2 (raw data are shown in Supplementary Fig 1). The syllables emitted from AFO exhibited the reduced number, shorter duration and different trajectory of the interval between two adjacent syllables (Fig. 2A, 2B and 2E). The number of syllables emitted from all offspring reached a peak at P9 and was affected significantly by main effects of paternal aging [father's age: $F(1, 236) = 12.79$, $p = 0.00127$] and postnatal day [day: $F(3, 236) = 4.54$, $p = 0.006$], but not by interaction of paternal aging × postnatal day effect (Fig. 2A). The syllable duration altered during postnatal development and showed a similar downward trend curve in both groups, showing the lowest point at P6 (Fig. 2B). The duration of overall syllables was influenced significantly by main effects of paternal aging [father's age: $F(1, 236) = 27.16$, $p < 0.001$] and postnatal day [day: $F(3, 236) = 10.83$, $p < 0.001$],

but not by interaction of paternal aging × postnatal day effect. In our observation, no significant paternal age effect was detected in the syllable maximum frequency and amplitude (Fig. 2C, D). During development, the interval between two adjacent syllables kept decreasing in the YFO, while in the AFO, the interval peaked at P6, then decrease from P6 to P12 (Fig. 2E). A significant interaction between the main effects of the paternal aging and postnatal day was observed [father's age × day: $F(3, 236) = 10.18$, $p < 0.001$]. Taken together, USV from AFO showed significantly different developmental patterns in the overall syllable number, duration and interval between two adjacent syllables, but not in maximum frequency and maximum amplitude across development. These results indicate that paternal aging may alter the developmental trajectory of overall syllables in quantitative and qualitative aspects.

2. Detailed analyses of syllable types

For deeper understanding of the paternal aging effects on the syllable patterns across the early postnatal period, all USV syllables induced by maternal separation were classified into twelve types (Fig. 1B) based on the shapes of spectrograms according to a previous report [24]. The USV features of twelve types' syllables were transformed into Z score and showed in Fig. 3. In general, a total of nine types of syllables (upward, downward, short, chevron, wave, complex, one jump, more jump and more jump + harmonics) showed significant alterations in the AFO. Among the altered syllables, six types of syllables (upward, chevron, wave, complex, more jump and more jump + harmonics) exhibited the reduced number only; the alterations of two features were detected in the downward syllable (shorter duration and different development trajectory in interval) and 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 interval). Therefore, paternal aging influences distinct syllables at different levels.

2.1. The Number of individual types of syllables

After classification of the syllable types, we found that the main effect of paternal aging significantly decreased the number of eight types of the syllables such as "upward", "short", "chevron" "wave", "complex", "one jump", "more jump", and "more jump + harmonics" (Fig. 3A, 3D-I and 3L, Supplementary Fig. 2A, 2D-I, 2L, and Table 2). We did not detect "wave" syllables in the AFO at P3. These eight types of syllables may contribute to the reduced number of the overall syllables emitted by the AFO.

2.2. Duration of individual types of syllables

We found that the main effect of paternal aging significantly affected duration of "downward" [father's age: $F(1, 234) = 22.74$, $p < 0.001$] and "one jump" [father's age: $F(1, 215) = 7.99$, $p = 0.008$] syllables during development (Fig. 3B and 3H, Supplementary Fig. 3B and 3H). Moreover, the significant interaction of two main effects of the paternal aging and postnatal day was exhibited in the duration of "one jump" syllables [father's age × day: $F(3, 215) = 2.97$, $p = 0.033$]. The shorter duration of overall syllables in the AFO may attribute to the changes of "downward" and "one jump" syllables.

2.3 Maximum frequency of individual types of syllables

Although we did not find a significant alteration of maximum frequency in the overall syllables emitted from the AFO, detailed analyses indicated that “short” [father’s age: $F(1, 219) = 11.66$, $p = 0.002$] and “one jump” [father’s age: $F(1, 215) = 7.49$, $p = 0.02$] syllables were influenced significantly by the main effect of paternal aging (Fig. 3D and 3H, Supplementary Fig. 4D and 4H); they showed lower maximum frequency in the AFO. Moreover, two main effects of paternal aging and postnatal day were interacted significantly in “one jump” syllables [father’s age × day: $F(3, 215) = 3.03$, $p = 0.046$]. Paternal aging thus affected the maximum frequency at the level of two syllables.

2.4 Maximum amplitude of individual types of syllables

After the classification of all syllables, there were again no significant main effects of paternal aging and postnatal day in maximum amplitude (Fig. 3 and Supplementary Fig. 5). The alteration of syllable maximum amplitude by paternal aging was not observed after detailed analyses.

2.5 Interval of individual types of syllables

We further analyzed the interval data in all of 12 different syllable types to identify any alteration in the interval of specific syllables. Significant interaction between the two main effects of paternal aging and postnatal day was detected in the interval of “downward” syllable [father’s age × day: $F(3, 223) = 7.22$, $p < 0.001$] and “one jump” syllable [father’s age × day: $F(3, 197) = 3.15$, $p = 0.039$] (Fig. 3B and 3H, Supplementary Fig. 6B and 6H). These data imply that relatively simple syllables such as “downward” and “one jump” may contribute to the altered trajectory of the syllable interval in overall syllables.

2.6 The percentage composition of the twelve types syllables

Next, we analyzed the composition of the twelve syllable types induced by maternal separation (Fig. 4A and Table 3). MANOVA was performed to detect the difference of syllable percentage composition between the two groups in each postnatal day we observed. At P3, no statistical difference was detected. From P6 to P12, the AFO emitted USV calls with a significantly different syllable component (Table 3). Compared with the syllables of YFO in 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 *t*-test). By contrast, other minor syllables such as “chevron”, “wave”, “complex”, “one jump”, “more jump”, “one jump + harmonics” and “more jump + harmonics” showed lower percentage in the AFO during postnatal development (Table 3). Therefore, the syllables emitted from the AFO had a significantly different composition of the distinct syllables across the four postnatal days.

2.7 Variation of syllables in offspring groups

Furthermore, we focused on the variation of syllables. Our results showed that YFO developed to emit more types of syllables along with postnatal days. In contrast, the AFO expanded the variation of the syllable types from P3 to P9, which became narrower from P9 to P12 (Fig. 4B). Likewise, significantly fewer syllable types were produced from the AFO from P3 to P12. The syllable types were affected significantly by two main effects of paternal aging [father’s age: $F(1, 236) = 58.32$, $p < 0.001$] and postnatal day [day: $F(3, 236) = 13.64$, $p < 0.001$], but not by interaction of paternal aging × postnatal day effect. To evaluate the different diversity of the

syllable types between the two groups, we calculated the entropy scores (see Methods for the formula) as an indicator of production uniformity across the syllable types in individual offspring (Fig. 4C). As we expected, entropy scores were also significantly influenced by two main effects of paternal aging [father's age: $F(1, 224) = 65.22$, $p < 0.001$] and postnatal day [day: $F(3, 224) = 40.55$, $p < 0.001$], but not by interaction of paternal aging × postnatal day effect. During development, a continuous rise of the entropy scores was observed in all the offspring, but 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 diversity of syllables, meaning that they had a narrower vocal repertoire.

3. Difference in individual developmental trajectories of vocal communication

In this study, we addressed the "individual" differences among pups belonging to YFO and AFO because ASD children exhibit "atypical" development. In order to understand the longitudinal syllable development in individuals, clustering analyses with Gaussian mixture models were applied first. Based on the Akaike Information Criterion (AIC), the cluster number was determined objectively (see Methods for details).

3.1 Clusters of the syllable number and duration

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. AIC implied that the choice of five clusters is optimal (Fig. 5A).

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 (Fig. 5B-5G). 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. Repeatedly, AFO showed less variation in the syllables.

3.2 Cluster of the diversity in the syllable types

For the identification of individual development, we next classified the syllable types into four clusters based on AIC (Fig. 6A). Interestingly, the syllables emitted from YFO were classified into three clusters, while those from AFO were classified into four clusters. Most of the syllable emitted from the YFO belonged to the second cluster, whereas most of those from the AFO belonged to the first cluster (Fig. 6B-6F). The cluster distribution was significantly different between the two groups (chi-squared test, $p = 0.003$). Furthermore, we clustered the normalized entropy to clarify individual development in syllable diversity. 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, $p < 0.001$). The YFO occupied four clusters and dominated the fourth cluster, while the AFO occupied five clusters and dominated the first cluster (Fig 7A-7G). Data showed here indicated that the developmental patterns of syllable diversity between the YFO, which differs from those of AFO.

Through the clustering analyses, individual differences of the developmental patterns in the syllable number, duration, types and diversity were clearly revealed. Therefore, the longitudinal development patterns of offspring were found to be significantly influenced by paternal aging.

3.3. Principal Component Analysis (PCA) of overall syllables

We further analyzed the vocal communication of offspring in regard with developmental diversity. We extracted principal components (PC) contributing to the individual character of overall syllables to pick up different features of individual YFO and AFO. The PC1 and PC2 that summarized more than 80% of the variability of the original data in overall syllables (Table 4) were plotted in Fig. 8. At P3, the circles including 90% of the offspring's data were large, indicating that each offspring exhibits a wide variety of individual difference in vocal communication regardless of the paternal age. It is of note that the circle areas were almost overlapping between the two groups of YFO (gray) and AFO (red) at P3. From P6 to P12, however, YFO showed a developmental convergence in vocal communication patterns, while the variability regions of AFO were kept wider, suggesting that the difference of individual difference in the AFO was still large. In other words, there were more individuals with "atypical development" within the AFO group. Through the horizontal comparison of each postnatal day, the present data clearly indicated that paternal aging resulted in greater individual diversity in regard with the syllable patterns.

4. Correlations between body weight and USV

We wondered above described difference in vocal communication could secondarily affect offspring's development. Therefore, we measured the body weight of offspring after the USV recording on P3, P6, P9 and P12. Statistical analyses clearly showed that offspring's body weight was affected significantly by two main effects of paternal aging [father's age: $F(1, 236) = 35.84$, $p < 0.001$] and postnatal day [day: $F(3, 236) = 874.91$, $p < 0.001$], but not by interaction of paternal aging × postnatal day effect (Supplementary Fig. 7). AFO exhibited a lower body weight gain throughout the four stages from P3 to P12. This result suggested that paternal aging, in general, induced lower body weight development in offspring during early postnatal development.

To clarify the correlations between body weight and USV, we first draw scatter plot graphs to visualize the data (Fig. 9). In general, the circles involving 90% of the offspring's data of correlations between body weight and USV were almost overlapping between the two groups at P3 (Fig. 9A-9F). From P6 to P12, however, the correlation between body weight and the number of syllable types gradually became diverse between the YFO and AFO; again, AFO displayed the wider variation than the YFO along with development (Fig. 9A). Furthermore, the correlation coefficients and *p*-value were calculated by the Pearson correlation coefficient (Table 5). The body weight of YFO had positive correlations with the number of syllables, the number of syllable types, and syllable duration at P6, as well as with syllable frequency at P12. By contrast, the body weight of AFO had a positive correlation with the number of syllables at P3, as well as with the number of syllable types at P6 and P9. The strongest positive correlation (correlation coefficient $\geq |0.5|$) we found was the correlation between body weight and the number of syllables in YFO at P6, whereas other correlation coefficient scores were not obvious; we could not find a

strong positive correlation between the syllables and body weight in AFO. For deeper analyses, the correlations between distinct types of syllables and body weight were examined at four developmental stages (Supplementary Table 1). In general, all types of syllables showed some correlations (positive or negative) with body weight at different stages, while more strong correlations (correlation coefficient ≥ |0.5|) were found in YFO. These data revealed again that AFO included more diverse and atypical individuals and abnormal developmental patterns.

