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AFFECT OF POPULATION SIZE ON ULTRASONIC VOCALISATIONS OF WILD-TYPE HOUSE MICE

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Abstract House mice (*Mus musculus*) like other rodents emit ultrasonic vocalisations (USVs) as a form of communication; their call types are diverse and form a vital role in their everyday lives. In January 2019 wild strain mice housed within a mixed pen environment showed an observed shift in the length, frequency and type of USVs emitted after a population cull. This paper presents data to provide an explanation for the observed change in behaviour and motivators behind USV production. The shift in USVs was observed across all mice demographics with little influence from social experiences, external temperature fluctuations or psychological implications from the cull. The population size caused an initial significant difference ($p=0.000000000000004$) in number of USVs emitted, with less than 35% of the original number of USVs produced after the population cull. Subsequent interactions between the number of USVs and population size revealed that it was not the population size but the impact of the population dynamics on the colony's genetic diversity causing the shift in USVs. The type of calls emitted also significantly changed, with the production of complex calls occurring after the first genetic bottleneck. The progression of complex call types thereafter revealed insights into the development of USVs and the effect of genetics on USV evolution.

Key words *Mus musculus,* USVs, genetics, temperature, call types

INTRODUCTION

Wild strain house mice (*Mus musculus*) emit ultrasonic vocalisations (USVs) as a crucial form of communication (von Merten et al., 2014; Warren et al., 2018). These calls are predominately inaudible to the human ear, at frequencies above 20kHz (Peleh et al., 2019). The function of USVs and the content in which they are emitted has been widely researched (Screven and Dent, 2019), primarily in respect to laboratory mice (Chabout et al., 2012). USVs have been specifically attributed to their role in social interactions, aggression, mating, courtship and pup-separation (Moles et al., 2007; Chabout et al., 2012; Matsumoto and Okanoya, 2018).

Early studies demonstrated that USVs were an essential component for male courtship and mating (Musolf et al., 2010); with male mice emitting USVs in the presence of females or female urine with a urinary pheromone component (Nyby et al., 1979). It was therefore initially considered that USVs were mainly attributed to male mice (White et al., 1998); however more recent studies such as Warren et al., (2018) identified that females also emit USVs during opposite-sex interactions just to a lesser extent. It is now widely acknowledged that both male and female mice display an array of USV calls that are vital for conspecific communication with the same and opposite sex (Moles and D'amato, 2000).

As well as the importance of USVs for adult mice, the role that they play in pup communication has also been extensively researched. During the first 2 to 3 weeks of a pup's life, if they become separated from their mother they automatically start producing ultrasonic vocalisations (Barnes et al., 2017). The principal purpose of these calls is for pup retrieval (Liu et al., 2003) and is associated to a drop in pup's body temperature (Peleh et al., 2019).

Although USVs have been widely researched for their role within social communications, the motivators and development of USVs and the different call types remains ambiguous

MATERIALS AND METHODS

A series of soundproof test booths were designed to enable recordings to be undertaken without interference of background noise; ensuring that the recordings were clean and eliminating the potential for external noise to be a variable. The booths consisted of a wooden structure (50 cm x 60 cm x 50 cm) lined with 38.1 mm thick acoustic

foam; no light was present in the booths to stimulate their natural nocturnal behaviour. A test arena containing a partition was placed inside the booth (Figure 1); this allowed for a dual side option where test mice could be separated from either other mice or a stimulus. The partition was perforated and transparent to allow for scents, noise and visual interactions to remain.

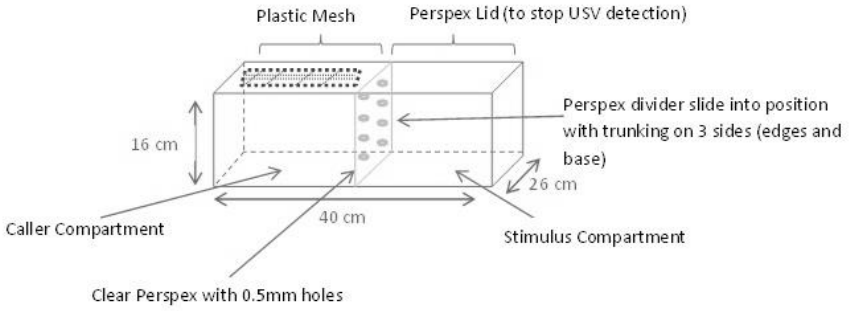

Figure 1. Test arena for mice during recordings

A tailored microphone was suspended above the plastic mesh, picking up all USVs emitted by mice within the caller compartment, connected externally to bespoke test equipment containing software designed for recording and analysing USVs. Throughout the trial mice were housed in a mixed pen, all were taken from the same pen to remove variables, the size of the pen was 1.5m x 2m.

