Paternal aging affects the developmental patterns of ultrasonic vocalization induced by maternal separation in neonatal mice individually

Lingling Mai [1], Ryuichi Kimura[1], Hitoshi Inada[1], Kouta Kanno[2], Takeru Matsuda[3], Ryosuke O. Tachibana[4], Kaichi Yoshizaki[5], Fumiyasu Komaki[3], 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]Department of Life Science, Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan

[5]Department of Pathology, Institute for Developmental Research, Aichi Human Service 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 focused on ultrasonic vocalization (USV) induced by maternal separation of pups corresponding to baby's cry. 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 12 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. Interestingly, from an individual perspective, there were more individuals with atypical developmental patterns in the offspring group derived from aged fathers. Taken together, we demonstrated 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 the 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 are 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 pups emit USV calls when they are separated from mothers and littermates, 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 component[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 has not been addressed yet.

Here we measured the maternal separation-induced USV of individual mouse pups during postnatal development up to 12 days. Not only comparing the developmental patterns of USV emitted from offspring derived from young (YFO) and aged (AFO) father, but also comprehensive analyses were performed by paying a 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 in the AFO.

Results

Offspring were obtained by mating young female mice together with either young (3 monts) or aged (>12 months) male mice (Fig 1A). Average litter size was not significantly different between the offspring groups; i.e., 7.00 ± 0.35 (n = 32) and 6.6 ± 0.97 (n = 29) mated with young and aged fathers, respectively (*t*-test, $p = 0.68$). Therefore, we assume that our experimental setup was suitable to evaluate paternal aging effects on offspring's vocal communication without considering other influence such as the number of siblings during postnatal development.

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 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 ultrasonic vocalization that are classified into 12 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.

1. USV measurement

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

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.

1.1. Number of syllables

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). Those emitted by AFO were significantly less through the developmental stages.

Fig 2. The developmental trajectories of acoustic features in overall syllable. The numbers (A), durations (B), maximum frequency (C), maximum amplitude (D), and interval (E) of syllables emitted from YFO and AFO. 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 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. YFO: young father-derived offspring; AFO: aged father-derived offspring.

1.2. Duration of syllables

Syllable duration altered during postnatal development and exhibited a similar downward trend curve in both groups, showing the lowest point at P6. Average 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 (Fig 2B). Paternal aging significantly decreased the duration of overall syllables.

1.3. Maximum frequency of syllables

Regarding tones of syllables, the maximum frequency (i.e., tone indicated with hertz; Hz) was altered significantly by the main effect of postnatal day [day: $F(3, 236) = 27.84$, $p < 0.001$], but not by the main effect of paternal aging and interaction of paternal aging × postnatal day effect (Fig 2C). A developmental change was observed from P3 to P12.

1.4. Maximum amplitude of syllables

A significant main effect of postnatal day was observed in development of maximum amplitude (i.e., loudness) of syllables [day: $F(3, 236) = 18.22$, $p < 0.001$] (Fig 2D), but no significant main interaction of paternal aging and interaction of paternal aging × postnatal day effect was detected.

1.5. Interval between adjacent two syllables

A significant main effect of postnatal day for interval of the overall syllables was detected [day: $F(3, 236) = 13.64$, $p < 0.001$] (Fig 2E). Interestingly, a significant interaction between the main effects of paternal aging and postnatal day was observed [father's age × day: $F(3, 236) = 10.18$, $p < 0.001$]. During development, the interval

kept decreasing in the YFO, while in the AFO, the interval peaked at P6, then decrease from P6 to P12. A *t*-test of *post-hoc* comparisons with Benjamini-Hochberg correction (BH correction) indicated that paternal aging significantly decreased the interval at P3, yet increased it at P6 (P3, 155.6 ± 6.56 ms: YFO vs 125.8 ± 9.99 ms: AFO, $p = 0.0012$; P6, 141.15 ± 3.47 ms: YFO vs 165.55 ± 5.56 ms: AFO, $p = 0.026$).

Taken together, USV from AFO showed significantly different developmental patterns in the overall syllable number, average duration and interval, but not in maximum frequency and maximum amplitude across development. These results suggested that paternal aging may alter the developmental trajectory of overall syllables in quantitative and qualitative aspects.

2. Classification of syllable types

For deeper understanding the paternal aging effects on the syllable patterns across the early postnatal period, all USV syllables induced by maternal separation were classified into 12 types (Fig 1B) based on the shapes of spectrograms according to a previous report[24].

2.1. The Number of individual types of syllables

After classification of syllable types, we found that the main effect of paternal aging significantly decreased the number of eight types of syllables such as “upward”, “short”, “chevron” “wave”, “complex”, “one jump”, “more jump”, and “more jump + harmonics” (Fig 3A, 3D, 3E, 3F, 3G, 3H, 3I, 3L, and Table 2). We did not observe “wave” syllable in the AFO at P3. Because the significant interaction of two main effects of paternal aging and postnatal day effect was found in the “wave” and

"complex" syllables, a *t*-test of *post-hoc* comparisons with BH correction was applied and revealed that at P9 and P12, the number of "wave" syllables significantly decreased in the AFO (P9, 6 ± 0.88: YFO vs 1.83 ± 0.56: AFO, $p < 0.001$; P12, 11.97 ± 1.53: YFO vs 3.79 ± 1.42: AFO, $p < 0.001$). In addition, "complex" syllables were significantly decreased at P9 (4.91 ± 1.11: YFO vs 0.76 ± 0.28: AFO, $p = 0.004$) and P12 (10.72 ± 2.31: YFO vs 3.41 ± 1.52: AFO, $p = 0.024$). These eight types of syllables may contribute to the reduced number of the overall syllables emitted by the AFO.

Fig 3. The Number of individual types of syllables

(A)-(L) Developmental trajectory in regard with 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 groups 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 significant decrease of the syllable number in the AFO at individual postnatal day (*t*-test with BH correction). 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, $p = 0.002$

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 4B and 4H). Moreover, the significant interaction of two main effects of paternal aging and postnatal day was exhibited in the duration of "one jump" syllables [father's age × day: F (3, 215) = 2.97, $p = 0.033$]. A *t*-test of *post-hoc* comparisons with BH correction showed that duration of "one jump" syllables significantly decreased in the AFO at P6 (27.84 ± 1.85 ms: YFO vs 18.79 ± 2.18 ms: AFO, $p = 0.010$). The shorter duration of overall syllable in the AFO may be attributed to the changes of "downward" and "one jump" syllables.

Fig 4: Duration of individual types of syllables

(A)-(L) Developmental trajectory in regard with 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 two groups 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 significant decrease of the syllable number in the AFO at individual postnatal day (*t*-test with BH correction). YFO: young father-derived offspring; AFO: aged father-derived offspring.

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 5D and 5H); they showed lower maximum frequency in the AFO (Fig 5D and 5H). Moreover, two main effects of paternal aging and postnatal day were interacted significantly in "one jump" syllables [father's age × day: $F(3, 215) = 3.03$, $p = 0.046$]. A *t*-test of *post-hoc* comparisons with BH correction revealed that at P12, maximum frequency of "one jump" syllable produced by the AFO was significantly lower (82.58 ± 1.25 kHz: YFO vs 76.26 ± 1.72 kHz: AFO, $p = 0.014$). Paternal aging affected the maximum frequency at the level of two syllables.

Fig 5. Maximum frequency of individual types of syllables

(A)-(L) Developmental trajectory in regard with 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 groups. 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 significant decrease of the syllable number in the AFO at individual postnatal day (*t*-test with BH correction). YFO: young father-derived offspring; AFO: aged father-derived offspring.

2.4 Maximum amplitude of individual types of syllables

After classification of all syllables, there were again no significant main effects of paternal aging and postnatal day in maximum amplitude (Fig 6). The alteration of syllable maximum amplitude by paternal aging still was not observed after detailed analyses.

Fig 6. Maximum amplitude of individual types of syllables

(A)-(L) Developmental trajectory in regard with the maximum amplitude of the twelve types of syllables. Two-way ANOVA was used to compare the syllable number between the two groups. Data are presented as means ± SEM. No significant differences were detected. YFO: young father-derived offspring; AFO: aged father-derived offspring.

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 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 7B, 7H). A *t*-test of *post-hoc* comparisons with BH correction showed significantly longer interval of "downward" syllables in aged father-derived offspring at P6 (133.71 ± 4.4 ms: YFO vs 162.47 ± 6.38 ms: AFO, $p = 0.002$) and P9 (121.75 ± 2.93 ms: YFO vs 140.09 ± 5.03 ms: AFO, $p = 0.004$). These data imply that "downward" and "one jump" syllables may contribute to the altered trajectory of the syllable interval in overall syllables.

Fig 7: Interval of individual types of syllables

(A)-(L) Developmental trajectory in regard with 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 two groups 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 significant interaction of father's age × day effect. * $p < 0.05$ indicates significant increase of the syllable interval in the AFO at individual postnatal day (*t*-test with BH correction). YFO: young father-derived offspring; AFO: aged father-derived offspring.

2.6 The percentage composition of the twelve types syllables

Next, we analyzed composition of the twelve syllable types induced by maternal separation (Fig 8 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 demonstrated the USV with a significantly different syllable component (Table 3). Compared with the syllables of YFO in each postnatal day, the syllables emitted from AFO with higher percentage of "downward" syllable at P6, P9 and P12, along with higher "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.

Fig 8. The percentage composition of the twelve types syllables

Pie graphs exhibited the percentage composition of the twelve type of syllables from P3 to P12 in the YFO and AFO. Data are presented as means. The colors of magenta, blue and grey spectrum indicate the syllables with increased, decreased and nonspecifically changed percentages, respectively, during development of the YFO. YFO: young father-derived offspring; AFO: aged father-derived offspring.

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.

2.7 Variation of syllables in individual offspring

Next, we focused on variation of syllables. Our results demonstrated that YFO developed to emit more types of syllables according to postnatal days. In contrast, the AFO expanded variation of the syllable types from P3 to P9, which became narrower from P9 to P12 (Fig 9A). Likewise, significantly less 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 further evaluate the different diversity of 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 9B). 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 less types and diversity of syllables, meaning that they had a narrower vocal repertoire.

