

**UNLEASHING THE POTENTIAL OF STUDYING THE CORRELATIONS  
BETWEEN ULTRASONIC VOCALIZATIONS AND BEHAVIOUR IN RATS**

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UNLEASHING THE POTENTIAL OF ANALYSES OF ULTRASONIC  
VOCALIZATIONS AND BEHAVIOUR

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## **Abstract**

This thesis explores how rats use various categories of ultrasonic vocalizations in social contexts. In the first study male juvenile rats were socially isolated and trained to anticipate play with a conspecific. During the anticipatory period the rats emitted a high frequency of 50-kHz ultrasonic vocalizations. Further, the 50-kHz vocalizations emitted were tightly linked to active behaviours while the rats anticipated conspecifics. The next two studies focused on social interaction between devocalized and vocal animals paired with intact animals in both juveniles and adults. These studies demonstrated a close linkage between active social behaviours and specific categories of vocalizations at both ages. Among juveniles trills seem important for soliciting playful interactions and in adulthood, flat type calls appear to be especially critical for preventing playful interactions from escalating to serious fighting. Thus, different categories of calls may have different communicatory functions. These novel findings provide new insights into the social interactions of rats, which may be applied to developing more sensitive rodent models for studying depression, autism and other pathologies that affect social behaviour.

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## **List of Abbreviations**

AMPH	Amphetamine
CCBN	Canadian Centre for Behavioural Neuroscience
FM	Frequency modulated
Hz	Hertz
kHz	Kilohertz
M	Mean
NSERC	Natural Science and Research Council
S.D	Standard Deviation
USVs	Ultrasonic vocalizations
VTA	Ventral Tegmental Area

## **Chapter 1**

### **Introduction to the Usage of Ultrasonic Vocalizations**

## **1.1. Background Information**

The purpose of ultrasonic vocalizations (USVs) in rats has puzzled researchers since the discovery that rats had a diverse range of such vocalizations. Because of their strong association with social interactions, it has been hypothesized that they play a role in communication. If so, what is the language that the animals are using? In other words how are these vocalizations used to elicit a response in conspecifics? Potential ways in which USVs could be used are 1) as a general indication of mood, like humans use facial expressions or laughter; 2) as a specific indicator of intent, such as to distinguish between aggression and play; and 3) as contact calls to establish contact with a conspecific. To help understand rat vocalization usage, this thesis reports on a series of experiments that explored whether USVs are tied to specific behaviours in three situations: juvenile rats anticipating arrival of a play partner, juvenile rats actively playing, and adult rats both playing and fighting.

## **1.2. History of Ultrasonic Vocalization Usage in Rats**

The study of ultrasonic vocalizations (USV's) started in 1954 when it was discovered that rats emitted vocalizations at a frequency of 40kHz for 1-2 seconds when isolated. It was hypothesized that these vocalizations occurring in the ultrasonic range were used for either echolocation or communication purposes (Anderson, 1954). Further research was done to explore the idea of echolocation in rats and, although some evidence to support this phenomenon was found, the field turned to look at other purposes for these calls (Bell et al., 1971, Rosenzweig, Riley, & Krech, 1955). The next discovery in the field of ultrasonic vocalizations was that pups separated from their mothers emit 40-kHz

calls (Allin & Banks, 1972). Further, 40-kHz calls are also made when the pups experience a temperature drop and it was proposed that these calls were a by-product of the rat's homeothermic regulation (Okon, 1970). Evidence to support this theory was the tight linkage between the animals change in breathing to increase oxygen delivery and warmth, and the production of ultrasounds (Blumberg, 1990). However, it was discovered that rat pups emit these ultrasonic calls when they are separated from their mothers and that the mothers respond to these calls (Smith, 1976). These maternal responses hinted of a potential communicatory purpose for these vocalizations. Subsequently, it was discovered that USV's were emitted in a variety of frequencies ranging from 20 to 90-kHz. The short, flat 40 kHz calls are now recognized as unique to pups. Adult vocalizations range from 20-70 kHz, but are often split into two groups: low frequency (around 22 kHz) and high frequency (ranging from 30-60 kHz but most common around 50 kHz) (Wöhr et al., 2007). Characteristically, the 22 and the 50-kHz calls are emitted in situations that are associated with different affective states.

### **1.3. 22-kHz Vocalizations**

#### ***1.3.1. Social Usage***

The 22-kHz category of USV's is most commonly associated with negative contexts, both, social and non-social. Socially, rats will emit these calls during generally aversive interactions such as fighting or confrontations with dominant conspecifics (Panksepp et al., 2004; Thomas et al., 1983). It has also been observed that 22-kHz calls are predominately emitted by the losing animal in these interactions, perhaps acting as a stop signal or to appease the dominant animal (Thomas et al., 1983). Male animals will

also emit these calls in sexual situations. For example, these calls are commonly seen at the end of a series of copulations, when the animal is in an absolute refractory period. In this situation, the calls are hypothesized to either demonstrate a change in overall motivation, from approach to withdrawal, or to indicate cessation of the interaction to the female (Barfield & Geyer, 1972). Further, males also emit these calls when the female is making the sexual interaction difficult via antagonistic displays (Brown, 1979) or if they experience unsuccessful mounting attempts (McIntosh et al., 1984). Finally, it has also been shown that the position in the social hierarchy also plays a role in the emission of these calls with the submissive animals making significantly more calls than their dominant counterparts (Inagaki et al. 2005).

### ***1.3.2. Non-Social Usage***

The 22-kHz calls are also emitted in situations that elicit high levels of anxiety. The presence of a predator or odour from a predator such as a cat, will elicit these calls (Blanchard et al., 2005). Further, other stimuli such as light puffs of air, a startling sound, or even an unfamiliar human handler can also cause the animal to emit these calls (Blanchard et al. 1986; Brudzynski & Ociepa 1992; Brudzynski & Holland 2005; Kaltwasser 1990, 1991). Further, rats that are exposed to inescapable shock, which elicits high levels of anxiety, emit significantly more 22-kHz calls than rats given escapable shock (Kikusui et al. 2003). It should be noted that the 22-kHz calls are not emitted while the animal is in pain; instead, rats generally emit audible vocalizations during painful events such as electric shock (Jourdan et al. 1995). Overall, 22 kHz calls appear to have a direct relationship to negative affective states indicating that they could serve as a way to communicate general distress or warn other rats about a specific danger.

## **1.4. 50-kHz Vocalizations**

### ***1.4.1. Anticipatory Vocalizations***

#### *1.4.1.1. Non-social stimuli*

The 50-kHz category of rat calls, which will be the main focus of this thesis, is broadly associated with positive affective states, including anticipation of a variety of rewarding, non-social stimuli. Pharmacologically, there is a significant increase in 50-kHz vocalizations emitted when rats are placed in a chamber associated with the delivery of amphetamine (AMPH) or morphine (Knutson et al., 1999; Burgdorf et al., 2001, Knuston et al., 1997). Interestingly, the amount of AMPH administered has a direct relationship with the amount of 50-kHz USVs produced (Wintink & Brudzynski, 2001). Anticipation of self-administration of electrical stimulation to brain reward centers, such as the ventral tegmental area (VTA) and lateral hypothalamic area, also elicits high rates of 50-kHz calls. The animals will show a marked increase in 50-kHz USVs to cues associated with the electrical stimulation as well as to the stimulation itself (Burgdorf et al., 2000). Finally, 50-kHz calls have also been associated with cues indicating food reward (Burgdorf et al., 2000). These findings suggest that 50-kHz USVs signal positive affective states associated with rewarding contexts, independent of social context.

#### *1.4.1.2. Social Stimuli*

Fifty kHz USVs are also emitted in anticipation of a variety of social interactions. Significant increases in 50-kHz calls have been found in males during the anticipatory period before introduction of a female. The increase in the number of vocalizations appears to reflect the rewarding value of sexual contacts (Bialy et al., 2000). Juvenile

male rats will also make 50-kHz vocalizations when anticipating the presence of a conspecific, and these vocalizations will increase over days of testing (Kisko et al., 2015b; Kunston et al., 1998). Rats will also make these vocalizations when entering an area frequented by other rats (Brudzynski and Pniak 2002).

#### ***1.4.2. Social Contact***

During positive social contact, 50-kHz USV's are common, fitting well with the concept of these calls being used in appetitive situations. Fifty kHz vocalizations are extremely common during rough and tumble play in juvenile males, a highly rewarding behaviour, and are most common before the interaction takes place (Himmler et al., 2014). Further, these calls have also been elicited by rats tickled by human handlers, and are more common in isolated than socially housed animals, possibly reflecting the greater value of this hetero-species contact when other social interactions are lacking (Burgdorf & Panksepp, 2001). During copulation, both male and female rats produce 50-kHz vocalizations (Burgdorf et al., 2008; Thomas & Barfield, 1985). Interestingly, the number of 50-kHz vocalizations elicited appears to relate to the level of sexual motivation in the respective vocalizing party (Borner et al., 2016; Bialy et al., 2000). Rats will also produce 50-kHz vocalizations when introduced to an immobilized and, therefore, easily approachable conspecific and when being introduced to a conspecific after separation (Brudzynski, 2009). All of these social interactions are generally positive, however, 50-kHz calls have also been demonstrated in negative social contexts such as during aggression and when initially meeting a resident intruder (Kisko et al. 2015b, Brudzynski, 2009). Thus, this category of calls may not be exclusively positive in nature.

#### ***1.4.3. Eliciting a behavioural response in conspecifics***



Finally, 50-kHz calls are associated with eliciting a response in conspecifics indicating that these calls could be used for social communication. During mating, the 50-kHz USVs emitted by male rats produces solicitation behaviour in female rats. When the males make these vocalizations the females respond with darting and ear wiggling specifically toward the male caller (Thomas et al., 1982). Playback studies also provide several lines of evidence that 50-kHz vocalizations are used to modulate the behaviour of other animals. In infancy, a marked increase in maternal search and care behaviour is seen when mothers are played 40-kHz USVs (distress calls) thus indicating that these calls play a critical communicative role during this period (Wohr et al., 2008a). During the juvenile period, when rats in a radial arm are played 50-kHz USVs, they will approach these calls over pure tones of the same frequency. Further, in adulthood, rats will show the same preference for the 50-kHz USVs and in some cases will call back (Wohr & Schwarting, 2007). All of these pieces of evidence provide support for the 50-kHz USVs as a communicative signal.

In summary, 50 kHz USVs have been found during anticipation of both social and non-social reward and during both positive and negative social contexts. They can also elicit a behavioural response in other animals. The diverse usage of this category of calls begs the question: why are these calls used in so many different situations? To answer this question it is necessary to explore both the diversity and classification of these calls.

### **1.5. Classifying Ultrasonic Vocalizations**

As stated above two general categories of USV's exist in adult rats, 50-kHz and 22-kHz, and these are generally defined in regards to the affective state of the animal.

However, as demonstrated above, the usage of 50-kHz USV's spans several different contexts: positive, negative, social and non-social. One possible explanation for this broad range of usage is that 50 kHz calls are actually a collection of diverse call types with different meanings. Indeed, as many as 14 separate call types have been identified in the 50 kHz range (Wright et al., 2010). There are a variety of classification schemes of varying specificity used to group these calls. The first and broadest classification is the divide between frequency modulated (FM) and constant frequency (flat) type calls. Behaviourally, FM calls have been related to positive and rewarding situations (Burgdorf et al., 2008). In contrast the 50 kHz flat type calls appear to play a role in social coordination as the calls are seen in aggressive and socially ambivalent situations (Wohr et al., 2008). This classification starts to depict a clearer picture of how different 50-kHz calls are used in different behavioural situations. Others have suggested categorizing USVs by frequency. Takahashi et al. divided FM calls into 25kHz, 40kHz and 60kHz ranges and found associations with fighting, feeding and moving, respectively (Takahashi et al., 2010).

The most detailed categorization scheme, created by Wright and colleagues, identified 14 distinct categories of 50-kHz calls based on acoustic parameters and call profile (Figure 1.1) (Wright et al., 2010). In addition to defining call categories, the researchers also compared social and amphetamine (AMPH) reward with the percentages of vocalizations found in each context. It was found that trill type calls, the most common of all calls, significantly increased when the rats were given either a social play partner or AMPH indicating that these type of calls are likely related to reward. Conversely, flat type calls were emitted by isolated rats, which supports the idea that these calls are

potentially used to maintain and re-establish contact (Wright et al., 2010; Wohr et al., 2008). This categorization identifies the rich variety of differences shown in the 50-kHz range and now provides a rationale for the diversity of behavioural correlates. Perhaps the different categories of calls are actually associated with different behaviours.

## **1.6. Behavioural Analysis**

### ***1.6.1 Anticipation of Play***

As stated above, anticipation of social stimuli, especially in the juvenile period, elicits high levels of 50-kHz USV's. However, besides an increase in time spent performing locomotor activities (Kunston, 1998), little is known about what actions rats are performing during this anticipatory period. Therefore, a detailed analysis of the animals' behaviour and vocalizations while anticipating a conspecific could be used to assess the role 50-kHz USV's play in anticipation of social stimuli.

### ***1.6.2. Juvenile Play***

Juvenile rodents commonly interact through rough and tumble play. This type of play involves the attack and defense of the nape of the neck when nuzzled by the snout of the conspecific (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). The peak of this play period occurs from 30 – 40 days old and is marked by increase in play tactics that are designed to keep the interactions dynamic with a high amount of role reversals (Pellis & Pellis, 1997; Pellis & Pellis, 2007). The fluid and reciprocal nature of this phase of play allows for both animals to experience and establish novel movements and configurations with their partner (Pellis & Pellis, 2007). Rough and tumble play shares many behavioural

similarities to aggressive behaviours; however, several criteria exist to distinguish play from overt aggression. Play is characterized by the fact that, during play: 1) no resources are being gained or protected; 2) no injuries are incurred during combat; 3) role reversals are common; and, 4) further contact is likely (Smith, 1997).

What keeps playful interactions from becoming aggressive? Not all pairs of animals will escalate into aggression. Juveniles, in particular, almost never escalate to aggression. But even in adults, aggression is rare. The tight linkage between 50-kHz USV's and social interaction suggest that these calls may act as signals to facilitate play. Indeed FM calls, specifically trill calls, appear to have a strong association with positive interactions such as those during juvenile play (Himmler et al., 2014). The effects of devocalization on juvenile pairs indicates that devocalization alters how the animals play, changing both their attack and defensive strategies and decreases the rate at which play occurs (Kisko et al., 2015a). This evidence and the diverse range of 50-kHz USV's indicate that these calls could have many potential usages such as signaling imminent attack, coordinating reciprocal exchanges or simply to maintaining a playful atmosphere. More research will need to be done to parse out the exact usage.

### ***1.6.3. Antagonistic Behaviours***

As discussed above, several criteria distinguishes play from overt aggression, the most prominent of which is the presence of bite-inflicted wounds. Generally fighting involves bites directed at the flanks and lower dorsum in contrast to the nape attacks in play (Blanchard et al., 1977; Pellis & Pellis, 1987). Along with bites, aggression can be identified by the presence of agonistic displays, such as tail rattling, piloerection, and

threatening an opponent by adopting a lateral orientation with an arched body. Finally, both animals will participate in mutual uprights in overtly aggressive situations where both will stand on their hind feet and vigorously push one another (Barnett, 1975; Grant, 1963). When unfamiliar adult male rats encounter one another in a neutral arena, the play involves vigorous pushing and defense, and these encounters can involve agonistic displays, but rarely do they escalate to outright aggressive biting (Smith et al., 1999). This means these unfamiliar animals have a way to de-escalate these potentially dangerous situations and continue to play.

### **1.7. Summary: Thesis Objectives**

The present studies were designed to enhance our understanding of USV usage in various behavioural paradigms. Together with my advisors, I developed a new analytical method for correlating behaviours with vocalizations with high temporal precision (i.e., within fractions of a second). We applied this method to a variety of situations involving either the anticipation of social interaction or actual interaction in both juvenile and adult rats.

We sought to answer three specific questions:

1. Are vocalizations tied to specific behaviours? To address this we studied the behaviours and vocalizations tied to the anticipation of play. To do this we studied vocalizations and behaviours of lone rats awaiting the arrival of a play partner. This paradigm allows for the exploration of a socially-related task without the problem of attributing vocalizations to specific rats during a dyadic encounter.
2. How are calls used during juvenile play? To study this we used juvenile pairs with in which one animal was devocalized, thus allowing for the determination of the

exact behaviour and corresponding vocalizations of the vocal animal. Therefore, we could determine which calls were made by the actor and recipient during dyadic interactions.

3. What calls are used to de-escalate adult aggressive encounters? Previously it was discovered that pairings of adult rats in which one partner was devocalized consistently escalated to aggression while intact pairings did not (Kisko et al., 2015b). This finding suggests that de-escalating calls exist, but to answer the questions of which calls are used and how they are used, we analyzed the behaviour and vocalizations of both the intact and devocalized pairs to discover the differences.

## Chapter 2

### **Specific 50-kHz vocalizations are tightly linked to particular types of behaviour in juvenile rats anticipating play\***

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## **2.1. Abstract**

Rat ultrasonic vocalizations have been suggested to be either a by-product of physical movement or, in the case of 50-kHz calls, a means to communicate positive affect. Yet there are up to 14 distinct types of 50-kHz calls, raising issues for both explanations. To discriminate between these theories and address the purpose for the numerous 50-kHz call types, we studied single juvenile rats that were waiting to play with a partner, a situation associated with a high number of 50-kHz calls. We used a Monte-Carlo shuffling procedure to identify vocalization-behaviour correlations that were statistically different from chance. We found that certain call types (“split”, “composite” and “multi-step”) were strongly associated with running and jumping while other call types (those involving “trills”) were more common during slower movements. Further, non-locomotor states such as resting and rearing were strongly predictive of a lack of vocalizations. We also found that the various sub-types of USVs can be clustered into 3–4 categories based on similarities in the way they are used. We did not find a one-to-one relationship between any movements and specific vocalizations, casting doubt on the motion byproduct theory. On the other hand, the use of specific calls during specific behaviours is problematic for the affect communication hypothesis. Based on our results, we suggest that ultrasonic calls may serve to coordinate moment-to-moment social interactions.