Discussion

Our present study is the first report to analyze detailed vocal communication of early postnatal stages in a mouse model of paternal aging. We revealed qualitative and quantitative alterations of syllable development not only by comparison between YFO and AFO groups but also at the individual perspective, across the first two postnatal weeks. We applied comprehensive mathematical analyses because we can collect big data by recording USV (e.g. hundreds of syllables per one pup in five minutes). This has a great advantage compared with other conventional behavior tests that can collect only a small number of scores. Considering epidemiological observation in human studies suggesting paternal aging as one of the risk factors associated with autism in offspring[7-10, 27], our study in mice indeed suggests that paternal aging actually plays an important role in the alterations of communicative behavior in infant mice, a consistent result with alterations of social behaviors in adult offspring in mice derived from aged fathers[28-30].

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[31, 32]. We reported here that the USV features of AFO were generally consistent with other ASD models with the decreased vocal numbers[26, 33, 34] and shorter syllable durations[34, 35]. In our study, YFO emitted the diverse types of syllables during the early postnatal periods, which is consistent with a previous study[23]. However, compared with YFO, the AFO emitted a narrower spectrum of the syllable types. The restricted syllable diversity has also been described in genetic ASD models, i.e., *Cd157* KO mice[36] and *Tbx1*-deficient heterozygous mice[37]. Additionally, we observed the different composition of the syllables in AFO, which has previously been reported in another genetic ASD mouse models such as *Reelin* mutant[38], *fmr1* knockout[39] and *ScSn-Dmd*^{mdx}*/J* mutant[40]. It is thus reasonable to assume that paternal aging leads to impairment in syllable development, diversity and composition as shown in other genetic ASD models.

We noticed that the AFO exhibited lower body weight during the postnatal two weeks. The difference was not observed when they became adults, according to our unpublished data from different experiments used for behavior analyses in adult mice. In human studies, lower birth weight is reported to be associated with several neurodevelopmental difficulties such as learning disabilities[41], speech and language problems[42], and social problems[43]. Indeed, paternal aging-associated low birth weight has been considered as a high-risk factor of ASD[27, 44]. Therefore, it is assumed that paternal aging might cause offspring developmental delay at the physical level related to lower body weight, which could impact on early communicative problems in the offspring.

Another possibility that can explain the difference in body weight might be influenced by altered maternal care due to difference in vocal communication of offspring. It is known that pup's USV has communicative significance[45-49], which is crucial for maternal behaviors and preserves the social bonds between mother and infant that are essential for the healthy development of offspring[50, 51]. In our study, even though there were no strong correlations (correlation coefficient ≥ |0.5|) between the body weight and overall syllables, some strong correlations were found between distinct types of syllables and body weight. Expectedly, more correlations existed in YFO, meaning "typical" developmental trajectories. It is, however, difficult to understand at this moment what kind of information was transmitted by individual syllables or syllable sequences and how these syllables impact maternal behaviors. In the future, we need a more advanced method for syllable analyses to explore the deeper biological meaning of pup's vocalization and examine the possibility of whether maternal care may change due to altered USV of pups. So far, one of the available methods to investigate the behaviors of maternal care and mother-infant interaction is the playback and foster mother experiments which may help to understand whether the abnormal syllables of AFO have an actual influence on their mothers. A previous USV playback experiment indicated that the altered syllables in *Tbx1*-deficient heterozygous mice led to fewer maternal responses to pups' vocalization[37]. Thus, the alteration of USV development in AFO could be affected by not only the primary factor of paternal aging, but also the secondary factor coming from maternal care.

Interestingly, we have previously noticed that pup's USV is a potent predictor for sociability in adulthood; the number of USV at P6 was positively correlated with sociability (in three-chamber social interaction test) and negatively with spatial

memory (in Morris water maze)[52]. In human infant's cry, wider variation of the fundamental frequency is significantly reported to associate with better language and cognitive outcomes[53]. Because of no detailed syllable classification to understand the infant's cry, the variation of the fundamental frequency is considered as the complexity of vocal control. In this case, since the AFO model exhibited poorer vocal abilities than the YFO, the AFO might develop abnormal sociability (including vocal behavior) in adulthood. We did not include adult behavior analyses in this study because we noticed that repeated USV recording (including maternal separation) could affect behavior in later stages (unpublished results). Actually, infancy is a sensitive and crucial period to understand the behavioral and neuronal complications at the beginning of life. The childhood emotional and behavioral problems often correspond with early abnormal development in infants[54]. For instance, the infant nonoptimal neuromotor development might predict the emotional problems in childhood[55]; the problems of infant crying and feeding are prognostic to poor social skills [56, 57] and adverse cognitive development[58] at the age of preschool. Therefore, the infant's cry could be developed as an effective and noninvasive tool to evaluate and diagnose neurodevelopmental disorders such as ASD in the future.

The highlight of this study is that we successfully modeled the "atypical" USV development in the individual mouse by dimensional comparisons. Compared with typically behavioral development in normal children (so called "neurotypical"), ASD children show the variety of "atypical" behavioral phenotypes[1, 59, 60]; they do not uniformly exhibit impaired scores in various criteria. In regard to our mouse model during horizontal observation, the cluster preference of USV development expressed unique patterns in each individual mouse pup and also "atypical" patterns caused by

paternal aging. In humans, 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[54, 61, 62]. Through the vertical comparison at each postnatal day, we also found that the USV patterns of PCA analyses were not the same in individuals but exhibited a gradual convergence during development in the YFO. However, more diversities were observed and remained in the AFO. At P3 (early infancy), each offspring emitted USV calls in different variations, but after P6, the cohort of YFO gradually obtained "typical" variations in their communication patterns by P12 (later infancy). By contrast, the AFO still showed great variations at P12. For early intervening ASD, to identify the "atypical" behaviors and elucidate the underlying neural basis, especially at the early infancy (under 6 months in human and under P3 in mouse), would be the next challenge.

The current study clearly demonstrates that detailed qualitative and quantitative syllable analyses can be a useful tool for communicative phenotypes in a sensitive period during postnatal stages of mice. We notice that complex syllables seemed to be more affected in the AFO. The cerebellum is not fully developed during the first week, and in another study of ours[30], we observed impairments in the structure of the motor cortex and neuronal activity indicated with c-fos expression in the paraventricular thalamus related to anxiety. Therefore, we should pay more attention to various brain areas, e.g., including the brainstem that is important for motor control to emit USV. Because the early postnatal period is critical for the development of the central as well as peripheral neural systems in regard with communication behaviors, studies on infant crying and rodent USV could synergistically contribute to understand the

neural basis for typical and atypical development, which could provide a cue for early diagnosis and interventions for ASD.

Materials and methods

1. Animals and ethic statement

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 (Fig. 1A). 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). 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.

2. USV measurement

According to previously described protocols[32, 52, 63], each offspring 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). After a 5-min recording session, offspring were measured their body weight and returned to the nest. This procedure was repeated in sequence until all offspring had completed the recording phase. Both male and female pups were analyzed. Room temperature was maintained at 22˚C.

(3) Syllable segmentation and classification

Acoustic waveforms were processed using a GUI-based MATLAB script ("usvseg") originally developed for segmenting rodents' ultrasonic vocalizations[64]. 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 typical mice USVs (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 those previously described by Scattoni *et al.*[24]. 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 1B):

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 jump 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 jump + harmonics syllables were contained more jump syllable and

harmonics syllable together and there was no time interval between each other.

3. Statistical analysis

For better visualization, the acoustic features of syllables are presented as Z score in Fig 2 -3 (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, as well as body weight between the YFO and AFO across P3, P6, P9 and P12. 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 between the YFO and AFO in each postnatal day with the independent variables of father's age and dependent variables of the percentage of twelve syllable types. *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) for Supplementary Fig. 1-6. 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) [65-66]. 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. 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 used as input data for the PCA to generate principal components. The first principal component (PC1) alone is able to explain more than 50% of the variability of all input values, and PC1 plus second principal component (PC2) can illustrate more than 80% of the variability.

For all comparisons, significance was set at $p = 0.05$. JMP V13 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.

Acknowledgements

The authors thank Ms. Sayaka Makino for animal care. The authors also appreciate all members of their laboratory for contributive discussions. This work was supported by KAKENHI in the Innovative Areas (Grant Number 16H06530) from MEXT.

References

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders: DSM-5 (5th ed.). Washington, DC: American Psychiatric Association; 2013.

2. Folstein SE, Piven J. Etiology of autism: genetic influences. Pediatrics. 1991;87(5 Pt 2):767-73. PubMed PMID: 1708492.

3. Lotspeich LJ, Ciaranello RD. The neurobiology and genetics of infantile autism. Int Rev Neurobiol. 1993;35:87-129. PubMed PMID: 8463065.

4. Schaefer GB, Mendelsohn NJ. Genetics evaluation for the etiologic diagnosis of autism spectrum disorders. Genet Med. 2008;10(1):4-12. doi: 10.1097/GIM.0b013e31815efdd7. PubMed PMID: 18197051.

5. Tordjman S, Somogyi E, Coulon N, Kermarrec S, Cohen D, Bronsard G, et al. Gene x environment interactions in autism spectrum disorders: role of epigenetic mechanisms. Frontiers in Psychiatry. 2014;5. doi: 10.3389/fpsyt.2014.00053. PubMed PMID: 25136320

6. Nardone S, Sams DS, Zito A, Reuveni E, Elliott E. Dysregulation of cortical neuron DNA methylation profile in autism spectrum disorder. Cereb Cortex. 2017;27(12):5739-54. doi: 10.1093/cercor/bhx250. PubMed PMID: 29028941.

7. Reichenberg A, Gross R, Weiser M, Bresnahan M, Silverman J, Harlap S, et al. Advancing paternal age and autism. Arch Gen Psychiatry. 2006;63(9):1026-32. doi: 10.1001/archpsyc.63.9.1026. PubMed PMID: 16953005.

8. Hultman CM, Sandin S, Levine SZ, Lichtenstein P, Reichenberg A. Advancing paternal age and risk of autism: new evidence from a population-based study and a meta-analysis of epidemiological studies. Mol Psychiatry. 2011;16(12):1203-12. doi: 10.1038/mp.2010.121. PubMed PMID: 21116277.

9. Lundstrom S, Haworth CM, Carlstrom E, Gillberg C, Mill J, Rastam M, et al. Trajectories leading to autism spectrum disorders are affected by paternal age: findings from two nationally representative twin studies. J Child Psychol Psychiatry. 2010;51(7):850-6. doi: 10.1111/j.1469-7610.2010.02223.x. PubMed PMID: 20214699.

10. Lampi KM, Hinkka-Yli-Salomaki S, Lehti V, Helenius H, Gissler M, Brown AS, et al. Parental age and risk of autism spectrum disorders in a Finnish national birth cohort. J Autism Dev Disord. 2013;43(11):2526-35. doi: 10.1007/s10803-013-1801-3. PubMed PMID: 23479075.

11. Acebo C, Thoman EB. Crying as social behavior. Infant Mental Health Journal. 1992;13(1):67-82.

12. Acebo C, Thoman EB. Role of infant crying in the early mother-infant dialogue. Physiology and Behavior. 1995;57(3):541-7.

13. St James-Roberts I, Halil T. Infant crying patterns in the first year: normal community and clinical findings. J Child Psychol Psychiatry. 1991;32(6):951-68.

PubMed PMID: 1744198.