Fig 9. Variation of syllables in distinct types.

(A) The developmental trajectories in regard with variation of the syllable types in YFO and AFO. (B) 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 father's age. YFO: young father-derived offspring; AFO: aged father-derived offspring.

3. Separation of individual patterns in different clusters

In order to understand the longitudinal syllable development in individuals, clustering analyses with Gaussian mixture models were applied. Based on Akaike Information Criterion (AIC), the cluster number was determined objectively (see Methods for detail).

3.1 Clusters of the syllable number and duration

Next, we addressed the individual differences among YFO and AFO. 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 10A). 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 10B-10G). Chi-square independence test revealed that the clustering pattern, i.e., the proportion of individuals in each cluster, was significantly different between the YFO and AFO

(p = 0.02). These results exhibited that the dominant developmental pattern of the syllable number and duration was different between YFO and AFO. Importantly, AFO showed less variation in the syllables.

Fig 10. Separation of individual patterns according to the syllable number and duration

(A) Choosing the number of clusters for the syllable number and duration using Akaike Information Criterion (AIC). Because the AIC reached a minimum with the number of five, the optimal number of clusters was determined as five. Minimum of AIC of On X-axis number of clusters and on Y-axis AIC values were showed. (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. YFO and AFO were arranged to each cluster with significantly different populations. (G) The different percentage of offspring in each cluster. The heat map summarized the percentage of YFO and AFO that was distributed to each cluster. YFO: young father-derived offspring; AFO: aged father-derived offspring.

3.2 Cluster of the diversity in the syllable types

For identification of individual development, we first classified the number of syllable types into four clusters based on AIC (Fig 11A). Interestingly, YFO were classified into three clusters, while AFO were classified into four clusters. Most of the YFO belonged to the second cluster, whereas most of the AFO belonged to the first cluster (Fig 11B-11F). The cluster distribution was significantly different between the two groups (chi-squared test, p = 0.003). Furthermore, we clustered the normalized

entropy to clarify the 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, $p = 0.002$). The YFO occupied four clusters and dominated the fourth cluster, while the AFO occupied five clusters and dominated the first cluster (Fig 12A-12G). Data showed here indicated that the different developmental patterns of syllable diversity between the YFO and AFO.

Fig 11. Separation of individual patterns according to syllable types

(A) Choosing the cluster number of syllable types using AIC. Because the AIC reached a minimum with the number of four, the four was determined as optimal number of clusters. Minimum of AIC of On X-axis number of clusters and on Y-axis AIC values were showed. (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 days. The YFO and AFO were arranged to each cluster with significantly different populations. (F) The percentage of offspring in each cluster. The heat map summarized the percentages of YFO and AFO that were distributed in each cluster. YFO: young father-derived offspring; AFO: aged father-derived offspring.

Fig 12. Separation of individual patterns according to normalized entropy

(A) Choosing the cluster number of normalized entropy using AIC. Number of clusters was determined by AIC. Because the AIC reached a minimum with the number of five, the five was determined as optimal number of clusters. Minimum of AIC of On X-axis number of clusters and on Y-axis AIC values were showed. (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 YFO and AFO were arranged to each cluster with significantly different populations. (G) the percentage of offspring in each cluster. The heat map summarized the percentage of the YFO and AFO that was distributed to each cluster. YFO: young father-derived offspring; AFO: aged father-derived offspring.

Through the clustering analyses, individual differences of 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.

4. Principal Component Analysis (PCA) of overall syllables

Finally, we summarized the developmental diversity in individual offspring in regard with vocal communication. We extracted principal components (PC) contributing to the individual character of overall syllables to analyze 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 13. At P3, the circles including 90% of the offspring's data were almost overlapping between the two groups of YFO (gray) and AFO (red). PC analyses demonstrated that the syllable variety of offspring showed a great individual difference regardless of the paternal age, as the consequence, the two groups were not clearly distinguished at P3. From P6 to P12, however, the variability regions became smaller and restricted in the YFO. In the AFO, by contrast, the variability regions were kept wider, suggesting that the difference of individual variability in the AFO was still large. Through the horizontal

comparison of each postnatal day, the present data clearly indicated that paternal aging resulted in the greater individual diversity in regard with the syllable patterns.

Fig 13. Principal component analysis of overall syllables.

PCA plots showing individual differences of syllable development among the individual YFO and AFO. At P3, PCA of syllable patterns, where the *Y*-axis (PC1) explained 55.6% variance, whereas the *X*-axis (PC2) explained 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 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 black or red included 90% population of YFO and AFO. 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%

5. Correlations between body weight and USV

After the USV recording on P3, P6, P9 and P12 we measured body weight of offspring to know paternal aging influence. 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 (Fig 14). AFO demonstrated a lower body weight gain from P3 to P12. This result suggested that paternal aging, in general, induced lower body weight development in offspring during early postnatal development.

Fig 14. Body weight development.

Body weight of the YFO and AFO. Two-way ANOVA followed 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 father's age. YFO: young father-derived offspring; AFO: aged father-derived offspring.

To clarify the correlations between body weight and USV, we first draw scatter plot graphs to visualize the data (Fig 15). In general, at P3, the circles involving 90% of the offspring's data of correlations between body weight and USV were almost overlapping between the two groups (Fig 15A -15F). From P6 to P12, especially, in the correlation between body weight and number of syllable types gradually demonstrated the differences between the YFO and AFO (Fig 15A). Again, AFO displayed the wider variation than the YFO with aging. Next, the correlation coefficients and *p*-value were calculated by Pearson correlation coefficient (Table 5). The body weight of YFO had positive correlation with the number of syllables, the number of syllable types, and syllable duration at P6, and positive correlation with syllable frequency at P12. By contrast, the body weight of AFO had positive correlation with the number of syllables at P3, with the number of syllable types at P6

and P9. The strongest positive correlation (correlation coefficient = 0.5) we found was the correlation between body weight and the number of syllable in YFO at P6, whereas other correlation coefficient scores were not obviously strong; we could not find a strong positive correlation between the number of syllables and body weight in AFO. These data revealed again that AFO had more atypical individuals and abnormal developmental patterns.

Fig 15. Correlation between body weight and USV

The correlation between body weight and USV parameters was detected by 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 the YFO and AFO. 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, respectively. The circles in black or red included 90% population of YFO or AFO. YFO: young father-derived offspring; AFO: aged father-derived offspring.

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 ($p < 0.05$)

Discussion

Our study is the first to analyze detailed syllable features in a mouse model of paternal aging. We revealed the 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 (e.g. hundreds of syllables per one pup in five minutes) from the USV test. This has great merit compared with other 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 also plays an important role in the alterations of communicative behavior in infant mice, a consistent result with alterations of social behaviors in adult offspring derived from aged father[28-30].

Previous studies demonstrated 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 report here that the USV features of AFO are generally consistent with other ASD models with the decreased vocal numbers[26, 33, 34] and shorter syllable durations[34, 35]. The YFO acquired diverse syllable types during early postnatal periods in consistent with a previous study[23]. However, compared with YFO, the AFO emitted a narrower spectrum of syllable types. This deficit of syllable diversity has also been described in a genetic ASD model, i.e., *Cd157* KO mice[36]. Additionally, we observed the different percentage composition of syllables in AFO. Altered compositions of syllables have previously been reported in another

genetic ASD mouse models such as *Reelin* mutant[37], *fmr1* knockout[38] and *ScSn-Dmd*^{mdx}*/J* mutant[39]. 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. This gap was not observed when they became adult according to our unpublished data of different cohorts of mice used for behavior analyses in adult stages. In human studies, lower birth weight is reported to be associated with several neurodevelopmental difficulties such as learning disabilities[40], speech and language problems[41], and social problems[42]. Paternal aging associated low birth weight has been considered as a high-risk factor of ASD[27, 43]. Therefore, it is assumed that paternal aging might cause offspring developmental delay at the physical level related with lower body weight, which could impact on early communicative problems in the offspring.

Another possibility that could explain the difference in body weight might be altered maternal care due to difference in vocal communication of offspring. It is known that pup's USV has communicative significance[44-48], which is crucial for maternal behaviors and preserves the social bonds between mother and infant that are essential for the healthy development of offspring[49, 50]. To examine the behaviors of maternal care and mother-infant interaction, playback and foster mother experiments may help to understand whether the abnormal syllables of AFO have actual influence on their mothers. Indeed, a previous USV playback experiment indicated that the altered syllables in *Tbx1*-deficient heterozygous mice led to less maternal responses to pups'

vocalization[51]. Thus, the alteration of USV development in AFO could to be affected by not only the primary factor of paternal aging, but also the secondary factor coming from maternal care. Since the correlation coefficients were less than 0.5 in our data, there was no strong correlation between body weight and syllable development. Therefore, we need further analyses to explore the possibility whether maternal care may change due to altered USV of pups.

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 (three-chamber social interaction test) and negatively with spatial memory (Morris water maze)[52]. In this case, the AFO might show abnormal sociability in adulthood, although we did not include adult behavior analyses in this study because repeated USV recording (including maternal separation) could affect behavior in adult. In human observations, the childhood emotional and behavioral problems are often corresponded with early abnormal development in infant[53]. For instance, the infant nonoptimal neuromotor development might predict the emotional problems in childhood[54]; the problems of infant crying and feeding are prognostic to poor social skills [55, 56] and adverse cognitive development[57] at the age of preschool. Therefore, importance of study in infancy should be emphasized also in rodent models; infancy is a sensitive and crucial period to understand the behavioral and neuronal complication at the beginning of life.

The highlight of this study is that we successfully modeled the "atypical" USV development in individual mouse by horizontal and vertical comparison. Compared with typically behavioral development in normal children (so called "neurotypical"),

ASD children show variety of “atypical” behavioral phenotypes[1, 58, 59]; they do not uniformly exhibit impaired scores in various criteria. In regard to our mice model, the cluster preference of USV development exposes the unique pattern in each individual mouse pup and also “atypical” patterns shaped by paternal aging. In addition, 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[53, 60, 61]. In the current study, we found that the USV patterns of PCA analyses were not the same in individuals during development even in the YFO, but more diverse in the AFO. At P3 (early infancy), each offspring emitted USV calls in different variation, but after P6, the cohort of YFO gradually obtained “typical” variation in their communication patterns by P12 (later infancy). By contrast, the AFO still showed great variation at P12. To elucidate the neural basis underlying the “atypical” development would be the next challenge.