## **2.2 Introduction**

Rats and related rodents emit a large number of ultrasonic vocalizations in the 50-kHz range (Brudzynski, 2009). There have been two major hypotheses advanced to account for these calls: the movement by-product hypothesis and the affective



communication hypothesis. The movement byproduct hypothesis posits that the 50-kHz vocalizations emitted by rodents are largely produced by the biomechanical forces on the thoracic area generated during movement (Blumberg, 1992). In contrast, the affective communication hypothesis posits that these vocalizations convey the animal's affective state to surrounding animals. The fact that 50-kHz calls are highly varied, with some authors recognizing as many as 14 different kinds involving distinct patterns of frequency modulation (FM) (Wright et al., 2010) raises questions about how the two hypotheses can account for such variability.

Thiessen and collaborators pioneered the movement byproduct hypothesis after finding a strong correlation between locomotion and the production of some USVs in gerbils (Thiessen et al., 1980). Particularly striking was that the calls were emitted most often when the animal landed on its forefeet when jumping, when the lungs are presumably compressed and air expelled. Similarly, vocalizations have been linked to the landing phase of hops and darts in female rats during mating (Blumberg, 1992). Vocalizations necessarily occur on the expiratory phase of respiration and hence, behaviours which modulate breathing could also modulate USVs (Roberts, 1975). For example, USVs are frequently seen during sniffing behaviour and show periodicity in the 6–8 Hz range, synchronous with the sniffing respiratory rhythm (Sirotin et al., 2014). Locomotor movements are known to entrain respiration in many quadrupeds, including rats (Bramble & Carrier, 1983; Alves et al., 2016). Hence, it is plausible that vocalizations are entrained to locomotion.

Several lines of evidence argue against the movement byproduct hypothesis. First, although sniffing and walking entrain respiration, rats can sniff and move without vocalizing (Sirotin et al., 2014; Alves et al., 2016). In fact, electromyographic studies suggest that vocalizations require the active contraction of the larynx muscles during the exhalation (Riede, 2013; Riede, 2011). Concordantly, rats with transected laryngeal nerves do not vocalize even though they actively move about (Kisko et al., 2015a; Kisko et al., 2015b). Second, respiration and locomotion can be decoupled during vocalizations as the rat prolongs the expiratory phase to produce a call (Alves et al., 2016). Third, the periodic frequency modulations produced during trills require active oscillatory adduction of the laryngeal muscles, suggesting that complex calls are not produced by accident (Roberts, 1975; Riede, 2013). Fourth, a recent study reported that vocalizations often occur at the onset of locomotion, not when the animal is stopping (Laplagne & Elias Costa, 2016). Finally, the presence of another rat can influence vocalization rate independent of the rate of movement (Laplagne & Elias Costa, 2016). Despite this evidence, it is clear that vocalizations are strongly associated with movements, leaving open the possibility that at least some vocalizations are motion byproducts (Alves et al., 2016; Laplagne & Elias Costa, 2016).

The alternate hypothesis is that USVs are indicators of affective state (Knutson, Burgdorf, & Panksepp, 2002), with 22 kHz calls indicating negative affect and 50-kHz calls indicating positive affect. The 22 kHz calls are emitted in situations where the rat may experience anxiety (Brudzynski, 2009). For example, 22 kHz calls are elicited by predators (such as a cat) (Blanchard, Blanchard, Agullana, & Weiss, 1991), foot shock (Tonoue, Ashida, Makino, & Hata, 1986), fear conditioning (Antoniadis & McDonald,

1999; Kikusui, Nishizawa, Takeuchi, & Mori, 2003; Wohr & Schwarting, 2008), a dominant conspecific (Panksepp, Burgdorf, Beinfeld, Kroes, & Moskal, 2004; Sales, 1972; Thomas, Takahashi, & Barfield, 1983), acoustic startle (Kaltwasser, 1990), handling by an unfamiliar handler (Blanchard, Flannelly, & Blanchard, 1986; Brudzynski & Ociepa, 1992), and social defeat (Panksepp et al., 2004; Thomas et al., 1983). In contrast, 50-kHz calls are present during anticipation of rewards such as a rewarding drug (amphetamine or methamphetamine) (Knutson, Burgdorf, & Panksepp, 1999), sexual encounters (Bialy, Rydz, & Kaczmarek, 2000), stimulation of brain reward pathways (Burgdorf, Knutson, & Panksepp), social play (Burgdorf et al., 2008) and “tickling” by human handlers (Panksepp & Burgdorf, 2000). Further distinctions have been drawn between different categories of 50-kHz calls, such as flat and frequency modulated (FM) calls [25-28]. Flat 50-kHz calls have been proposed as a social coordination signal based on the finding that rats in their home cage emit these calls when a partner is removed (Wohr, Houx, Schwarting, & Spruijt). These calls are also associated with aggressive interactions and, therefore, could be an indicator of social ambivalence (Burgdorf et al., 2008). In contrast, FM calls have been associated with a variety of positive situations such as social interactions or rewards (Bialy et al., 2000; Burgdorf et al.; Knutson et al., 1999). However, the affective communication hypothesis does not predict nor offer a particularly compelling account for why there should be as many as 14 categories of vocalizations.

One of the issues complicating the understanding of ultrasonic calls is that it is not yet clear where to draw categorical boundaries between calls. As mentioned, a distinction is often made between flat and FM calls (Burgdorf et al., 2008; Ciucci et al., 2009; Wohr

et al., 2008; Wright et al., 2010). FM calls are sometimes further subdivided. For example, Takahashi et al. (Takahashi, Kashino, & Hironaka, 2010) divided FM calls by the frequencies around which they range (25kHz, 40kHz or 60kHz). The most extensive categorization scheme is that proposed by Wright et al. (Wright et al., 2010) who identified 14 distinct categories of 50-kHz calls based on acoustic parameters and call profile. Similarly detailed schemes have been devised for mouse vocalizations (Grimsley, Monaghan, & Wenstrup, 2011; Hanson & Hurley, 2012). However, it has been argued that these categorization schemes are more detailed than necessary (Brudzynski, 2009). Exactly which call categories are needed to capture the essential features of rat communication remains an open question.

In this experiment, we sought to discriminate between the movement byproduct and affective communication hypotheses by examining the correspondence between specific subtypes of 50-kHz calls and specific movements. We examined behaviour and vocalizations while juvenile rats awaited the arrival of a play partner, a period associated with a large number of 50-kHz calls (Knutson, Burgdorf, & Panksepp, 1998) which, owing to the lack of a partner, can be uniquely ascribed to one animal. The movement byproduct hypothesis predicts that vocalizations should be tightly correlated with actions involved in locomotion, not those in which the rat is stationary. Further, because each movement produces its own characteristic airflow pattern, there should be a one-to-one correspondence between specific movements and vocalization types. The affective communication hypothesis, on the other hand, simply predicts a high number of positively valenced calls, presumably intended to elicit play from the expected partner. In this view, calls need not be associated with any particular behaviour. Hence, a finding

that 50-kHz vocalizations are decoupled from behaviours would strongly favor the affective communication hypothesis over the movement byproduct hypothesis.

## **2.3. Methods**

### **2.3.1. Ethics**

All procedures reported here were in accordance with the Canadian Council on Animal Care guidelines. This study was specifically approved by the University of Lethbridge institutional Animal Welfare Committee.

### **2.3.2. Subjects**

A total of 36 male, juvenile Long Evans rats were used in this study. All animals were bred at the Canadian Centre for Behavioural Neuroscience at the University of Lethbridge and were housed in pairs until testing. Nine subjects were used in Analysis 1 and 12 subjects in Analysis 2. The remaining 18 animals were cage-mates of the target animals, whose data only became relevant during social interactions, not reported here. Testing occurred from post-natal days 26 – 38, spanning the age when play is most frequent (30 – 40 days from birth) (Pellis & Pellis, 1997).

### **2.3.3. Behavioural procedure**

#### **2.3.3.1. Apparatus**

The testing enclosure was a Plexiglas box (50x50x50 cm), which was situated inside a soundproof chamber (61x61x83 cm) lined with acoustic foam. The floor of the chamber was covered with 2 cm of paper-based bedding (Softzorb, Nepco, Warrensburg, NY), which we found to facilitate play while causing very low levels of ultrasonic interference. Ultrasonic vocalizations were collected using a specialized microphone

(Model 4939, Brüel & Kjaer, Denmark) with a frequency response of 4-Hz to 100-kHz. The microphone was located in the ceiling of the chamber and was approximately 15 cm above the middle of the play enclosure. The microphone was connected to a Soundconnect™ amplifier (Listen, Inc, Boston, MA) and sound waves were recorded at 195,313 Hz using 16-bit resolution via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, FL). Video was recorded using two cameras. One was a USB webcam (Microsoft Lifecam Studio, Redmond, WA) with its infrared filter removed, positioned directly above the animal and the second was a Sony digital video camera, also with infrared sensitivity, with a view through the side of the testing chamber at a downward angle of 45 degrees. Illumination was provided by an array of 16 100 mW infrared LEDs (Osram SFH 4557, Regensburg, Germany) mounted on the ceiling of the soundproof chamber and from the in-built infrared light source in the Sony camera.

#### *2.3.3.2. Anticipation of play test*

Data presented here were taken from a 2-minute anticipation period during which a target animal waited in the testing enclosure for the arrival of a familiar play partner (i.e., his former cage mate). Once the play partner was introduced, then animals were allowed to play for 5 minutes, following previously established methods (Himmler, Pellis, & Kolb, 2013). Prior to testing, pairs were habituated to the enclosure for 30 min each day for 6 consecutive days. On the 6<sup>th</sup> day all subjects were socially isolated from their cage mates for 24 hours prior to play testing and isolation continued until after all 7 days of testing were complete, in order to increase overall playfulness (Niesink & Van Ree, 1989; Panksepp & Beatty, 1980; S. M. Pellis & Pellis, 1990). Both habituation and testing sessions occurred immediately at the end of the light cycle and were conducted in

complete darkness, as this has been shown to facilitate USV production (Knutson et al., 1998). Audio and video recordings began after the target rat was placed in the test enclosure. Because both audio and video data were recorded on separate devices, a custom-made beeper with an LED light was used to emit a simultaneous light/sound cue at the beginning and end of each recording session and these times were used to synchronize audio and video recordings during subsequent analysis. Following each session, rats were returned to their home cages, and the apparatus was thoroughly cleaned with Virkon, a broad-spectrum disinfectant (Virkon Disinfectant Technologies, Sudbury, United Kingdom), and bedding was replaced to avoid any odors from other subjects.

#### ***2.3.4. Scoring***

##### *2.3.4.1. Behaviour*

For each animal the type of behaviour and duration of that particular behaviour were scored during the 2-minute testing session. Importantly, we assigned a behavioural category at every time point, so that no time was left unaccounted for. Knowing the rats' behaviour at every time point was important because in our analysis, that lack of a vocalization during a specific behaviour was informative (e.g., a call which occurs for 8 of 8 instances of a behaviour is more tightly coupled to that behaviour than one which occurs only 3 times out of 8). Behaviours were coded via frame-by-frame analysis of video taken either at a 45-degree angle relative to vertical or via an overhead camera. A switch from a camcorder (45 degree angle) to an infrared webcam (overhead) occurred mid-way through data collection due to the greater ease-of-use of the webcam. We have found that either view is sufficient to distinguish the behaviours we studied here.

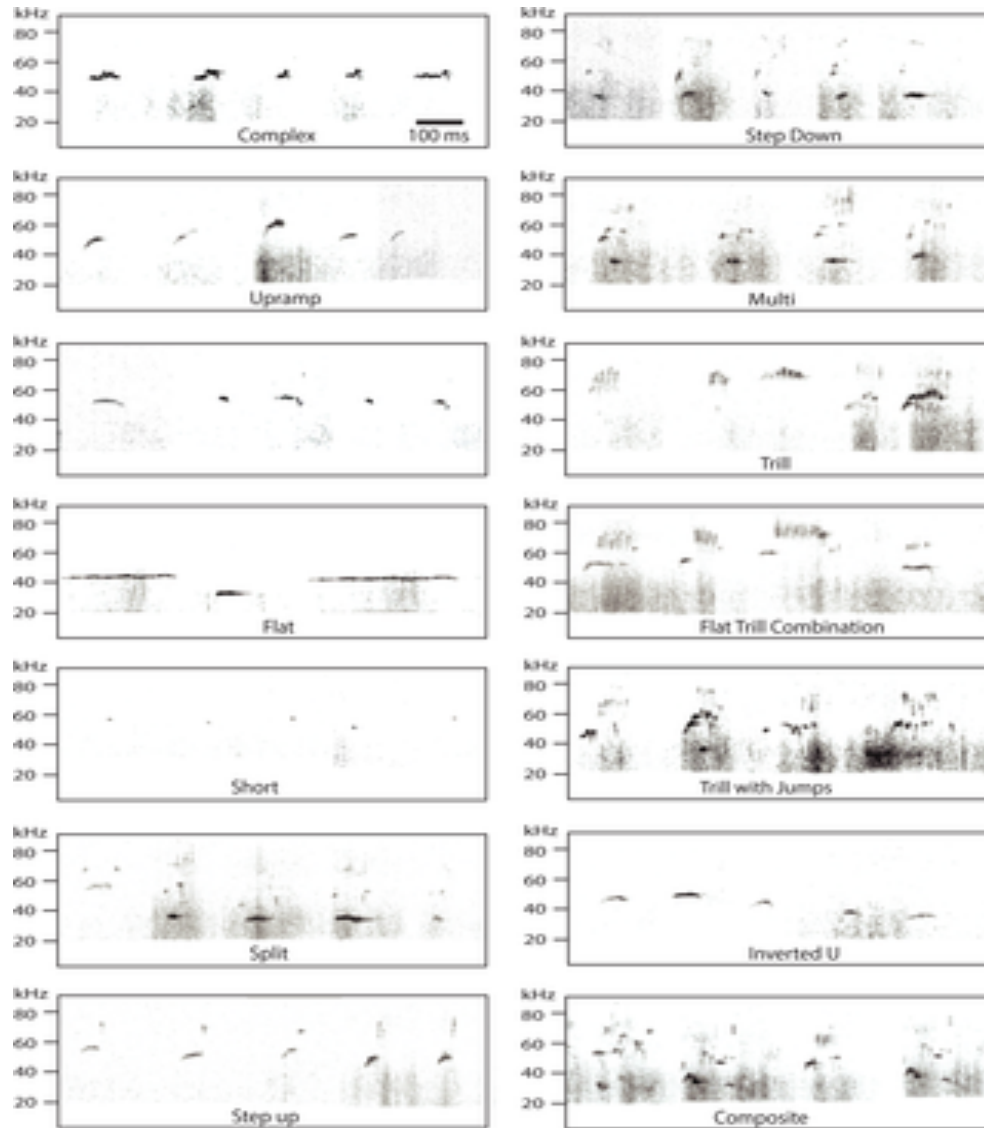
The behaviours included in the analyses are described in Table 1, which also describes the criteria used in categorization. Separate coders performed behavioural (and vocal) coding for each of the analyses. In the first analysis, overlapping behaviours were allowed (e.g., if the animal turned while walking, that time interval would be coded as both a turn and a walk). In the second analysis, each time interval was assigned only to one behaviour, thus simplifying our vocal-behavioural correlation analysis. In the case of turning and walking simultaneously, the behaviour was coded as a walk. Most other behaviours were mutually exclusive by their nature.

#### *5.3.4.2. Vocalizations*

Acoustic data was analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. Fifty kHz vocalizations were included if they followed Wright et al.'s (Wright et al., 2010) call criteria, specifically: 1) temporal continuity, 2) fundamental frequencies between 35- and 90-kHz, and 3) a local peak in sound intensity distinct from background noise that exhibited patterned temporal structure. Some putative vocalizations were not perceptually distinct from the background noise and were hence removed from further analyses. After manual scoring, the Raven Pro software provided the beginning and end times of each vocalization as well as the manually determined category of each call. The call classifications were done according to the 14 categories identified by Wright et al. (Wright et al., 2010). Although these calls were identified from slightly older rats (60-70 days of age), most of our calls were highly similar to at least one of the 14 adult call types, suggesting that the call structure of Long-



Evans rats does not change much between 1 and 2 months of age. Examples of vocalizations in each category are shown in Fig 1. As with behavioural coding, categorization of vocalizations for each experiment was by one experimenter and the coders differed for analysis 1 and 2.



**Figure 2.1.** Examples of categorized 50-kHz calls (as originally described by Wright and colleagues (Wright et al., 2010)) taken from our data.

**Table 2.1.** Description of Rat Behaviours

<b>Behaviour</b>	<b>Description</b>
Step	Removal of at least two paws from the ground in an alternating manner
Walk	Removal of all four limbs off the ground in an alternating manner (left paw and right hind limb move simultaneously followed by right paw and left hind limb) OR significant shift from one location to another (if all limbs are not visible)
Run	Only two limbs touch the ground at any given time; the rat may alternate two limbs at a time (as is seen during walking behaviour) OR the rat may move two paws followed by two hind limbs at any given time; such movement is accompanied by the extension of the torso as the front limbs reach forward followed by flexion of the torso as the hind limbs are removed from the ground and placed under the body (Muir, 2005)
Jump	Up jump: the two front limbs leave the ground followed by the hind limbs while body is lifted into the air, then all limbs touch the ground simultaneously or closely one after the other (Sergio M. Pellis & Pellis, 1983); Forward jump: the two front limbs are extended forward and removed from the ground followed by the removal of the hind limbs from the ground; this behaviour is accompanied by the extension of the torso as the front limbs reach forward followed by flexion of the torso as the hind limbs are removed from the ground
Turn	Turn with one or both front limbs at a 45-, 90-, or 180-degree angle OR turn with three or more limbs at a 360-degree angle. Turning may also be preceded by a stepping or walking pattern or followed by a rear (see below for the operational definition of rearing behaviour)
Explore	Immobile; may extend one front limb; turning of head so as to examine the surrounding area
Dig	Vigorous forward and backward motion of front limbs while significantly displacing bedding
Rear	Standing on rear limbs with both front paws off ground (either free standing or against wall)
Shake	Vigorous side-to-side shudder of head, neck, and trunk (Gorzalka & Hanson, 1998)
Groom	Licking of paws; wipes/rubs face and nose; wipes behind ears, neck, and/or downward to either side of the body; may grab fur and nibble with teeth. Grooming may consist of a variation of these behaviours many consecutive times. However, grooming is typically initiated by wiping of the nose or face and followed by grooming of the neck and body (Aldridge, 2005)
Scratch	Rapid movement of hind limb with the claws rubbing against head, neck, or side
Rest	Immobile; may turn head, but significantly less than is seen during exploration

### **2.3.5. Analyses**

#### *2.3.5.1. Overview*

We present data from two separate analyses (meaning a full coding of all behaviours and vocalizations) taken during anticipation of play in 18 rats. The purpose of the first analysis (Analysis 1) was to explore the feasibility of the analysis methods. It included data recorded at multiple time-points in the experiment (testing days 1, 5, 6, and 7) and, in some cases, included two data sets from the same animal because that is the data that we had available at the time. In light of the results of the first analysis, a second, better controlled, analysis (Analysis 2) was conducted by a different researcher (C.B.) using only data from day 7 of testing and with each animal contributing only one session. Day 7 was used because this is when the most vocalizations occurred and it is also the session most likely to have calls of a social nature (relevant to the affective communication hypothesis). Three animals from the first analysis were included in the second, but using different sessions so that there was no overlap between the data used in the two analyses. As part of Analysis 2, we also quantified the total number of calls and time spent in locomotor behaviours (turning, walking, running and jumping) between the first and last day of training (Day 1 vs Day 7).

