Age-dependent changes in dogs' (*Canis familiaris*) separation-related behaviours in a longitudinal study

András Marx[1*], Rita Lenkei[1], Paula Pérez Fraga[1,2], Lisa Wallis[1,3], Enikő Kubinyi[1], Tamás Faragó[1]

1 Department of Ethology, ELTE Eötvös Loránd University, Budapest, Hungary

2 MTA-ELTE 'Lendület' Neuroethology of Communication Research Group, Hungarian Academy of Sciences

3 Department of Livestock and One Health, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

*Corresponding author, contact: marxandras@gmail.com; present address: Department of Ethology, ELTE Eötvös Loránd University, Pázmány Péter stny. 1/C Budapest, Hungary, H-1117

Abstract

Separation related problems (SRP) caused by distress associated with separation from the preferred member of the social group, can be characterised by their symptoms e.g., excessive vocalisation. In dogs' separation whines, nonlinear phenomena (NLP) (abrupt changes in the resonance of the vocal folds) might occur, which could be adaptive in communicating aroused inner states. Previously, using a separation test we found that more dogs that were classified as having SRP by their owner have NLP in their whines than nonaffected dogs and that NLP ratio increases with age, which suggests that separation stress might intensify with age.

We repeated the separation test 21.19±9.37 months later with 32 dogs from the previous study to investigate longitudinally how separation behaviour and vocalisations change with age. Beside behaviour, we measured the acoustic structure of the whines (jitter - small-scale irregularity of the pitch, entropy - vocal harshness, call length, pitch (f_0) related parameters, and the spectral components) and calculated the NLP ratio. We formed clusters based on the dogs' behaviour changes from the first test to the second, to see individual ageing patterns. Finally, we compared the dogs' behaviour and the acoustic structure of their whines in the two occasions.

We found that dogs could be clustered by the changes in their separation behaviour. 41 % of the dogs were stable over time, 38 % improved, and 16 % showed an increase in their separation behaviours. 3 % switched from barking to whining. Interestingly, SRP dogs were stable, some of them even showed improvement in their separation behaviour. On the contrary, we also found that SRP dogs tended to have an increased NLP ratio with age from test 1 to test 2 (p=0.09), showed less escape-related behaviour (p=0.01), but tended to spend more time passively whining at the door through which the owner left the room (p=0.05), than non-SRP dogs.

The behavioural and vocal results suggest elevated stress levels in SRP dogs with age, although they did not decline but mostly stayed stable in their separation behaviour, confirming that there could be a connection between SRP status, age, and NLP. However, together with the results of the clustering that showed that there are different patterns in dogs' separation behaviour, we emphasise the importance of individual level longitudinal investigations in order to facilitate the early diagnosis of SRP and to provide a solid basis for the development of individualised treatment plan for SRP dogs.

Keywords: *Canis familiaris*, ageing, separation, stress, separation-related problems, NLP

Introduction

Ageing is a complex phenomenon, which results in age-related changes in cognitive and physiological functions, leading to a decline in these functions and a decrease in survival and reproduction rate in old age (Aunan et al., 2016; Bonduriansky et al., 2008; Rose et al., 2012). Most of the dog ageing research has concentrated on the pathological aspects of ageing (for more details see: Szabó et al., 2016). However, not every individual suffers from the same age-related symptoms and some successful agers are less or not affected at all by these declines (Rowe and Kahn, 1997, 1987; Rowe and Minaker, 1985).

Dogs have always been popular as companion animals in the urbanised world (McConnell et al., 2011). Due to increased levels of care and advanced veterinary medicine, more and more dogs are living a longer life: e.g., in Tokyo, dogs have experienced a 1.67-fold increase in longevity over the past three decades (Inoue et al., 2018). This means that the physiological and behavioural changes associated with advancing age and related problems might compromise welfare and affect the owner-dog relationship (Davies, 2012; Sándor and Kubinyi, 2019). However, studying ageing should start at an early age, with continuous monitoring throughout the lifespan to be able to differentiate between the typical change trajectories during ontogenesis, and to validate the results of cross-sectional studies.

One of the major behavioural problems that might occur in dogs is the separation related problems (SRP) (Mongillo et al., 2013; Schwartz, 2003). Distress associated with s*eparation* from the preferred member of the social group has been described in many species e.g. pigs (Schrader and Ladewig, 1999), birds (Davis, 1991; Sufka and Hughes, 1991), cats (Guyot et al., 1980; Schwartz, 2002), and nonhuman primates (Gust et al., 1994; Laudenslager et al., 1990; Norcross and Newman, 1999), and is one of the most common problems found in companion dogs (Bamberger and Houpt, 2006; Borchelt and Voith, 1982a; Denenberg et al., 2005; Konok et al., 2011, 2015, 2019; Lenkei et al., 2018; Lund and Jørgensen, 1999; Pongrácz et al., 2017) and seems to worsen with advancing age ((Marx et al., 2021; Mongillo et al., 2013)).

Anxiety is defined as an emotion of apprehension to an anticipated danger or threat. The terms ’separation distress’ or ‘separation-related behaviour problems’ describe the phenomenon more precisely in the case of dogs, and both incorporate signs consistent with anxiety, fear, frustration, and phobic behaviour (de Assis et al., 2020; Lenkei et al., 2018; Lund and Jørgensen, 1999; Sherman and Mills, 2008). The signs related to SRP are only observable in the owner’s real or perceived absence or shortly after her/his departure (McCrave, 1991), and include excessive vocalisation (barking and whining), destructive behaviour (scratching, chewing), and urinating and defecating at inappropriate places (King et al., 2000; Konok et al., 2011; Overall et al., 2001; Schwartz, 2003; Sherman and Mills, 2008). The different physiological problems that occur in adulthood or in old age might affect the level of stress dogs experience during separation (Schwartz, 2003). Older dogs in particular adapt harder to changes in general, so even small disturbances can cause distress to the dog, and they cope less efficiently with emotional distress caused by a mild social challenge. Accordingly, (Mongillo et al., 2013) found that aged dogs sought more physical contact with their owners than adult dogs, and showed a significant increase in salivary cortisol concentrations after they were separated from their owner, even though they were more passive during the separation.