Control recordings of individual mice were undertaken to observe the spontaneous rate of USV generation. Mice were then exposed to a series of stimuli and social interactions; recordings were taken for 30 minutes and then analysed. In January 2019 upon detection of an observed shift in frequency, length and type of USVs emitted after a sudden reduction in population size, an investigation was instigated. In order to identify the exact motivators behind this shift in USV communication, the process was replicated; taking into account the time of year/season, equipment used for recording, the population of mice (including the pen) and population size. Recordings were retaken in September and December 2019, a reduction in population size was undertaken in the latter half of December 2019 and further recordings were then taken in January 2020.

RESULTS

The initial USV shift detected was observed in female, male and pup recordings; a preliminary investigation concluded that this shift was across all social contexts; therefore for this study in order to reduce variability, only recordings related to females have been used for further analysis. A total of 63 hours of recordings were completed.

Classification of sounds during analysis was grouped into 3 subheadings: whistles, unclassified and noise. A variety of different USV call types have previously been identified (Vogel et al., 2019), for the purpose of this initial investigation all USVs categorised by Vogel et al., (2019) were grouped under one subheading of 'Whistles'. Any other USVs that did not fall under this subheading were grouped as 'unclassified', this term also included any faint detections of USVs elicited from individuals in the stimulus compartment of the test-booth. Further classification within the 'Whistle' and 'Unclassified' headings were undertaken for the 'females with pup' files; details of these categories can be found in Figure 2.

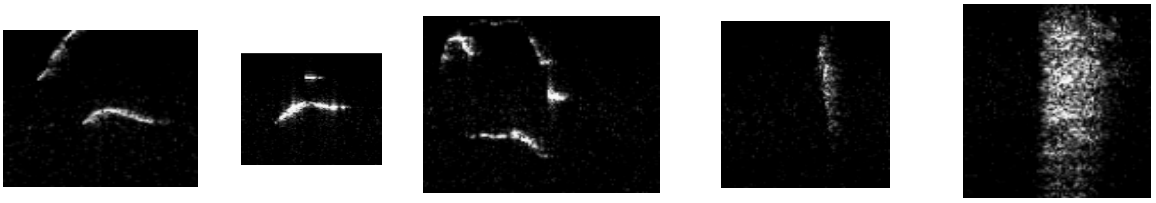

Figure 2. Examples of calls for classification under the sub-headings of '2-Tone calls' and '3-Tone calls' within 'Whistles' and 'Wide Calls' and 'Shriek' under 'Unclassified'.

A comparison between the recordings of 'One female and pup together' and 'One female separated from pup' showed a direct correlation between the average numbers of calls over a period of time (Figure 3); therefore all further analysis for this study concentrated only on those recordings with a female and pup together.

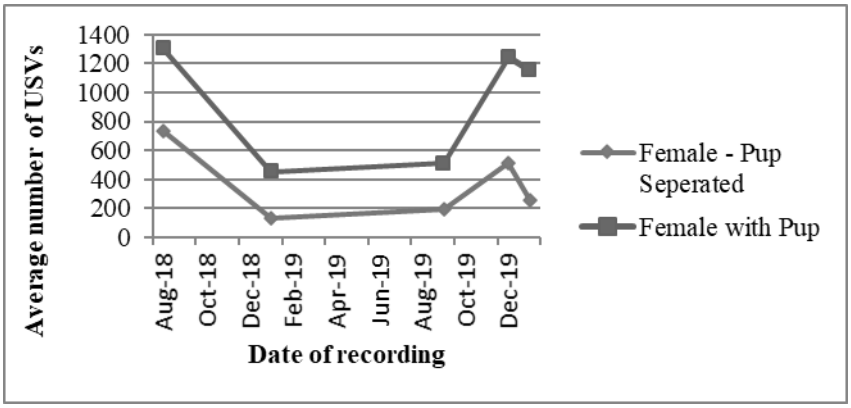

Figure 3. Average number of USVs emitted during 30 minute recordings of female-pup together and female-pup separated at different time periods.