#### *2.3.5.2. Monte-Carlo shuffling*

The total number of behavioural and vocal events differed substantially between different categories, meaning that a simple correlation of behaviours with vocalizations would have been problematic to interpret. For example, there were a very large number of trills that occurred during walking, but this could be simply because trills were the most

common vocalizations and walking was one of the most common behaviours. A statistical analysis that accounted for these differences in event baselines was necessary to determine whether a particular vocalization co-occurred with a particular behaviour above chance levels. Therefore, a Monte-Carlo technique was used to calculate the probability distribution of each unique combination of vocalizations and behaviours. We first counted the number of co-occurrences of each vocalization type with each of the coded behavioural categories. Next, for each session, vocalizations were repeatedly shuffled and the number of behaviour-vocalization co-occurrences computed. A vocalization was counted as occurring during a particular behaviour if the mid-point of the call occurred between the start and stop time of the behaviour. To allow for errors in the coding of the behaviour times, we expanded the time window for each behaviour by 200 ms in each direction. Shuffling was achieved by assigning each vocalization a random time within the duration of the 2-minute observation period. Hence, the relative frequency of vocalizations was kept the same for each shuffle. This shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behaviour was tabulated. Based on the distribution of these counts, we assigned a z-score to the actual number of occurrences. The higher the z-score, the less likely that specific combination of vocalization and behaviour could have occurred by chance (i.e. for the number of sessions included here, a z-value of 2.8 gives a  $p$ -value of  $<0.05$  and a z-value of 3.3 gives a  $p$ -value of  $<0.01$ ). Shuffling was performed separately for each session and the z-scores averaged across session to generate the final, average z-score values.

#### *2.3.5.3. Proportion of movements associated with vocalizations*

To further assess the link between movement and vocalizations, we measured the proportion of times that each movement type was accompanied by a vocalization. A 200 ms window was created, centered on the onset or offset of each movement (i.e., 100 ms before to 100 ms after the movement onset or offset). We then tabulated the number of times each vocalization type overlapped with this window. For this analysis, each vocalization type was counted either as an occurrence or non-occurrence (i.e., two trills before a walk would be treated as a single occurrence). The results were expressed as a proportion of the total number of movements. For example, if there were 80 instances of the movement type “run” and 60 of them were accompanied by a “trill” call during the onset of that movement, we reported that 75% of runs began with a trill. The total proportion of movements accompanied by a call was calculated by summing the proportions of co-occurrence across each of the vocalization types.

#### *2.3.5.4. Dendrogram of vocalization similarity*

To assess the similarity of vocalizations based on their behavioural correlates, we created a dendrogram (see Fig 5 below). Similarity was based on the z-score matrix describing the relative frequency of occurrence of each vocalization and behaviour, as described above under Monte Carlo Shuffling, with vocalizations in rows and behaviours along the columns. We measured the distance between each pair of rows of this matrix as  $1-r$  where  $r$  is the correlation between rows. A distance of near zero meant that the behavioural correlates for those two vocalizations were nearly identical whereas a distance of 1 meant that the behavioural correlates were unrelated. Values above 1 indicated that the behavioural correlates were anti-correlated. A dendrogram was then

generated from this data using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm, implemented in the BMEToolbox (<http://www.bioinformatics.org/mbetoolbox/>) (Cai, Smith, Xia, & Yuen, 2005) written for Matlab (The Mathworks, Natick, MA).

#### *2.3.5.5. Student's t-tests*

A 2-tailed paired *Student's t-test* was used to assess whether there was a significant increase in vocalizations from day 1 to day 7 for all animals in Analysis 2. Each rat contributed two measures to this analysis, one for the total number of calls during the two-minute anticipation period on day 1 and another for the total number of calls in the same period on day 7 of training. A similar analysis was performed on the total amount of time spent in locomotor behaviours. Time was used rather than counts because behaviours are extended over seconds in contrast to vocalizations which are so short that they can be considered discrete events

#### *2.3.5.6. Inter-rater reliability analysis.*

To assess inter-rater reliability of both behavioural and vocal data, data from three sessions were coded by both of our coders. To compute reliability, each session was broken down into 10 ms bins and the behaviour or vocalization within that bin tabulated for each coder using an arbitrary numbering scheme (e.g., turn was assigned a 1, walk was assigned as a 2, and so on). We then ran a simple correlation between the ratings of the two coders, including all bins from all three sessions. In the case of vocalizations, bins with no vocalization coded were excluded before running the correlation because there were many long stretches of silence. Note that judicious assignment of numbers to

behaviours or vocalizations (i.e., giving similar calls/behaviours closer numbers) would improve the reliability ratings, so the reported values should be considered a conservative estimate of inter-rater reliability. Further, independent of the numbering scheme, a correlation of one would always mean total agreement.

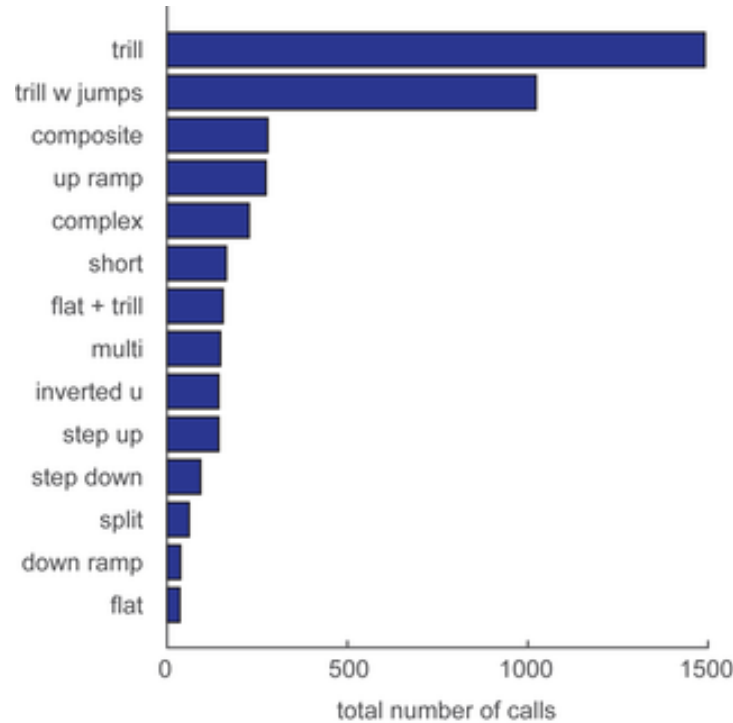
## **2.4. Results**

### **2.4.1. *Vocalization counts during anticipation of play***

To investigate the behavioural correlates of vocalizations, we ran juvenile rats in an anticipation of play paradigm, analyzing vocalizations during the two-minute period before the introduction of a play partner. Animals were tested over the course of 7 anticipation periods, one per day, each followed by pairing with the same play partner (see Methods for details). In the first analysis, we used data from 9 animals and a total of 14 play anticipation sessions (some animals contributed two sessions recorded on different days). To test the robustness of our results, a second analysis was conducted, using data from 12 rats and a total of 12 test sessions, all from testing day 7.

Previous studies have shown that FM 50-kHz calls are most common during appetitive situations. For the purposes of comparison with previous studies, we counted the frequency of occurrence of each of the 14 call sub-types identified in this study, using categories previously suggested by Wright et al. (Wright et al., 2010). As shown in Fig 2, calls containing trills (trills, trills with jumps, and composites) were by far the most frequent types of calls. In comparison, flats were the least frequent call. These data contrast with those of Wright et al, who found that almost half of the calls emitted by young adults tested one at a time were flats, but the data agrees with Himmler et al. (Himmler, Kisko, Euston, Kolb, & Pellis, 2014), who found that the most frequent

vocalizations during play by juveniles were trill type calls, accounting for 77% of the total calls.



**Figure 2.2. Total number of vocalizations by call category.** Categories correspond to those shown in Fig 1. Bars indicate the total raw counts of vocalizations across Analysis 1 and Analysis 2 combined.

#### **2.4.2. Training effects on vocalizations and behaviours**

To address predictions made by the locomotor byproduct and affective state hypotheses, we examined changes in vocalization and behaviour prevalence between training days 1 and 7. First, we compared the overall number of 50-kHz calls on both day 1 and day 7 for all rats included in analysis 2 (analysis 1 could not be used due to missing data). A 2-tailed paired *Student's t-test* showed a significant increase in 50-kHz calls from day 1 (M = 92.9 calls; SD = 82.6) to day 7 (M = 182; SD = 95.3;  $t = 3.04$ ,  $df = 14$ ,  $p = 0.009$ ), suggesting that at least some of the calls observed were due to anticipation of a



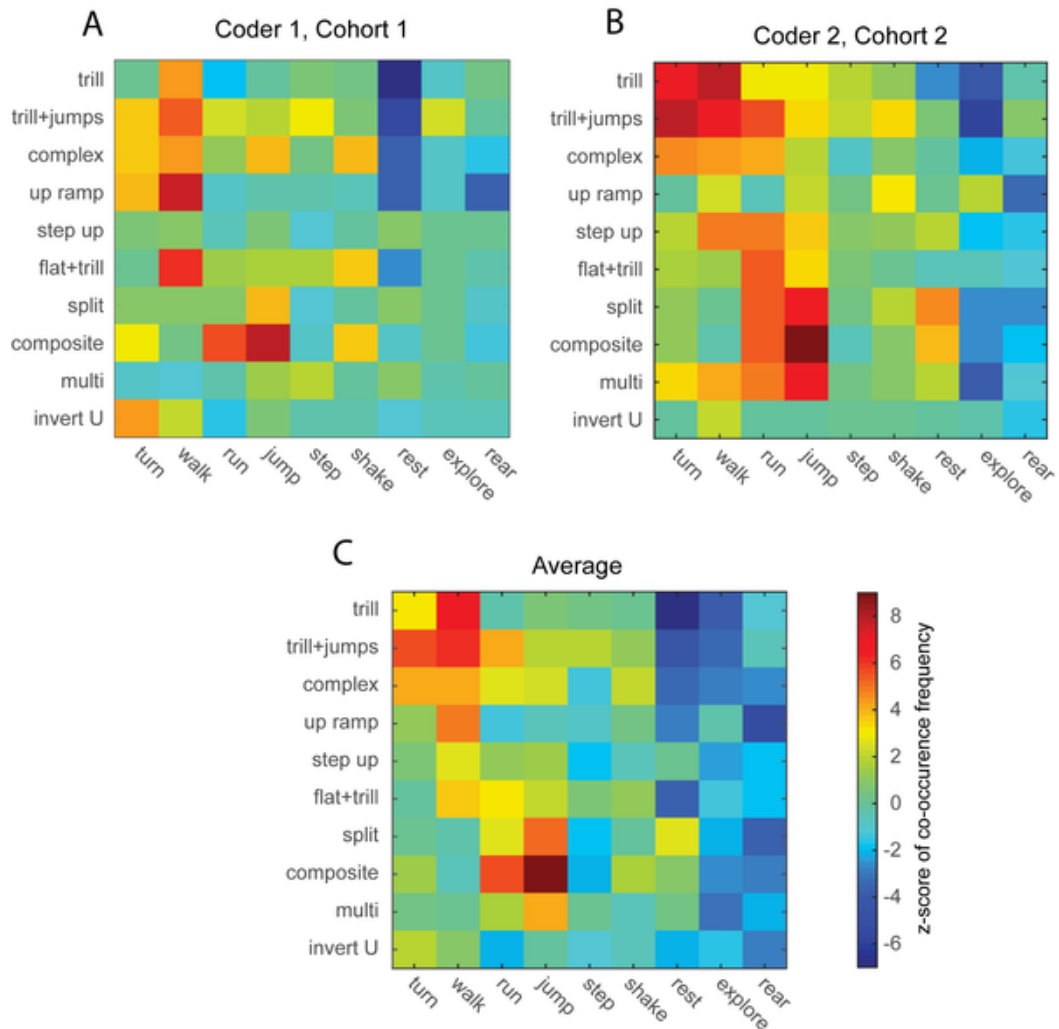
play partner. Could increased movement account for the increased number of vocalizations? To address this, we compared the total time spent in locomotion (walking, running or jumping) between day 1 and day 7. A repeated measures *Student's t-test* revealed that locomotion time actually decreased with training (Day 1: M = 56.7 sec; SD = 19.1; Day 7 M = 28.4 sec; SD = 10.6;  $t(10) = 5.59$ ,  $p < .001$  (2 tailed)). On the other hand, the total number of jumps and runs both increased significantly from day 1 to day 7 (Jumps: Day 1: M = 2.00; SD = 2.57; Day 7: M = 9.18; SD = 6.27;  $t(10) = -3.70$ ,  $p = .004$  (2 tailed); Runs: Day 1: M = 0.64; SD = 1.03; Day 7: M = 7.27; SD = 7.48;  $t(10) = -2.85$ ,  $p = .017$  (2 tailed)). In other words, rats trained to expect a play partner spend less overall time moving, but those movements they do make are more vigorous. Because both vocalizations and locomotion increase with training, these data do not discriminate between the locomotor byproduct and affective state hypotheses.

#### **2.4.3. Specific correspondences between vocalizations and behaviours**

To further address the locomotor byproduct and affective state hypotheses, we counted the number of co-occurrences of each vocalization type with each of the coded behavioural categories. A Monte-Carlo shuffling technique was then used to estimate how likely the observed frequency of coincidence between a behaviour and a vocalization was to have occurred by chance, expressed as a z-score (a z-score above 2.3 is significant at  $p < .01$ ). Negative z-scores indicated that the call was much less likely to occur during the specified behaviour than if calls were randomly distributed.

As shown Fig 2.3 some actions are clearly more likely to be associated with certain vocalizations than others. Our first analysis was a preliminary study to establish

the feasibility of the method, and hence the behavioural sessions were taken from different days during testing. Despite this variability, the z-scores from this study show clear evidence of certain calls being tied to certain behaviours. As shown in Fig 3A jumping behaviour is consistently associated with the composite, split, and complex call categories with  $z$ -values of 7.9, 3.7, and 3.7 respectively. Running appears to be consistently associated with the composite and trill-with-jumps calls. Walking was associated with the trill-with-jump, trill, complex and flat-with-trill calls. Further, several calls were associated with turning (invert U, trill-with-jumps, complex and ramp) and shaking (complex, flat-with-trill, and composite). Finally, rearing, resting, and exploring are among the least likely behaviours to be coupled with any type of vocalizations. Z-scores for the most statistically significant vocalization-behaviour correspondences are shown in



**Figure 2.3. Z-scores showing the probability of occurrence of each of the vocalization-behavioural combinations are graphically presented.**

(A) Data from Analysis 1. (B) Data from Analysis 2. (C) Grand average of the results from Analysis 1 and 2. Colors indicate the z-score and the same color scheme is used for all subplots. Note that vocalizations and behaviours with very low rates of occurrence have been omitted, as it was not possible to run statistical analysis for these low-frequency events.

**Table 2.2** Ten strongest associations between behaviours and vocalizations are shown for Analysis 1. Values are z-scores.