The current study clearly demonstrates that detailed qualitative and quantitative syllable analysis can be a useful tool for communicative phenotypes in the sensitive period to understand etiology of ASD. 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 impairment in the structure of the motor cortex and neuronal activity indicated with c-fos expression in the paraventricular thalamus related with 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 for up to one week and separated from the female mice to minimize possible confounding factors against 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 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, 62], 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[63]. Briefly, the script computed the spectrograms from each waveform (60 second/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 12 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 which 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 continuously with a frequency modification ≤ 3 kHz.
4) Short syllables were displayed as a dot and shorter than or equal to 5 milliseconds.
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 component respectively. There was on time interval between adjacent components.
10) Harmonics syllables were displayed as one main component stacking with other harmonically components of different frequency.
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.

(4) Statistical analysis

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 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 percentage of 12 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 Figure 2 – 7, 9, and 14. The correlation between body weight and USV parameters was detected by Pearson correlation coefficient.

To 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 Akaike Information Criterion (AIC)[64-65]. Based on the fitted GMM, we classified each individual mouse pup into the cluster with maximum posterior probability. Then 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) along 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.

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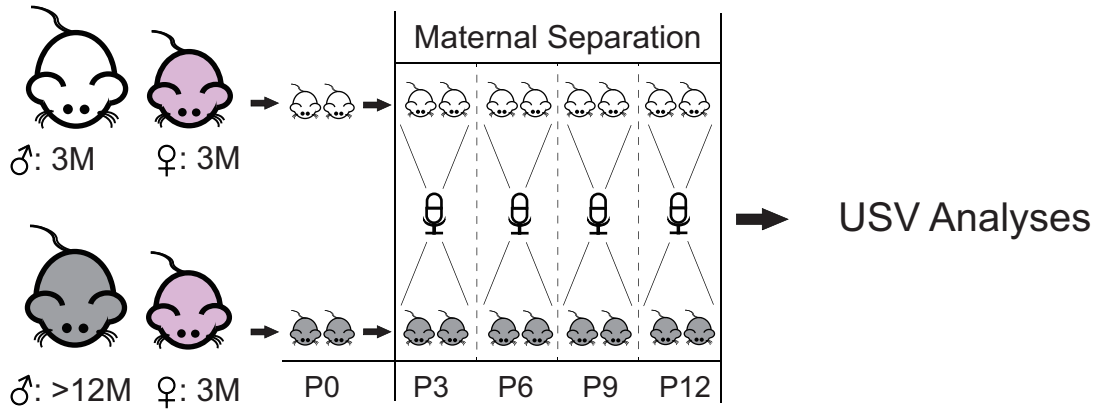
Fig 1

A


Maternal Separation
♂: 3M
♀: 3M
♂: >12M
♀: 3M
P0
P3
P6
P9
P12
USV Analyses

B

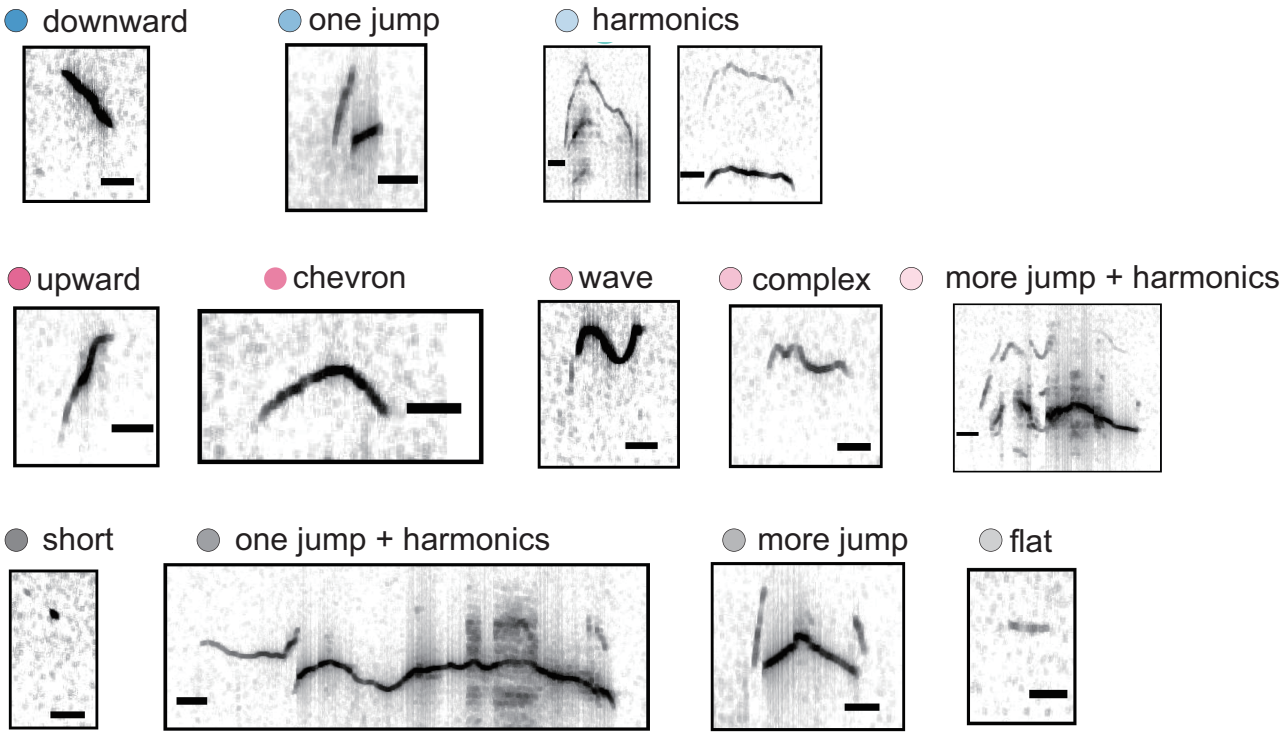

downward
one jump
harmonics
upward
chevron
wave
complex
more jump + harmonics
short
one jump + harmonics
more jump
flat

Fig 2

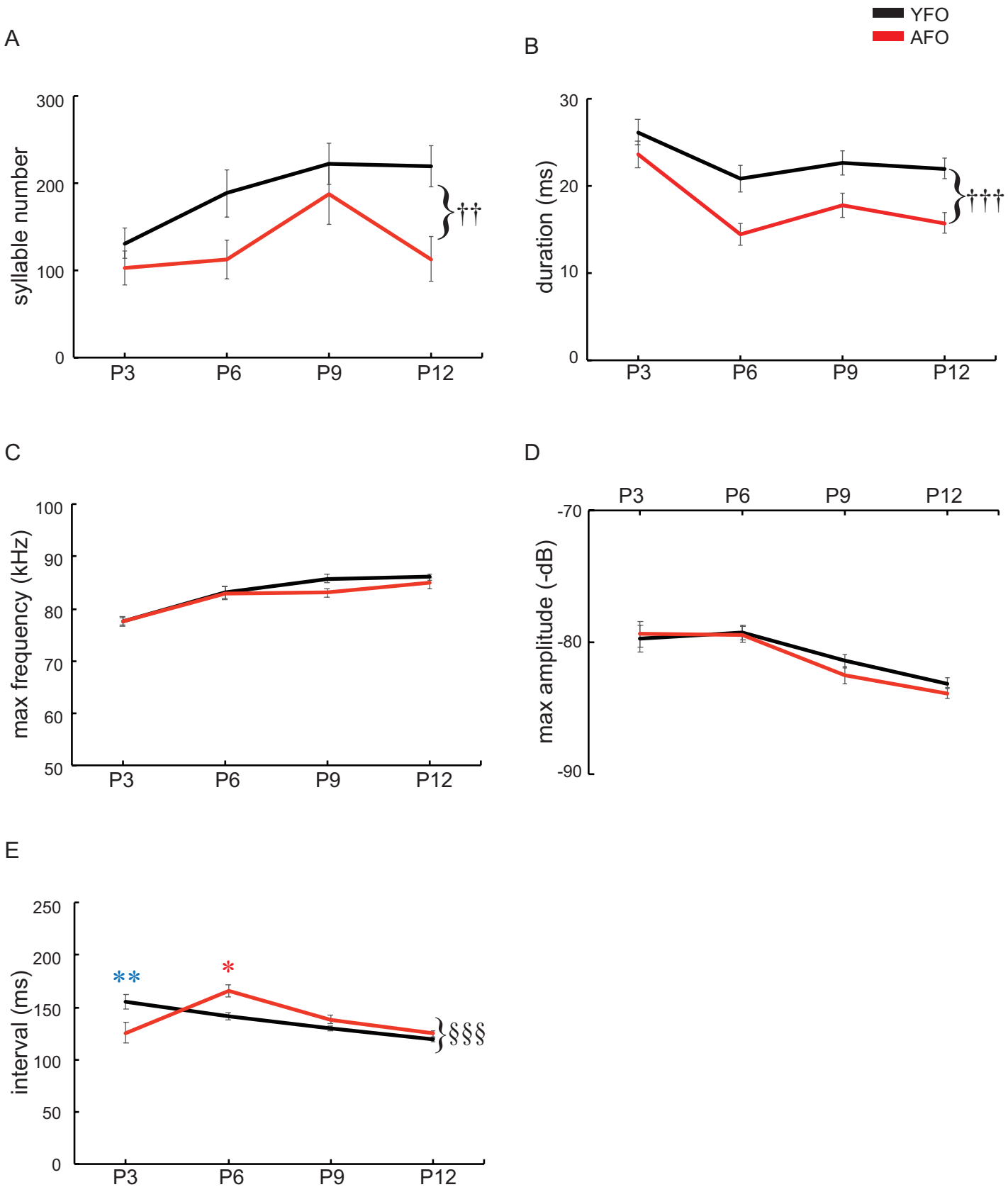

YFO
AFO
A
syllable number
300
200
100
0
P3
P6
P9
P12
††
B
duration (ms)
30
20
10
0
P3
P6
P9
P12
†††
C
max frequency (kHz)
100
90
80
70
60
50
P3
P6
P9
P12
D
max amplitude (-dB)
-70
-80
-90
P3
P6
P9
P12
E
interval (ms)
250
200
150
100
50
0
**
*
§§§
P3
P6
P9
P12