14. Soltis J. The signal functions of early infant crying. Behav Brain Sci. 2004;27:443-90. doi: 10.1017/s0140525x0400010x.

15. Dudek J, Faress A, Bornstein MH, Haley DW. Infant cries rattle adult cognition. PLoS One. 2016;11(5):e0154283. doi: 10.1371/journal.pone.0154283. PubMed PMID: 27191845; PubMed Central PMCID: PMCPMC4871531.

16. Sheinkopf SJ, Iverson JM, Rinaldi ML, Lester BM. Atypical cry acoustics in 6-month-old infants at risk for autism spectrum disorder. Autism Res. 2012;5(5):331-9. doi: 10.1002/aur.1244. PubMed PMID: 22890558; PubMed Central PMCID: PMCPMC3517274.

17. Unwin LM, Bruz I, Maybery MT, Reynolds V, Ciccone N, Dissanayake C, et al. Acoustic properties of cries in 12-Month old infants at high-risk of autism spectrum disorder. J Autism Dev Disord. 2017;47(7):2108-19. doi: 10.1007/s10803-017-3119-z. PubMed PMID: 28425020.

18. Crawley JN. Designing mouse behavioral tasks relevant to autistic-like behaviors. Ment Retard Dev Disabil Res Rev. 2004;10(4):248-58. doi: 10.1002/mrdd.20039. PubMed PMID: 15666335.

19. Kazdoba TM, Leach PT, Yang M, Silverman JL, Solomon M, Crawley JN. Translational mouse models of autism: advancing toward pharmacological therapeutics. Curr Top Behav Neurosci. 2016;28:1-52. doi: 10.1007/7854_2015_5003. PubMed PMID: 27305922; PubMed Central PMCID: PMCPMC5116923.

20. D'Amato F, Scalera E, Sarli C, Moles A. Pups call, mothers rush: does maternal responsiveness affect the amount of ultrasonic vocalizations in mouse pups? . Behav Genet. 2005 Jan;35 (1):103-12. doi: 10.1007/s10519-004-0860-9. PubMed PMID: 15674537.

21. Hahn ME, Lavooy MJ. A Review of the methods of studies on infant ultrasound production and maternal retrieval in small rodents. Behav Genet. 2005;35 (1):31-52. doi: 10.1007/s10519-004-0854-7. PubMed PMID: 15674531.

22. Esposito G, Hiroi N, Scattoni ML. Cry, baby, cry: expression of distress as a biomarker and modulator in autism spectrum disorder. International Journal of Neuropsychopharmacology. 2017;20(6):498-503. doi: 10.1093/ijnp/pyx014.

23. Grimsley JM, Monaghan JJ, Wenstrup JJ. Development of social vocalizations in mice. PLoS One. 2011;6(3):e17460. doi: 10.1371/journal.pone.0017460.

24. Scattoni ML, Gandhy SU, Ricceri L, Crawley JN. Unusual repertoire of vocalizations in the BTBR T+tf/J mouse model of autism. PLoS One. 2008;3(8):e3067. doi: 10.1371/journal.pone.0003067. PubMed PMID: 18728777; PubMed Central PMCID: PMCPMC2516927.

25. Wohr M. Ultrasonic vocalizations in Shank mouse models for autism spectrum disorders: detailed spectrographic analyses and developmental profiles. Neurosci Biobehav Rev. 2014;43:199-212. doi: 10.1016/j.neubiorev.2014.03.021. PubMed PMID: 24726578.

26. Dougherty JD, Maloney SE, Wozniak DF, Rieger MA, Sonnenblick L, Coppola G, et al. The disruption of Celf6, a gene identified by translational profiling of serotonergic neurons, results in autism-related behaviors. J Neurosci. 2013;33(7):2732-53. doi: 10.1523/JNEUROSCI.4762-12.2013. PubMed PMID: 23407934; PubMed Central PMCID: PMCPMC3711589.

27. Itzchak BE, Lahat E, Zachor DA. Advanced parental ages and low birth weight in autism spectrum disorders--rates and effect on functioning. Res Dev Disabil. 2011;32(5):1776-81. Epub 2011 Apr 17. doi: 10.1016/j.ridd.2011.03.004. PubMed PMID: 21498045.

28. Foldi CJ, Eyles DW, McGrath JJ, Burne TH. Advanced paternal age is associated with alterations in discrete behavioural domains and cortical neuroanatomy of C57BL/6J mice. Eur J Neurosci. 2010;31(3):556-64. doi: 10.1111/j.1460-9568.2010.07074.x. PubMed PMID: 20105239.

29. Janecka M, Manduca A, Servadio M, Trezza V, Smith R, Mill J, et al. Effects of advanced paternal age on trajectories of social behavior in offspring. Genes Brain Behav. 2015;14(6):443-53. doi: 10.1111/gbb.12227. PubMed PMID: 26096767.

30. Yoshizaki K, Koike T, Kimura R, Kikkawa T, Oki S, Koike K, et al. Paternal age affects offspring's behavior prossibly via an epigenetic machanism recruiting a transciptional repressor REST. bioRxiv. 2019. doi: 10.1101/550095.

31. Branchi I, Santucci D, Alleva E. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav Brain Res. 2001;125(1-2):49-56. doi: 10.1016/S0166-4328(01)00277-7. PubMed PMID: 11682093.

32. Scattoni ML, Crawley J, Ricceri L. Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci Biobehav Rev. 2009;33(4):508-15. doi: 10.1016/j.neubiorev.2008.08.003. PubMed PMID: 18771687; PubMed Central PMCID: PMCPMC2688771.

33. Wohr M, Roullet FI, Hung AY, Sheng M, Crawley JN. Communication impairments in mice lacking Shank1: reduced levels of ultrasonic vocalizations and scent marking behavior. PLoS One. 2011;6(6):e20631. doi: 10.1371/journal.pone.0020631. PubMed PMID: 21695253; PubMed Central PMCID: PMCPMC3111434.

34. Yang L, Faraone SV, Zhang-James Y. Autism spectrum disorder traits in Slc9a9 knock-out mice. Am J Med Genet B Neuropsychiatr Genet. 2016;171B(3):363-76. doi:

10.1002/ajmg.b.32415. PubMed PMID: 26755066.

35. Gaub S, Groszer M, Fisher SE, Ehret G. The structure of innate vocalizations in Foxp2-deficient mouse pups. Genes Brain Behav. 2010;9(4):390-401. doi: 10.1111/j.1601-183X.2010.00570.x. PubMed PMID: 20132318; PubMed Central PMCID: PMCPMC2895353.

36. Lopatina OL, Furuhara K, Ishihara K, Salmina AB, Higashida H. Communication Impairment in Ultrasonic Vocal Repertoire during the Suckling Period of Cd157 Knockout Mice: Transient Improvement by Oxytocin. Frontiers in Neuroscience. 2017;11. doi: 10.3389/fnins.2017.00266.

37. Takahashi T, Okabe S, Broin PO, Nishi A, Ye K, Beckert MV, et al. Structure and function of neonatal social communication in a genetic mouse model of autism. Mol Psychiatry. 2016;21(9):1208-14. doi: 10.1038/mp.2015.190. PubMed PMID: 26666205; PubMed Central PMCID: PMCPMC4909589.

38. Romano E, Michetti C, Caruso A, Laviola G, Scattoni ML. Characterization of neonatal vocal and motor repertoire of reelin mutant mice. PLoS One. 2013;8(5):e64407. doi: 10.1371/journal.pone.0064407. PubMed PMID: 23700474; PubMed Central PMCID: PMCPMC3660261.

39. Lai JK, Sobala-Drozdowski M, Zhou L, Doering LC, Faure PA, Foster JA. Temporal and spectral differences in the ultrasonic vocalizations of fragile X knock out mice during postnatal development. Behav Brain Res. 2014;259:119-30. doi: 10.1016/j.bbr.2013.10.049. PubMed PMID: 24211451.

40. Miranda R, Nagapin F, Bozon B, Laroche S, Aubin T, Vaillend C. Altered social behavior and ultrasonic communication in the dystrophin-deficient mdx mouse model of Duchenne muscular dystrophy. Mol Autism. 2015;6:60. doi: 10.1186/s13229-015-0053-9. PubMed PMID: 26527530; PubMed Central PMCID:

PMCPMC4627616.

41. Johnson EO, Breslau N. Increased risk of learning disabilities in low birth weight boys at age 11 years. Biol Psychiatry. 2000;47(6):490-500. doi: 10.1016/S0006-3223(99)00223-1.

42. Aram DM, Hack M, Hawkins S, Weissman BM, Borawski-Clark E. Very-low-birthweight children and speech and languange development. Journal of Speech, Language, and Hearing Research. 1991 34:1169-79. doi: 10.1044/jshr.3405.1169. PubMed PMID: 1749247.

43. Hack M, Taylor HG, Klein N, Eiben R, Schatschneider C, Mercuri-Minich N. School-age outcomes in children with birth weights under 750g. N Engl J Med. 1994;331:753-9. doi: 10.1056/NEJM199409223311201. PubMed PMID: 7520533.

44. Kolevzon A, Gross R, Reichenberg A. Prenatal and perinatal risk factors for autism: a review and integration of findings. Arch Pediatr Adolesc Med. 2007;161(4):326-33. doi: 10.1001/archpedi.161.4.326. PubMed PMID: 17404128.

45. Hashimoto H, Saito TR, Furudate S, Takahashi K. Prolactin levels and maternal behavior induced by ultrasonic vocalizations of the rat pup. Exp Anim. 2001;50(4):307-12. doi: 10.1538/expanim.50.307. PubMed PMID: 11515093.

46. Liu RC, Miller KD, Merzenich MM, Schreiner CE. Acoustic variability and distinguishability among mouse ultrasound vocalizations. J Acoust Soc Am. 2003;114(6):3412-22. doi: 10.1121/1.1623787. PubMed PMID: 14714820.

47. Liu RC, Linden JF, Schreiner CE. Improved cortical entrainment to infant communication calls in mothers compared with virgin mice. Eur J Neurosci. 2006;23(11):3087-97. doi: 10.1111/j.1460-9568.2006.04840.x. PubMed PMID: 16819999.

48. Ehret G, Bernecker C. Low-frequency sound communication by mouse pups

(Mus musculus): wriggling calls release maternal behaviour. Animal Behaviour. 1986;34(3):821-30. doi: 10.1016/S0003-3472(86)80067-7.

49. Kikusui T, Hiroi N. A Self-Generated Environmental Factor as a Potential Contributor to Atypical Early Social Communication in Autism. Neuropsychopharmacology. 2017;42(1):378. doi: 10.1038/npp.2016.225. PubMed PMID: 27909329; PubMed Central PMCID: PMCPMC5143512.

50. Mogi K, Takakuda A, Tsukamoto C, Ooyama R, Okabe S, Koshida N, et al. Mutual mother-infant recognition in mice: The role of pup ultrasonic vocalizations. Behav Brain Res. 2017;325(Pt B):138-46. doi: 10.1016/j.bbr.2016.08.044. PubMed PMID: 27567527.

51. Mogi K, Nagasawa M, Kikusui T. Developmental consequences and biological significance of mother-infant bonding. Prog Neuropsychopharmacol Biol Psychiatry. 2011;35(5):1232-41. doi: 10.1016/j.pnpbp.2010.08.024. PubMed PMID: 20817069.

52. Yoshizaki K, Koike K, Kimura R, Osumi N. Early postnatal vocalizations predict sociability and spatial memory in C57BL/6J mice: Individual differences in behavioral traits emerge early in development. PLoS One. 2017;12(11):e0186798. doi: 10.1371/journal.pone.0186798. PubMed PMID: 29091920; PubMed Central PMCID: PMCPMC5665508.