<b>Association Type</b>	<b>z-value</b>
Jump – Composite	7.9
Walk - Upward Ramp	7.6
Walk - Flat-Trill Combination	6.1
Walk - Trill with Jumps	5.5
Walk - Step Down	5.5
Explore - Downward Ramp	4.6
Walk - Trill	4.4
Turn – Inverted U	4.4
Shake – Composite	3.8
Turn – Upward Ramp	3.8

A second analysis was conducted to test the reliability of our methodology and to insure that our results actually reflected calls emitted during the anticipation of a play partner. While analysis 1 focused on a variety of testing days, the second analysis used data from of testing. As the vocal and behavioural scoring for each analysis involved a different coder, an inter-observer reliability analysis was conducted. To do this, the two experimenters coded three data sets in common. The raw behavioural counts showed a 92% agreement between coders. For vocalizations, the raw vocalization counts showed only a 36% agreement. Upon closer inspection, it was determined that the discrepancies were due to disagreement over how to categorize four highly similar call types: trills, complex, composite and trill-with-jumps. Accounting for this confusion gave 98% agreement in call categorization. Despite the less than complete agreement between coders, the data from analysis 2 (Fig 3B) look strikingly similar to those from analysis 1 (Fig 3A). Again, jumps were highly correlated with composite and split calls, but now also with multistep calls. Runs were now associated with a much wider range of call types, including multi-step, split, flat-trill, step up, and trill-with-jumps. Walking was

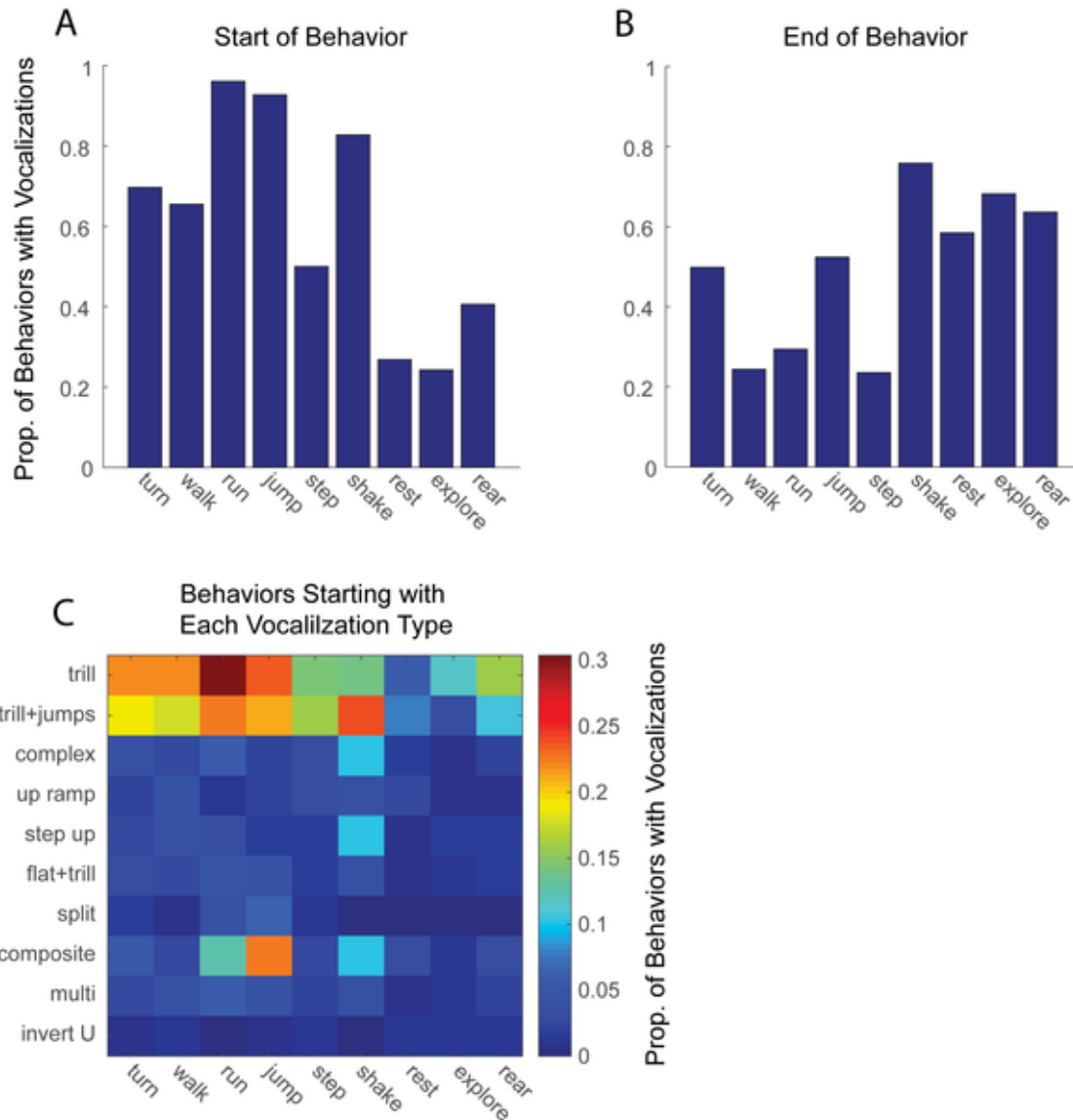
now more associated with trill and step-up but not flat-trill calls. Turning was now associated with trill but not up ramp calls. Some splits occurred during rest, but again, resting, exploring and rearing were either unrelated to most calls or even anti-correlated. Z-scores for the most statistically significant vocalization-behaviour correspondences are shown in Table 3.

**Table 3.** Ten strongest associations between behaviours and vocalizations are shown for Analysis 2. Values are z-scores.

<b>Association Type</b>	<b>z-value</b>
Jump – Composite	8.8
Walk – Trill	8.3
Turn – Trill with Jumps	7.6
Jump – Multi	7.4
Turn – Trill	6.8
Walk – Trill with Jumps	6.6
Jump – Split	6.3
Run – Trill with Jumps	6.0
Run – Split	5.4
Rest – Split	5.4

Given the gross similarities between the results of analysis 1 and 2 we averaged the results (Fig 3C) to present a summary of our findings. Speaking broadly, slower locomotion (turning and walking) is associated with calls that include trills or warbles (trill, trill with jump, and complex) as well as up ramps. As locomotion becomes more vigorous (running and jumping), vocalizations shift to composite and sometimes split and multi-step calls. Again, less active states such as resting, exploration and rearing are associated with a marked reduction in the probability of calls, suggesting that these are receptive states for the rat (i.e., they are quiet, perhaps listening, rather than emitting calls).

The highly significant statistical relationship between jumps and composite calls is consistent with the idea that composite calls are a byproduct of jumping. Similarly, calls with trills might be byproducts of walking and running. To investigate these possibilities further, we examined the consistency of the coupling between calls and behaviours, collapsing across data from Analyses 1 and 2. If a call was caused by a particular movement, then we'd expect a one-to-one correspondence between that call (or group of similar calls) and that movement. To address this, we first computed the proportion of times that each behaviour was associated with any call. We focused on calls either within 100 ms of the onset or offset of the movement because these are the times of the most dramatic postural change and hence the most likely to lead to thoracic compression and involuntary calls. As shown in Fig 4A there were three behaviours which were frequently accompanied by calls: runs, accompanied by calls 96% of the time, jumps, accompanied by calls 93% of the time, and shakes, accompanied by calls 83% of the time. In comparison, with the possible exception of shakes, the offset of behaviours was less frequently accompanied by a call (Fig 4B). Note that the relatively high number of calls after a rest, explore and rear are likely onset responses to the subsequent movement, which are often locomotive.



**Figure 2.4. Proportion of each behaviour associated with vocalizations.** (A) Bars show the proportion of times each behaviour type was accompanied by any vocalization within 100 ms of the onset of that behaviour. (B) Same as A but vocalizations are counted within 100 ms of the offset of the behaviour. (C) The proportion of times each behaviour was accompanied by vocalization, broken down by the specific type of vocalization.

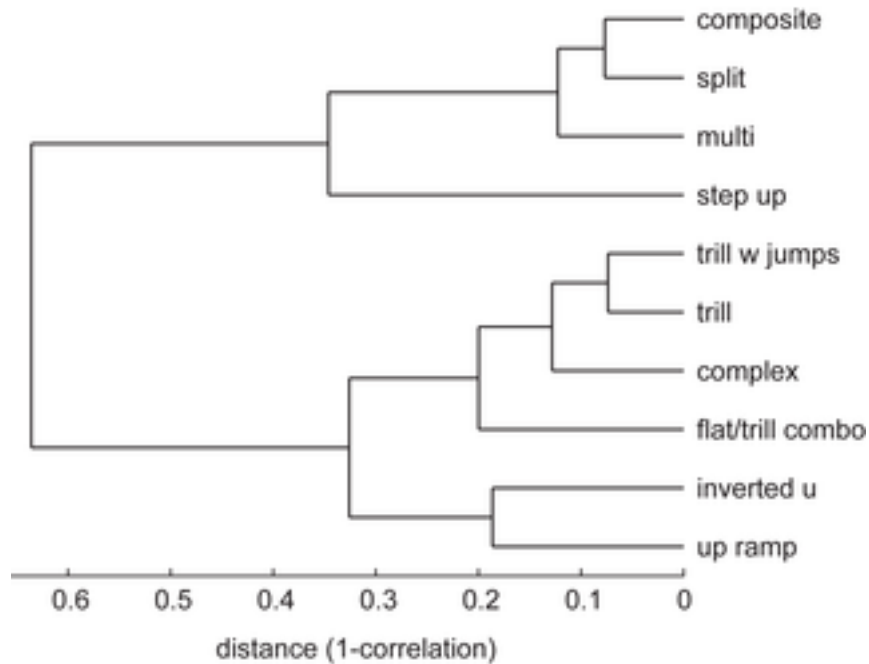
The locomotor byproduct hypothesis also predicts that a given movement should elicit the same (or highly similar) call. As seen in Fig 4C this is not the case, even for running, which is associated with composite calls 13% of the time, trill with jumps 23%

of the time, and trills 30% of the time. As shown in Fig 1 these calls are fairly distinct. Similarly, shakes are accompanied by trills, trills with jumps, complex, step-up and composite calls. These findings are hence not consistent with the idea that the movement obligatorily causes a specific type of call.

#### ***2.4.4. Similarities between calls based on behavioural correlates***

It is apparent from Fig 3 that certain calls have similar behavioural correlates. For example, trill and trill with jump calls are almost equally likely to be emitted during turning and walking, whereas split, composite and multi-step calls are all associated with jumping. To assess this further, we performed a clustering analysis, based on the pair-wise correlations of the behavioural z-scores shown in Fig 3C for each vocalization. The results, shown in Fig 5 indicate that the ten most frequent calls types can be grouped into three or perhaps four larger categories. The first consists of composite, split, and multi-step, calls that are generally associated with vigorous activities such as jumping. The second group consists of calls with trills (trill, trill-with-jumps, complex and flat-trill calls), which are generally associated with slower locomotion. A third group consists of the inverted U and up ramp calls, whose behavioural correlates were not well defined. The step-up calls may form a fourth group that seems to be solely associated with walking. As we discuss below, it is important to bear in mind that these results can be influenced by the perceptual similarity of the calls. Two calls that are often confused by coders will end up having highly similar behavioural correlates. However, they also point the way to a more objective method for determining a categorization scheme that reflects the natural communicative purposes used by rats.





**Figure 2.5. Dendrogram showing the relative similarity of different call types based on behavioural correlates.**

The x-axis shows the distance between pairs of vocalizations based on the correlation between their behavioural z-scores (i.e., the rows of Fig 3) The dendrogram was generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. As with Figs 3 and 4, data from the 4 least frequent vocalizations have been omitted.

## 2.5 Discussion

Our results show that specific sub-types of rat 50-kHz vocalizations are strongly associated with specific behaviours during play anticipation. Vocalizations frequently co-occurred with active behaviours, such as running, walking and jumping. Less active behaviours, such as exploratory sniffing and rearing were strongly predictive of a lack of any vocalizations. Our findings shed light on two prominent hypotheses thought to account for the emission of 50-kHz calls: the locomotion byproduct hypothesis (Blumberg, 1992) and the affect state hypothesis (Brudzynski, 2009, 2013; Wohr & Schwarting, 2007).

The motor byproduct hypothesis predicts a much more specific relationship between movements and vocalizations than we have observed. Certainly, locomotor behaviours were far more likely to be accompanied by a call than stationary behaviours. Further, a closer inspection showed that three vigorous movements, runs, jumps and shakes, were almost always (83-96%) associated with a call and these calls occurred at the onset of the movement. However, if the calls are a consequence of the movement, the similar mechanism of production should cause all calls associated with a particular movement to be highly similar. This was not the case. Runs, for example, were strongly associated with trills, trills with jumps, and composite calls. If calls are a physiological by-product the relationship between body movements and vocalization must be very complex. Our data strengthen the arguments made by Laplante et al. (2016), discussed in the Introduction, against the notion that vocalizations are physiological coupled to movement.

Consistent with the idea that 50-kHz calls communicate positive mood, rats emitted more of these calls when prior experience led them to expect a play partner. This is consistent with previous reports (Knutson et al., 1998; Laplante & Elias Costa, 2016). Rats also exhibited more runs and jumps under these conditions. The relationship between calls and movement in this case might arise not because the locomotion is causally producing the calls, but because both locomotion and calling are indicative of elevated positive affective. This explanation is consistent with the effects of amphetamine and cocaine, which elicit robust increases in 50-kHz vocalizations and activity levels (Brudzynski et al., 2011; Mu et al., 2009), with high frequencies of calls being emitted whether the rats are moving or not (Maier, Ma, Ahrens, Schallert, & Duvauchelle, 2010).

On the other hand, the affective communication hypothesis offers no explanation for the specific relationships between calls and actions shown in Fig 3. It might be the case that rats shift from trills to composite calls to communicate both increasing speed and positive mood. But ultimately, this is a *post hoc* explanation.

If neither the motion byproduct nor the affective communication hypotheses can fully account for our observed results, what are the alternatives? It seems plausible that the different call types have specific meaning. Indeed, several studies have shown a distinction between 50-kHz flat and frequency-modulated calls, based on both behavioural context and pharmacological treatment (Burgdorf et al., 2008; Ciucci et al., 2009; Wohr et al., 2008; Wright et al., 2010). Further, vocalizations may be temporally locked to on-going behaviour on a sub-second time scale. Specifically, during juvenile play, Himmler et al. (Himmler et al., 2014) showed that 50-kHz calls were more frequent in a 300 ms window before contact is made compared to a 300 ms window after contact is terminated. Our results show that there is far more detail in both the specificity of calls and to their temporal coupling to behaviour than has previously been suspected. The finding that rats emit specific calls tied to their ongoing behaviour raises the possibility that such calls may be used to coordinate moment-by-moment social interactions (i.e., to warn a playmate of an impending attack, communicate that the contact is friendly, beg for mercy, etc.). An important caveat is that our findings come from single animals awaiting a play partner, which undermines the idea that the observed vocalizations serve a social role. However, the number of vocalizations increased significantly over 7 days of repeated reunions with a play partner in the test chamber, suggesting that vocalizations may be intended for the soon-to-arrive play partner. To pursue the possible

communicative role of specific vocalizations, the positive and negative associations between specific call types and behaviours found in the present study in the absence of a partner need to be compared with the associations that are produced when a social partner is present.

How do our results compare with previous studies? Our results are consistent with the high association between movements and 50-kHz vocalizations reported by Laplagne and Costa (2016). They showed a striking increase in what they called “step” calls during high-speed locomotion. Closer inspection of their examples suggests that their “step” includes Wright et al.’s step, split and complex types. This is entirely consistent with our finding of a strong association between these same three calls and jumps. Takahashi et al. (2010) also found a strong relationship between movement and high-frequency calls. In their case, calls above 60 kHz, which included trills, upward ramp and part of the step call were strongly associated with locomotion. Our results agree, but also include several other 50-kHz calls associated with movement including trills-with-jumps, complex, composite and multi-step calls. Contrasting with previous reports in gerbils (Thiessen, Davenport, & Graham, 1978) and rats (Blumberg, 1992), which claim that vocalizations were frequently tied to the landing of the forepaws after a jump, we find that vocalizations are most often associated with the onset of a movement. Our results agree with the more recent observations of Laplagne and Costa (2016), who found a dramatic increase in call density peaking 140 ms before movement onset.

An important issue is whether the results obtained from our analysis method are stable across repeated observations. To this end, we replicated our analysis twice using different testing days and different coders. Based on a subset of data coded by both

coders, we found that agreement for behavioural categories was quite high (92%) while agreement on vocal categories was much lower (36%). The issue was that several of the most frequent call types (trills, complex, composite and trill-with-jumps) are highly similar, leading to different decisions by the two coders. By temporarily treating these four categories as identical, we found a much higher agreement between coders (98%). Despite the less-than-perfect agreement between coders, the overall pattern of results is strikingly similar between analyses (compare Fig 3A to 3B), helping to establish that our results are not due to sampling or statistical errors. The slight differences between analysis 1 and analysis 2 may be due to coding differences but also suggest that slightly more observation time may be necessary to obtain truly stable correspondence between vocalizations and behaviours. The limitation here is not minutes of observation, of which we had an easily manageable 24-28 minutes per analysis, but the time it takes to manually identify and categorize behaviours and calls (2946 behaviours and 4272 vocalizations for the combined analyses). Our best guess as to the true call-behaviour correspondence is provided by the averaged data from our two analyses (Fig 3C).

As mentioned in the Introduction, it is currently unclear how many call categories should be used in the analysis of rat USVs. Several researchers have broken 50-kHz calls into flat and FM calls (Burgdorf et al., 2008; Ciucci et al., 2009; Wohr et al., 2008; Wright et al., 2010). Takahashi et al. (Takahashi et al., 2010) found evidence for two categories within the 50-kHz range, a group of 60 kHz calls broadly associated with movement and another of 40 kHz calls associated with feeding. Brudzynski (2009) has suggested that four categories are probably sufficient to capture the important distinctions between 50-kHz calls (flats, trills, step-trills or “other”) while Wright et al. (Wright et al.,

2010) found evidence for 14 distinct calls within the 50-kHz range. Our study offers a possible answer to this question. A clustering algorithm was used to identify groups of calls with similar behavioural correlates among the call categories originally identified by Wright et al. (Wright et al., 2010). It is perhaps worth emphasizing that our categorization is based on the way the calls are used, not their acoustic similarity. As shown in Fig 5, this clustering algorithm identifies three categories of calls: Multi-part calls (including composite, split, and multi-step) were all associated with running and jumping; calls with trills were associated with slower locomotion; and inverted U and up ramp calls had similar, though less well-defined, behavioural correlates. Step-up calls may form a fourth distinct category associated with walking. The similarities in the usage of the calls we've identified are based on objective criteria, although admittedly it is not clear what level of similarity should be used as a criterion. An important caveat is that certain distinctions between calls may become relevant in other behavioural circumstances. For example, flat calls are likely to form a separate behavioural category, such as re-establishing social contact with cage mates (Brenes et al., 2016), but we did not even include them in our clustering because they were so infrequent in our paradigm. Another caveat is that the perceptual similarities among certain call types may have artificially increased our estimates of their behavioural similarities. For example, as revealed by our coder reliability data, calls with trills (trill, trill-with-jumps, complex and flat-trill) were easily confused, reducing our ability to discriminate any differences in their usage. Studies of categorical perception in human speech show that acoustic parameters of a sound do not necessarily predict their perceptual similarity (Liberman, Cooper, Shankweiler, & Studdert-Kennedy, 1967). Hence, we cannot assume that calls

that we perceive as similar based on a spectrogram are, in fact, similar to the rat. It remains possible that trills, trills-with-jumps, and complex calls have different meanings that will manifest once a more objective method of vocal categorization is implemented. In sum, our study provides a method to identify groupings of calls but further studies using different behavioural contexts and, perhaps, automated recognition of vocalizations, will be necessary to definitively identify the behaviourally relevant call categories.