Fig 3

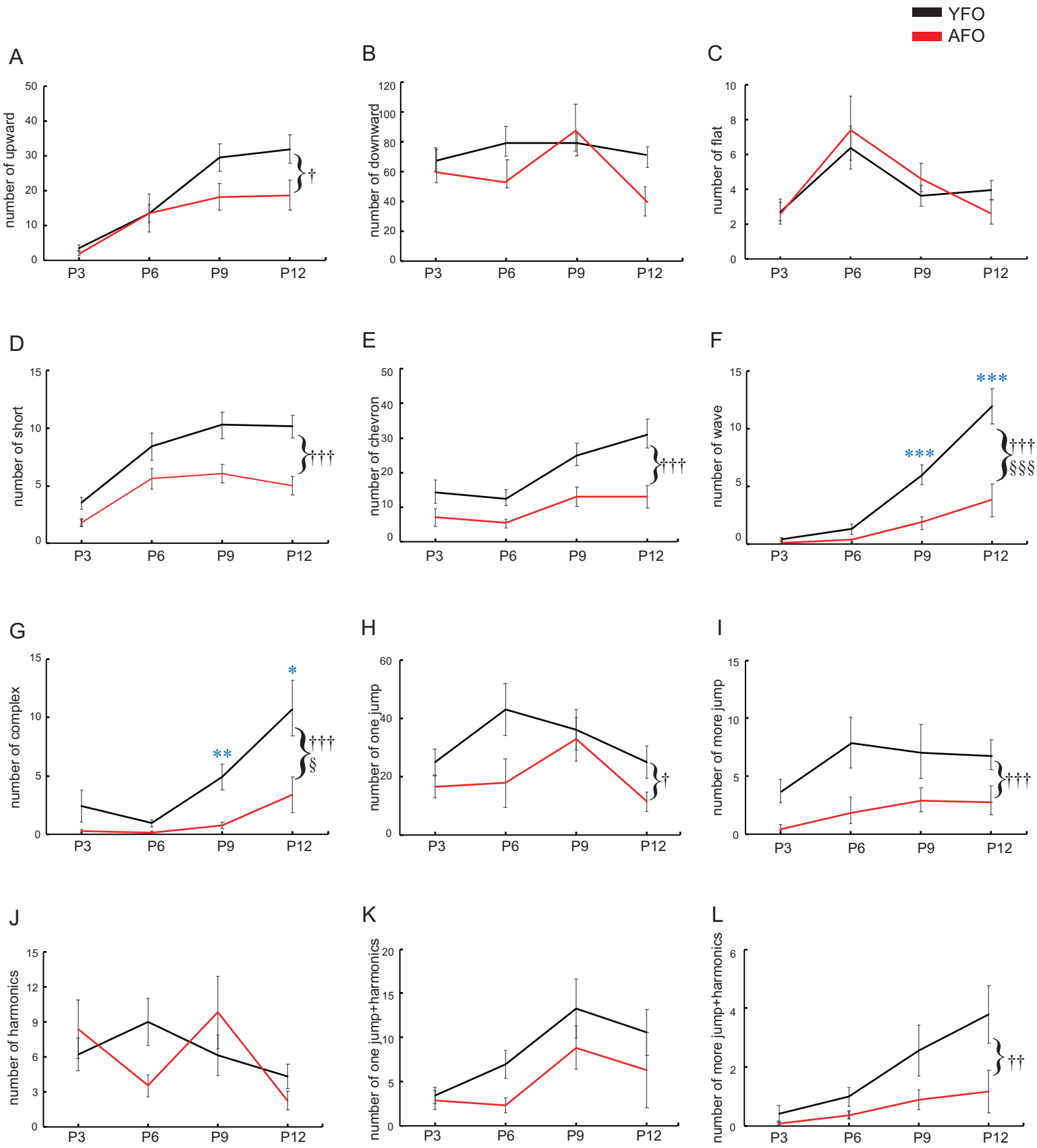

YFO
AFO
A
number of upward
B
number of downward
C
number of flat
D
number of short
E
number of chevron
F
number of wave
G
number of complex
H
number of one jump
I
number of more jump
J
number of harmonics
K
number of one jump+harmonics
L
number of more jump+harmonics
P3
P6
P9
P12

Fig 4

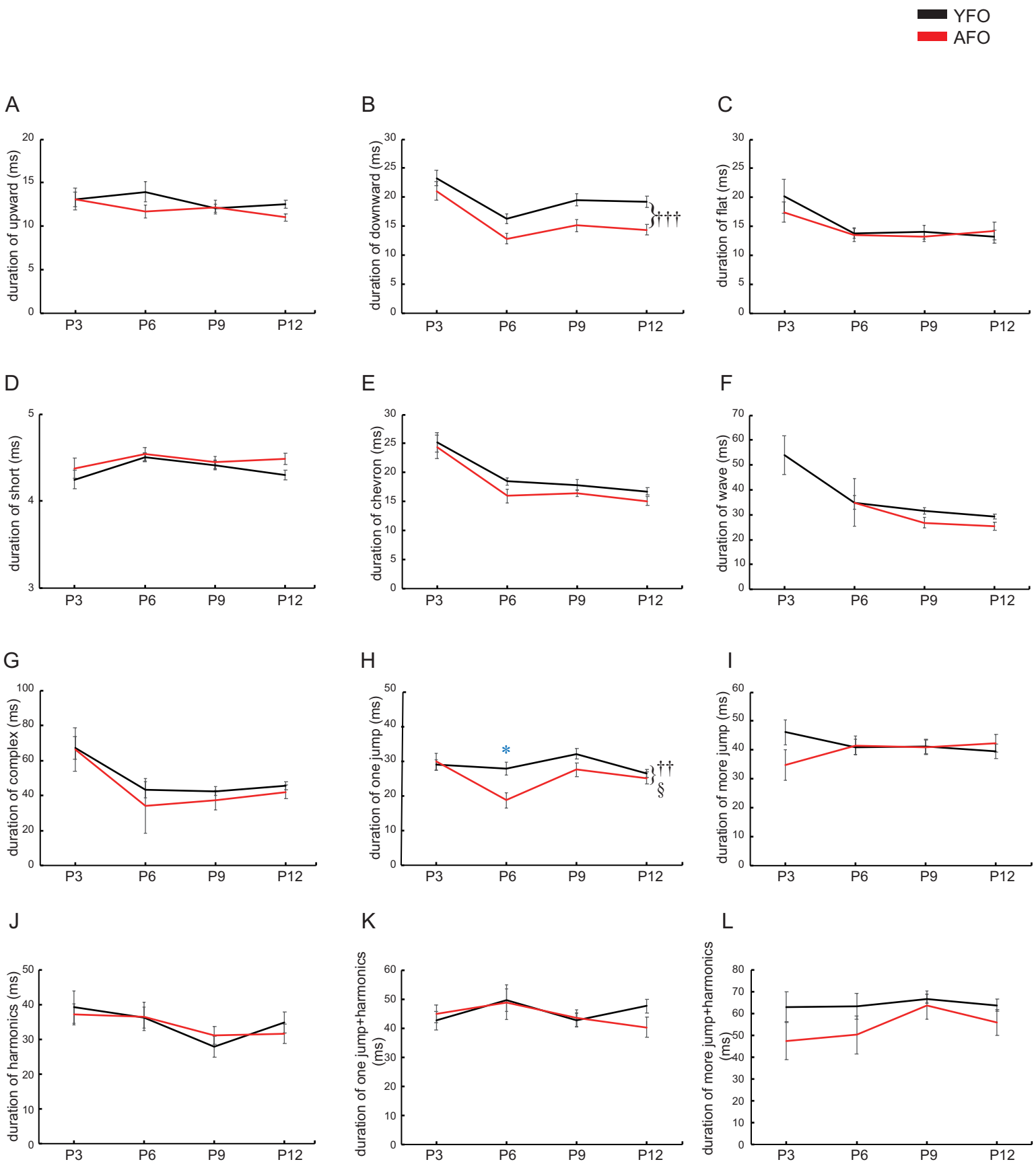

YFO
AFO
A
duration of upward (ms)
B
duration of downward (ms)
C
duration of flat (ms)
D
duration of short (ms)
E
duration of chevron (ms)
F
duration of wave (ms)
G
duration of complex (ms)
H
duration of one jump (ms)
I
duration of more jump (ms)
J
duration of harmonics (ms)
K
duration of one jump+harmonics (ms)
L
duration of more jump+harmonics (ms)
P3
P6
P9
P12
*
†††
††
§