53. Shinya Y, Kawai M, Niwa F, Imafuku M, Myowa M. Fundamental Frequency Variation of Neonatal Spontaneous Crying Predicts Language Acquisition in Preterm and Term Infants. Front Psychol. 2017;8. PubMed PMID: doi:10.3389/fpsyg.2017.02195; PubMed Central PMCID: PMCPMC5744644.

54. Zwaigenbaum L, Bryson S, Rogers T, Roberts W, Brian J, Szatmari P. Behavioral manifestations of autism in the first year of life. Int J Dev Neurosci. 2005;23(2-3):143-52. doi: 10.1016/j.ijdevneu.2004.05.001. PubMed PMID:

15749241.

55. Serdarevic F, Ghassabian A, van Batenburg-Eddes T, Tahirovic E, White T, Jaddoe VWV, et al. Infant Neuromotor Development and Childhood Problem Behavior. Pediatrics. 2017;140(6). doi: 10.1542/peds.2017-0884. PubMed PMID: 29138362.

56. Schmid G, Schreier A, Meyer R, Wolke D. A prospective study on the persistence of infant crying, sleeping and feeding problems and preschool behaviour. Acta Paediatr. 2010;99(2):286-90. doi: 10.1111/j.1651-2227.2009.01572.x. PubMed PMID: 19886897.

57. Winsper N, Wolke D. Infant and Toddler Crying, Sleeping and Feeding Problems and Trajectories of Dysregulated Behavior Across Childhood. Journal of Abnormal Child Psychology. 2014;42(5):831-43. doi: 10.1007/s10802-013-9813-1).

58. Wolke D, Schmid G, Schreier A, Meyer R. Crying and feeding problem in Infancy and cognitive outcome in preschool children born at risk: a prospective population study. J Dev Behav Pediatr. 2009;30(3):226-38. doi: 10.1097/DBP.0b013e3181a85973. PubMed PMID: 19433987.

59. Grzadzinski R, Huerta M, Lord C. DSM-5 and autism spectrum disorders (ASDs): an opportunity for identifying ASD subtypes. Mol Autism. 2013;4(12). doi: 10.1186/2040-2392-4-12. PubMed PMID: 2375638.

60. Wozniak RH, Leezenbaum NB, Northrup JB, West KL, Iverson JM. The development of autism spectrum disorders: variability and causal complexity. Wiley Interdiscip Rev Cogn Sci. 2017;8(1-2):e1426. doi: 10.1002/wcs.1426. PubMed PMID: 27906524.

61. Piven J, Elison JT, Zylka MJ. Toward a conceptual framework for early brain and behavior development in autism. Mol Psychiatry. 2017;22(10):1385-94. doi:

10.1038/mp.2017.131. PubMed PMID: 28937691; PubMed Central PMCID: PMCPMC5621737.

62. Ozonoff S, Losif AM, Baguio F, Cook IC, Hill MM, Hutman T, et al. A Prospective study of the emergence of early behavioral signs of autism. J Am Acad Child Adolesc Psychiary. 2010;49(3):256-66. PubMed PMID: 20410715.

63. Yoshizaki K, Furuse T, Kimura R, Tucci V, Kaneda H, Wakana S, et al. Paternal Aging Affects Behavior in Pax6 Mutant Mice: A Gene/Environment Interaction in Understanding Neurodevelopmental Disorders. PLoS One. 2016;11(11):e0166665. doi: 10.1371/journal.pone.0166665. PubMed PMID: 27855195; PubMed Central PMCID: PMCPMC5113965.

64. Tachibana RO, Kanno K, Okabe S, Kobayasi KI, Okanoya K. Usvseg: A robust segmentation of rodents' ultrasonic vocalization. bioRxiv. 2019. doi: 10.1101/572743.

65. Konishi S, Kitagawa G. Information Criteria and Statistical Modeling. Springer-Verlag New York Press; 2008.

66. Mclachlan G, Peel D. Finite Mixture Models. 1st ed. Wiley-Interscience Press; 2000.

Figure Legends

Fig. 1. Experiment design and syllable types.

(A) 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 to obtain offspring. On P3, P6, P9 and P12, each offspring was separated from the mother to record the USV for 5 minutes. After data collection, detailed analyses were performed. (B) Typical sonograms of USV are classified into twelve types of syllables such as

downward, one jump, harmonics, upward, chevron, wave, complex, more jump + harmonics, short, one jump + harmonics, more jump, and flat. Scale bar = 50 kHz, 20 ms. The colors of magenta, blue and grey spectra indicate the syllables with increased, decreased and non-specifically changed percentages, respectively, during the development of offspring derived from young father.

Fig. 2. The Z score trajectories of USV features in overall syllable.

Z scores of syllable number (A), duration (B), maximum frequency (C), maximum amplitude (D) and interval (E) were obtained from USV data of offspring derived from young (YFO) and aged (AFO) fathers. Two-way ANOVA was used to compare the differences between the two groups. Data are presented as means ± SEM. $^{††}p < 0.01$, $^{†††}p < 0.001$ indicates a significant main effect of father's age, $^{§§§}p < 0.001$ indicates a significant interaction of father's age × day effect.

Fig. 3. The Z score trajectories of USV features in individual type of syllables.

The syllable number, duration, maximum frequency, maximum amplitude and interval of individual type of syllables from offspring derived from young (YFO) and aged (AFO) fathers are transformed to Z score. (A)-(L) show the developmental trajectories of five USV features in the individual type of syllables. Two-way ANOVA was used to compare the differences between the two groups. Data are presented as means ± SEM. $^{†}p < 0.05$, $^{††}p < 0.01$, $^{†††}p < 0.001$ indicates a significant main effect of father's age. $^{§}p < 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.001$ indicates a significant interaction of father's age × day effect.

Fig. 4. The composition of the twelve types syllables

(A) Pie graphs exhibited the percentage composition of the twelve types of syllables from P3 to P12 in offspring derived from young (YFO) and aged (AFO) fathers. Data are presented as means. The colors of magenta, blue and grey spectra indicate the syllables with increased, decreased and non-specifically changed percentages, respectively, during the development of the YFO. (B) The developmental trajectories in regard to the variation of the syllable types in YFO and AFO. Data are presented as means ± SEM. ††† $p < 0.001$ indicates a significant main effect of the father's age. (C) Normalized entropy scores of syllables emitted from YFO and AFO. Two-way ANOVA was used to compare the differences between the groups across development. Data are presented as means ± SEM. ††† $p < 0.001$ indicates a significant main effect of the father's age.

Fig. 5. Separation of individual patterns according to the syllable number and duration

(A) The cluster number of syllable number and duration were chosen as five by using the Akaike Information Criterion (AIC) as the minimum number (shown on X-axis) with AIC values (Y-axis). (B)-(F): Clustering the syllable number and duration. The clustering analyses with Gaussian mixture models (GMMs) were applied and separated the individual offspring into five clusters according to the syllable number and duration across four postnatal days. Offspring derived from young (YFO) and aged (AFO) fathers were arranged to each cluster with significantly different populations. (G) The different percentages of offspring in each cluster. The heat map summarized the percentages of YFO and AFO that were distributed to each cluster.

Fig. 6. Separation of individual patterns according to syllable types

(A) The cluster number of syllable types was chosen as four by using AIC as the minimum number (shown on X-axis) with AIC values (Y-axis). (B)-(E) Clustering the syllable types. The clustering analyses with Gaussian mixture models (GMMs) were applied and separated the offspring into four clusters according to the syllable types across four postnatal stages. The young father-derived offspring (YFO) and aged father-derived offspring (AFO) were arranged to each cluster with significantly different populations. (F) The percentages of offspring in each cluster. The heat map summarized the percentages of YFO and AFO that were distributed in each cluster.

Fig. 7. Separation of individual patterns according to normalized entropy

(A) The cluster number of syllable types was chosen as five by using AIC as the minimum number (shown on X-axis) with AIC values (Y-axis). (B)-(F) Clustering the normalized entropy. The clustering analyses with Gaussian mixture models (GMMs) were applied and separated the offspring into five clusters according to the normalized entropy across four postnatal days. The young father-derived offspring (YFO) and aged father-derived offspring (AFO) were arranged to each cluster with significantly different populations. (G) The percentages of offspring in each cluster. The heat map summarized the percentages of the YFO and AFO that were distributed to each cluster.

Fig. 8. Principal component analysis of overall syllables.

PCA plots showing individual differences of syllable development among the individual offspring derived from young (YFO) and aged (AFO) fathers. At P3, PCA of syllable patterns, where the Y-axis (PC1) explained 55.6% variance, whereas the X-axis (PC2) explained a 25.6% variance of the data. At P6, PCA of syllable patterns,

where the Y-axis (PC1) explained 62.3% variance, whereas the X-axis (PC2) explained a 21.2% variance of the data. At P9, PCA of syllable patterns, where the Y-axis (PC1) explained 62.6% variance, whereas the X-axis (PC2) explained 20.3% variance of the data. At P12, PCA of syllable patterns, where the Y-axis (PC1) explained 59.2% variance, whereas the X-axis (PC2) explained 23.4% variance of the data. The circles in gray or red included a 90% population of YFO and AFO.

Fig. 9. Correlation between body weight and USV

The correlation between body weight and USV parameters was detected by the Pearson correlation coefficient. The correlation coefficient and *p*-value were showed in Table 5. Scatter plot showing the correlation between body weight and USV in individuals from P3 to P12 among offspring derived from young (YFO) and aged (AFO) fathers. Correlation between body weight and the number of syllable types (A), the number of syllables (B), syllable duration (C), syllable maximum frequency (D), syllable maximum amplitude (E), and syllable interval (F), respectively. The circles in gray or red included a 90% population of YFO or AFO.

Supplementary Fig. 1. The developmental trajectories of USV features in overall syllable.

The numbers (A), durations (B), maximum frequency (C), maximum amplitude (D), and interval (E) of syllables emitted from offspring derived from young (YFO) and aged (AFO) fathers. Two-way ANOVA followed by *post-hoc* comparisons (*t*-test with BH correction) was used to compare the differences between the two groups. Data are presented as means ± SEM. $^{††}p < 0.01$, $^{†††}p < 0.001$ indicates a significant main effect of father's age, $^{§§§}p < 0.001$ indicates a significant interaction of father's

age × day effect. ** $p < 0.01$ indicates a significant decrease in AFO; * $p < 0.05$ indicates a significant increase in AFO.

Supplementary Fig. 2. The Number of individual type of syllables

(A)-(L) The developmental trajectory in regard to the number of the twelve types of syllables. Two-way ANOVA followed by *post-hoc* comparisons *t*-test with BH correction was used to compare the syllable number between the offspring derived from young (YFO) and aged (AFO) fathers at each postnatal day. Data are presented as means ± SEM. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ 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 significant interaction of father's age × day effect. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates a significant decrease of the syllable number in the AFO at individual postnatal day (*t*-test with BH correction).

Supplementary Fig. 3: Duration of individual types of syllables

(A)-(L) The developmental trajectory in regard to the duration of the twelve types of syllables. Two-way ANOVA followed by *post-hoc* comparisons *t*-test with BH correction was used to compare the syllable duration between the offspring derived from young (YFO) and aged (AFO) fathers at each postnatal day. Data are presented as means ± SEM. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ 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. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicates a significant decrease of the syllable number in the AFO at individual postnatal day (*t*-test with BH correction).