Recordings at different periods of time showed a significant shift in the number of USV calls elicited by female mice housed with a pup (Figure 4), with the highest number of calls being detected in September 2018 (1305) and the lowest number in January 2019 (450.25). A statistical analysis using ANOVA was applied to the data, showing a statistically significant difference of $p<0.05$ ($p=0.000000000000004$) between the number of USVs emitted and period of time that they were recorded. Further statistical analysis was applied to this data to determine where, within these time periods, the significant variation occurred; paired t-tests were applied to each group combination. In order to allow for the reduced effectiveness of multiple t-tests on the same data set, a sequential Bonferroni adaptation was undertaken to amend the p value; corrected p-value of $p<0.005$ was considered statistically different.

A significant difference was found between six combinations of dates (Table1); the first significant change in number of USVs occurred between September 2018 and January 2019 ($p=0.0000000003$) with a sudden decrease in the number of USVs being emitted. After January 2019 there was no significant change again until December 2019 where the number of USVs was not only significantly different from January 2019 ($p=0.0000000072$) but it had reverted to a level of USV call rate that was no longer significantly different from the original recordings in September 2018 ($p=0.6749793235$). Post December 2019 there was no significant change in number of USVs emitted.

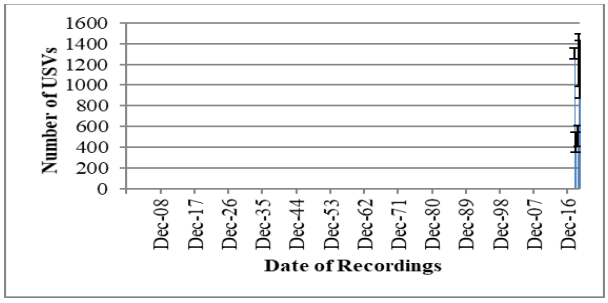

Figure 4. Average number of USVs detected during 30 minute recordings of female-pup together in comparison to the date that they were recorded; error bars show the standard deviation of USV frequency.

Table 1. Results from multiple t-tests evaluating effect of date of recordings on number of USVs emitted. P value was amended using sequential Bonferroni adaptation to compensate for effect of multiple tests. P value for significance = 0.005, any groups showing a significant difference are indicated with a *

	Sep-18	Jan-19	Sep-19	Dec-19	Jan-20
Sep-18		P = 0.0000000003*	P = 0.0000000010*	P = 0.6749793235	P = 0.3109126305
Jan-19			P = 0.4555055694	P = 0.0000000072*	P = 0.0000000754*
Sep-19				P = 0.0000000395*	P = 0.0000004289*
Dec-19					P = 0.5220917602

An investigation into other possible sources of variation was undertaken to see if there were any factors that may have contributed or caused the significant changes in number of USVs; the population size and temperature

within the pens during these testing periods were explored in more detail. Figure 5 shows how the temperature fluctuations change throughout the year, prior to September 2018 the variation in temperature was minimal, with the average temperature fluctuating only 0.67°C and the minimum temperature reaching 13.8°C. Although temperature does not show a direct correlation with the number of USVs emitted, in the months preceding the initial shift in USVs (observed in January 2019) there was a sudden change in the variation of temperature recorded. The average temperature between September 2018 and January 2018 varied by 1.7°C and reached a minimum of 11.5°C in December 2018; January 2019 alone was the lowest recorded average temperature (18.7°C).