Several cross-sectional studies have concentrated on the different risk factors and causes of SRP both on the genetic and environmental levels, from attachment theories through sex, age, and or early negative experiences of the dogs, to a low level of frustration tolerance, and high fearfulness and anxiety (Appleby and Pluijmakers, 2004; Borchelt and Voith, 1982b, 1982a; Flannigan and Dodman, 2001; Konok et al., 2011; Lenkei et al., 2018; Pongrácz et al., 2017). However, due to the lack of longitudinal individual level investigations, individual differences and risk factors could only be assessed on population level patterns, in which affected individuals are the outliers. As a consequence, these studies could only have a small impact in veterinary medicine that aims to diagnose and treat the individuals, who are suffering from separation distress (de Assis et al., 2020).

The intensification of separation-related problems due to coping ability change through the development and ageing of dogs (Bishop, 2007; Fast et al., 2013; Landsberg et al., 2012), may be detectable in their vocal behaviour too. The acoustic signals of mammals are the result of an interaction between the nervous system and the vocal apparatus (Fitch et al., 2002). The Source-filter theory (Fant, 1960; Titze, 1994) explains this connection between inner states and vocalisations' acoustic structure. The vocal apparatus has two different functional parts: the source, including the lungs and the larynx, that affects the pitch related parameters and call length, and the filter, that includes all the cavities (pharynx, oral and nasal cavity) and obstacles (tongue, teeth) from the larynx to the nose and lips, which modifies the spectral components of the vocalisation. Neural changes, due to emotion arousal (low or high emotional intensity) and valence (positive or negative emotional load), affect different aspects of sound production, resulting in different vocal outputs that provide the basis of general emotion encoding rules (Briefer, 2020, 2012).

The vocal folds normally produce a tonal, periodic sound with harmonic structure. But as vocal folds are coupled oscillators, they can generate nonperiodic signals as well as periodic ones (Berge et al., 1986; Glass and Mackey, 1988) leading to noisy, irregular, atonal sections in the vocalisations, called nonlinear phenomena (NLP). Each vocal fold movement affects the other's, thus even small differences between their movements can lead to complex vibration patterns and abrupt, unpredictable transitions between periodic, quasiperiodic and nonperiodic vibratory states (Berry et al., 1994). Simply put, the asynchronization of the vocal folds' oscillations causes abrupt changes in the harmonic structure of the sound produced in the larynx (Wilden et al., 1998). For more information on NLP see Fitch et al., 2002; Marx et al., 2021; Riede et al., 1997; Wilden et al., 1998. Despite the fact that NLP are by-products of vocalisations (Fitch et al., 2002), they could have an adaptive function in vocal communication during social interactions (Anikin et al., 2020; Blumstein et al., 2012; Fitch et al., 2002; Karp et al., 2014; Manser, 2001; Townsend and Manser, 2011), and in distress vocalisation e.g.: separation calls (Marx et al., 2021).

The occurrence of NLP might be connected to elevated spectral noise and less regular pitch (Briefer, 2012). This spectral noise can be measured by harmonics-to-noise ratio (the ratio of amplitude peaks of detectable harmonics to noise threshold (HNR); higher HNR indicates more tonal vocalisations, Briefer, 2012) or wiener entropy (from here entropy: the ratio of the geometric mean to the arithmetic mean of the spectrum; higher entropy indicates noisier and harsher vocalisations, Briefer, 2012). Jitter, the frequency variation from cycle to cycle is an accurate measure of regularity or stability of pitch of vocalisations (higher jitter - less regular or less stable pitch, Teixeira et al., 2013).

We can assume that vocal harshness (measured in the current study by entropy), small-scale irregularity or instability of pitch (jitter) and the occurrence of NLP (Anikin et al., 2020) are an honest signal of negative arousal (Briefer, 2012; Faragó et al., 2014) as an aroused inner state causes an intensified tension in the vocal folds, favouring the occurrence of NLP and/or elevated spectral noise in the signal. Communicating arousal level could be adaptive, as these calls occur in contexts when the caller is in need and dependent on another individual or social group (Lingle et al., 2012). The arousal level of the caller causes higher jitter, indicating less regular vocal cycles, leading to unstable pitch (f_0), higher entropy, and lower harmonic-to-noise ratio (HNR), resulting in less tonal vocalisations and an elevated NLP ratio, which provides an indication of the aroused inner state of the caller. As excessive vocalisation, especially whining, is one of the salient symptoms of SRP in dogs (Konok et al., 2019; Lund and Jørgensen, 1999; Marx et al., 2021; Pongrácz et al., 2017), in which NLP are quite prominent and common (Marx et al., 2021), we can assume that NLP and spectral noise might be an honest indicator of dogs' inner state. Accordingly, in a separation study we investigated the connection between stress and the acoustic structure of vocalisations (Marx et al., 2021), and found that dogs with SRP produced NLP whines more likely than non-SRP dogs. Interestingly, we also found that there are more NLP in the whines of older dogs, than in younger dogs' whines prompting us to further study the age-related change of NLP ratio in dogs' whines.

Our main goal here was to test how ageing affects the dogs' separation related behaviour with a repeated separation test, in a longitudinal study. We have very little knowledge about the different aspects of age-related changes in separation behaviours, and how dogs' arousal level is coded in occurrences of NLP and spectral noise (Azkona et al., 2009; Neilson et al., 2001; Osella et al., 2007; Salvin et al., 2010). Some authors in cross-sectional studies (Marx et al., 2021; Mongillo et al., 2013) have addressed age effects on separation behaviour, but it is important to support these findings in repeated, longitudinal studies, as these have the potential to indicate the direct effects of ageing

(Salthouse, 2019). Our study, besides being interesting from an ethological and animal welfare point of view, could also be a step forward in developing a system for diagnostical purposes, which could help the veterinarians and the owners in diagnosing, monitoring and treating separation behaviour problems.