Supplementary Fig. 4. Maximum frequency of individual types of syllables

(A)-(L) The developmental trajectory in regard to the maximum frequency of the twelve types of syllables. Two-way ANOVA followed by *post-hoc* comparisons *t*-test with BH correction was used to compare the syllable number between the offspring derived from young (YFO) and aged (AFO) fathers. Data are presented as means ± SEM. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ 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. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ indicates a significant decrease of the syllable number in the AFO at individual postnatal day (*t*-test with BH correction).

Supplementary Fig. 5. Maximum amplitude of individual types of syllables

(A)-(L) The developmental trajectory in regard to the maximum amplitude of the twelve types of syllables. Two-way ANOVA was used to compare the syllable number between the offspring derived from young (YFO) and aged (AFO) fathers. Data are presented as means ± SEM. No significant differences were detected.

Supplementary Fig. 6: Interval of individual types of syllables

(A)-(L) The developmental trajectory in regard to the interval of the twelve types of syllables. Two-way ANOVA followed by *post-hoc* comparisons *t*-test with BH correction was used to compare the syllable number between the offspring derived from young (YFO) and aged (AFO) fathers at each postnatal day. Data are presented as means ± SEM. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ indicates a significant interaction of father's age × day effect. $^{*}p < 0.05$ indicates a significant increase of the syllable interval in the AFO at individual postnatal day (*t*-test with BH correction).

Supplementary Fig. 7. Body weight development.

Body weight of offspring derived from young (YFO) and aged (AFO) fathers. Two-way ANOVA was used to compare the body weight between the groups. Data are presented as means ± SEM. ††† $p < 0.001$ indicates a significant main effect of the father's age.

Table 1. The number of syllables was collected from the offspring at each postnatal day.

	P3	P6	P9	P12
YFO	4212	6041	7122	7039
AFO	2990	3276	5454	3296

YFO: young father-derived offspring; AFO: aged father-derived offspring.

Table 2. Eight types of syllables with decreased numbers in the AFO from P3 to P12.

Syllable types	Results of ANOVA
Upward	father's age: $F(1, 236) = 6.78$, $p = 0.015$
Short	father's age: $F(1, 236) = 30.50$, $p < 0.001$
Chevron	father's age: $F(1, 236) = 28.91$, $p < 0.001$
Wave	No wave syllable at P3 in the AFO, from P6 to P12:
	father's age: $F(1, 177) = 30.76$, $p < 0.001$
	father's age × day: $F(2, 177) = 6.69$, $p = 0.002$
Complex	father's age: $F(1, 236) = 17.64$, $p < 0.001$
	father's age × day: $F(3, 236) = 2.77$, $p = 0.042$
One jump	father's age: $F(1, 236) = 7.67$, $p = 0.018$
More jump	father's age: $F(1, 236) = 16.03$, $p < 0.001$
More jump + harmonics	father's age: $F(1, 236) = 10.47$, $p = 0.002$

Table 3. The percentage composition of syllable types was significantly different between the YFO and AFO.

Postnatal day	Results of MANOVA	Syllable types	Results of *t*-test
P3	F (11, 49) = 1.65, p = 0.114	Chevron	8.88 ± 1.34%: YFO vs 4.33 ± 0.88%: AFO, p = 0.006
		Wave	0.26 ± 0.11%: YFO vs 0%: AFO, p = 0.026
P6	F (11, 49) = 2.92, p = 0.005	Downward	46.76 ± 2.27%: YFO vs 56.29 ± 4.21%: AFO, p = 0.046
		Chevron	6.01 ± 0.63%: YFO vs 3.32 ± 0.57%: AFO, p = 0.003
		Complex	0.70 ± 0.27%: YFO vs 0.08 ± 0.043%: AFO, p = 0.037
		One jump	16.27 ± 2.01%: YFO vs 8.03 ± 2.01%: AFO, p = 0.006
		More jump	2.87 ± 0.74%: YFO vs 0.71 ± 0.28%: AFO, p = 0.011
		One jump + harmonics	3.05 ± 0.5%: YFO vs 1.35 ± 0.34%: AFO, p = 0.008
P9	F (11, 49) = 5.37, p < 0.001	Upward	15.54 ± 1.57%: YFO vs 10.63 ± 1.74%: AFO, p = 0.039
		Downward	36.54 ± 1.49%: YFO vs 46.03 ± 2.34%: AFO, p < 0.001
		Flat	1.86 ± 0.27%: YFO vs 4.78 ± 1.36%: AFO, p = 0.031
		Short	5.6 ± 0.67%: YFO vs 9.76 ± 2.05%: AFO, p = 0.050
		Chevron	11.46 ± 0.78%: YFO vs 5.77 ± 0.87%: AFO, p < 0.001
		Wave	3.08 ± 0.43%: YFO vs 0.62 ± 0.16%: AFO, p < 0.001
		Complex	2.08 ± 0.4%: YFO vs 0.29 ± 0.1%: AFO, p < 0.001
		More jump	2.22 ± 0.47%: YFO vs 0.87 ± 0.22%:AFO, p = 0.015
		Harmonics	2.15 ± 0.46%: YFO vs 4.39 ± 0.99%: AFO, p = 0.038
		More jump + harmonics	0.86 ± 0.2%: YFO vs 0.2 ± 0.07%: AFO, p = 0.004
P12	F (11, 49) = 2.73, p = 0.008	Downward	33.14 ± 1.53%: YFO vs 40.57 ± 2.52%: AFO, p = 0.013
		Wave	5.51 ± 0.71%: YFO vs 2.00 ± 0.47%: AFO, p < 0.001
		Complex	4.41 ± 0.64%: YFO vs 2.38 ± 0.66%: AFO, p = 0.030
		More jump	2.88 ± 0.4%: YFO vs 1.31 ± 0.47%: AFO, p = 0.012
		Harmonics	1.98 ± 0.36%: YFO vs 1.05 ± 0.93: AFO, p = 0.047
		More jump + harmonics	1.45 ± 0.31%: YFO vs 0.31 ± 0.14%: AFO, p = 0.002

YFO: young father-derived offspring; AFO: aged father-derived offspring.

Table 4. The variabilities of PC1 and PC2 from P3 to P12.

	P3	P6	P9	P12
PC1	55.6%	62.3%	62.6%	59.2%
PC2	25.6%	21.1%	20.3%	23.4%
PC1 + PC2	81.2%	83.4%	82.9%	82.6%

Table 5. The correlation coefficients and *p*-value between the body weight and USV.

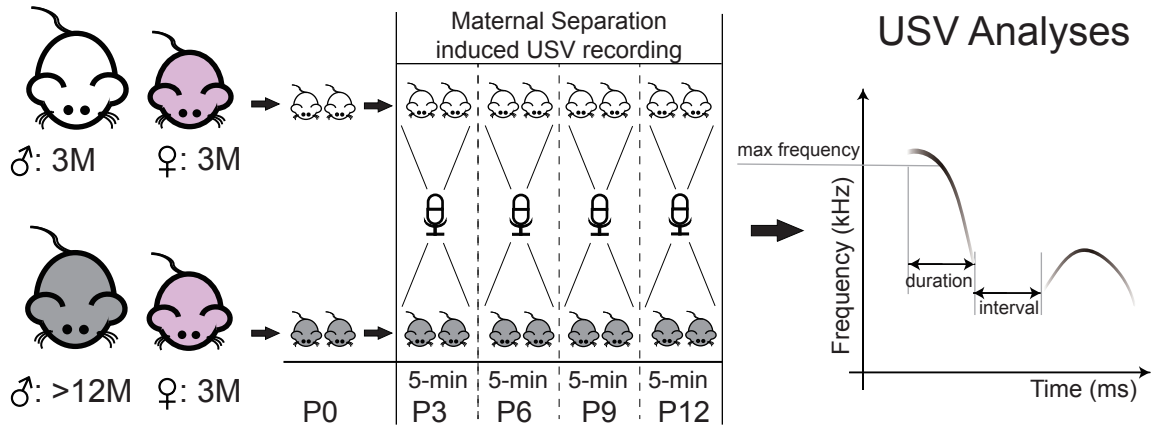
		YFO	AFO
		Body weight	Body weight
P3	Number of types	0.03 (0.890)	0.32 (0.095)
	Number of syllables	0.23 (0.211)	**0.39 (0.037)**
	Duration	-0.14 (0.453)	-0.00 (0.999)
	Max frequency	0.27 (0.143)	-0.04 (0.848)
	Max amplitude	-0.12 (0.531)	0.06 (0.757)
	Interval	0.14 (0.500)	0.18 (0.384)
P6	Number of types	**0.42 (0.016)**	**0.40 (0.032)**
	Number of syllables	**0.50 (0.004)**	0.37 (0.051)
	Duration	**0.41 (0.019)**	0.13 (0.517)
	Max frequency	0.04 (0.838)	0.22 (0.249)
	Max amplitude	0.16 (0.373)	0.05 (0.807)
	Interval	0.22 (0.227)	-0.35 (0.107)
P9	Number of types	-0.15 (0.427)	**0.40 (0.031)**
	Number of syllables	0.01 (0.971)	0.31 (0.101)
	Duration	0.08 (0.666)	0.32 (0.090)
	Max frequency	0.05 (0.770)	0.07 (0.725)
	Max amplitude	-0.10 (0.601)	0.27 (0.158)
	Interval	-0.18 (0.317)	-0.24 (0.219)
P12	Number of types	-0.26 (0.151)	0.14 (0.456)
	Number of syllables	-0.31 (0.086)	0.23 (0.222)
	Duration	-0.15 (0.416)	0.36 (0.057)
	Max frequency	**0.44 (0.012)**	-0.15 (0.433)
	Max amplitude	-0.31 (0.080)	0.30 (0.111)
	Interval	-0.01 (0.951)	-0.25 (0.221)

Correlation coefficients (p-value)

The bold values are statistically significant ($p < 0.05$).

Fig 1

A


♂: 3M
♀: 3M
♂: >12M
♀: 3M
Maternal Separation
induced USV recording
P0
5-min
P3
5-min
P6
5-min
P9
5-min
P12
USV Analyses
max frequency
Frequency (kHz)
duration
interval
Time (ms)