Fig 5

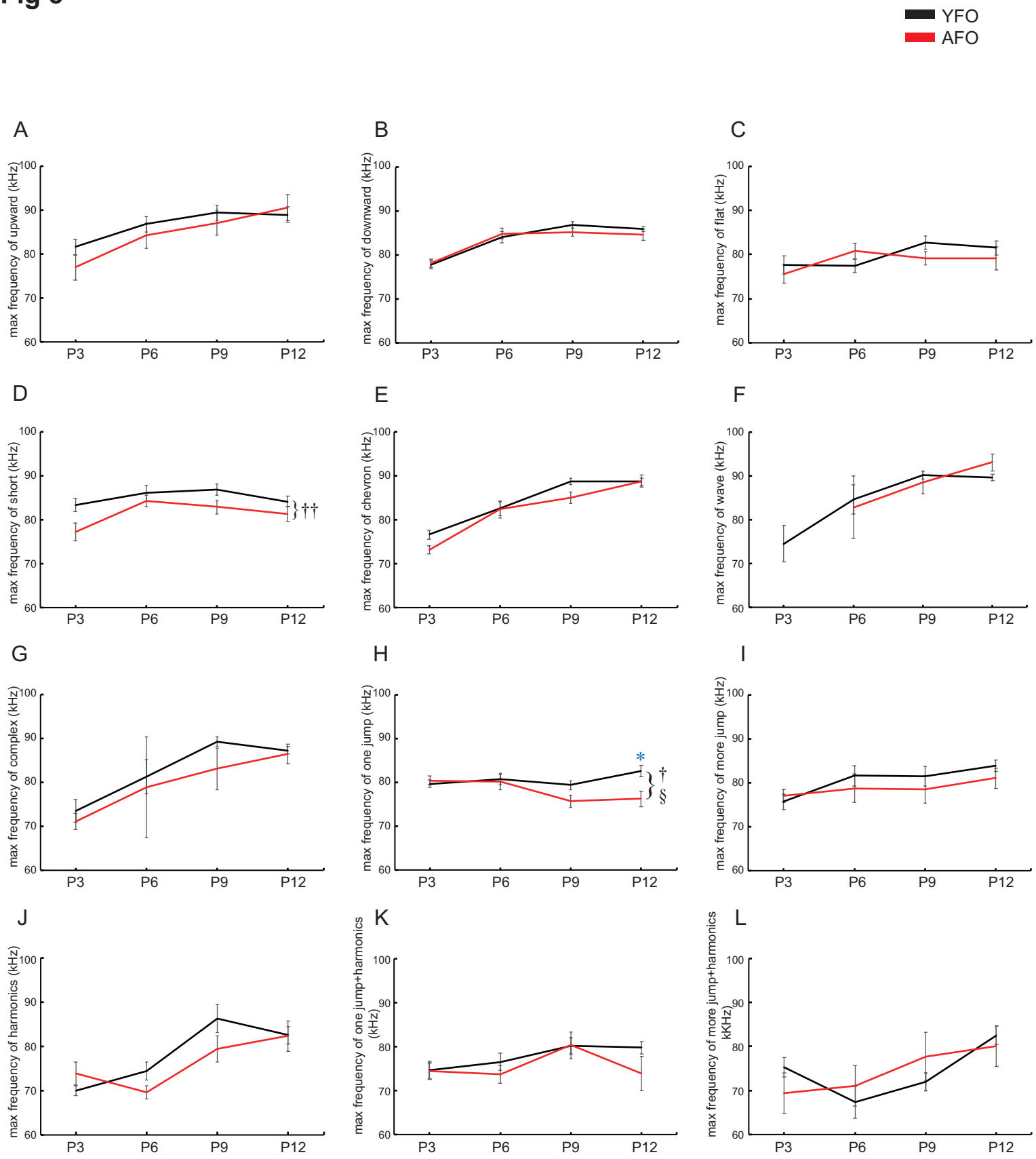

YFO
AFO
A
max frequency of upward (kHz)
B
max frequency of downward (kHz)
C
max frequency of flat (kHz)
D
max frequency of short (kHz)
††
E
max frequency of chevron (kHz)
F
max frequency of wave (kHz)
G
max frequency of complex (kHz)
H
max frequency of one jump (kHz)
*
†
§
I
max frequency of more jump (kHz)
J
max frequency of harmonics (kHz)
K
max frequency of one jump+harmonics (kHz)
L
max frequency of more jump+harmonics kKHz)
100
90
80
70
60
P3
P6
P9
P12

Fig 6

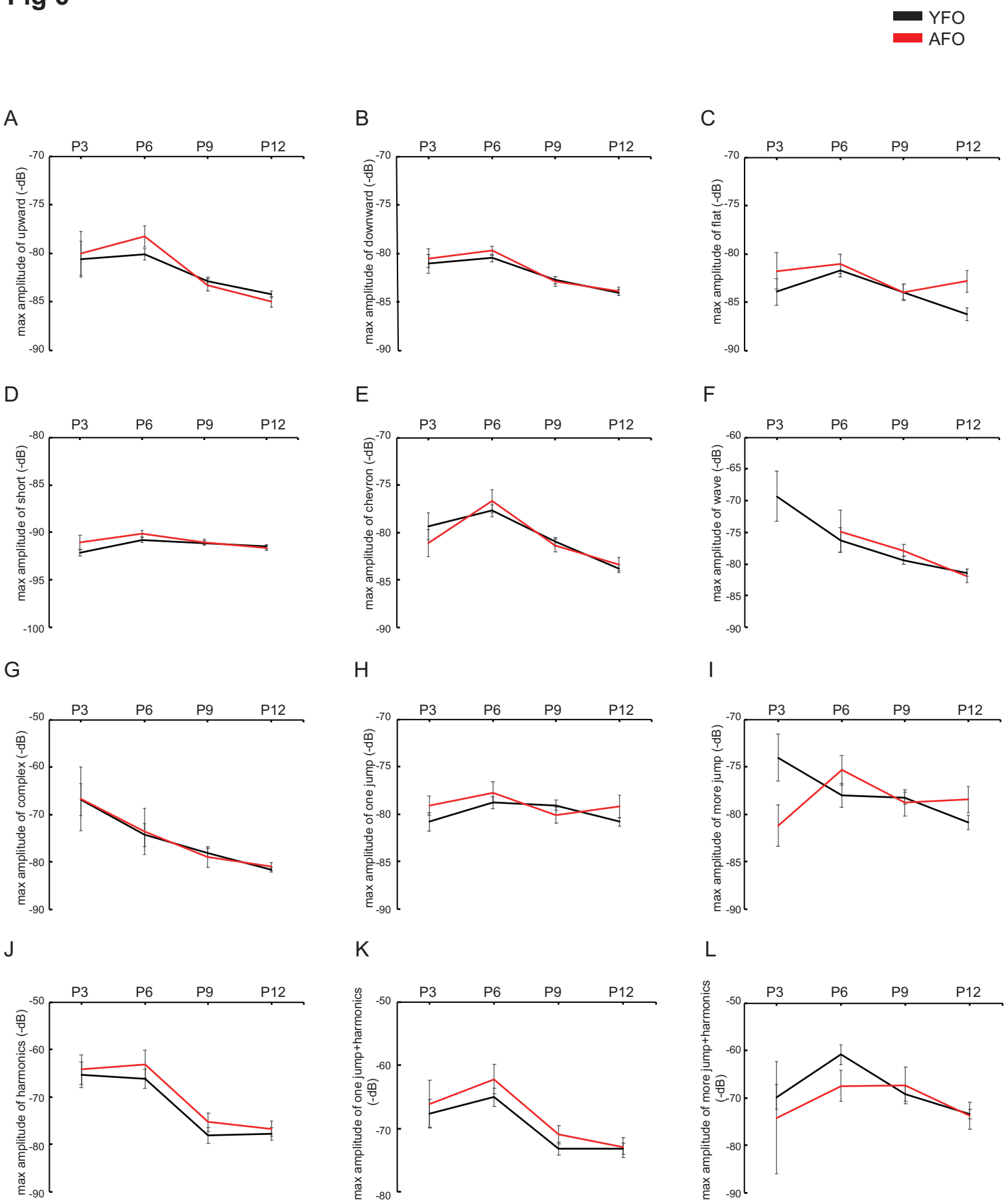

YFO
AFO
A
B
C
D
E
F
G
H
I
J
K
L
P3
P6
P9
P12
max amplitude of upward (-dB)
max amplitude of downward (-dB)
max amplitude of flat (-dB)
max amplitude of short (-dB)
max amplitude of chevron (-dB)
max amplitude of wave (-dB)
max amplitude of complex (-dB)
max amplitude of one jump (-dB)
max amplitude of more jump (-dB)
max amplitude of harmonics (-dB)
max amplitude of one jump+harmonics (-dB)
max amplitude of more jump+harmonics (-dB)