## **2.6. Acknowledgments**

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## **Chapter 3**

### **Do Rats Use Specific Ultrasonic Calls to Coordinate their Social Play?**



### **3.1. Abstract**

Rough and tumble play, a critical behaviour for juvenile rats, is associated with a high occurrence of 50-kHz vocalizations. Do these ultrasonic calls facilitate play, serving as play signals? In the present study, unfamiliar juvenile male rats, paired with either an intact or devocalized partner, were introduced in a neutral enclosure and their social interactions were observed. Behaviourally, the devocalized animals initiated more playful encounters and, when receiving a nape attack, fully rotated significantly more than their intact counterparts. A Monte Carlo shuffling technique was used for the analysis of the correlations between the playful actions performed and the types and frequencies of various 50-kHz calls that were emitted. The analysis revealed that during nape attacks, flat type calls are emitted by the intact pairs, indicating that this call may change the type of response the rats perform in response to nape attacks. Moreover, coordinating calls reciprocally with complementary calls emitted by participants as they engage in complementary actions (e.g., attacking the nape, being attacked on the nape), appeared to be a way in which calls could potentially be used as play signals to influence the quality of the interaction.

### **3.2. Introduction**

Rough-and-tumble play, or play fighting, in rats involves attack and defense of the nape of the neck, which is nuzzled by the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). Although such play can be distinguished from serious fighting, which involves biting attacks directed at the face and lower flanks and dorsum (Blanchard et al., 1977; Pellis & Pellis, 1987), in some situations play fighting can be quite rough and

escalate to serious fighting (Smith et al., 1999; Takahashi, 1986; Takahashi & Lore, 1983). A widely held view is that many species use play signals to inform the recipient that the upcoming contact is playful (Bateson, 1956; Bekoff, 1975). Several play signals such as the canine play bow (Bekoff, 1995), head shaking in spider monkeys (Eisenberg & Kuehn, 1966) and the open mouth play face of primates (van Hoof, 1967) and some carnivores (Poole, 1982) have been shown to promote engagement in play (Palagi et al., 2016 a or b). Hops (Pellis & Pellis, 1983) and open mouth facial gesturing (Panksepp & Burgdorf, 2003) have been identified as possible play signals in the play fighting of rats, but given that play fighting in this species can occur in darkness or low light levels (Himmler et al., 2013), it is unlikely that any visual signal is critical in signaling play. However, rats emit many ultrasonic vocalizations during play (Burgdorf et al., 2008) and it is possible that some of these may be used as play signals (Himmler et al., 2014). Indeed, vocalizations that facilitate play have been identified in some primates (Kipper & Todt, 2002) and keas parrots, which upon hearing the vocalizations of conspecifics engaged in more social and object play (Schwing et al., 2017).

Two categories of ultrasonic vocalizations (USV) exist in juvenile and adult rats. Calls in the 22 kHz frequency are emitted in situations that might lead to an animal's demise (Knutson et al., 2002), such as in the presence of predator odor (Blanchard et al., 1991), foot shock (Tonoue et al., 1986), fear conditioning (Antoniadis & McDonald, 1999; Kikusui et al., 2003; Wöhr & Schwarting, 2008), a dominant conspecific (Sales, 1972; Panksepp et al., 2004; Thomas et al., 1983), acoustic startle (Kaltwasser, 1990), handling by an unfamiliar handler (Blanchard et al., 1986; Brudzynski & Ociepa 1992) and social defeat (Panksepp et al., 2004; Thomas et al., 1983). Calls that occur at a

frequency of 33 kHz and higher are typically regarded as 50 kHz calls (Wöhr et al., 2015), and these are associated with positive affective states, induced in circumstances such as anticipating an addictive drug (amphetamine or methamphetamine) (Knutson et al., 1999), sexual encounters (Bialy et al., 2000), stimulation of brain reward pathways (Burgdorf et al., 2000), social play (Burgdorf et al., 2008) and “tickling” by human handlers (Panksepp & Burgdorf, 2000). The 50-kHz category can be further divided into flat type calls and frequency modulated (FM) calls (Burgdorf et al., 2008). Moreover, the FM calls can be further divided into several different kinds (Wright et al., 2010). Given that many of these varieties of USV calls are emitted during play (Himmler et al., 2014), there are many possible ways that different calls could be used to facilitate play.

The role of USV usage in social paradigms is clearly affected by age. Introducing adult male rats to one another in a neutral arena leads to a rougher version of play fighting which occasionally may escalate to aggression (Smith et al., 1999). If one of the partners is devocalized the risk of escalation from play to aggression is greatly increased (Kisko et al., 2015b). However, neither deafening (Siviy & Panksepp, 1987) nor devocalizing (Kisko et al., 2015a,b) juvenile rats prevents play, nor increases the risk of escalation to aggression, suggesting that play can proceed without such signaling. Although play can occur in the absence of USVs, pairs of devocalized juvenile rats play less (Kisko et al., 2015a). Exposing a devocalized juvenile to an intact partner increases its frequency of initiating playful attacks to that of vocally competent animals (Kisko et al., 2015b). The latter finding suggests that the calling by an intact partner can motivate the devocalized partner to become more playful. Therefore, in juveniles vocalizations appear to invigorate play where as in adults they appear to mitigate aggression.

Not only may USVs act as general motivators of play, but they may also be important to negotiate particular actions with the partner. Particular types of 50-kHz calls are associated with particular actions when moving about an enclosure in which the rat was trained to anticipate the arrival of a play partner (Burke et al., 2017a). During actual play fighting, calls are most likely to be emitted immediately prior to contact (Himmler et al., 2014; Kisko et al., 2015a), and some types of calls appear to be associated with particular defensive actions during play fights (Himmler et al., 2014). Such calling may thus help to coordinate the actions of the partners.

A key distinguishing feature of play fighting is that to sustain such encounters as playful, animals have to follow rules that ensure some degree of reciprocity (Pellis & Pellis, 2017). In juvenile rats, the rules followed lead to a 30% likelihood that a play fight will involve a role reversal, whereby the original attacker becomes the defender (Himmler et al., 2016). When both partners are devocalized the rate of role reversals is halved (Kisko et al., 2015a). Therefore, while USVs may not be necessary for play to occur, they may facilitate its occurrence and help partners negotiate cooperative actions. As already noted, the risk that the play of unfamiliar adult male rats encountering one another in a neutral arena escalates to serious fighting is greatly increased if one of the partners is devocalized (Kisko et al., 2015b), and that risk appears to be associated with a failure to reciprocate particular USVs (Burke et al., 2017b).

Because both different calls and different behavioural actions are produced at markedly different frequencies, it is difficult using standard statistical approaches to detect significant associations (Himmler et al., 2014). Therefore, we developed a scoring scheme that samples both actions and calls every second and then uses a Monte Carlo

shuffling technique to analyse the associations. This method has robustly revealed strong associations between particular calls and particular actions in both solitary and social situations (Burke et al., 2017a, b). To test the hypothesis that juvenile rats use specific USVs to coordinate their play, we applied our new method to analyze the correlations between calls and behaviour in pairs of juvenile rats in which both could vocalize and pairs in which one partner was devocalized. The use of a devocalized animal allows for the assignment of vocalizations to a particular individual when engaged in specific behaviours.

### **3.3. Methods**

#### ***3.3.1. Subjects and experimental procedures***

Video and audio files of juvenile male Long Evans rats playing in a neutral arena were obtained from our library of data that had been collected in a previous study (Kisko et al., 2015b). The testing and data collecting procedures followed have been detailed elsewhere (Himmler et al., 2013, 2014; Kisko et al., 2015a,b; Burke et al. 2017a). In summary, 24 juvenile rats, age 30 – 40, housed in quads after weaning at 24 days were used. Between 28-30 days two members each from three quads were devocalized by bilateral transections of the recurrent laryngeal nerves following (Snoeren & Amgo, 2013) and maintained in the same quads until testing occurred. Then pairs of rats unfamiliar with one another were tested in pairs in a 50 cm x 50 cm x 50 cm Plexiglas box, lined with approximately 1-2 cm of bedding for trials lasting 10 min. Each animal was habituated to the testing enclosure for 30 min per day for three consecutive days, then, after testing, housed in isolation for 24 h.

For testing, unfamiliar pair mates were placed in the test enclosure and videotaped in the dark using cameras with night shot capability for 12 min (for further procedural details see B. Himmler, Pellis, & Pellis, 2013). One rat was introduced first and allowed to roam freely for two minutes while awaiting the arrival of his partner. Ultrasonic vocalizations were collected using a high frequency microphone (Model 4939, Brüel & Kjaer, Denmark), with sensitivity to frequencies ranging from 4-Hz to 100-kHz. The microphone was located in the roof of the chamber, approximately 65cm above the play surface. The microphone was connected to a Soundconnect<sup>TM</sup> amplifier (Listen, Inc, Boston, MA) and recordings were processed via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, CA) using a self-developed MATLAB acquisition program. All files were then converted to .wav files and were analyzed using the RavenPro system (Cornell, MA). To synchronize video and audio recordings, a device emitting a simultaneous light and sound cue was used prior to play (Himmler et al., 2014; Kisko et al., 2015a).

### ***3.3.2. Behavioural analyses***

Interactions during the first four minutes after introduction of the second rat were analyzed. The playful encounters were coded using videotaped sequences and were evaluated at normal speed, slow motion, and frame-by-frame to manually code these behaviours (Kisko et al., 2015b; Smith et al., 1999). To capture the range of possible actions in the encounters, behaviour patterns associated with exploration, social investigation, and physical contact between partners were scored (Table 1).

**Table 3.1.** Description of the social behaviours that were scored

<b>Behaviour</b>	<b>Description</b>
Nape	By slowly approaching or by pouncing, one rat moves toward the nape of its partner's neck with its snout
Chase	Following an interaction, one of the animals chases its fleeing partner
Pin Active	One animal stands over its supine partner, which by squirming, pushing with the forepaws and kicking with its hind feet actively attempts to free itself or attack its partner. Conversely, the partner standing on top moves to block the supine animal's maneuvers.
Pin Passive	One animal stands over its supine partner, but the supine animal remains relatively immobile.
Sniff	One animal sniffs the face and flanks of its partner.
Sniff (Genital)	One animal sniffs the anogenital area of its partner.
Evade	The recipient of a nape attack protects against contact on its nape by dodging, running or jumping away.
Approach	One animal moves toward its partner, but without any clear indication that the nape is being targeted.
Follow	One animal moves in tandem or directly follows its partner. Unlike chasing, such following need not be preceded by an interaction.
Mutual Upright	Both rats face one another while rearing up on their hind feet, usually holding one another with their forepaws. From this position, they can sniff one another or actively push one another.

As described elsewhere (Kisko et al., 2015b; Smith et al., 1999), when the rats were introduced into the test arena they investigated one another, and then began to play.

### ***3.3.3. Vocalization Analyses***

Acoustic data was analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. 50-kHz vocalizations were included if they followed Wright et al., 2010 criteria (see Figure 2.1.). The occurrence of these calls was used to

compare rates of calling, types of calling and whether different types of calls were associated with particular types of actions.

### ***3.3.4. Statistical analyses***

To evaluate the associations between all the behaviours and call types a Monte-Carlo Shuffling method was used (Burke et al., 2017a). This method was used because the total number of behavioural and vocal events differed substantially between different categories, making a simple correlation between behaviours and vocalizations difficult to interpret. A statistical analysis that accounted for these differences in event baselines was necessary to determine whether a particular vocalization co-occurred with a particular behaviour above chance levels. Therefore, a Monte-Carlo technique was used to calculate the probability distribution of each unique combination of vocalizations and behaviours. We first counted the number of co-occurrences of each vocalization type with each of the coded behavioural categories. Next, for each animal pair, vocalizations were repeatedly shuffled and the number of behaviour-vocalization co-occurrences computed. A vocalization was counted as occurring during a particular behaviour if the mid-point of the call occurred between the start and stop time of the behaviour. To allow for errors in the coding of the behaviour times, we expanded the time window for each behaviour by 200 ms in each direction. Shuffling was achieved by assigning each vocalization a random time within the duration of the 4-minute observation period. Hence, the relative frequency of vocalizations was kept the same for each shuffle. This shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behaviour was tabulated. Based on the distribution of these counts, we assigned a



z-score to the actual number of occurrences. The higher the z-score, positive or negative, the less likely that specific combination of vocalization and behaviour, or its absence, could have occurred by chance (i.e. for  $p \leq 0.05$  the z score is 1.96 (or -1.96) and for  $p \leq 0.01$  the z score is 2.58 (or -2.58). Shuffling was performed separately for each animal pairing and the z-scores averaged across play pairings to generate the final, average z-score values.

### **3.4. Results**

#### ***3.4.1. Vocalization Analysis***

The mean frequency of overall calling by the two animals in the vocal–vocal pairs ( $M = 417.8$ ,  $SD = 253.3$ ) and the one animal able to vocalize in the vocal–devocalized pairs ( $M = 378.0$ ,  $SD = 200.6$ ) did not differ significantly ( $t(10) = -0.30$ ,  $p = 0.42$ ) (using a 2-tailed comparison). With regard to types of calls emitted, the four most common calls—trills, trills with jumps, flat/trill combinations and flat calls—represented 88% and 78% of calling in vocal-vocal and the vocal–devocalized pairs, respectively.

#### ***3.4.2. Behavioural Analysis***

As shown in Kisko et al. (2015b), the devocalized rats differed from the intact controls in several ways with regard to their play fighting behaviour: 1) They initiated more playful attacks, 2) they were more likely to defend themselves against playful attacks, and 3) they were more likely to use the supine defense tactic and less likely to use evasive tactics. Using the total amount of time engaged in play (i.e., maintaining contact) as a measure, we confirmed that pairs with a devocalized rat spent a greater proportion of the sample period playing in active pins although the difference was not significant

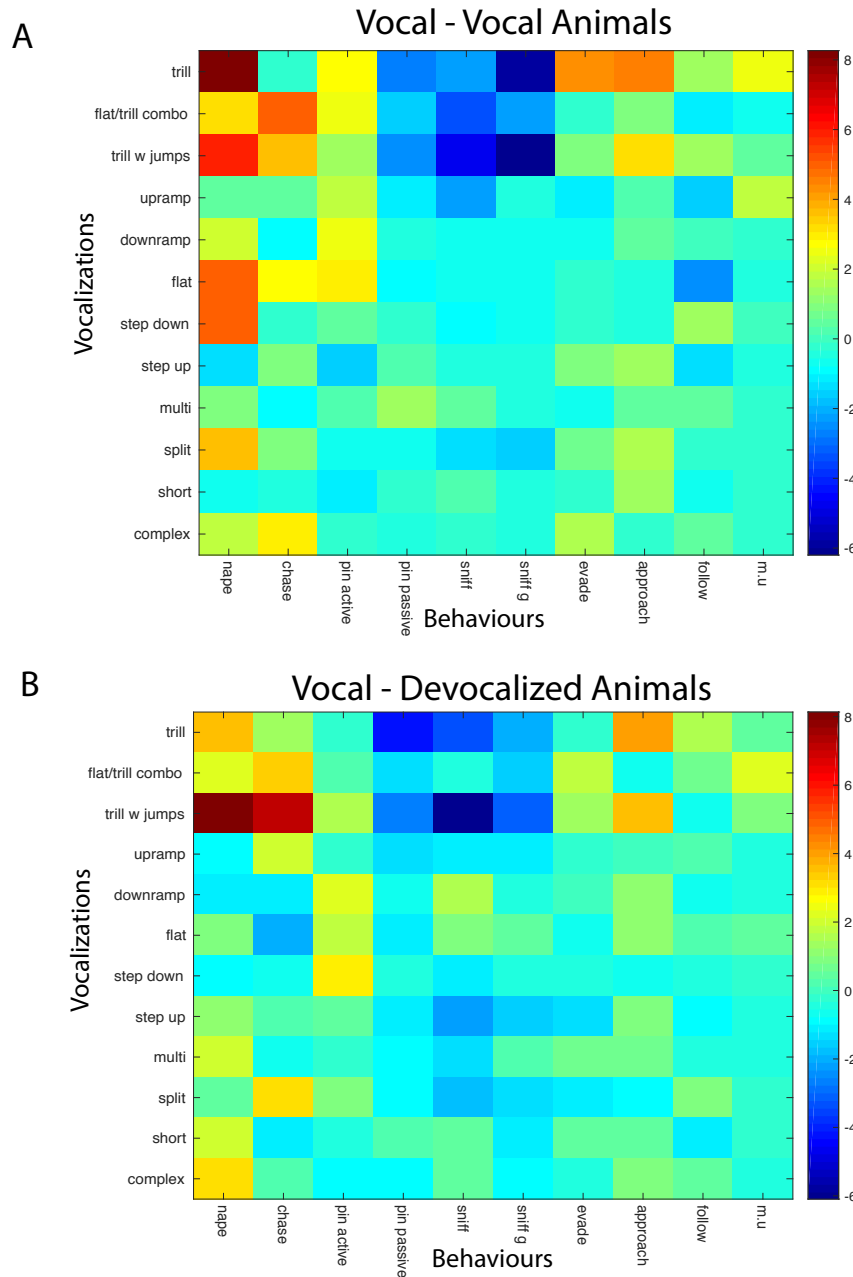
(vocal-vocal:  $M = 23.48$ ,  $SD = 32.71$ ; vocal-devocal:  $M = 46.49$ ,  $SD = 19.88$ ;  $t(10) = 1.47$ ,  $p = 0.074$ ).

### ***3.4.3. Vocal-Behavioural Analysis***

The Monte Carlo shuffling procedure revealed that some vocalizations are clearly more likely to be associated with certain actions than are others, while other associations are highly unlikely (Fig. 3.1A). The pattern of association was altered when one partner was devocalized (Fig. 3.1B). To simplify the comparison between the groups, significant positive associations, at the extreme end of the distribution ( $z \geq \pm 2.58$ ), are shown side-by-side in Table 3.2. The most striking difference is the higher usage of flat type calls (50-kHz flats, flat/trill combos, and step-down) during the nape and chase behaviours in the intact pairs, compared to pairs with a devocalized partner. The other major points of interest are: 1) the trill call is associated with evasions in the intact pairs and not in the pairs with a devocalized partner, 2) in both groups, social sniffing directed both to the anogenital area and the body more generally is negatively associated with trill type calls, 3) in both groups both approach behaviours and nape contact were positively associated with trill type calls, 4) in both groups passive pins were negatively associated with trill type calls, and finally, 5) active pins had a different profile of associated vocalizations in the intact pairs than in the pairs with a devocalized partner. Whereas intact pairs used trills, trills with jumps, flats, and down ramps during active pins, vocal-devocal pairs primarily used step-downs and down ramps.