Hypothesis

Due to the stress caused by separation at an unfamiliar place we expected that SRP dogs will show increased duration of vocalisations, and stable or decreasing activity during separation (rearing up against the door, scratching at the door, pacing etc.) with advancing age compared to non-SRP dogs with the same age distribution. In the acoustic structure of whines of SRP dogs, we expected to find a positive corelation between age (from younger to older SRP dogs) and increasing NLP ratio, entropy, and jitter; pitch, which might be connected to an elevated stress level. In the case of non-SRP group we did not expect similar behavioural and acoustic changes.

We anticipated that groups can be formed based on the behaviour of the dogs in the two tests, that represent different trajectories of change (stable, improving and declining) in separation behaviour and inner states of the different individuals as they age.

An alternative hypothesis to explain the connection between age and acoustic parameters would be to take ageing tissues into account. The physiological changes connected to ageing, such as deteriorating neural control, and the ageing tissues involved in vocalisations could cause similar vocal phenomena. Similarly, in the long term, the more the dog vocalises generally, the more its phonatory apparatus wears out, which could result in more NLP. Nonetheless, to a certain extent we can exclude these alternative hypotheses, if the dogs’ behaviour also implies an elevated stress level, and if we find differences between the groups of SRP status on stress -related behaviours and the acoustical structure of their whines. Furthermore, owners might perceive their dogs’ separation status in the light of the noisiness of their separation whines, which could magnify the connection between NLP and SRP. The clusters emerging based on the separation behaviour could show how strong this bias could be.

In this paper we concentrate on the clustering and the acoustic changes during ontogenesis. For the results of breed, sex, reproductive status, and traumatic background see the Supplemental Information. In this paper we are not going to discuss these results in detail.

Methods

Ethical statement

The owners of the dogs were informed about the goals and circumstances of the experimental procedure before the test, they were present during the test, and they could watch their dogs' behaviour on the computer screen in real time whilst separated from their dog after leaving the testing room. We also informed them that they could interrupt the experiment and reconsider their participation if at any point they believed the test was too stressful for the dog. The Animal Welfare Committee of the Eötvös Loránd University reviewed and accepted the protocol of the experiment (Ref. no.: PEI/001/1056-4/2015).

Subjects

We retested 32 dogs from our earlier separation study (Marx et al., 2021 N_{male}= 17, N_{female}= 15; mean $age_{1st\ test}$= 6.1 years; ranging from 11 months to 11.5 years old, mean $age_{2nd\ test}$= 7.9 years ranging from 2.7 to 13.4 years old) in the same test procedure on average 21.2 ± 9.4 months later. In our sample there were nine mixed breed dogs, and 23 purebred dogs, 22 dogs had no traumatic background, ten had trauma, 15 dogs were neutered and 17 were intact. Eight dogs had been reported to have SRP by their owner (mean age_{SRP1st}=5.64 +/- 3.92 years, mean age_{SRP2nd}=7.68 +/- 3.69 years), and 24 were considered to not have SRP (mean $age_{NON-SRP1st}$=6.24 +/- 2.94 years, mean $age_{NON-SRP2nd}$=7.91 +/- 2.79 years).

Separation questionnaire

For more details on the questionnaire see (Konok et al., 2019). We collected demographic data about the dogs prior to the first test: age (in months), sex, breed (purebred or mixed breed), reproductive status, along with their traumatic life history, based on the origin of the dog and coded it into a traumatic or nontraumatic category. The following were considered as traumatic life events: spent time in a shelter, being a stray, and being rescued from a harmful environment. Finally, we asked the owners to assess their dogs' SRP status in a yes-no question. Based on their answers we categorised the dogs into SRP and non-SRP groups and used this categorisation in the further analysis.

Procedure

The set-up of the testing room (size: 6.27 m x 5.40 m) is presented in Figure 1. There was a chair for the owner to sit on during the first phase in the testing room. The experimenter was in the next room, where he could oversee the whole procedure. The computer recorded the six digital cameras' video stream (Basler sca640-120gc; Basler AG, Ahrensburg, Germany) and the sound from two microphones placed in the room: an omnidirectional microphone (Sennheiser ME62 with K6 power module; Sennheiser electronic GmbH & Co. KG, Wedemark, Germany) was hung from the ceiling in the middle of the room to record the ambient sound of the room, and a shotgun microphone (Sennheiser ME65 with K6 power module; Sennheiser electronic GmbH & Co. KG, Wedemark, Germany) was fixed above the door used by the owner when leaving to provide a more focused recording of the dogs' vocalisations. The two different signals were recorded (Zoom H4n; Zoom Corporation, Tokyo, Japan) on separate stereo channels synchronized with the video streams, and during the analysis the signal of the shotgun microphone was used, because of the better signal-to-noise ratio of the recordings.

The *1st phase* of the test started when the owner entered the room with the dog on a leash. Prior to the experiment, all tags and accessories were removed from the dogs' collar to avoid any unwanted noise. The owner sat down on the chair and released the dog. From this point the dog could move and behave freely, and the owner ignored the dog, even if the dog initiated any interactions with the owner. This phase was 1 minute long.

The *2nd phase* started after this initial 1 minute, when the owner stood up and left the room with minimal interaction with the dog. The dog was alone for 3 minutes.

In the 3^{rd} *phase* of the test the owner went back into the room and greeted the dog and played with it for at least 30 seconds to calm it and release stress.