B

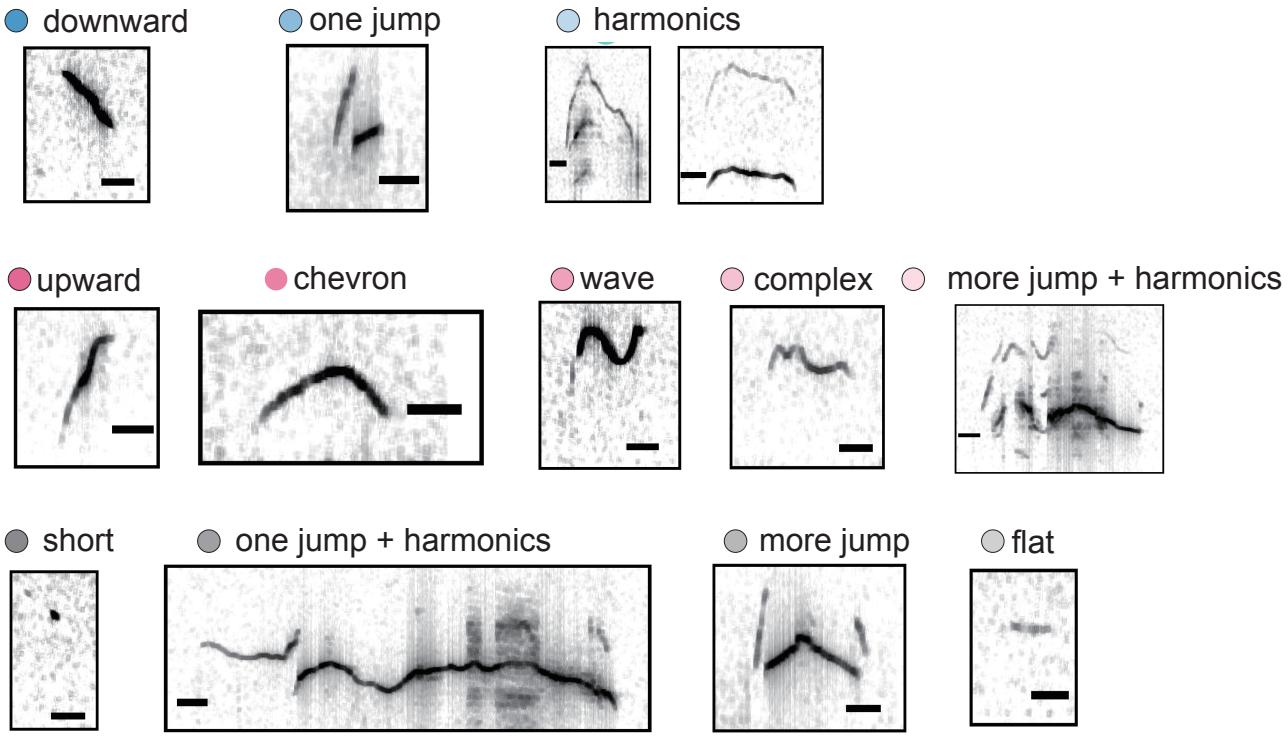

downward
one jump
harmonics
upward
chevron
wave
complex
more jump + harmonics
short
one jump + harmonics
more jump
flat

Fig 2

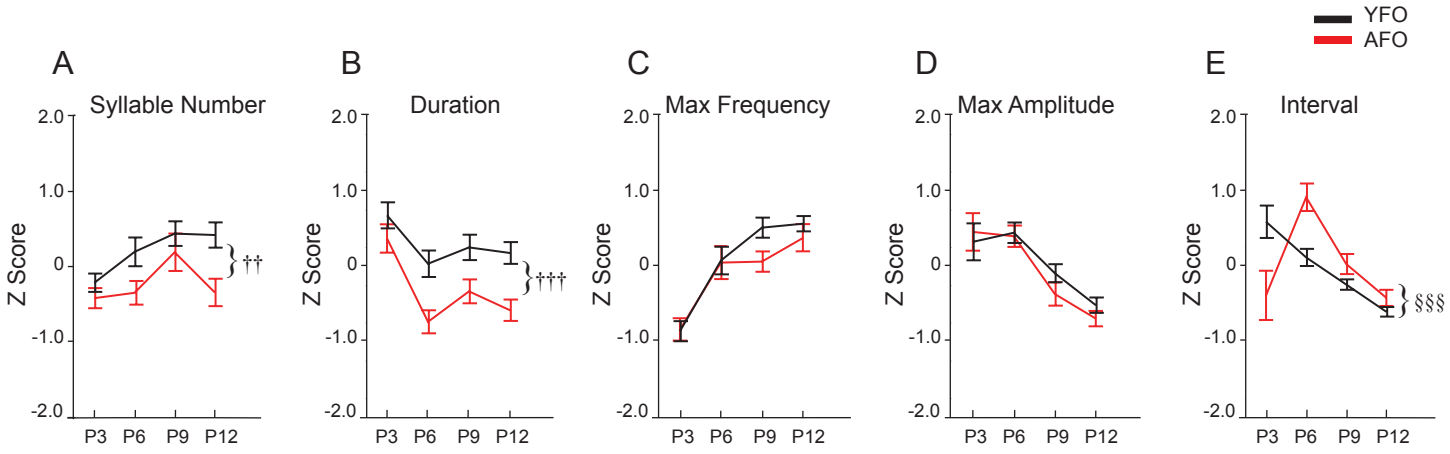

YFO
AFO
A
Syllable Number
B
Duration
C
Max Frequency
D
Max Amplitude
E
Interval
Z Score
2.0
1.0
0
-1.0
-2.0
P3
P6
P9
P12
††
†††
§§§

Fig 3

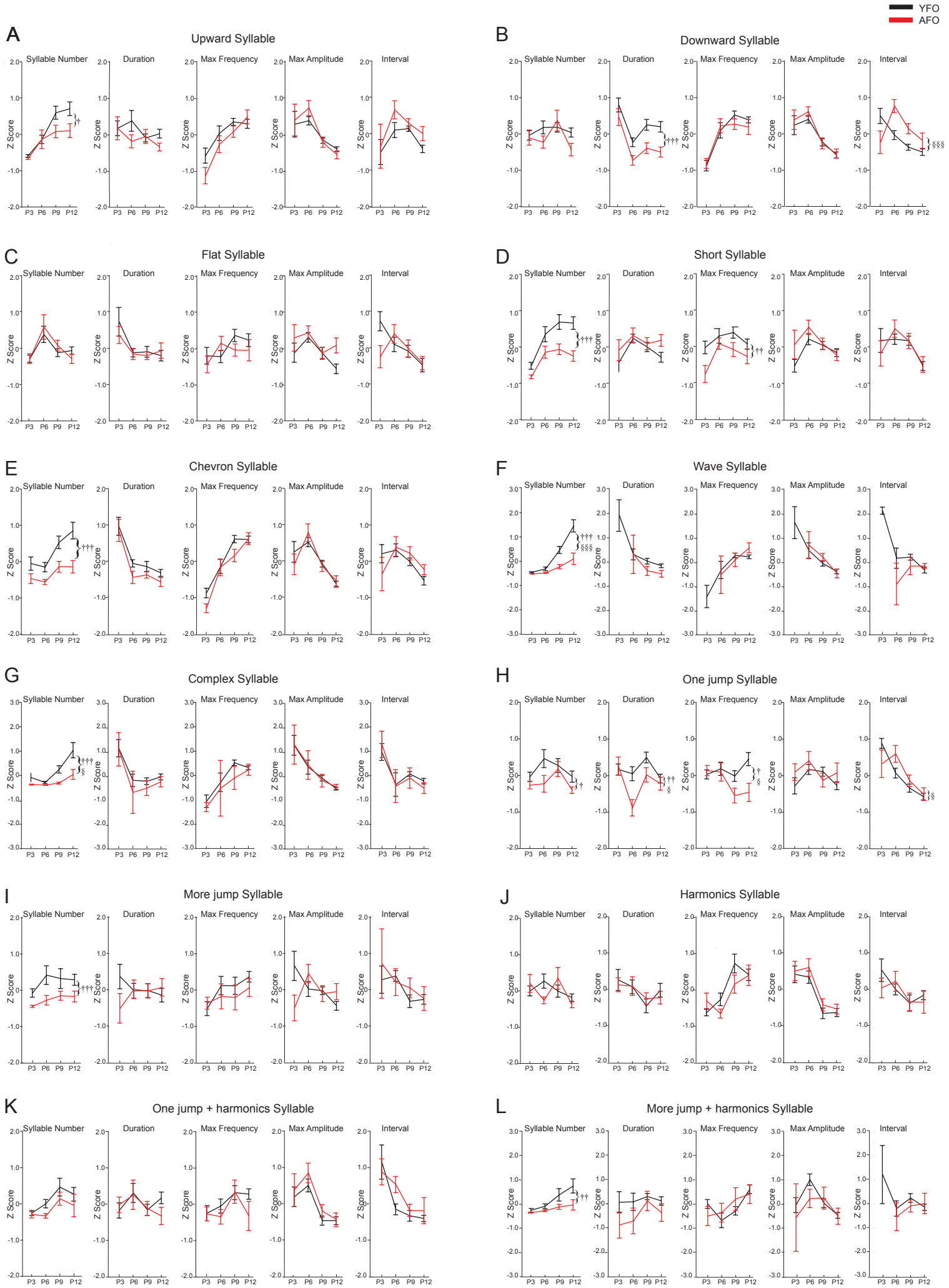
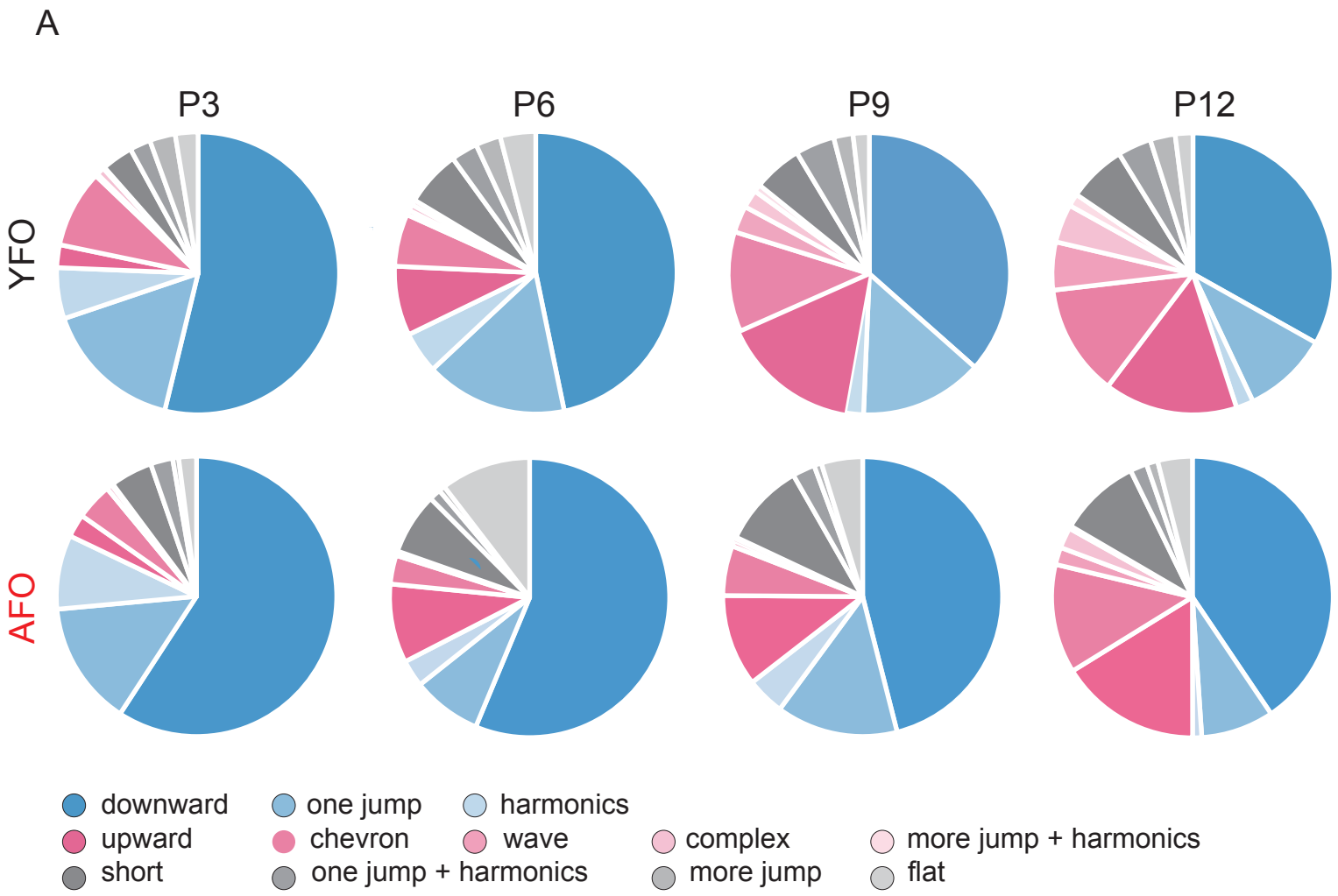
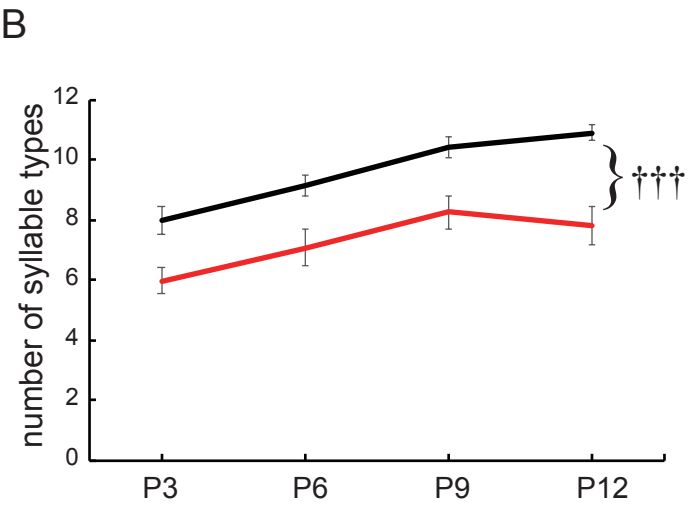