**Table 3.2.** Comparison of the significant positive associations ( $p \leq 0.01$ ;  $z \geq 2.58$ ) present in the two groups of dyads. The numbers in parentheses shows the z-scores for the association.

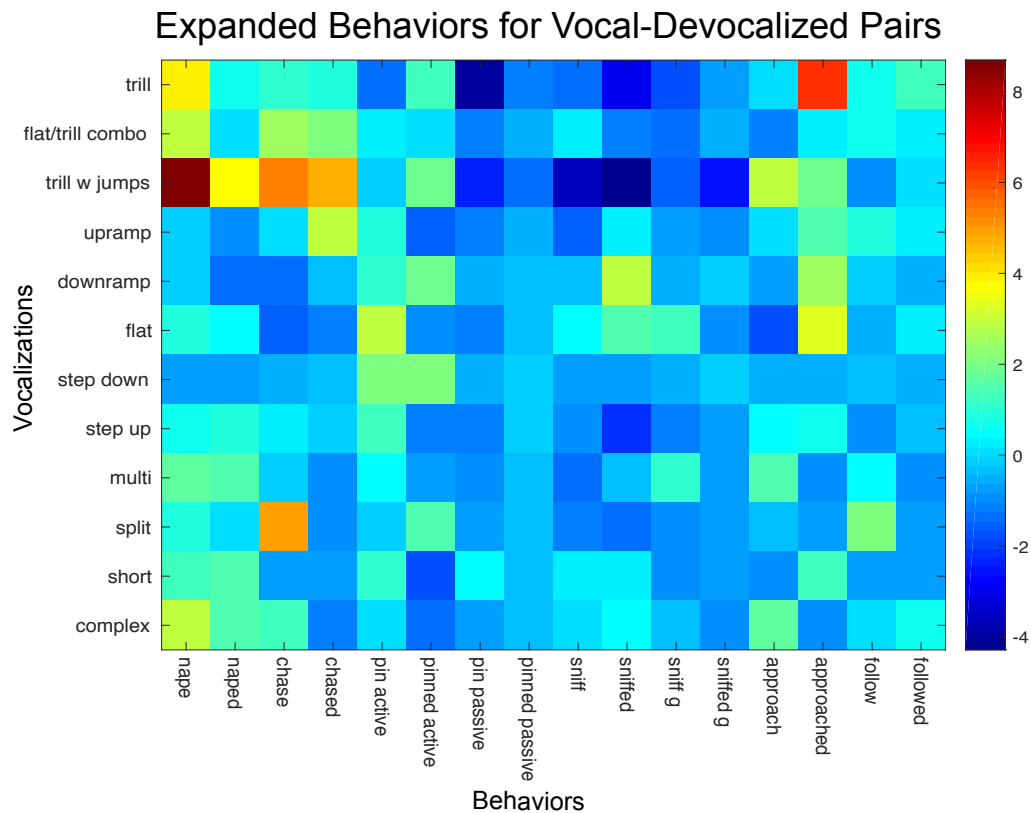
<b>Vocal-vocal pairs</b>	<b>Vocal-devocalized pairs</b>
Nape-trill (8.3)	Nape-trill w jumps (8.1)
Nape- trill w jumps (5.7)	Chase-trill w jumps (7.2)
Nape- step down (5.2)	Approach-trill (4.1)
Nape-flat (5.0)	Nape-trill (3.6)
Chase-flat/trill combination (4.9)	Approach-trill w jumps (3.5)
Approach-trill (4.6)	Chase-flat/trill combination (3.3)
Evade-trill (4.4)	Chase-split (3.2)
Chase-trill w jumps (3.8)	Nape-complex (3.1)
Nape-split (3.7)	Pin Active-step down (3.0)



**Figure 3.1.** The matrix shows call types on one axis and the types of behaviour on the other, with z-scores showing the strength of the associations between particular calls and particular behaviours, spanning from deep red for the strongest and deep blue for the weakest. The pattern for the vocal pairs is shown in A and the pattern for the pairs with a devocalized partner is shown in B.

#### ***3.4.4. Detailed Vocal-Behavioural Associations***

In the vocal-devoval pairs, it was possible to attribute calls to either the actor or recipient in any given interaction, allowing a more detailed analysis of vocal-behavioural correlations. A number of asymmetries were observed in the usage of vocalizations (Figure 3.2). Instigating a nape attack is strongly associated with trill with jumps ( $z = 8.7$ ), trills ( $z = 4.0$ ) and flat/trill combination calls ( $z = 3.0$ ), while being the recipient of a nape attack is associated solely with trill with jumps ( $z = 3.4$ ). Chasing a partner is associated with trill with jumps ( $z = 5.3$ ) and split calls ( $z = 4.8$ ), whereas being chased is only associated with trill with jumps calls ( $z = 4.8$ ). Being approached is associated with trills ( $z = 6.3$ ) and flat calls ( $z = 3.3$ ) while approaching is associated with trill with jumps calls ( $z = 2.9$ ). Finally, during active pins, being pinned is not significantly associated with any vocalizations, whereas pinning is associated with flat calls ( $z = 2.8$ ).



**Figure 3.2:** The matrix shows call types on one axis and the types of behaviour on the other, with z-scores showing the strength of the associations between particular calls and particular behaviours, spanning from deep red for the strongest and deep blue for the weakest. This matrix is the behavioural - call profile from the one vocal animal paired with a devocalized animal. The behaviours of the vocal animal alone were recorded allowing for the discrimination between reciprocal behaviours while the animals played. For example we can now explore both the behaviour of the vocal animal as attacker and recipient of nape attacks.

### 3.5. Discussion

#### 3.5.1. Calling Frequency

The frequency of play when both partners are devocalized is greatly reduced (Kisko et al., 2015a), but when devocalized rats are exposed to a vocal partner, the frequency of play initiation is increased (Kisko et al., 2015b). The present analysis showed that the frequency and types of calls emitted in the intact pairs and the pairs with a devocalized partner did not differ. In this way, the vocal partner appears to have

increased its emission of calls, compensating for the lack of calling by its devocalized partner. The vocal pairs do play more, and this would be expected to increase the number of vocalizations, but the increase in play is small compared to the nearly two-fold increase in vocalizations from the vocal partner. Thus, it seems likely that the increase in calls is a compensatory mechanism employed by the vocal partner to elicit more play from the devocalized partner. Such a possibility is consistent with the motivating effects shown for the playful vocalizations of keas parrots (Schwing et al., 2017) and some non-vocal play signals (Pellis et al., 2011; Scopa & Palagi, 2016).

### ***3.5.2. Trill Calls***

The similarities between intact dyads and dyads with a devocalized animal in the usage of trills is striking. Nape contact, chasing, pins evasion and approaching a partner are all associated with a type of trill call in both groups of animals. This is consistent with reports of high rates of frequency modulated USVs during social play (Burgdorf, 2008). Trills are also common during adult social interaction, despite the fact that these often involve high levels of aggression (Burke et al., 2017b). Further, juvenile animals emit trill calls while running, jumping and walking when anticipating a partner (Burke et al., 2017a). The tight linkage between vigorous behaviours and trills is not a by-product of breathing (Burke et al., 2017a) and, therefore, likely serves other purposes. Given the high frequency of emission of these calls and their consistent usage in both groups, these calls may be affective signals, emitted to increase or maintain the playful mood of the partners. Consistent with this supposition is the finding that the number of 50-kHz vocalizations emitted appears to relate to the level of sexual motivation (Borner et al.,

2016; Bialy et al., 2000). Playback experiments (Seffer et al., 2014) mimicking the kea study (Schwing et al., 2017) could be used to test this hypothesis.

### ***3.5.3. Flat Calls***

Some of the differences in call-behaviour associations between the vocal-vocal and vocal-devocal groups suggest that calls may be used tactically to negotiate particular interactions during play, as has been demonstrated for play signals in other species (Bekoff, 1995). The two calls that differed most markedly in usage between groups were the flat and step down calls, which occurred during nape attacks. These two calls are present in the vocal pairs when initiating nape attacks, but largely absent in the pairs with a devocalized partner. Other studies have shown that 50-kHz flat calls are emitted when a rat is separated from their cage mate, suggesting that these calls could have a role in social coordination (Wöhr et al., 2008). The flat calls could also be useful in coordinating playful interactions. Indeed, for adult males, vocal pairs emitted flat calls at times in the encounter which could have escalated to serious fighting but ultimately did not. Pairs with a devocalized partner, on the other hand, did not emit as many flat calls and, in several instances, did fight (Burke et al., 2017b).

The overall duration of play was higher for vocal-devocal pairs, but there were also qualitative differences in their play (Kisko et al., 2015b). The previous analysis confirmed that not only do devocalized rats initiate more playful attacks, but they also engage in defensive actions that are more likely to lead to wrestling and pinning. This means that the flat calls could potentially be responsible for this difference and these calls could therefore be a cue to establish dominance or submission, which, in turn, might lead



to cessation of the interaction. This idea is consistent with data showing high usage of flats in the mutual upright behaviours among adult males, which may be used to establish dominance (Burke et al, 2017b). Closer analysis of the vocalizations used by the intact partner in these situations, compared to the condition where both partners can vocalize is needed to identify if changes in how the partners coordinate the use of particular calls, such as the step down, accounts for these altered patterns of play.

#### ***3.5.4. Reciprocity***

Ultrasonic vocalizations could also function as play signals via reciprocal exchange. As stated above different calls are associated with complementary behaviours such as approaching and being approached. This opens the possibility that the temporal coordination of either similar or complimentary ultrasonic calls among rats engaged in play may facilitate the coordination of movements that ensure the continuation of play. Indeed, adult data suggests that pairs with a devocalized conspecific quickly escalate into aggression where intact pairs do not (Burke et al., 2017b; Kisko et al., 2015b).

### **3.6. Conclusions**

In conclusion, this paper has demonstrated a tight linkage between specific behaviours and vocalizations during juvenile play. This study identifies USVs as a candidate for facilitating play in juvenile rats. Potentially, both increasing the frequency of emission of trill type calls and delivering them at moments when bodily contact is imminent may function to elevate the playful mood of both caller and the recipient. Flat calls may have a role in coordinating the partners' actions during the most vigorous

phases of the encounter. Finally, some of these functions, especially those involving coordinating the partners' actions, may be achieved by calling in a reciprocal manner. The latter usage is lost when one partner is devocalized and thus more detailed study will require the development of techniques in which the vocalizations of both vocal partners can be monitored simultaneously. Overall, though, even with the data currently available, it would seem that rats use ultrasonic vocalizations as signals that facilitate and coordinate play interactions.

### **3.7. Acknowledgements**

We thank Vivien Pellis for her comments on the paper and the animal care staff and veterinarian for their help with the care of the rats. The work was supported by grants from the Natural Science and Engineering Research Council of Canada to DRE and SMP and by a grant from Alberta Innovates Health Solutions to DRE.

## **Chapter 4**

### **Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls\***

\*Burke CJ, Kisko TM, Pellis SM, Euston DR (2017) Avoiding escalation from play to aggression in adult male rats: The role of ultrasonic calls. Under Revisions with Behavioural Processes

#### **4.1. Abstract**

Play fighting is most commonly associated with juvenile animals, but in some species, including rats, it can continue into adulthood. Post-pubertal engagement in play fighting is often rougher and has an increased chance of escalation to aggression, making the use of play signals to regulate the encounter more critical. During play, both juvenile and adult rats emit many 50-kHz calls and some of these may function as play facilitating signals. In the present study, unfamiliar adult male rats were introduced in a neutral enclosure and their social interactions were recorded. While all pairs escalated their playful encounters to become rougher, only the pairs in which one member was devocalized escalated to serious biting. A Monte Carlo shuffling technique was used for the analysis of the correlations between the overt playful and aggressive actions performed and the types and frequencies of various 50-kHz calls that were emitted. The analysis revealed that lower frequency (20-30 kHz) calls with a flat component maybe particularly critical for de-escalating encounters and so allowing play to continue. Moreover, coordinating calls reciprocally, with either the same call mimicked in close, temporal association or with complementary calls emitted by participants as they engage in complementary actions (e.g., attacking the nape, being attacked on the nape), appeared to be ways with which calls could be potentially used to avoid escalation to aggression and so sustain playful interactions.

#### **4.2. Introduction**

Play fighting involves partners competing for some advantage, such as biting a particular body area (Aldis, 1975; Symons, 1978). While superficially similar, play

fighting can be distinguished from serious fighting by several criteria (Smith, 1997): (1) a resource, such as a piece of food, is not gained or protected; (2) the contact is restrained, or, at least, there are no combat-induced injuries; (3) there are frequent role reversals between a pair, with partners alternating as to which is the attacker and which is the defender; (4) even if chasing ensues following contact, further affiliation is likely; and (5) the presence of play signals. However, play fighting can escalate into serious fighting because either one partner is excessively forceful in its attempts to gain the advantage or, because the recipient fails to recognize that the action performed by the partner is a playful one (Aldis, 1975; Fagen, 1981; Pellis & Pellis, 1998). In these situations in which play fighting can be ambiguous, play signals, such as the primate play face (van Hoof, 1967) and the canid play bow (Bekoff, 1974), can be critical in alleviating the misunderstanding and so maintain the encounters as playful (Palagi et al., 2016b). For example, dogs are more likely to perform play bows before initiating playful bites (Bekoff, 1995), with mutual signaling by the partners leading to more prolonged play bouts (Cordoni et al., 2016).

Although play fighting is most commonly associated with juveniles (Fagen, 1981), in many species it can continue past puberty into adulthood (Pellis, 2002). The play fighting among post-pubertal animals is often used as a means of social assessment and manipulation (Brueggeman, 1978; Palagi, 2011; Pellis & Iwaniuk, 1999, 2000), such as testing their relationships with either peers in established social groups or with strangers to gain or maintain dominance (e.g., Antonacci et al., 2010; Erhlich, 1977; Erhlich, 1975; Jones, 1983; Mills, 1990; Newell, 1971). The rougher play typical of post-pubertal play fighting (Fry, 2005; Pellis, 2002) can make such utilitarian use of play both more difficult

to distinguish from serious fighting and increase the risk of escalation to serious fighting, raising the importance of play signals in navigating the encounter.

In rats, play fighting is most common in the juvenile period (Meaney & Stewart, 1981; Thor & Holloway, 1984), but continues into adulthood (Pellis & Pellis, 1990, 1991b), when adult males use it as a means of both reinforcing and testing dominance relationships (Pellis & Pellis, 1991a; Pellis et al., 1993). For practical purposes, the play fighting of rats can be readily distinguished from serious fighting at all ages. Play involves attack and defense of the nape of the neck which is nuzzled with the snout if contacted (Pellis & Pellis, 1987; Siviy & Panksepp, 1987), whereas serious fighting involves bites directed at the flanks and lower dorsum (Blanchard et al., 1977; Pellis & Pellis, 1987). In addition, serious fighting is associated with a variety of agonistic displays, such as tail rattling, threatening an opponent by such actions as adopting a lateral orientation with an arched body and piloerection, and standing on hind feet from which position the rats vigorously push one another (Barnett, 1975; Grant, 1963). When pairs of adult male rats, either familiar or unfamiliar, encounter one another in a neutral arena, the play can be very rough, with vigorous pushing and defense, and these encounters can involve the performance of agonistic displays, but rarely do they escalate to outright aggressive biting (Pellis & Pellis, 1992; Pellis et al., 1993; Smith et al., 1999; Yamada-Haga, 2002; Kisko et al., 2015b). This suggests that the rats are able to escalate the playful encounter to the brink of aggression, but then signal to one another to enable them to de-escalate, and so maintain it as playful or at least non-injurious.

Except for the possible use of hops (Pellis & Pellis, 1983) and an open mouth facial gesture (Panksepp & Burgdorf, 2003), rats do not have any obvious overtly performed

actions that can unambiguously serve as play signals, but they do emit many ultrasonic vocalizations (Burgdorf et al., 2008; Knutson et al., 1998), and some of these may potentially function as play promoting signals (Himmler et al., 2014). These calls are of two types, those around 50-kHz, that are associated with positive affective states such as sex and the administration of psychoactive drugs, and those at 22-kHz, that are associated with negative affective states, such as aggression and drug withdrawal (Brudzynski, 2013; Wöhr & Schwarting, 2013). There remains some ambiguity about whether 22 kHz calls are purely positive and 22 kHz calls exclusively negative. For example, 22 kHz vocalizations are emitted by male rats following ejaculation, suggesting an association with a positive affective state (Bialy et al., 2016); however, once the refractory period is over and the male approaches and mounts the female, the male switches back to emitting 50-kHz calls (Burgdorf et al., 2008). The latter findings suggest a switch from an initial, post-ejaculatory state, when the continued presence of the female may be negative, to one in which her presence is again positively rewarding. Conversely, the usage of 50 kHz calls is not always reflective of a positive state. For example, flat 50-kHz calls can occur in ambiguous situations reflecting at least a partially negative affective state (Wöhr et al., 2007). Thus, there may well be gradations in the usage of 22 kHz and 50 kHz emissions as encounters shift from being positively reinforcing to aversive and back again. This may be particularly evident in cases, such as the one studied in the present paper, in which playful encounters escalate to aggression.

Both 50-kHz and 22-kHz calls have been implicated in mitigating aggression. In the resident-intruder test, an unfamiliar adult male is placed in the home cage of another adult male, and the resident attacks the intruder, directing bites at the lower flanks and

dorsum (Blanchard & Blanchard, 1994). In these encounters, the intruders emit 50-kHz calls (Takahashi et al., 1983; Thomas et al., 1983), and the likelihood of a biting attack is reduced following the emission of 22-kHz calls by one or both animals (Lehman & Adams, 1977; Lore et al., 1976; Sales, 1972; Sewell, 1967). While the 22-kHz calls are found in a narrow frequency range and have a simple, long, flat profile, the 50-kHz emissions are more variable (Wright et al., 2010). This variability of 50-kHz calls provides the possibility that at least some of these calls may be used for more specific purposes than simply communicating affect (Pellis et al., 2017; Wöhr et al., 2015).