Fig 7

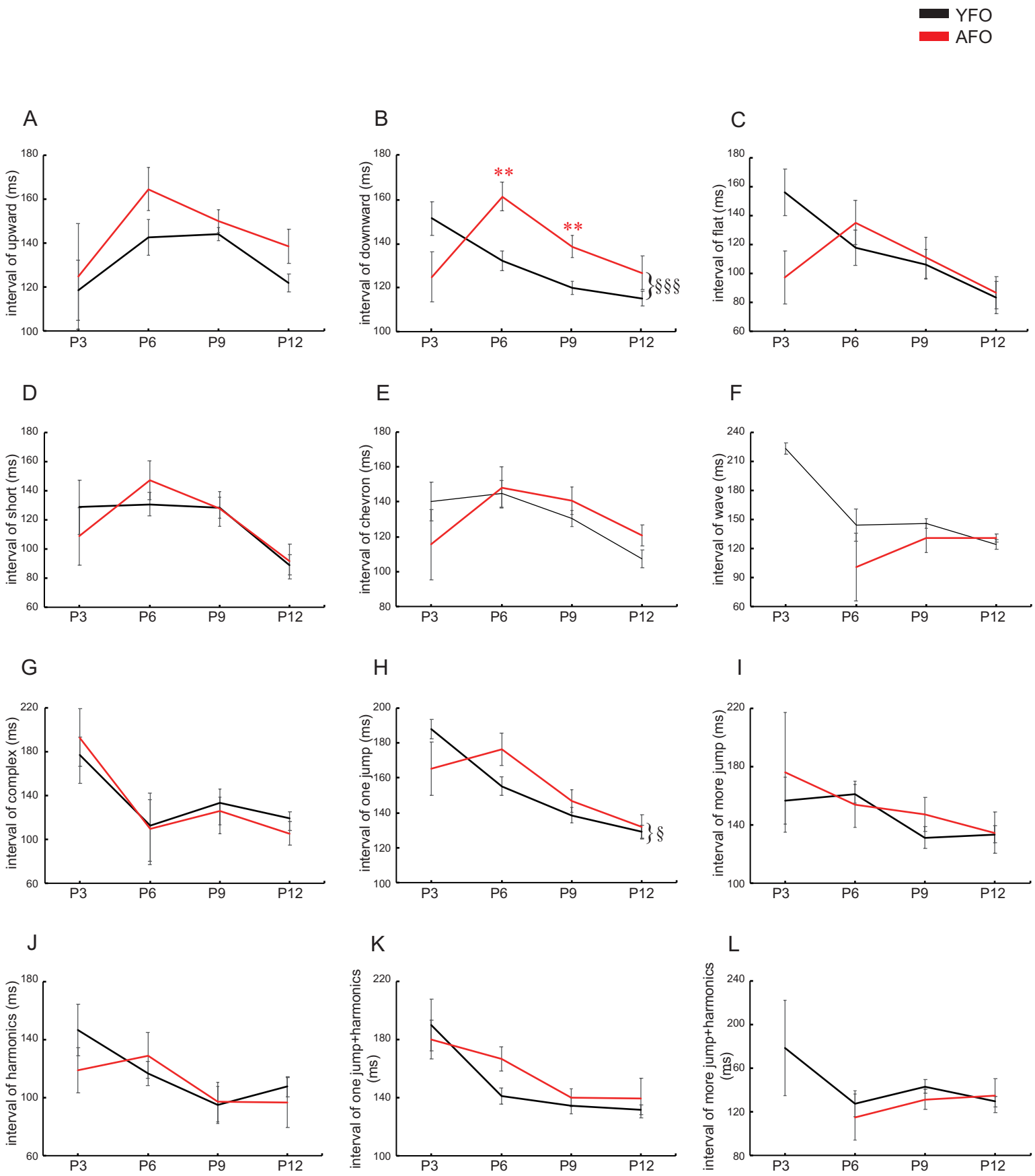

YFO
AFO
A
interval of upward (ms)
B
interval of downward (ms)
**
**
§§§
C
interval of flat (ms)
D
interval of short (ms)
E
interval of chevron (ms)
F
interval of wave (ms)
G
interval of complex (ms)
H
interval of one jump (ms)
§
I
interval of more jump (ms)
J
interval of harmonics (ms)
K
interval of one jump+harmonics (ms)
L
interval of more jump+harmonics (ms)
P3
P6
P9
P12

Fig 8

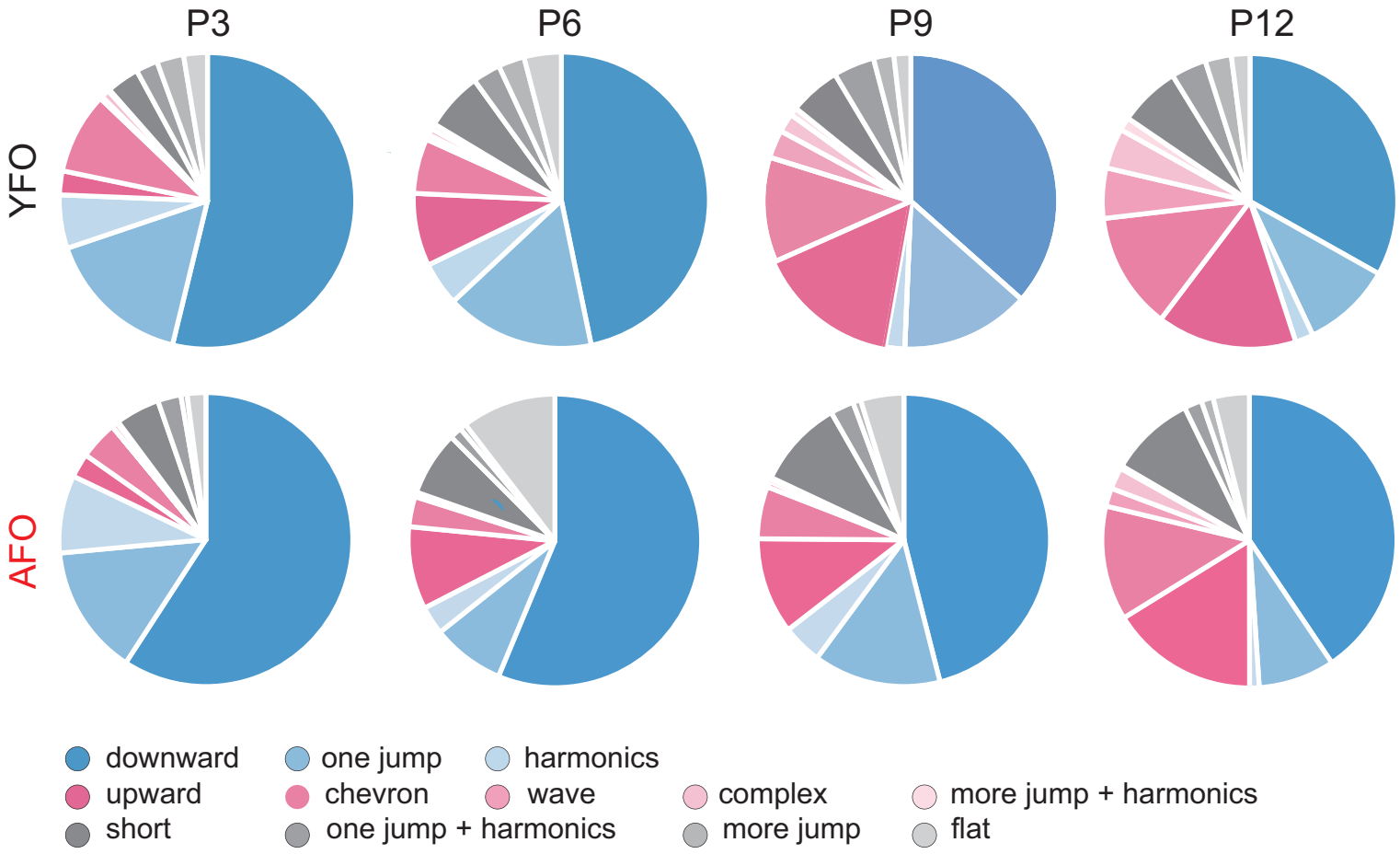

P3
P6
P9
P12
YFO
AFO
downward
one jump
harmonics
upward
chevron
wave
complex
more jump + harmonics
short
one jump + harmonics
more jump
flat

Fig 9

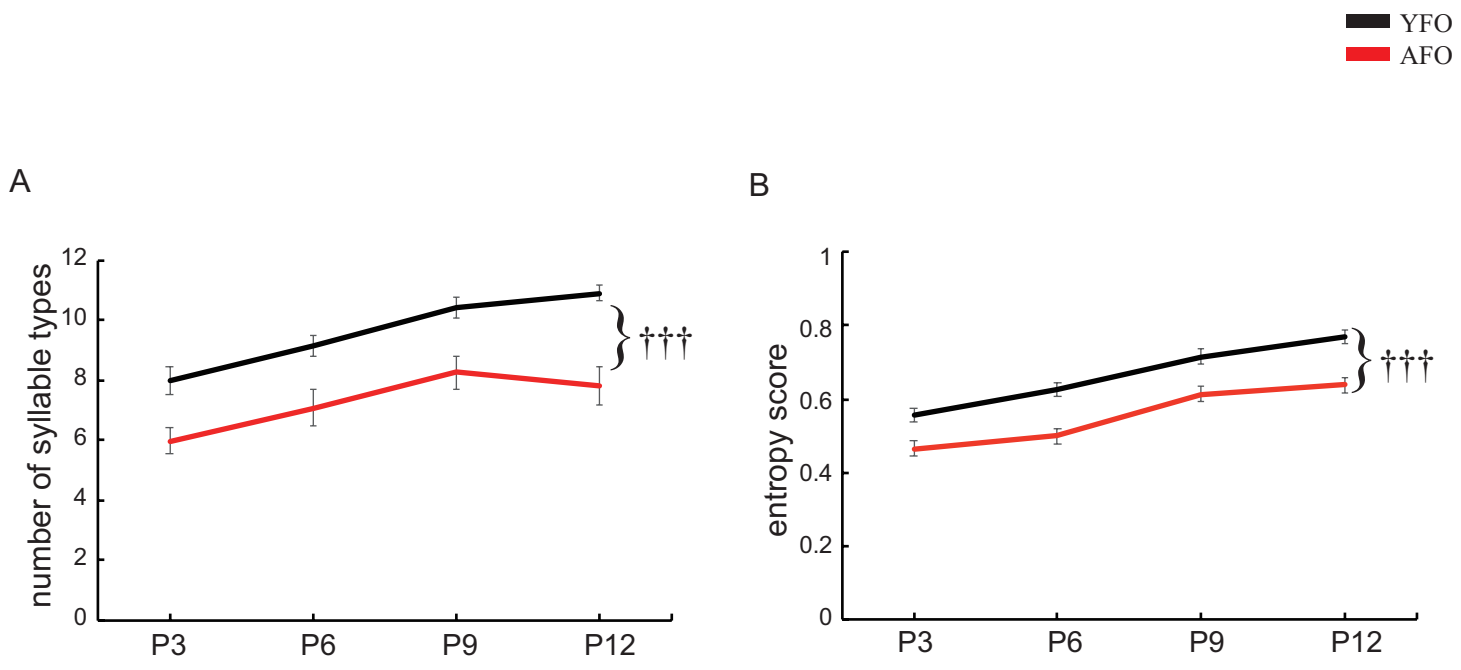

YFO
AFO
A
number of syllable types
12
10
8
6
4
2
0
P3
P6
P9
P12
†††
B
entropy score
1
0.8
0.6
0.4
0.2
0
P3
P6
P9
P12
†††