Analysis

We coded the behaviour of the dogs in the 2^{nd} phase of the separation test with a continuous sampling method using a 0.2 second long time-window, using the software Solomon Coder (Péter, 2014). We coded the position, proximity and distance to the chair and the door, rearing, scratching, orientation of the dog towards the chair and the door, exploration, tail-wagging, and vocalisations (for further details see SI. Table 1). We measured the duration of the dogs' behaviour, starting from the point when the owner left the room until opening the door upon their return. Reliability was tested on 20% of the sample with Kappa statistics between two independent coders: mean K = 0.7557, SD ±0.0794, which showed substantial agreement.

The sounds made by dogs during the separation were recorded as uncompressed PCM wav files (44.1kHz, 16bit) and analysed using a custom made Praat (versions 6.0 and 6.1 Boersma and Weenink, 2014) script (Marx et al., 2021). We segmented and annotated the recordings to mark each individual whine containing the first fundamental frequency, f_0 and omitted the high frequency squeaks, g_0, and secondary fundamental frequency. Then we measured the acoustic features of these whines, including the jitter, entropy, call length, f_0 related parameters, and spectral components and marked the whines containing NLP based on auditory and visual inspection of the calls. Reliability of NLP detection was tested on 10% of the sample with Pearson correlation between two independent coders: r=0.964. Finally, we calculated the ratio of NLP whines, the number of the NLP occurrences in all f_0 whines divided by the number of f_0 whines.

Statistics

Analyses were run in the R statistical environment using RStudio (R Core Team, 2020; RStudio Team, 2015).

We applied Principal Component Analysis (psych package, principal function, Revelle, 2020) with oblimin rotation to form behavioural scales from the duration of time data in our earlier analysis of the full sample of 167 dogs that participated in the first separation test. The number of extracted principal components (PCs) were determined with parallel analysis (paran package, Dinno, 2018). Five PCs were defined based on the first test, then the scores were calculated for each individual for further analysis. The PC structures from the first test were used as a template to calculate the dogs' scores in the second test, using the predict function of the psych package to make the two tests comparable.

To compare the results of individual dogs in test 1 and test 2 we used General Linear Mixed Models (GLMM) with AIC based backwards elimination to find the most parsimonious models. In the analysis we included the ID of the dog as a random factor, also the age (in both test occasions) as a covariate, as well as the fixed factors and their interactions. The ID connects the two datapoints from the two tests of the given individuals. We built separate models for all the PCA scales and the vocal parameters to test the effect of the fixed factors (sex, reproductive status, breed, traumatic background and SRP status) and the covariate (age) as well as the interactions (sex: reproductive status, age:sex, age:reproductive status, age:breed, age:traumatic background, age:SRP status) to see how potential ageing effects are modulated by individual features of the dogs on the behaviour data. The model fits lines for each individual through the two datapoints (with different intercepts but the same slope [we tested if random slope models are better, but its addition explained minuscule variance]), models the slope of these lines and finally based on these it gives back the overall estimates. As each individual has their age included for both tests, it accounts for the repeated testing and the age effects and in the interactions, we have incorporated both the effect of initial age, the time between the two tests and the repeated testing together. All dependent variables were normalized with box-cox transformation when necessary. For post hoc comparisons in the case of interactions between the continuous (age) and categorical variables, simple slopes analysis was used (interactions package, Long, 2019).

In the case of the NLP ratio, we ran Generalized Linear Mixed Models (GzLMM) with a binomial distribution and the number of f_0 whines produced was added as a weight. All other details were the same as above. For post-hoc comparisons, the Tukey test (emmeans function) was applied for pairwise comparisons of factor levels, while in the case of an interaction between the continuous (age) and categorical variables, simple slopes analysis was used (interactions package, Long, 2019).

Finally, we applied a trajectory analysis to investigate how the behaviour of the individual dogs changed over time, using Latent Class Linear Mixed Models (lcmm package, Proust-Lima et al., 2017). These heterogeneous linear mixed models look for different change patterns over the repeated measurements and group together similar ones, and provide new grouping variables that separate individuals with different change patterns of separation behaviour over time. The input of these models were the PC scores of the behaviour variables from the two test occasions, and we also added age as the independent variable. We always compared three different versions with one, two or three classes and decided which one better fits the sample based on the AIC and entropy comparisons. Using this clustering method, we investigated if the change is significant within the classes and exported the class membership of the individuals. To test the clustering performance, we ran a posterior classification that showed the ratio of individuals that can be differentiated between classes. Then we plotted the classes separately for each behavioural variable and then explored how these patterns in the different behaviours act together to separate individuals that are stable, improve or decline in their separation behaviour over time. We also inspected the distribution of the SRP dogs in the different groups.

Results

Principal Component Analysis

The PCA resulted in 5 PCs:

1. Chair proximity: dogs that had high scores on this component spent most of the time in close distance and interacting with the chair, which might have provided them with a safe haven during separation.
2. Escape: dogs responded to separation by trying to reduce the distance between them and their absent owners, or escape from an unpleasant situation, by trying to escape through the door

their owners used to exit the room. These dogs showed escape behaviours such as rearing up against the door and scratching the door.

3. Whining at the door: others produced contact calls such as whining to signal their stress to the owner, while waiting for the owners' arrival. They spent their time close to the door through which the owner left the room. This might be the outcome of experiencing fear. Their inner state might also influence their body position, they were less likely to lay down calmly.
4. Barking at the door: similarly to whining at the door, there were dogs that mostly barked (but could produce other vocalisations as well). They were more likely to wag their tail, and spent their time orienting towards the door. They might experience frustration during separation, which could be connected to their reduced exploring behaviour.
5. Sitting: dogs that sat during the test were more likely to remain sitting passively and not stand up.

For the results of the PCA, the loadings and Cronbach's alpha values see SI. Table 2.