YFO
AFO
A
Upward Syllable
B
Downward Syllable
C
Flat Syllable
D
Short Syllable
E
Chevron Syllable
F
Wave Syllable
G
Complex Syllable
H
One jump Syllable
I
More jump Syllable
J
Harmonics Syllable
K
One jump + harmonics Syllable
L
More jump + harmonics Syllable
Syllable Number
Duration
Max Frequency
Max Amplitude
Interval
Z Score
P3
P6
P9
P12

Fig 4


A
P3
P6
P9
P12
YFO
AFO
downward
one jump
harmonics
upward
chevron
wave
complex
more jump + harmonics
short
one jump + harmonics
more jump
flat


B
number of syllable types
0
2
4
6
8
10
12
P3
P6
P9
P12
†††

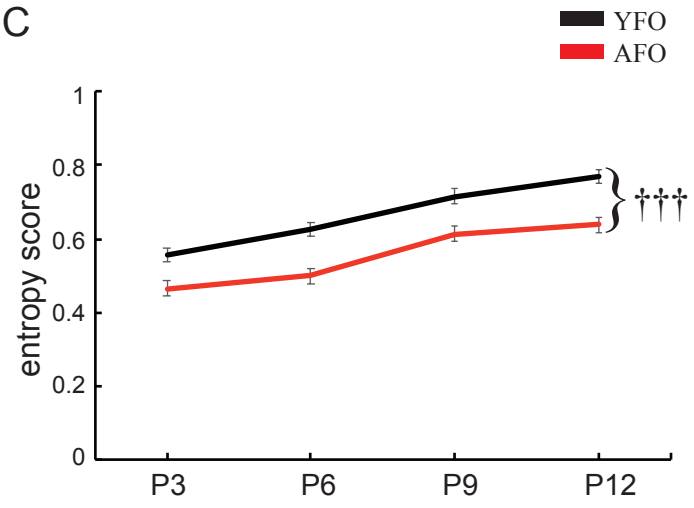

C
YFO
AFO
entropy score
0
0.2
0.4
0.6
0.8
1
P3
P6
P9
P12
†††

Fig 5

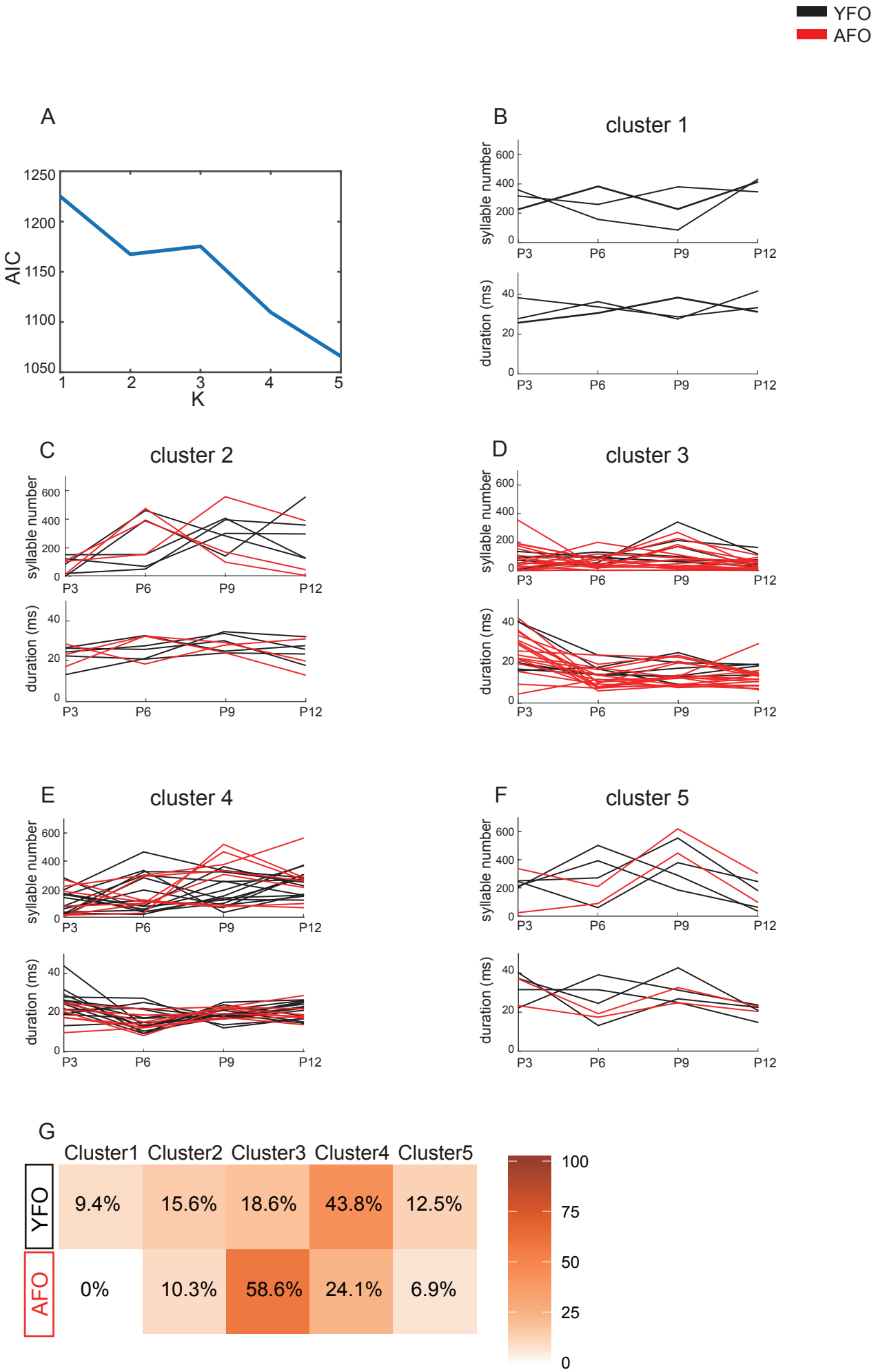

YFO
AFO
A
AIC
K
B
cluster 1
C
cluster 2
D
cluster 3
E
cluster 4
F
cluster 5
syllable number
duration (ms)
P3
P6
P9
P12
G
Cluster1 Cluster2 Cluster3 Cluster4 Cluster5
YFO
9.4% 15.6% 18.6% 43.8% 12.5%
AFO
0% 10.3% 58.6% 24.1% 6.9%

Fig 6

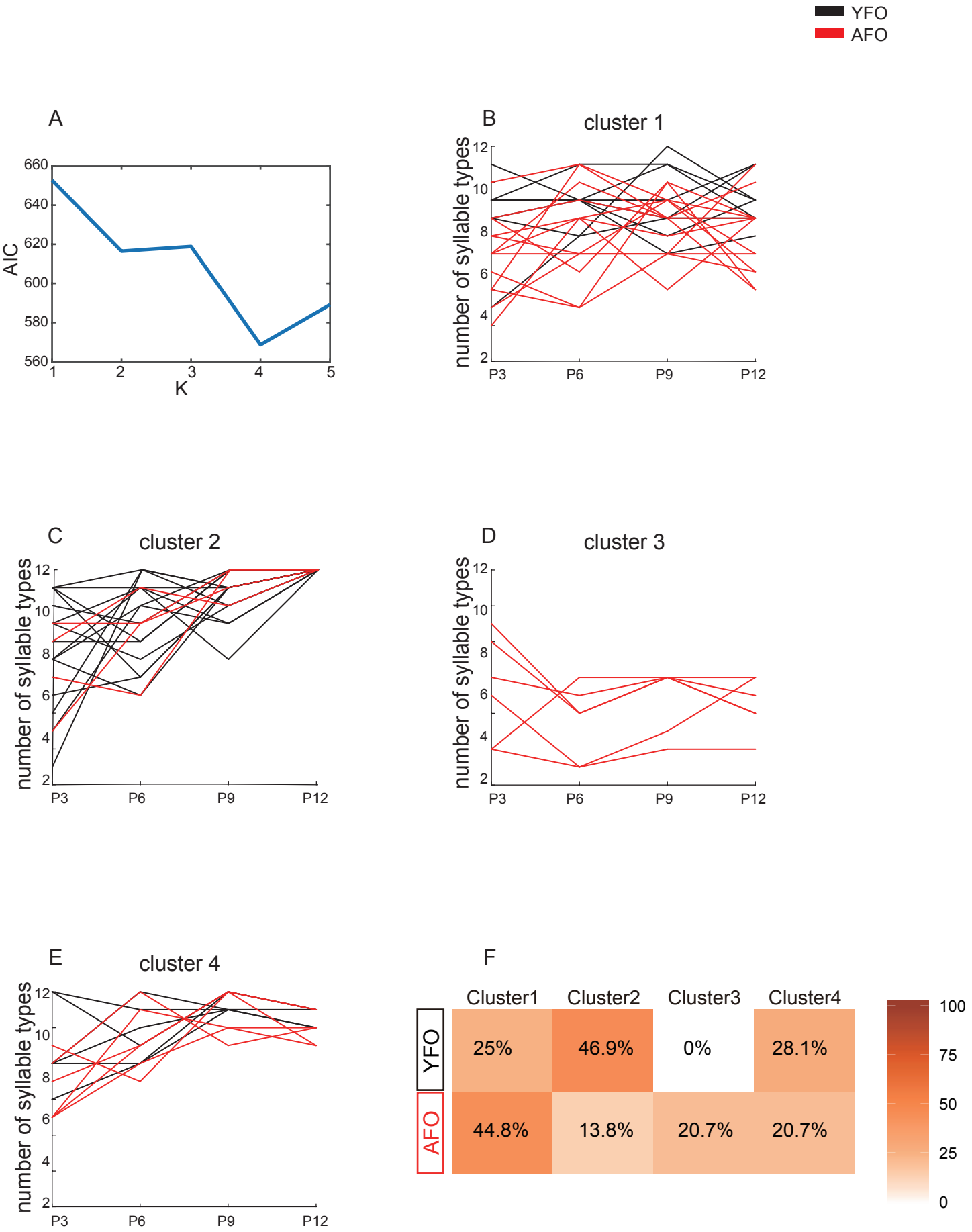

YFO
AFO
A
AIC
K
B
cluster 1
number of syllable types
P3
P6
P9
P12
C
cluster 2
D
cluster 3
E
cluster 4
F
Cluster1
Cluster2
Cluster3
Cluster4
YFO
AFO
25%
46.9%
0%
28.1%
44.8%
13.8%
20.7%
20.7%
100
75
50
25
0