Fig 10

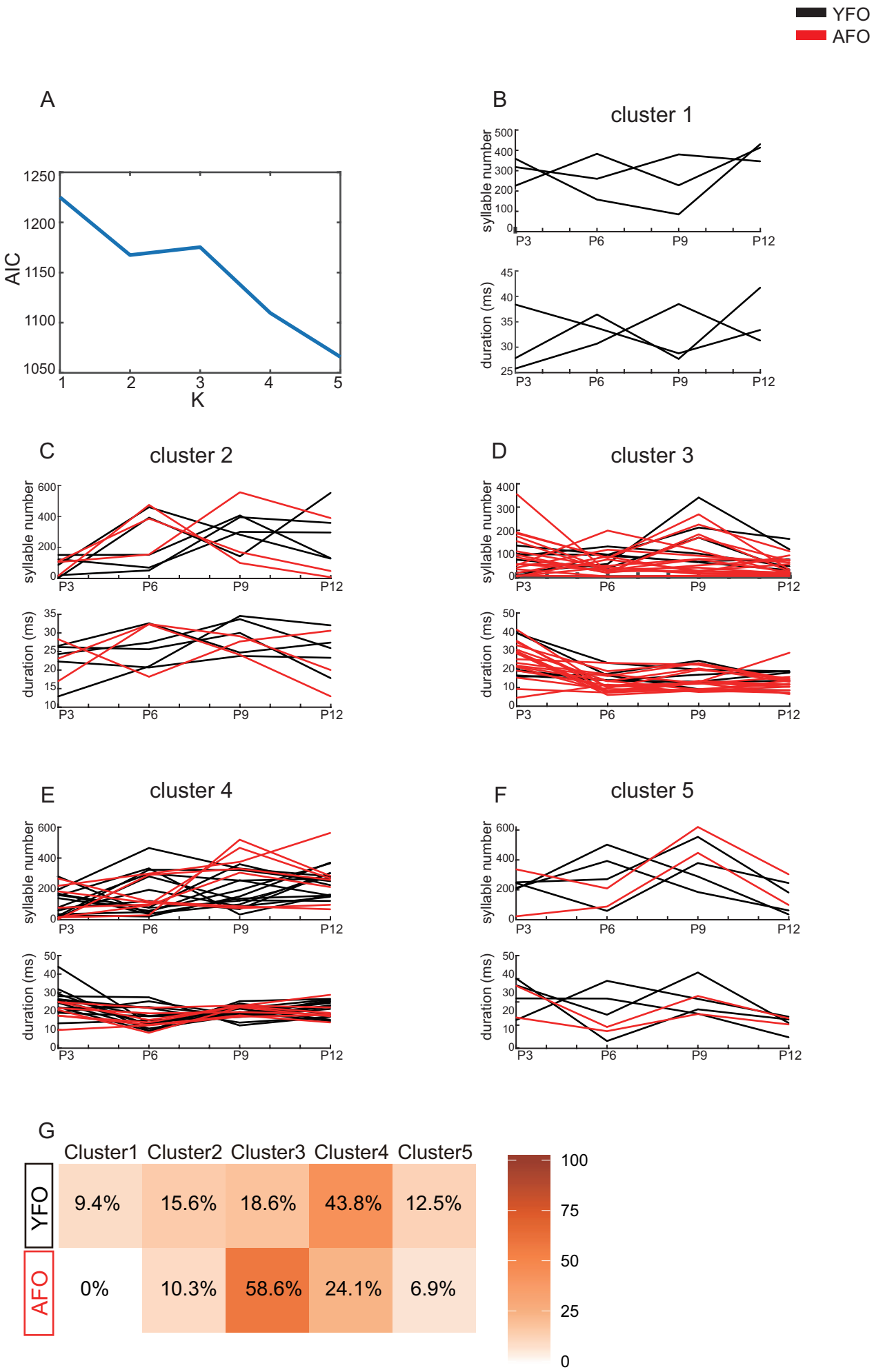

YFO
AFO
A
AIC
K
B
cluster 1
syllable number
duration (ms)
C
cluster 2
D
cluster 3
E
cluster 4
F
cluster 5
P3
P6
P9
P12
G
Cluster1 Cluster2 Cluster3 Cluster4 Cluster5
YFO
9.4% 15.6% 18.6% 43.8% 12.5%
AFO
0% 10.3% 58.6% 24.1% 6.9%
100
75
50
25
0

Fig 11

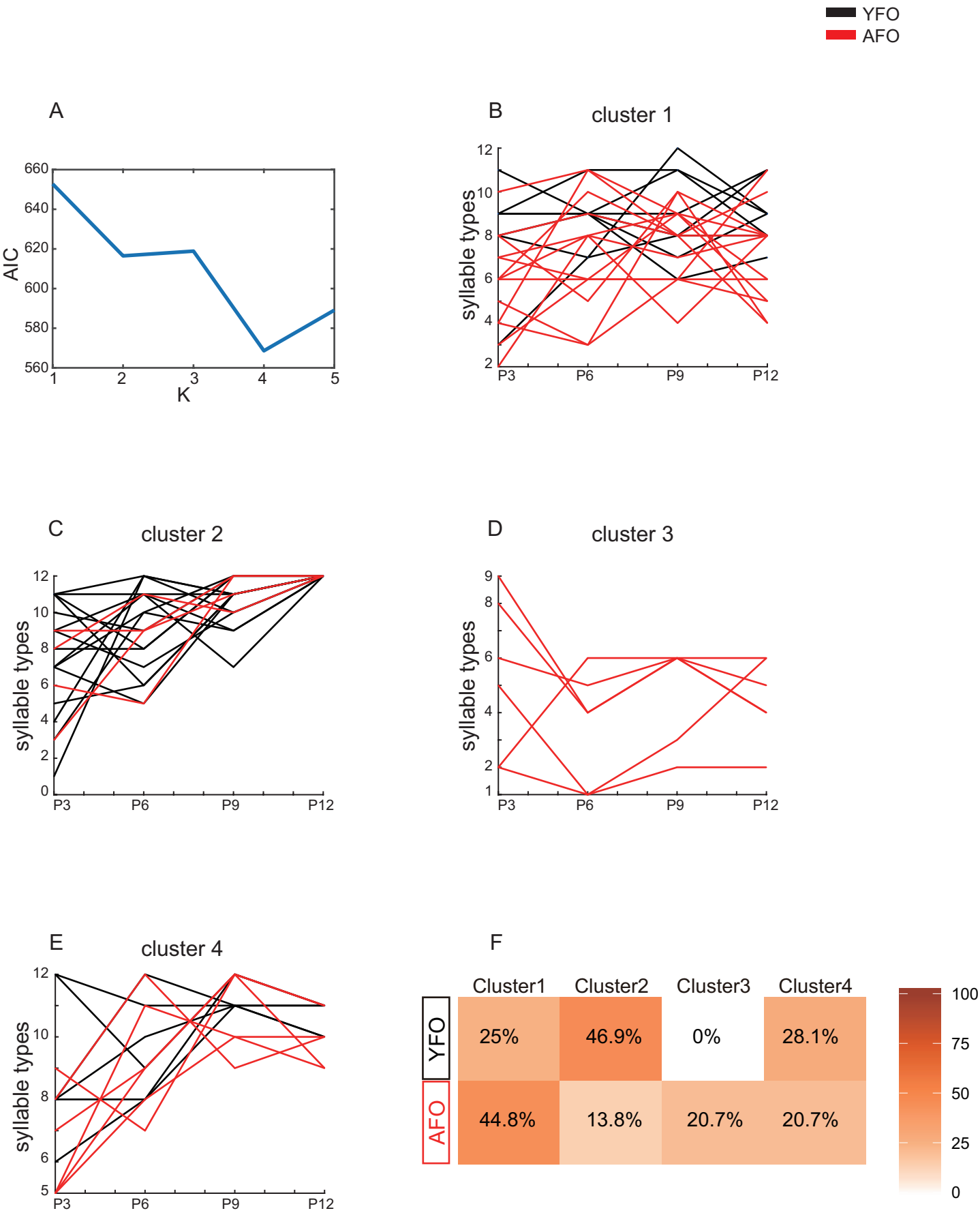

YFO
AFO
A
AIC
K
B
cluster 1
syllable types
C
cluster 2
syllable types
D
cluster 3
syllable types
E
cluster 4
syllable types
P3
P6
P9
P12
F
Cluster1
Cluster2
Cluster3
Cluster4
YFO
AFO
25%
46.9%
0%
28.1%
44.8%
13.8%
20.7%
20.7%
100
75
50
25
0