While some 50 kHz calls have a flat frequency profile, the most commonly emitted 50-kHz calls during play are those that are frequency modulated (FM) (Burgdorf et al., 2008), and of the FM 50-kHz calls, trills are the most common (Himmler et al., 2014; Wright et al., 2010; Burke et al., 2017). While such trilling may reflect the positive affective state induced by play (Panksepp & Burgdorf, 2008), some types of FM 50-kHz calls, though less frequently emitted than trills, are statistically associated with particular actions performed during play, such as when evading contact and when nosing a partner's nape (Himmler et al., 2014; Pellis et al., 2017), suggesting that the animals may be signaling in specific ways to influence the behaviour of their partner. Even so, among juveniles, the elimination of the ability to hear (Siviy & Panksepp, 1987) or produce (Kisko et al., 2015a, b) ultrasonic calls has only a modest effect on the frequency and form of play (Kisko et al., 2017). However, in the context of the rougher play present in adult rats, the ability to emit ultrasonic calls appears more critical (Kisko et al., 2015b).

Recently, we showed that the risk of aggression among unfamiliar male rats is greatly elevated when one of the pair has been devocalized (Kisko et al., 2015b). When



the rats were introduced into the test arena they investigated one another, and then began to play. The play, which could become increasingly rough, in some cases continued until the rats began to exhibit visual threat signals, and then finally deliver a bite. After a bite, the encounter would gradually become gentler until the animals played again (Kisko et al., 2015b, 2017; Pellis et al., 2017). The pairs with a devocalized member initiated just as much play (i.e., nuzzling attacks to the nape), but also more frequently displayed the various signs of aggression (e.g., tail rattling, piloerection). Most strikingly, while pairs from both groups vigorously pushed one another in the mutual upright position, none of the intact pairs escalated to actual biting, whereas all pairs with a devocalized member did so (Kisko et al., 2015b). These findings suggest that fully vocal pairs may be able to use ultrasonic vocalizations in a manner that can diffuse intense moments of the interaction, such as when they vigorously pushing one another in the mutual upright configuration, from escalating to all-out fighting (Kisko et al., 2017). In the present study, we categorized calls and examined their relationship to specific behaviours on a fine (sub-seconds) time-scale to determine how calls are used to mitigate the risk of playful encounters escalating to serious fights. Of particular interest was to identify whether particular calls were associated with moments in the encounters that risked escalation, including the period preceding each bite. Given that for the present sample of animals none of the vocal pairs escalated to biting and all the pairs with a devocalized member did (Kisko et al., 2015b), the moment of escalation to biting in the latter and the de-escalation from that moment in the former provided a particularly crucial context to identify the relevant calls involved.

### **4.3. Methods**

#### ***4.3.1. Subjects and experimental procedures***

Video and audio files of adult male Long Evans rats encountering one another in dyads in a neutral arena were obtained from our library of data that had been collected in a previous study (Kisko et al., 2015b). The testing and data collecting procedures followed have been detailed elsewhere (Himmler et al., 2013, 2014; Kisko et al., 2015a,b). In brief, 24 85-90 day old rats were used, which had been housed in quads since being weaned at 24 days. Between 28-30 days, two members each from three quads were devocalized by bilateral transections of the recurrent laryngeal nerves following Snoeren & Amgo (2013) and maintained in the same quads until testing. Then, rats unfamiliar with one another were tested in pairs in a 50 cm x 50 cm x 50 cm Plexiglas box, lined with approximately 1-2 cm of bedding for trials lasting 10 min. Each animal was habituated to the testing enclosure for 30 min per day for three consecutive days, then, preceding testing, rats were housed in isolation for 24 h.

For testing, the designated pairmates were placed in the test enclosure and videotaped in the dark using cameras with night shot capability for 12 min (for further procedural details see Himmler et al., 2013). Ultrasonic vocalizations were collected using a high frequency microphone (Model 4939, Brüel & Kjaer, Denmark), with sensitivity to frequencies ranging from 4-Hz to 100-kHz. The microphone was located in the ceiling of the chamber and was approximately 15 cm above the middle of the play enclosure. The microphone was connected to a Soundconnect<sup>TM</sup> amplifier (Listen, Inc, Boston, MA) and recordings were processed via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, CA) using a self-developed MATLAB acquisition

program. All files were then converted to .wav files and were analyzed using the RavenPro system (Cornell, MA). To synchronize the video and audio recordings, a device emitting a simultaneous light and sound cue was used (Himmler et al., 2014; Kisko et al., 2015a).

#### ***4.3.2. Behavioural analyses***

Encounters were a mixture of playful and serious fighting and several features of the animals' social behaviour were used to assess both of these components. Videoed sequences were evaluated at normal speed, slow motion, and frame-by-frame to code these behaviours manually (Kisko et al., 2015b; Smith et al., 1999). Attacks directed to the nape characterized playful attack and bites directed at the lower flanks and dorsum characterized serious attack (Blanchard et al., 1977; Pellis & Pellis, 1987). Some of the tactics used to attack and defend these targets are similar in both forms of fighting, except that, in using them, the animals are competing for different targets (Pellis & Pellis, 2015). In addition, as the rats switch from play to aggression, they emit visual signals associated with agonistic threat (Smith et al., 1999), such as tail rattling and lateral posturing with arched back and piloerection (Grant, 1963). Therefore, to capture the range of possible actions in the encounters, behaviour patterns associated with exploration, social investigation, and physical contact between partners were scored (Table 1).

**Table 4.1.** Description of the social behaviours that were scored

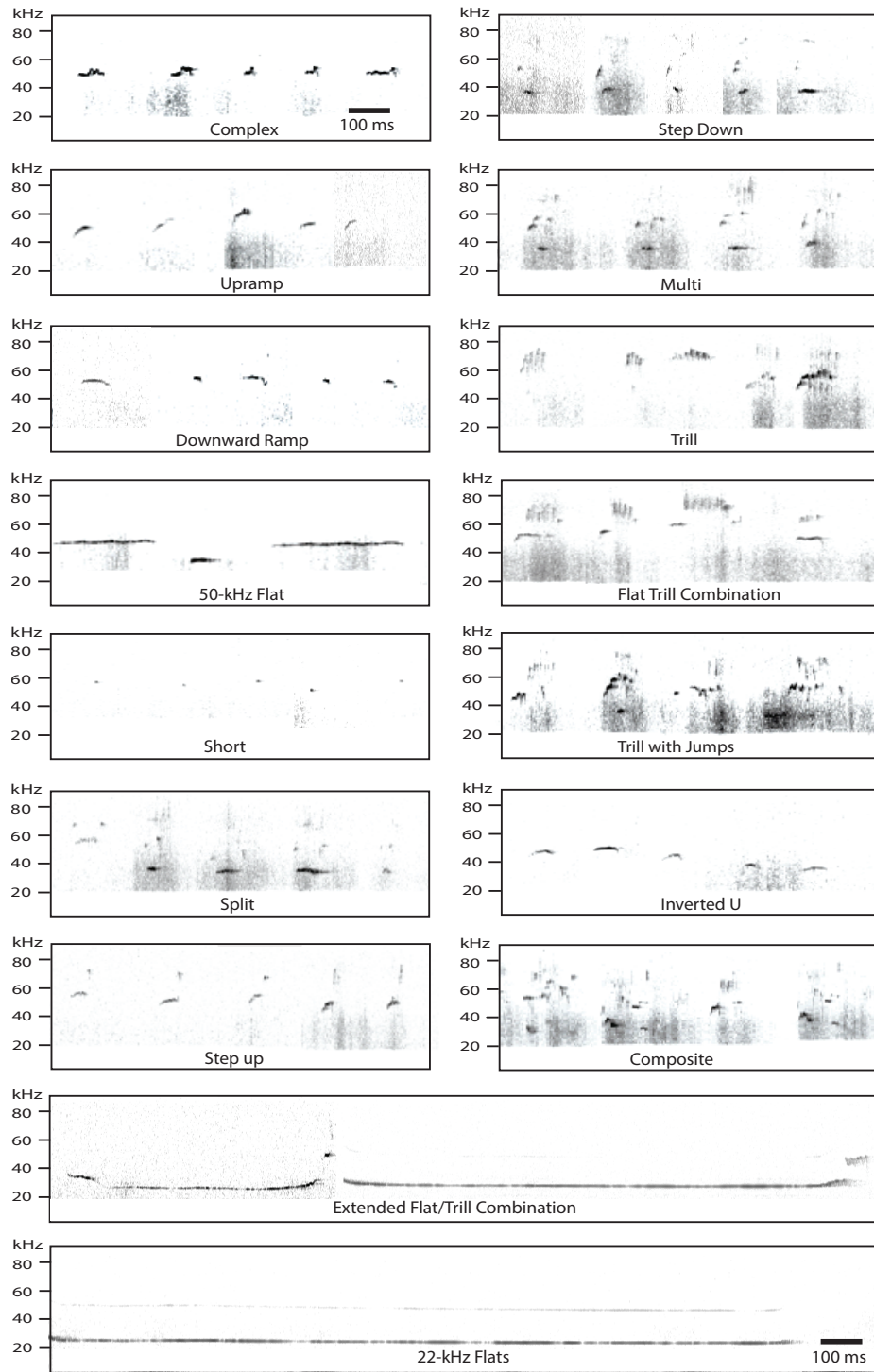
<b>Behaviour</b>	<b>Description</b>
Nape	By slowly approaching or by pouncing, one rat moves toward the nape of its partner's neck with its snout
Chase	Following an interaction, one of the animals chases its fleeing partner
Pin Active	One animal stands over its supine partner, which by squirming, pushing with the forepaws and kicking with its hind feet actively attempts to free itself or attack its partner. Conversely, the partner standing on top moves to block the supine animal's maneuvers.
Pin Passive	One animal stands over its supine partner, but the supine animal remains relatively immobile.
Sniff	One animal sniffs the face and flanks of its partner.
Sniff (Genital)	One animal sniffs the anogenital area of its partner.
Evade	The recipient of a nape attack protects against contact on its nape by dodging, running or jumping away.
Approach	One animal moves toward its partner, but without any clear indication that the nape is being targeted.
Follow	One animal moves in tandem or directly follows its partner. Unlike chasing, such following need not be preceded by an interaction.
Mutual Upright	Both rats face one another while rearing up on their hind feet, usually holding one another with their forepaws. From this position, they can sniff one another or actively push one another.
Bite	One animal delivers a bite on its partner, typically on the lower flanks and rump. Even if the bite cannot be directly seen, the rapid and exaggerated jump away by the recipient can be used to identify bites.

### ***4.3.3. Vocalization Analyses***

Acoustic data were analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. The different types of 50-kHz vocalizations were scored

according to the criteria established by Wright et al. (2010) (see Figure 1). The occurrence of these calls was used to compare rates of calling, types of calling and whether different types of calls were associated with particular types of actions. The first four minutes from each encounter was used to evaluate overall group differences in vocalizing.

During the analysis of the frequency of call types, a potentially novel sub-category of the flat/trill combination was noted. This new version appeared to have an extended flat component. To test the possibility that this is a new call, the duration and the average frequency of occurrence (kHz) of calls conforming to the Wright et al. (2010) categorization of the flat/trill combination, were compared to calls that we deemed had the extended flat phase. Four pairs of rats (two from the intact group and two from the devocalized group) were excluded for low levels of these calls, (i.e., less than ten instances of each call in the ten minutes analyzed). Therefore, eight pairs were used to analyze the difference in average duration and frequency (in kHz) of each call type. The first ten calls of each type were taken from each pair and the mean difference was analyzed with a matched sample t-test. The same analysis was then re-run on only the flat component of each of the target calls.



**Figure 4.1.** Examples of categorized 50-kHz calls and 22-kHz (as originally described by Wright and colleagues (Wright et al., 2010)) taken from our data.

#### ***4.3.4. Detailed Vocal-Behavioural Analysis***

To evaluate which partner is calling in association with particular behaviours, the vocal-devocalized pairs were re-analyzed. The behavioural coding scheme shown in Table 1 was modified so that each of the behaviours involving an interaction between the partners was divided into actor and recipient. For example, nape contact was scored as ‘nape’, when the rat emitting the calls was the one contacting its partner’s nape, and ‘naped’, when the rat emitting the calls was the one whose nape was being contacted.

#### ***4.3.5. Statistical analyses***

To evaluate the associations between all the behaviours and call types, a Monte Carlo Shuffling method was used (Burke et al., 2017). This method was used because the total number of behavioural and vocal events differed substantially between different categories, making a simple correlation between behaviours and vocalizations difficult to interpret. For example, there were a very large number of trills which occurred during walking simply because trills were the most common vocalizations and walking was one of the most common behaviours. A statistical analysis that accounted for these differences in event baselines was necessary to determine whether a particular vocalization co-occurred with a particular behaviour above chance levels. Therefore, a Monte Carlo technique was used to calculate the probability distribution of each unique combination of vocalizations and behaviours. First, we counted the number of co-occurrences of each vocalization type with each of the coded behavioural categories. Next, for each animal pair, vocalizations were repeatedly shuffled and the number of behaviour-vocalization co-occurrences computed. A vocalization was counted as occurring during a particular

behaviour if the mid-point of the call occurred between the start and stop time of the behaviour. To allow for errors in the coding of the behaviour times, we expanded the time window for each behaviour by 200 ms in each direction. Shuffling was achieved by assigning each vocalization a random time within the duration of the two minute observation period. Hence, the relative frequency of vocalizations was kept the same for each shuffle. This shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behaviour was tabulated. Based on the distribution of these random-shuffle counts, we assigned a z-score to the actual number of occurrences. The higher the z-score, the less likely that that specific combination of vocalization and behaviour could have occurred by chance (i.e. a z-value of 1.96 gives a  $p$ -value of  $\leq 0.05$  and a z-value of 2.58 gives a  $p$ -value of  $\leq 0.01$ ). Shuffling was performed separately for each animal pairing and the z-scores averaged across play pairings to generate the final, average z-score values.

To compare differences across the two groups (i.e., vocal-vocal pairs versus vocal-devocalized pairs), independent t-tests were used, and for comparisons of the same individuals within groups, matched-pairs t-tests were used. Differences were judged to be significant if  $p \leq 0.05$ . For example, this approach was used to evaluate whether there was an overall significant difference between the two groups in the number of vocalizations and whether particular calls were used differently at specific moments in the encounters.



## 4.4. Results

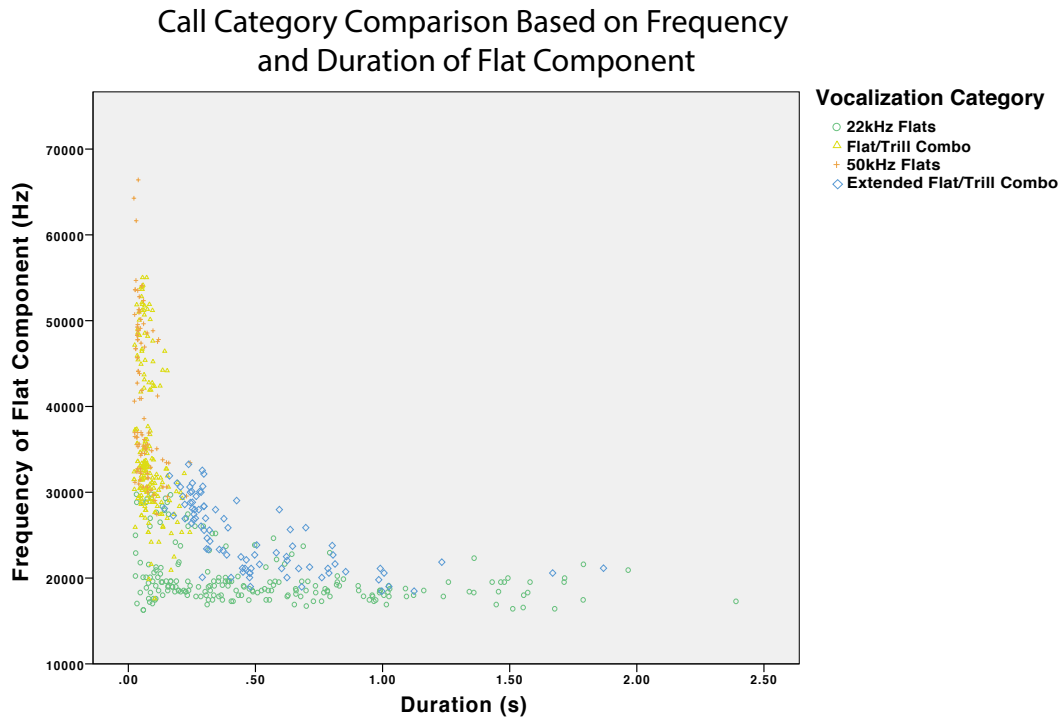
### 4.4.1. Vocalization Analysis

The mean frequency of overall calling by vocal–vocal pairs ( $M = 523.2$ ,  $SD = 178.3$ ) and vocal–devocalized pairs ( $M = 428.5$ ,  $SD = 205.0$ ) did not differ significantly ( $t(10) = -0.85$ ,  $p = 0.41$ ) (using a 2-tailed comparison). With regard to types of calls emitted, the four most common calls—trills, trills with jumps, flat/trill combinations and flat calls—represented 86% and 73% of calling in vocal-vocal and the vocal–devocalized pairs, respectively.

Both duration (s) and mean frequency (kHz), the extended flat/trill combination call differed significantly from the originally categorized flat/trill combination (Table 4.2). Characteristically, the flat component of the extended flat/trill combination has a frequency that is close to that of 22-kHz calls. To further test whether the extended flat/trill combination call may be a separate type of call, the frequency and duration of all calls with a flat component (22-kHz flats, 50-kHz flats, flat/trill combination) was plotted (Figure 4.2). Three clusters are evident. One cluster incorporates the 50-kHz flat calls and the originally described flat/trill combination and another involves the 22-kHz calls. The third cluster, incorporating the extended flat/trill combination, sits in a transitional position relative to the other two. The current findings cannot differentiate whether the extended flat/trill combination is truly a unique call or represents a graduated change from the 50-kHz flats to the 22-kHz flats, but whether a distinct category or a graduate set of calls, the variations may reflect different functional uses during interactions. As such, they were coded separately in our behavioural-vocalization analysis.