Parameters		Behaviour									
		Escape					Whining at the door				
		Est.	S.E.	t	d.f.	P	Est.	S.E.	t	d.f.	P
Intercept		0.04	0.37	0.11	33.60	0.91	0.05	0.44	0.10	35.82	0.92
Age		-0.00	0.00	-0.21	37.30	0.84	-0.01	0.01	-1.07	39.93	0.29
SRP		1.98	0.60	3.30	42.51	0.00	-0.85	0.82	-1.04	37.70	0.31
Age:SRP		**-0.02**	**0.01**	**-2.70**	**47.43**	**0.01**	*0.02*	*0.01*	*2.05*	*40.46*	*0.05*
Post hoc (simple slopes)											
Parameters		Est.	S.E.	t		P	Est.	S.E.	t		P
Age:SRP	Yes	**-0.01**	**0.00**	**-2.38**		**0.02**	0.01	0.01	1.65		0.11
	No	0.01	0.00	1.52		0.14	-0.01	0.00	-1.30		0.20

Table 1: The results of the models and the post hoc tests of the different behaviour components. Bold numbers represent significant results, and italic numbers show tendencies. We treated main effects as significant, based on p values, only if they were not in a significant interaction with another parameter. Blank cells represent non-significant results. Breed, sex, reproductive status and traumatic background and their interactions that are

excluded from the table can be found in the Supplemental Information. Chair proximity, barking at the door and sitting were excluded, the results were either not significant or they can be found in the Supplemental Information.

Behaviour Components

The results of the statistical models of the behaviour components are presented in Table 1. In the case of the component chair proximity, we found no significant effects of any tested variable or interaction. For the escape component (PseudoR^2 (total) = 0.63; Random intercept standard deviation (RISD) = 0.50) we found an interaction between age and the SRP status of the dog (Figure 2). SRP dogs showed less escape behaviours as they aged (from test 1 to test 2).

In the case of the whining at the door component (PseudoR^2 (total) = 0.66; RISD = 0.64) we found a tendency for an interaction between age and SRP status (Table 1, Figure 3). The post hoc test found this interaction between age and SRP status to be not significant.

	Acoustics								
Parameters	Jitter (POLY)					NLP ratio (GLMER)			
	Est.	S.E.	t	d.f.	P	Est.	S.E.	z	P
Intercept	0.03	0.00	13.79	19.17	0.00	-2.33	0.49	-4.77	0.00
Age (POLY 1,2)	-0.02 **-0.04**	0.02 **0.01**	-1.02 **-2.65**	27.21 **34.57**	0.32 **0.01**	0.01	0.01	1.22	0.22
SRP						-1.00	0.99	-1.01	0.31
Age:SRP						*0.02*	*0.01*	*1.77*	*0.09*

	Post hoc (simple slopes)								
Parameters		Est.	S.E.	t	P	Est.	S.E.	z	P
Age:SRP	Yes					**0.02**	**0.00**	**3.79**	**0.00**
	No					0.00	0.01	0.27	0.79

Table 2: The results of the models and the post hoc tests of the different acoustic parameters. Bold numbers represent significant results, and italic numbers show tendencies. We treated main effects as

significant, based on p values, only if they were not in a significant interaction with another parameter. Blank cells represent non-significant results. Parameters and their interactions that are excluded from the table can be found in the Supplemental Information or were not significant in any of the components (age:reproductive status). Pitch, call length and entropy were also excluded, the results were either not significant or they can be found in the Supplemental Information.

Acoustics

The results of the statistical models on the acoustic parameters are presented in Table 2. In the case of entropy and pitch we found no significant effects of any of the tested variables or interactions.

On jitter (PseudoR^2 (total) = 0.46; Rand. int st.d = 0.01) we found an effect of age (Figure 4, Table 2) that was better explained by a quadratic polynomial (LRT test: $\chi^2(1)=6.896$; $p=0.009$). Jitter increased with age at first, but after adulthood it started to decrease in old age.

Finally, on the NLP ratio of f_0 whines (PseudoR^2 (total) = 0.23; RISD = 0.00) we found an interaction between age and SRP which was a tendency (Figure 5, Table 2). Post hoc tests showed a significant increase in the NLP ratio of SRP dogs with advancing age.

	Escape component				Whining at the door component				Barking at the door component			
	Coef.	SE	Wald	P	Coef.	SE	Wald	P	Coef.	SE	Wald	P
Intercept class1	-3.700	0.478	-7.741	0.000	-1.392	0.570	-2.441	0.015	2.580	0.158	16.313	0.000
Intercept class2	-0.059	0.163	-0.361	0.718	0.597	0.229	2.605	0.009	1.628	0.076	21.388	0.000
Intercept class3	1.458	0.884	1.649	0.099	-0.344	0.521	-0.661	0.509				
Age-Class1	**0.035**	**0.005**	**7.242**	**0.000**	**0.025**	**0.006**	**4.009**	**0.000**	**-0.013**	**0.002**	**-7.490**	**0.000**
Age-Class2	-0.002	0.002	-1.144	0.253	**-0.005**	**0.002**	**-2.117**	**0.034**	0.001	0.001	1.409	0.159
Age-Class3	**-0.018**	**0.009**	**-2.073**	**0.038**	**-0.011**	**0.005**	**-2.194**	**0.028**				

Table 3: The results of the Latent Class Linear Mixed Models on the escape, whining at the door and barking at the door components. Bold numbers represent significant results. Behaviour components that are excluded from the table were not significant.

Trajectory analysis

The results of the trajectory analysis are presented in Table 3 and SI. Table 6. In the case of the chair proximity and sitting components we found no significantly different trajectories. In the case of the escape component we found a group of four, in which dogs' escape score got higher with ageing, and a group of seven, in which dogs' escape score got lower. However, in the majority (20 dogs) the escape score was low already at the time of the first test and stayed low with advancing age (Figure 6).

In the whining at the door component we found a group of three dogs that whined more, a group of eight that whined less and the majority (20 dogs) showed a slight but significant drop in whining behaviour (Figure 7).

Finally, for the barking at the door component we found a group of three in which dogs showed a drop in their barking behaviour, while the rest of the subjects showed no change (Figure 8).