Fig 12

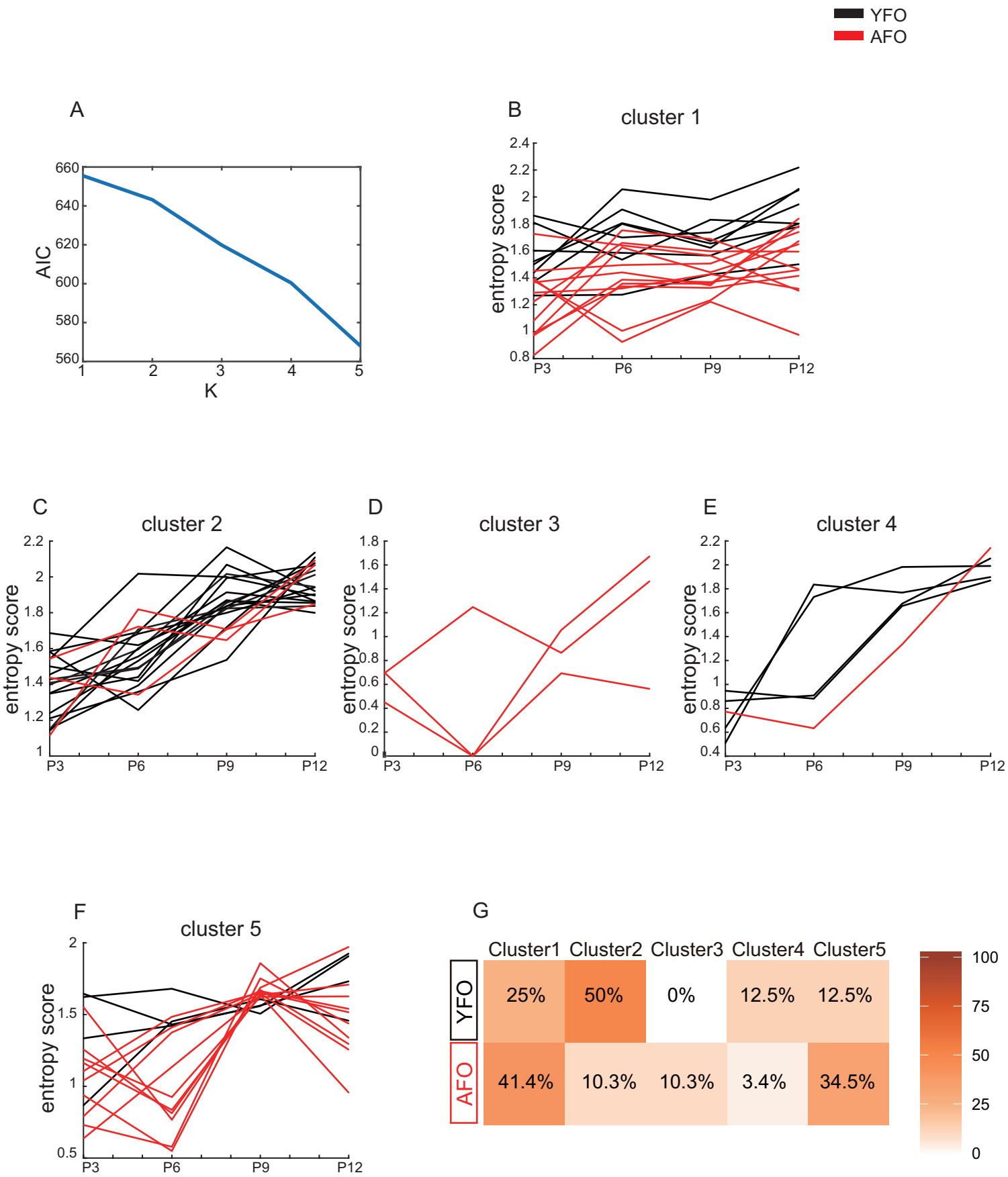

YFO
AFO
A
AIC
K
B
cluster 1
entropy score
C
cluster 2
D
cluster 3
E
cluster 4
F
cluster 5
P3
P6
P9
P12
G
Cluster1
Cluster2
Cluster3
Cluster4
Cluster5
YFO
AFO
25%
50%
0%
12.5%
12.5%
41.4%
10.3%
10.3%
3.4%
34.5%

Fig13

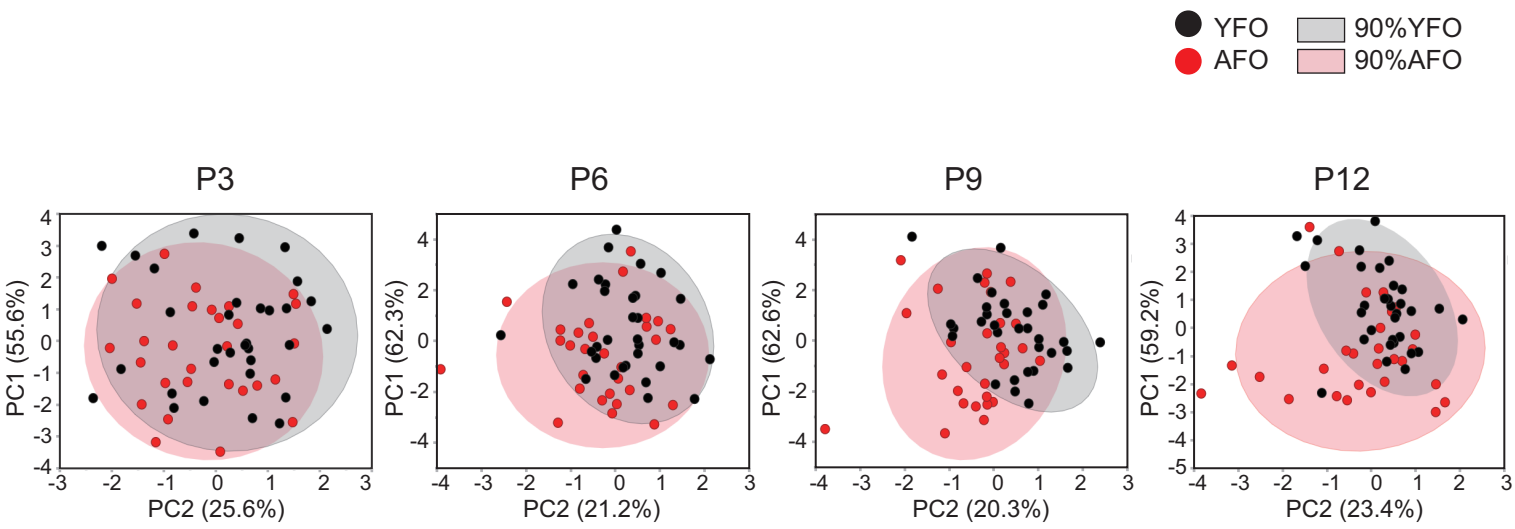

YFO
AFO
90%YFO
90%AFO
P3
P6
P9
P12
PC1 (55.6%)
PC2 (25.6%)
PC1 (62.3%)
PC2 (21.2%)
PC1 (62.6%)
PC2 (20.3%)
PC1 (59.2%)
PC2 (23.4%)

Fig 14

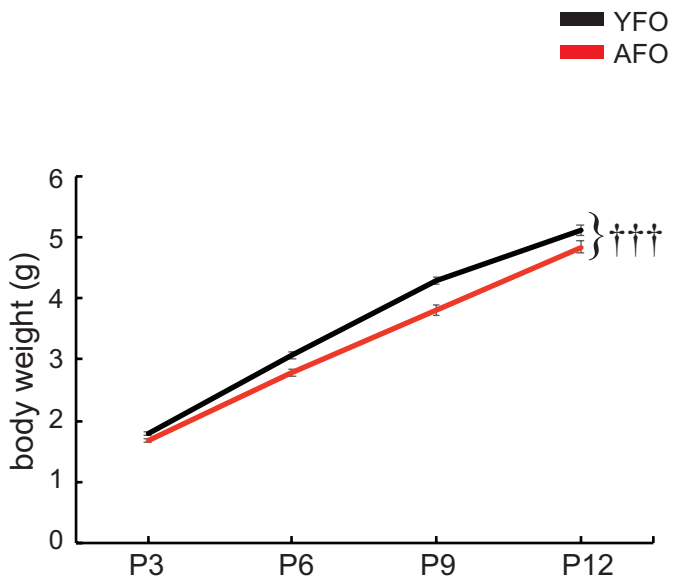
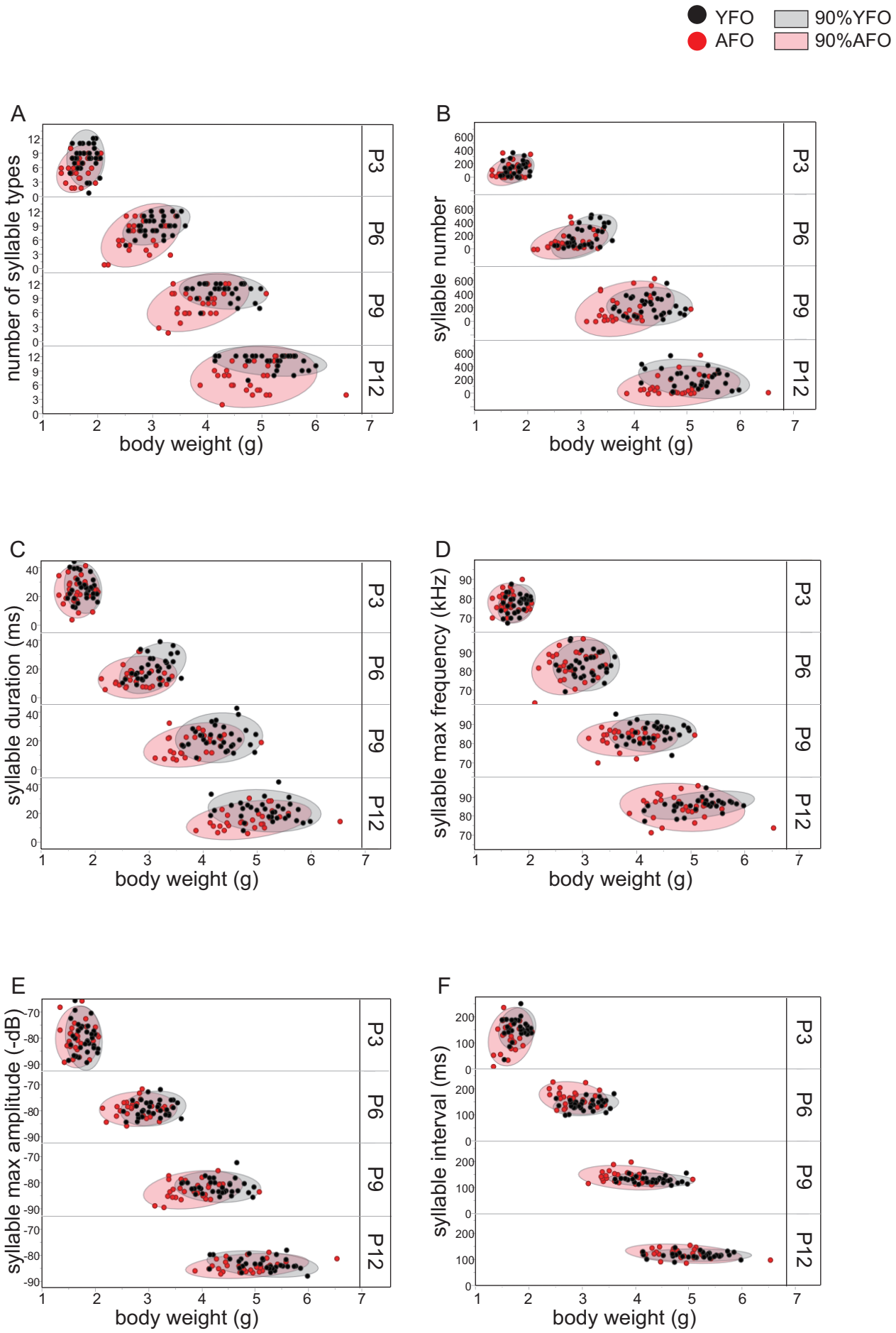


Fig 15


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)