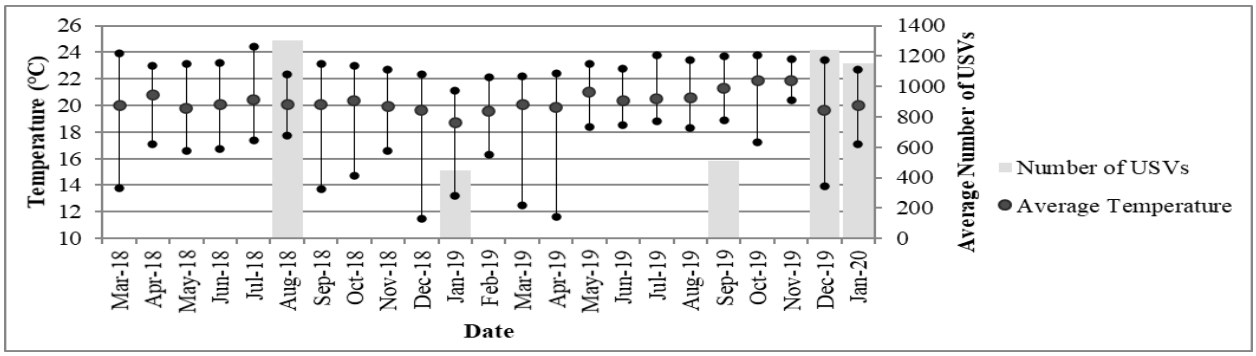

Figure 5. Average numbers of USV calls emitted by a female and pup together during 30 minutes of recordings at different periods of time and the temperature recorded for each month. The average temperatures are indicated along with error bars showing the minimum and maximum temperatures recorded during this month.

An evaluation between the population size and average number of USVs was undertaken (Figure 6); vertical lines in the number of mice on the graph represents a population cull. During 2018 there was a consistent population increase, with the number of mice in the pen starting off with 20 adults (10 male and 10 female) to a total of 160 individuals being present in November 2018. A cull in November 2018 dropped the population to 80, with equal males and females; however the pen underwent an additional cull in December 2018 dropping the number to 5 individuals, 2 were female. This drop in population numbers was accompanied by the initial significant decrease in USVs emitted. In April 2019, a population cull occurred again and although the overall total was 8, only 2 of these adults were females. When the population was allowed to grow naturally, limiting the culls to a minimum of 12 adult mice with an equal split of gender, the number of USVs started to increase retuning to a level in December 2019 that was no longer significantly different to the original recording in September 2018. After this point, even with the presence of another sudden reduction in population size at the end of December 2019 the number of USVs emitted was not significantly affected.

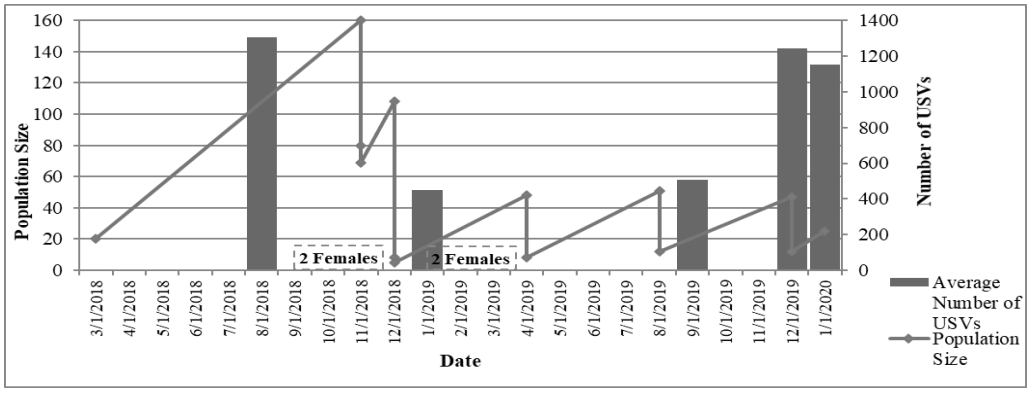

Figure 6. Number of mice within pen compared to the average number of USVs emitted by female mice with a pup. Vertical lines on graph represent a population cull and the two points where number of female mice was drastically reduced are indicated.

Although by December 2019 the number of USVs emitted returned to a level that was no longer significantly reduced, the call types and patterns remained different. Prior to the initial shift in rate of USVs observed in January 2019, the calls observed were classified as ‘simple single-tone calls’ relating to call and response between an adult and

pup. After this shift, the call patterns changed significantly; the majority of communications no longer presented as standard 'call and response' calls with the introduction of complex (2-tone and 3-tone) and 'unclassified' calls (Figure 7). There was no correlation between number of complex calls and total USVs; first occurrence of complex calls was recorded in January 2018 with incidences remaining hereafter.