When combining these results together we found a stable group of thirteen dogs, a group of twelve dogs that actually improved over time (their escape behaviour and separation related vocalisations dropped with age), and a group of five dogs that got worse (they showed more escape behaviour and/or whined more), and one dog that showed a different pattern from the rest of the subjects that switched from barking to whining (Table 4).

Number of dogs	**SRP**	**Escape**	**Whining at the door**	**Barking at the door**	**Summary**
3	0	Increase L-H	No change M	No change M	Escape behaviour got worse.
1	0	Increase L-H	Increase L-H	Decrease M-L	Escape behaviour got worse, barking switched to whining

1	0	No change L	Increase L-H	No change M	Whining behaviour got worse
1	1	No change L	Increase L-H	Decrease M-L	Barking switched to whining
13	3	No change L	No change M	No change M	No change
1	1	No change L	No change M	Decrease M-L	Barking behaviour got better
3	1	Decrease H-L	No change M	No change M	Escape behaviour got better
4	0	No change L	Decrease M-L	No change M	Whining behaviour got better
4	2	Decrease H-L	Decrease M-L	No change M	Escape and whining behaviour got better

Table 4: The combination of the three grouping variables, showing the different individuals that were stable, improved or declined in their separation behaviour over time. The number of individuals in the given group is presented in the 1st column and the SRP status distribution among the groups in the 2nd column. The change in directions of the behaviour component could be an increase, a decrease or no change and the scores could change between L-low, M- medium, and H-high in the given group (3rd-5th columns). The summary column gives an interpretation of the previous columns.

Discussion

Our results show that there are longitudinal age-related changes in the separation behaviour of dogs, and the acoustic structure in their whines, although these might be modified by individual features in the majority of cases.

Stress and NLP

SRP dogs showed less escape behaviour and tended to have a higher NLP ratio with advancing age. They also tended to whine more in close distance to the door, however, a post hoc test did not support this result, so a larger sample size is needed to confirm this. These results are connected to our earlier cross-sectional findings (Marx et al., 2021) that SRP status and age affect the dogs' separation behaviour and the occurrence of NLP in separation whines. SRP dogs are suffering from separation distress, but this appears mostly in their vocal behaviour (higher whining at the door component scores and NLP ratio) and not in their movement patterns, as they showed decreasing activity with age in their movement (lower escape component scores) during the separation, unlike non-SRP dogs. Mongillo et al., 2013 found similarly, that older dogs were more passive during the separation, and they showed a significant increase in salivary cortisol concentrations after the separation, which is in line with our results and suggests elevated stress, but lower activity in the case of older SRP dogs compared to younger SRP dogs and non-SRP dogs with the same age groups. The lower escape activity with age could originate from SRP dogs' motivational or inner state rather than issues in their motor system. They might have a long history with separation-related problems, and with age they have developed their own coping strategy, which does not include attempting to escape. This may be due to failed attempts in the past when separated from their owner, and so they rather strive to "whine their way out" from the stressful situation. They also whine mostly and not bark, which is in line with the results of Pongrácz et al., 2017, that showed that SRP dogs whine mainly when separated from the owner (regardless of breed type, Pongrácz et al., 2020), while non-SRP dogs mainly bark, which could explain our results on SRP dogs' vocalisations. In our study we did not find any effect of SRP and age on barking, in contrast to Pongrácz et al., 2020, which might be due to differences in test conditions (indoor, moving freely in the testing room, completely alone vs. outdoor, on leash, not completely alone, breed composition of the samples). Furthermore, Lenkei et al., (2018) found that the different vocalisation types might be connected to different inner states. The underlying mental state of barking might be frustration while whining dogs might experience fear. Together with our acoustic results on the NLP ratio of SRP dogs we suggest that dogs with SRP might experience higher levels of arousal or even fear during separation, which is reflected in the increased NLP ratio, that might intensify with advancing age.

However, an elevated NLP ratio might be reflected in the vocal harshness and instability of the whines, as NLP whines are noisy, irregular vocalisations, which could increase entropy and jitter. Our results show that jitter increases with age but after reaching 7.5 years it starts to decrease, meaning young and old dogs have more stable pitch in their whines than middle aged ones. This seemingly contradicts our result; however, we did not find a significant interaction of age and SRP status in the measurement of jitter, which might solve this disagreement. An entropy effect could also add further details to this dispute over the connection of NLP vocal harshness and instability and the arousal level of the calling individual. However, we did not find a significant effect of any of the predictors on the entropy of the whines. In our earlier study (Marx et al., 2021) we found a similar contradiction, in that entropy decreased with age, but NLP ratio increased. It seems that NLP are not directly correlated with vocal harshness (measured by entropy) and the instability of the pitch (measured by jitter) of the whines. These two parameters might be predictors of the occurrence of NLP; however, NLP could occur independently from them as well. The occurrence of NLP is sudden and often quick transitions between the resonance states of the vocal folds might not affect the acoustic structure of the whole call, and may be associated with another process, such as losing neural control over the vocal apparatus.

The influence of ageing tissues, such as the loss of elastic fibres, changes of the epithelium, and muscle atrophy on vocalisations, which was described in humans earlier (Awan, 2006; Baken, 2005; Gorham-Rowan and Laures-Gore, 2006; Mueller, 1997; Xue and Hao, 2003) might not be responsible for the higher ratio of NLP found in older SRP dogs in the current study. The behavioural parameters also suggested the dogs experienced an elevated stress response: SRP dogs, being exposed to a high level of stress, showed higher scores on stress related behaviours and acoustic parameters than non-SRP dogs, even during the first separation test (when we were not measuring longitudinal changes, Marx et al., 2021). Similarly, we find it unlikely that our results are caused by the wearing out of the phonatory apparatus, due to a higher rate of vocalisations in the long term, causing an elevated NLP ratio. SRP dogs might be more vocal in general, however, to our knowledge there is no evidence out of the context of separation suggesting that SRP dogs whine more than non-SRP dogs.