Fig 7

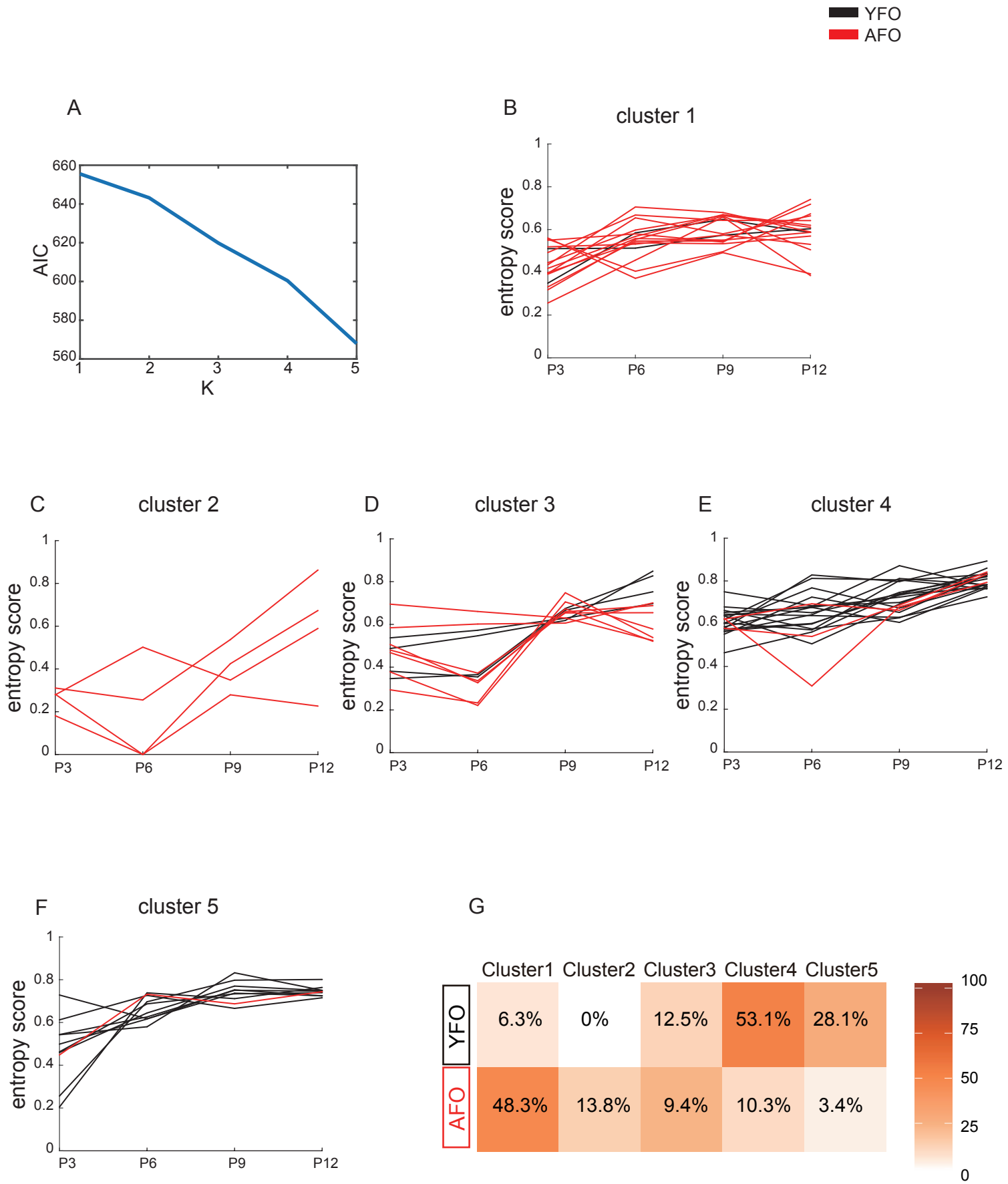

YFO
AFO
A
AIC
K
B
cluster 1
entropy score
C
cluster 2
D
cluster 3
E
cluster 4
F
cluster 5
P3
P6
P9
P12
G
Cluster1 Cluster2 Cluster3 Cluster4 Cluster5
YFO
6.3% 0% 12.5% 53.1% 28.1%
AFO
48.3% 13.8% 9.4% 10.3% 3.4%

Fig 8

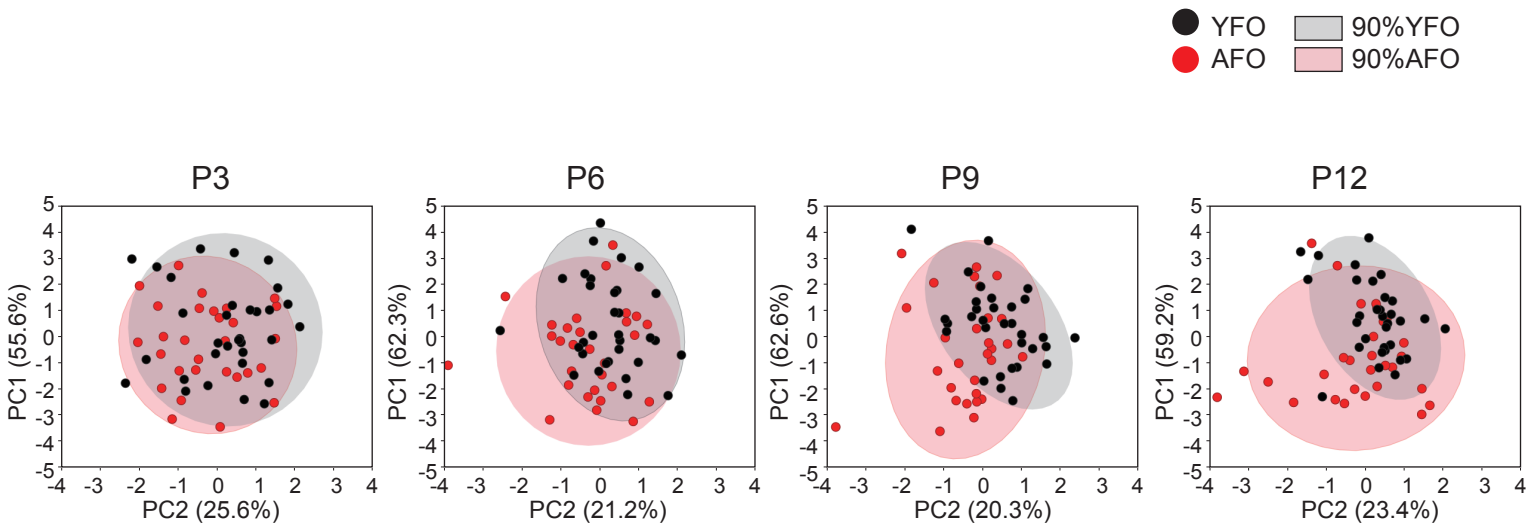
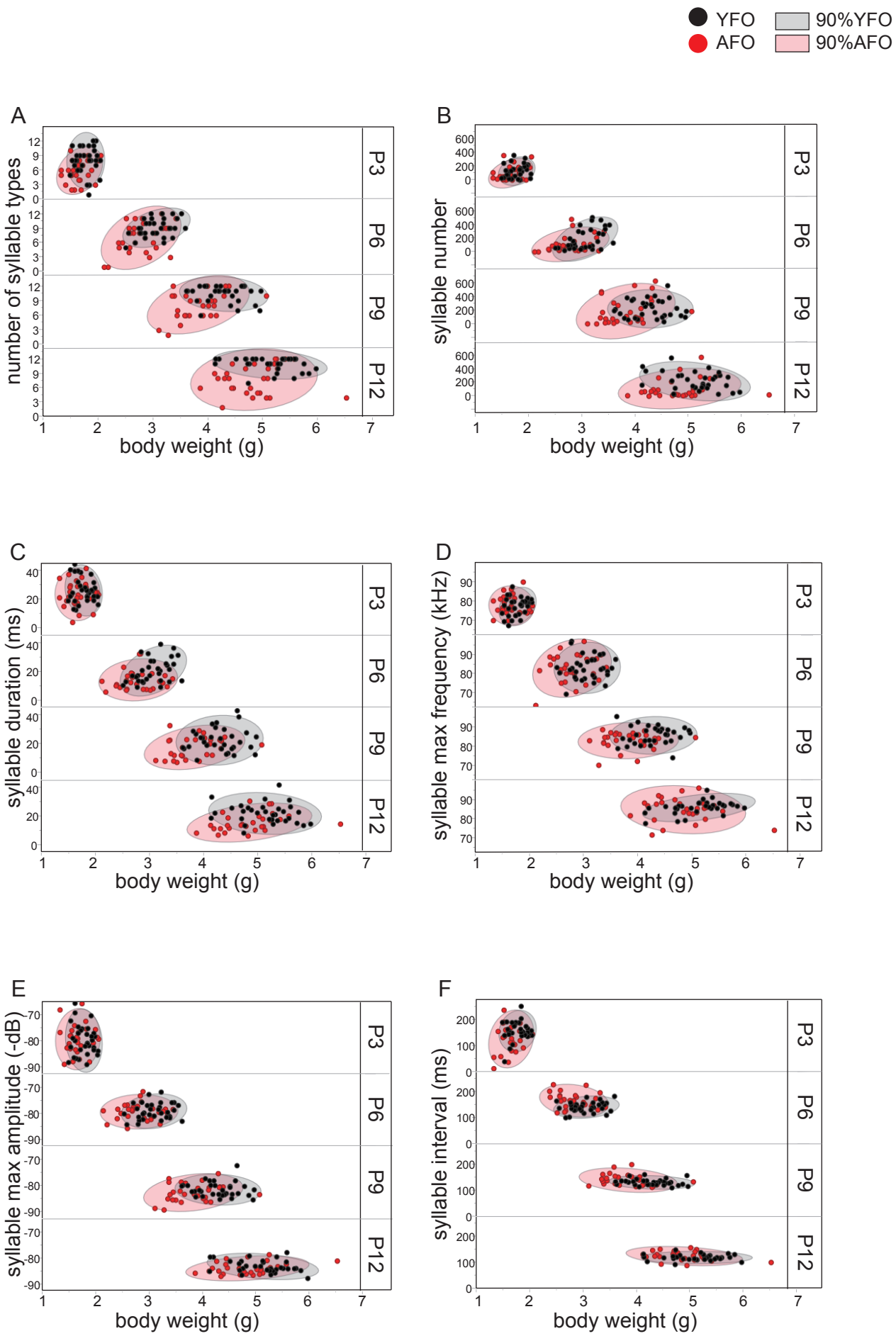

YFO
AFO
90%YFO
90%AFO
P3
P6
P9
P12
PC1 (55.6%)
PC2 (25.6%)
PC1 (62.3%)
PC2 (21.2%)
PC1 (62.6%)
PC2 (20.3%)
PC1 (59.2%)
PC2 (23.4%)

Fig 9


YFO
AFO
90%YFO
90%AFO
A
number of syllable types
B
syllable number
C
syllable duration (ms)
D
syllable max frequency (kHz)
E
syllable max amplitude (-dB)
F
syllable interval (ms)
P3
P6
P9
P12
body weight (g)