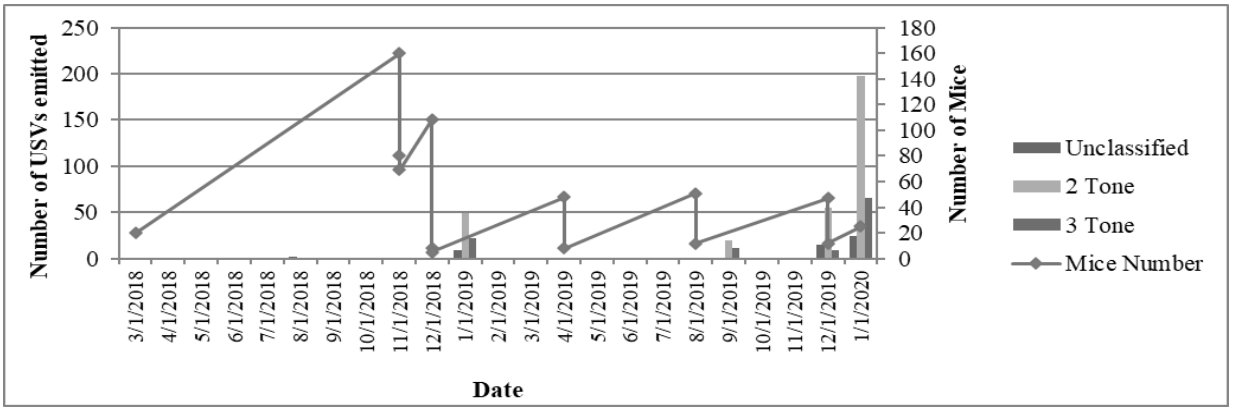

Figure 7. Number of mice in the pen over time in comparison to the number of USVs that fitted into the categories of 'Unclassified', '2 Tone' and '3 Tone'.

DISCUSSION

This is the first study, to the best of my knowledge, which has reviewed the effects of different factors that may influence production of USVs in mice within a laboratory environment. The phenomenon of shifts in mice USV patterns has been observed amongst multiple research establishments, but until now the motivation behind USV production has not been widely researched and the potential cause for this shift remained unknown.

The direct association of USVs produced between a female with pup and female separated from pup (Figure 3) negates the possibility that the shift in USVs emitted being related solely to the pups and a change in their communication. Gender cannot be responsible for this shift due to effects being seen across all demographics.

Although a significant temperature change was observed at the same time as the initial USV shift, this was not the primary cause due to the lack of association during repeat trials; USVs returned to a level that was not significantly different from September 2018 yet the temperature fluctuations remained and in December 2019 another significantly low minimum temperature was recorded (13.9°C). However, temperature could still have been a contributing factor of the initial change in USVs; low temperature has a negative impact on the physiological and psychological condition of pups causing a reduction in USV production (Shair, 2014). This effect may cause a lasting impact on the mice, potentially affecting their call frequency in adult life; further research would be required to confirm the lasting effect of stress on USV production.

This study identified that the factor preceding the initial significant shift in USV production was a sudden decrease in population size during November and December 2018; this effect could have been due to the adverse experience of a cull, prior social experience or the number of remaining individuals after population reductions.

Adverse Experience. USVs in mice are emitted during interactions with both positive and negative stimuli, which enable them to be a potential indicator of mental state (Finlayson et al., 2016); previous studies have found that mice under stressful situations produce a lower frequency of USVs (Chabout et al., 2012). However, although this could have accounted for the initial shift in January 2019 after the large population cull, this effect did not continue and despite additional culls the number of USVs returned to the original rate. The cull at the end of December 2019 is confirmation that the effect of the cull as an adverse experience was not the primary cause as despite the cull between the December 2019 and January 2020 recordings, the number of USVs emitted did not significantly change.