Furthermore, we have taken other types of vocalisations dogs produce into account, which could have a similar effect on other, not necessarily SRP dogs as well. In the lack of evidence suggesting that SRP dogs are more vocal in general, one would not expect to find any differences between SRP and non-SRP groups in their NLP ratio content. We cannot completely discount this alternative hypothesis, as if the wearing out of the phonatory apparatus is the cause behind the phenomenon, the NLP ratio and SRP status would still be connected, but not directly and not because of stress.

In addition, we did not find increasing vocal harshness (entropy) and/or an increasing instability of the pitch (jitter) with age, which besides stress could also be connected to ageing tissues, problems linked to muscles, joints, decreased resistance to fatigue and/or wearing out of the phonatory apparatus. This validates the hypothesis that NLP are most likely produced in relation with a higher level of arousal. In the case of humans, chronic stress (e.g. separation distress) causes higher cortisol levels in the saliva (Dickerson and Kemeny, 2004; Hellhammer et al., 2009; Pruessner et al., 1999, 2003; Wüst et al., 2000b, 2000a), which could affect the muscles involved in vocalisations and the vocal folds, and might be connected to the deterioration of the phonatory apparatus (Holmqvist-Jämsén et al., 2017). The same could be true in the case of dogs (Assia et al., 1989; Beerda et al., 1997, 1996; Broom and Johnson, 2019; Wiepkema and Koolhaas, 1992) and might be even more complex, if we consider evolutionary processes such as domestication, and the differently selected breed types (Pongrácz et al., 2020).

Different ageing patterns

Our aim was to find acoustic and behavioural differences between dogs utilising a longitudinal design, to take a step forward to be able to recognise typical change trajectories on an individual level. Our results could contribute to identifying these individuals and the factors behind their progress or decline in their separation behaviour during their ontogenesis. However, our groups are not to be mistaken for the successful, typical and unsuccessful agers. Dogs that show low escape whining and barking behaviour are more likely to age successfully, as they experience less stress in separation related contexts leading to an overall better life quality. However, this could also mean that these dogs have lower level of attachment to their owner, that resulted in different attachment and caretaking styles of dogs and owners (Konok et al., 2019), and which conversely for them, might lead to a lower owner-dog bond, and less care from the owner. The question of the optimal stress level that shows good stable bond remains unanswered for further studies.

Most of the dogs were stable in their separation behaviour, or they even improved with advancing age. A smaller group showed a decline in their escape activity and vocal behaviour. Escape attempts and vocal behaviour were found to be good indicators of the long-term changes in the dogs' separation behaviour, that might have an applied value. Based on these results, a diagnostic and monitoring system could be developed, which could identify different ageing patterns of dogs on an individual level, helping in the early recognition of dogs with SRP. This could assist in the identification of risk factors which could be associated with SRP (Flannigan and Dodman, 2001) and based on them, the development of treatment plans. Those dogs that are diagnosed earlier and treated from an early stage of SRP, could improve much and live a less stressful life, without separation problems disturbing the owner-dog relationship. Additionally, as we showed in this study, separation behaviours change during the life of the individuals, the behaviours are not always as they are expected to be at every age based on the population level cross-sectional results, making it harder to diagnose and treat the problem. Age-specific individual level and longitudinal data is needed.

Additionally, SRP dogs did not decline, they did not change in their escape, barking and whining behaviour during the separation test; however, one of them switched from barking to whining. Some of them even improved, which somewhat contradicts our hypothesis, that SRP intensifies with age. However, our subjects were not only old dogs, and we did not expect young dogs to decline in this short duration of time. Additionally, stability and improvement in the behaviour of an SRP dog is not necessarily equivalent to a recovered dog. Problematic dogs are more salient, and owners might actively work on the problematic behaviour, which could result in the stability or improvement of SRP (Takeuchi et al., 2000). Furthermore, the dogs that did not change and the dog that switched from barking to whining over time show that, besides individual differences, there are dogs that have SRP that remain stable regarding separation stress. These highlight the importance of longitudinal individual level studies, like ours, that aim to develop a monitoring and diagnosing system, to help veterinarians in the early diagnosis of the problem, and treating the affected dogs with an individual level intervention to avoid the chronic stress that comes with this condition (McCrave, 1991). Furthermore, it could also help us identify different factors behind these changes or the lack of these changes (e.g. how often and how long do owners of improving stable or declining dogs leave the dogs alone, keeping conditions, breed, sex, traumatic life events etc). We must note however, that our sample size is small, especially in the case of SRP dogs, which emphasises the importance of a repetition on a larger sample. We suggest that owners that bring their dogs to behavioural tests might be more aware of their dogs' behaviour and problems, so they could take good care of their dogs, which could result in this improvement. It might also be possible that owners perceive their dogs' SRP status in the light of the noisiness and NLP content of their whines. Additionally, we found that dogs that show an increase in separation related behaviours from test 1 to test 2 were not classified as SRP dogs by the owners. Based on Konok et al., 2019, 2015, 2011; and van Rooy et al., 2018 we suggest that owners can assess their dogs' separation behaviour and SRP status relatively accurately. We admit that the owners' opinion has to be taken with caution regarding their dogs' SRP status, because these behaviours occur in the absence of the owner. Furthermore, a recent study found that owners are usually aware of their dogs' behaviour during separation, but the correlation between owner-reported and empirically observed separation behaviours were only moderate (van Rooy et al., 2018), and that owners are different in their caretaking behaviour and the level of concern regarding their dogs' SRP (Konok et al., 2019). There is evidence that connects NLP in vocalisations to aroused inner states (Fitch et al., 2002), caused by for example separation stress, thus NLP might be an honest signal of arousal in separation whines of dogs. We argue that the owners' perception only magnifies these phenomena, and it is possible that owners perceive the SRP status of the dog correctly. However, future studies should examine owners' perception of SRP in their dogs, and the different clues in the dogs' behaviour and vocalisations that shapes the owners' perception, even extrinsic parameters such as complaining neighbours.

After confirming these results on a larger dataset, clarifying the connection between jitter, entropy and NLP, and corroborating the variables on which individual ageing patterns can be based, the results could make a useful tool in veterinary practice, to diagnose and treat dogs' separation issues at an early stage and monitor the changes of the dogs' separation behaviour throughout their lifespan, completing a full behavioural history analysis. This tool in case of dogs that vocalize in separation might be very precise and objective (less dependent on the specialist's experience), less time-consuming than a full behavioural analysis and could work even with dogs that show mostly physiological signs of stress (Konok et al., 2019).

Limitations

Firstly, the sample size is small, which could weaken our results: some results that are significant at this sample size, might not be in a larger sample and vice versa. Carrying out longitudinal studies has special challenges, e.g.: the subjects can die, owners change their contact addresses, and they might decide not to expose their dog to stressful situations in old age. Furthermore, the Covid-19 pandemic made it difficult to carry out such longitudinal studies. Additionally, we could not monitor the subjects between the two test occasions, which could have helped us in tracking the changes of the dogs' living conditions, behaviour, especially separation behaviour, medical history and give us more information about the background of these changes (traumatic life events, behavioural therapy etc.). For these purposes, involving practicing veterinary specialists could be beneficial, as they could make more precise diagnosis of the dogs' SRP status, and through them we might gain access to more, affected patients and monitor them more frequently. However, in Hungary the concept and the treatment of SRP nowadays, is not widely accepted and known yet, that made it impossible to involve such specialist.

Secondly, our results are based on data collected in a testing situation, which could only explore the age effect on SRP and vocal behaviour within this limitation, thus it is a first step in the research of changes in SRP with advancing age. The conditions at home or at a familiar place could be very different, which could have an effect on the dogs' behaviour as well.

Conclusion

Studies concentrating on spectral noise and arousal in contact calls have reported correlations between the two (e.g.: Blumstein et al., 2008; Liao et al., 2018; Linhart et al., 2015; Siebert et al., 2011). However, the connection between the occurrence of NLP in contact calls and stress related vocal communication is underrepresented in the literature. Here we would like to contribute to the studies highlighting the importance of NLP. Furthermore, in light of our results, it seems reasonable to suggest that longitudinal studies, although they are rare, have the potential to indicate the direct effects of ageing in relation to SRP, which emphasizes the necessity to support the results of cross-sectional studies in a repeated longitudinal study. Additionally, but no less importantly we would like to raise awareness regarding this topic. Despite the growing number of aged companion dogs in Western countries, very little is known about age-related changes in their separation-related behaviour. The relatively extended lifespan dogs are experiencing due to the current levels of extensive care, has artificially enhanced the proportion of dogs with physical and/or cognitive decline in the population, which is important when considering animal behaviour and welfare. However, helping our aged companion animals starts with understanding them, the process of their ageing in general, and the specific aspects of the individual in question, such as demographic parameters and life history effects on their ageing and welfare.

Authors' contribution

MA **-** recruited subjects, carried out the behaviour tests, coded the behaviour, ran the acoustic analysis, performed the statistical analysis, provided funding and drafted the manuscript.

LR **-** recruited subjects, carried out behaviour tests, coded the behaviour, and critically revised the manuscript.

PPF **-** recruited subjects, carried out behaviour tests, and critically revised the manuscript.

LW **-** critically revised the manuscript and English correction

KE **-** critically revised the manuscript, provided funding

FT - conceived and designed, coordinated the study, recruited subjects, carried out the behaviour tests, coded the behaviour, ran the acoustic analysis, performed the statistical analysis, provided funding, and drafted the manuscript.

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Figure 1: The set-up of the testing room during the separation test. The door which the dog and the chair is closer to was used during the test. The other door led to the corridor. This door was used only when the owner and the dog first entered the testing room at the beginning of the test. The position of the cameras and the microphones are also presented on the figure.

Figure 2: The interaction between age and SRP on the escape component. Note that the different y axis scales are due to showing partial residuals from the interaction effects. On the interaction plots the colours represent the different groups of the categorical variable, but the individuals' two test points are both presented on them.

Figure 3: The interaction between age and SRP status on the whining at the door component. Note that the different y axis scales are due to showing partial residuals from the interaction effects. On the interaction

plots the colours represent the different groups of the categorical variable, but the individuals' two test points are both presented on them.

Figure 4: The age effect on jitter (the colours represent the different individuals, a dot and a triangle joined by a line with the same colour are the same individuals' values in the two tests, the dot represents the first and the triangle represents the second test).

Figure 5: The interaction between age and SRP status on NLP ratio. The size of the dots represents the number of f_0 *whines that were included in the models as weights. Note that the different y axis scales are due to showing partial residuals from the interaction effects. On the interaction plots the colours represent the different groups of the categorical variable, but the individuals' two test points are both presented on them.*

Figure 6: The results of the escape component in (a) the group of dogs in which the escape score increased, (b) the dogs in which the escape scores decreased, (c) and the dogs which did not show any significant change. The colours represent the different individuals, a dot and a triangle joined by a line with the same colour are the same individuals' values in the two tests, the dot represents the first and the triangle represents the second test.

Figure 7: The results of the whining at the door component for the (a) dogs that whined more, (b) the dogs that whined less, (c) and the dogs that showed a slight but significant decrease in their whining behaviour. The colours represent the different individuals, a dot and a triangle joined by a line with the same colour are the same individuals' values in the two tests, the dot represents the first and the triangle represents the second test.

Figure 8: The results of the barking at the door component for (a) the dogs that barked less, (b) and the dogs that show no significant change. The colours represent the different individuals, a dot and a triangle joined by a line with the same colour are the same individuals' values in the two tests, the dot represents the first and the triangle represents the second test.