Social Experience. USVs have undergone significant research with respect to social communication (Ferhat et al., 2016); focus in this area of research is attributed to the translation of this information to studying human disorders such as autism (Peleh et al., 2019). Although the rate of USVs can be used as an index for sociability in mice (Moles et al., 2007; Matsumoto and Okanoya, 2018), the findings are still uncertain for the reversal of this. Many studies have found that social experience has no significant impact on USV production in mice (Screven and Dent, 2019); however, recent research has revealed some minor effects from previous social experience on USVs, with social isolation or

reduced social interaction reducing the frequency that they are emitted (Lefebvre et al., 2020). Therefore although social experience does not play a major role on USV production it can have some influencing properties (Screven and Dent, 2019). The effect of social experience in this study is likely to be minimal, especially as the recordings that underwent more detailed investigation were those containing females and prior research has revealed that social experience has more of an impact on USV production in males than females (Screven and Dent, 2019). In addition, the lack of direct association between number of mice in the pen and USV rates (Figure 6) confirms that this factor was neither the primary cause nor a major influencing factor, although it is important to note that this may have played some minor role within the USV shift.

Genetics. One final area to explore is the potential effects of genetics on the shift in USVs. Although the event preceding this initial shift in frequency of USVs was a reduction in population size, the correlation did not continue. The number of USVs returned to a level that was no longer significantly different in December 2019 regardless of the prior reductions in population size; the level in January 2020 was also not significantly affected despite the population decrease in the month directly preceding it. This suggests that the sudden reduction in population size in its own entity was not the cause; however the factor that was consistent with the changes in USVs was the number of mice that the population size was reduced down to. During the period where the population experienced reductions down to a dramatically restricted number of individuals, with only 2 females remaining at each incidence, the number of USVs remained significantly reduced (up to the recordings in September 2019); yet once the mice started to breed up more naturally with less drastic reductions in the population size the number of USVs significantly increased back to original levels (December 2019) suggesting a genetic influence.

It is well documented that a sudden drop in population size is akin to a genetic bottleneck, whereby the genetic diversity is significantly reduced (Broquet et al., 2020). In mice the genetic variation between strains significantly effects the number, length and type of USVs produced in adults (Sugimoto et al., 2011) as well as the pups (Barnes et al., 2017). The reduction of mice preceding the initial USV shift resulted in a population size of 5, a further reduction occurred 4 months after down to 8 individuals which only consisted of 2 females, each of these were responsible for causing a genetic bottleneck. It is therefore viable to conclude that the reason for the observed shift in number of USVs was not the reduction in the population size per se but in fact the effect that this had on the genetics within this population. In addition to the number of USVs significantly changing in response to the genetic bottleneck, a noteworthy shift in the type of calls was also experienced.

A fundamental advancement in USV research was the classification of different call types using sonograms (Vogel et al., 2019); further research has been undertaken to identify the contexts in which these are emitted (Warren et al., 2018). Complex calls were initially thought to be used by males for courtship (Holy and Guo, 2005), with more recent studies revealing their presence also in female communications (Matsumoto and Okanoya, 2018). It is now acknowledged that different types of USVs are present in a wider range of social contexts (Burke et al., 2017) with an element of individuality being ostensible (Chabout et al., 2012).

This study has revealed a novel insight into how USV call types are developed. Initial recordings in September 2018 found no instances of complex calls being emitted by females, males or pups; in January 2019 these calls started to occur and they remained present regardless of population dynamics. Development of the new call types persisted, with a significantly higher frequency of complex calls being emitted in January 2020; suggesting an association with population genetics. Although the exact process in which USV call types are learnt or developed remains uncertain, there is some degree of certainty that genetics played a pivotal role in the shift in USVs experienced in this study. The initial shift in USVs was caused by the sudden change in population genetics; the continued development of different call types was caused by the impact genetics has on the evolution of USVs and influenced by individuals learning from each other (Von Merten et al., 2014). This also provides an explanation for why even though the number of USVs returned to the original level the type of calls remained more diverse.

CONCLUSION

This study suggests that the observed shift in frequency of USVs was primarily the result of a genetic bottleneck affecting the colony's genetic diversity. This resulted in a reduced frequency of USVs but a greater repertoire of call types. Future studies are required to review and evaluate the 35 hours of male and pup recordings to determine whether they follow a similar pattern.

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