**Table 4.2.** The means and standard deviations for duration and call frequency are compared for three versions of the flat/trill combination call

Flat/trill combination call	Standard call	New version	t-test (2-tailed)
Average Duration (ms)	74.10 $\pm$ 16.34	321.00 $\pm$ 130.57	t(7) = 5.114, p < .001
Average Frequency (kHz)	49.90 $\pm$ 12.63	43.03 $\pm$ 0.97	t(7) = -4.03, p = .005
Flat Frequency	34.99 $\pm$ 5.70	28.05 $\pm$ 3.24	t(7) = -3.37, p = .012



**Figure 4.2.** A scatter-plot shows the distribution of calls involving or containing flat components, contrasting their frequency (kHz) against their duration (s). The 50-kHz flat calls and the flat/trill combination calls, originally described by Wright et al. (2010), are clustered together to the left of the graph, having a narrow time dimension, but highly variable in frequency. The 22-kHz flat calls are clustered near the floor of the graph, having a narrow frequency range, but are highly variable in duration. The newly described extended flat/trill combination call is intermediate, having a wider frequency range than the 22-kHz calls and wider range for duration than the other 50-kHz calls.

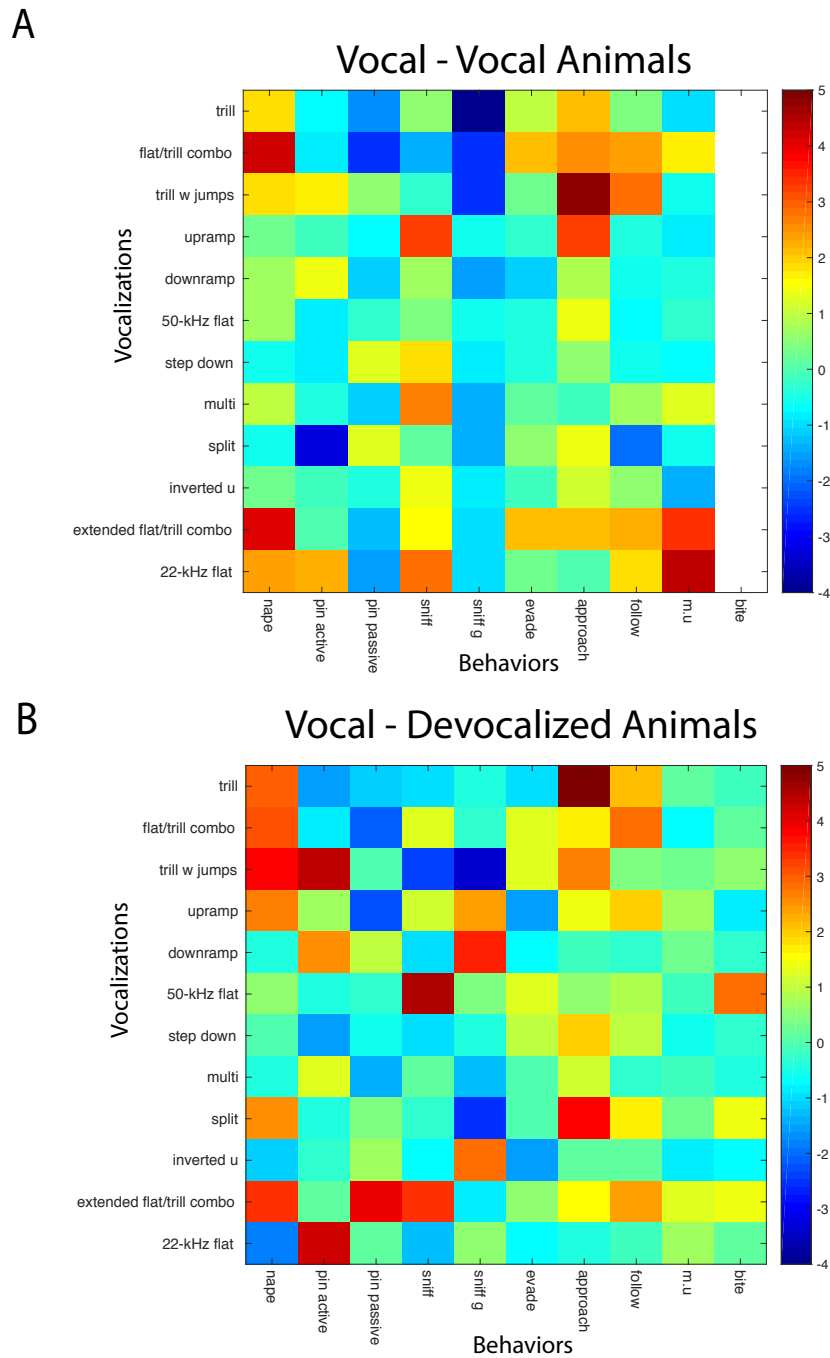
#### ***4.4.2. Vocal-Behavioural Analysis***

The Monte Carlo shuffling procedure revealed that some vocalizations are clearly more likely to be associated with certain actions than are others, while some associations are highly unlikely (Fig. 4.3A). The pattern of association was altered when one partner was devocalized (Fig. 4.3B). A major difference between groups was the usage of low flat type calls. 22-kHz flat calls and the extended flat/trill combinations had 10 positive behavioural associations in the vocal–vocal pairs ( $z \geq 1.65$ ) and only five in vocal-devocalized pairs (Figure 4.3). To simplify the comparison between the groups, significant positive associations, at the extreme end of the distribution ( $z \geq 2.58$ ), are shown side-by-side in Table 4.3. The table shows that the two groups differ in several ways: 1) the lack of flat type calls during mutual uprights in the vocal-devocalized pairs, 2) bites, only present in the vocal-devocalized pairs, were strongly associated with the 50-kHz flats, 3) the intact pairs used a greater variety of categories of calls during sniffs, compared to the vocal-devocalized group which only used the extended flat/trill call and the 50-kHz flats, and, 4) The vocal-devocalized group uses lower frequency calls during pins in comparison to the intact pairs.

**Table 4.3.** Comparison of the significant positive associations ( $p \leq 0.01$ ;  $z \geq 2.58$ ) present in the two groups of dyads

<b>Vocal-vocal pairs</b>	<b>Vocal-devocalized pairs</b>
Approach-trill with jumps (4.8)*	Approach-trill (6.5)
Mutual upright-22kHz flat (4.4)	Sniff-50kHz flat (4.6)
Nape- flat/trill combination (4.2)	Pin active-trill with jumps (4.4)
Nape-extended flat/trill combination (4.1)	Pin active-22kHz flat (4.2)
Mutual Upright - extended flat/trill combination (3.4)	Pin passive-extended flat/trill combination (4.0)
Approach-up ramp (3.3)	Approach-split (3.9)
Sniff-up ramp (3.2)	Nape-trill w jumps (3.8)
Sniff-22kHz flat (2.9)	Sniff g-down ramp (3.6)
Follow-trill w jumps (2.8)	Nape-extended flat/trill combination (3.4)

\*The numbers in parentheses shows the z-scores for the association



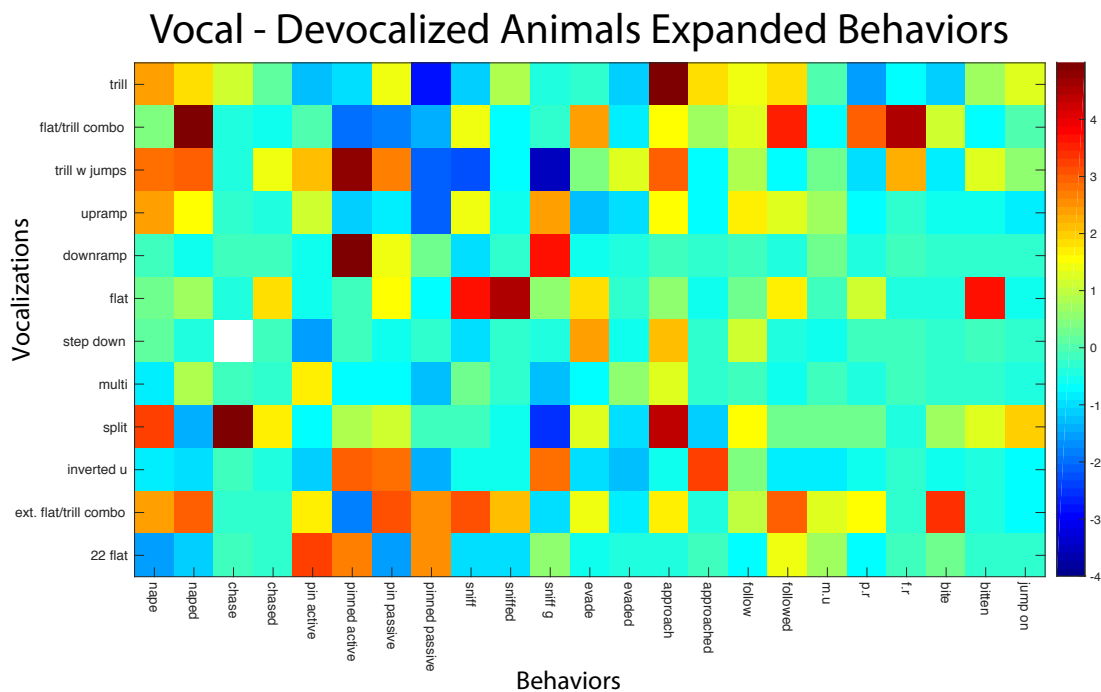
**Figure 4.3.** The matrix shows call types on one axis and the types of behaviour on the other, with z-scores showing the strength of the associations between particular calls and particular behaviours, spanning from deep red for the strongest and deep blue for the weakest. The pattern for the vocal–vocal pairs is shown in A and the pattern for the vocal–devocalized pairs is shown in B. Note that vocalizations and behaviours with very low rates of occurrence were omitted, as it was not possible to run statistical analysis for these ultra low-frequency events.

#### ***4.4.3. Mutual Uprights***

As both vocal-vocal and vocal-devocalized pairs engaged in mutual upright contests, and this moment in the encounter involved the most vigorous pushing and so likely the phase in the encounter most likely to escalate to aggression, these were further analyzed to assess the role of vocalizations in mitigating escalation from play to aggression. All cases of the mutual upright position in both groups were identified and re-analyzed frame-by-frame to characterize the differences and so the possible cues that are critical for escalation to biting. A total of 51 mutual uprights were analyzed (20 for the vocal-vocal pairs, 31 for the vocal-devocalized pairs). In 49 of these cases, one animal successfully pushed the other on its back or side. In all cases, the winner of these encounters (i.e., the one pushing its partner over) was also the one that was invariably in the on-top position, standing over its pinned partner in the subsequent play fights. In the context of the immediate outcome of being pushed over, in 76% of cases in the vocal-devocalized pairs the winner bit the loser. None of the cases of such pushing over led to biting in the vocal-vocal pairs. With regard to vocalizations, during the mutual upright position, the vocal-vocal pairs emitted a large number of 50kHz calls with flat components (see above), suggesting that these calls may be critical in mitigating the escalation to aggression in such situations.

A number of major differences were revealed for actors and recipients in the vocal-devocalized pairs (Figure 4). Delivering a bite, is strongly associated with the extended flat/trill combination ( $z = 3.4$ ), whereas being bitten is strongly associated with 50-kHz flats calls ( $z = 3.6$ ). Attacking the nape playfully is strongly associated with split calls ( $z = 3.3$ ), whereas receiving such a nape attack is strongly associated with flat/trill

combinations ( $z = 6.0$ ). Being pinned is strongly associated with down ramps ( $z = 7.9$ ), whereas the strongest association with pinning is with the 22-kHz flat ( $z = 3.3$ ). Finally, approaching a partner is associated with trills ( $z = 6.3$ ), whereas being approached is associated with inverted u calls ( $z = 3.3$ ). The complementary patterns of calling between partners suggest that both animals may need to reciprocate specific calls to facilitate social interactions.



**Figure 4.4.** The matrix shows call types on one axis and the types of behaviour on the other, with z-scores showing the strength of the associations between particular calls and particular behaviours, spanning from deep red for the strongest and deep blue for the weakest. This matrix is the behavioural - call profile from the one vocal animal paired with a devocalized animal. The behaviours of the vocal animal alone were recorded allowing for the discrimination between reciprocal behaviours while the animals played. For example we can now explore both the behaviour of the vocal animal as attacker and recipient of nape attacks.

That reciprocity of mutual calling may be important in coordinating actions is supported by the surprising absence of the use of the extended flat/trill combination call during the mutual upright position for the vocal-devocalized pairs (Figure 4.3B and 4.4). In contrast, this call and all other flat type calls were strongly associated with the mutual upright position in the vocal-vocal pairs (Figure 4.3A and Table 4.3). Reciprocal emission of this call during mutual upright contests may be critical in the vocal-vocal pairs to de-escalate encounters and so avoid biting.

#### **4.5. Discussion**

The most striking behavioural effect of devocalizing one partner in a pair of unfamiliar adult rats was that these pairs escalated to aggression and biting while intact pairs did not. Our findings suggest two mechanisms by which the coordinated use of vocalizations may help mitigate the risk of aggression. The first is the use of low-frequency flat calls (22 kHz and extended flat/trill), especially during mutual upright behaviours. While these calls were strongly associated with mutual upright interactions in vocal-vocal pairs, they were seldom used in the vocal-devocal pairs. The second mechanism to avoid aggression may be the mutual exchange of vocalizations. The vocalizations associated with several dyadic interactions are asymmetric (see Figure 4). For example, a rat attacking the nape of its partner often makes a split call while the rat receiving the nape attack is more likely to emit a flat/trill combination call. Similar asymmetric vocalizations were observed for pins and approaches. These observations are consistent with the view that the reciprocal exchange of vocalizations is important for normal social interactions.



Based on our findings, the mutual upright interaction appears to play an important role in adult social interactions. Rats adopt an upright posture to defend or attack (Grant, 1963; Blanchard, 1977). From this position, they can box with their forepaws and push one another (e.g., Miczek, 1979; Militzer, 1995; Suzuki & Lucas, 2009). The present analysis found that the ‘winner’ of mutual upright contests (i.e., the one that succeeded in pushing the other over), was also the one that then consistently pinned its partner in subsequent interactions, an indication that the winner became the more dominant animal, at least temporarily (e.g., Panksepp et al., 1985; Pellis & Pellis, 1992). The majority of the mutual upright positions in the vocal-devocalized pairs led to the winner biting the loser. One interpretation of these findings is that bites occur when the non-dominant rat subsequently approached the dominant rat, apparently violating some etiquette of rat behaviour.

Flat calls appear to play an important role in adult interactions that could potentially become aggressive. Our data help refine our understanding of previous findings about the role of these calls. Adult aggressive encounters between adult rats are known to be associated with increased rates of 22 kHz calling (Thomas, 1983; Panksepp, 2004; Burgdorf, 2008). Panksepp et al. (2004) have shown that 22 calls are emitted only when one rat exhibits submissive behaviour and occur almost exclusively after a bite has occurred. One interpretation of these data is that 22 kHz calls signal the expectation of an unpleasant encounter (Brudyznski, 2009). Our data agree with the alternate interpretation that the low-frequency flat calls are employed as a mechanism to mitigate aggression (Lehman & Adams, 1977; Lore et al., 1976; Sales, 1972; Sewell, 1967). We found that 22 kHz calls were associated with mutual upright interactions than resolved amicably,

suggesting they play a role in negotiating a non-aggressive end to such encounters. We also observed that both flat 22kHz calls and extended flat/trill combinations (in the 20 kHz range) were associated with a wider range of behaviours in the vocal-vocal pairs than in the vocal-devocalized pairs (Figure 3 and Table 3). This raises the possibility that the failure to integrate calls with low-frequency flat components, or overreliance on one version, the extended flat/trill combination, may have put the vocal-devocal pairs at greater risk of escalating their encounters. Finally, in our vocal-devocal pairs, the use of extended flat/trill calls by the biting rat, usually the dominant, suggest that these calls serve as a warning signal to the non-dominant rat to keep away. This contrasts with early reports that 22 kHz calls are emitted as a submission signal (Lore et al., 1976; Sales, 1972; Sewell, 1967).

Our data are less clear about the use of 50 kHz flat calls. One previous study showed that these calls are increased during aggressive encounters (Burgdorf, 2008). Another found an increase in such calls when rats were separated from their cage mates, suggesting a role as a contact call (Wohr, 2008). In our experiment, 50 kHz flat calls were emitted primarily by a rat around the time it was being bitten.

Another potential mechanism by which vocalizations could act to offset escalation from play to aggression is via reciprocal exchange. We have already mentioned the finding that calls associated with several dyadic interactions appear to be asymmetric (e.g., different calls for initiating and receiving a nape attack) and hence, potentially complementary. It is also possible that rats facilitate play via the reciprocal exchange of the same call. For example, during a mutual upright, both rats may emit low-frequency flat calls to facilitate play. The plausibility of this scenario is enhanced by

the finding that devocalizing one animal causes a dramatic reduction in all vocalizations during mutual upright interactions. Consistent with this idea, many species use rapid mimicry of play signals to increase the duration of play fights (e.g., Davila et al., 2008; Mancini et al., 2013; Palagi et al., 2015; Scopa & Palagi, 2016). That is, the temporal coordination of either similar or complimentary ultrasonic calls among rats engaged in play may facilitate the coordination of movements that ensure the continuation of play. This explanation is consistent with findings from the play fighting in juvenile devocalized rats. Among juvenile rats, about 30% of play fights involve role reversals (Himmler et al., 2016), and this reciprocity is essential to maintain play as playful (Dugatkin & Bekoff, 2003; Palagi et al., 2016a; Pellis et al., 2010). In the absence of either partner being able to vocalize, such reciprocation is halved (Kisko et al., 2015a). That is, in the absence of calls, the coordination necessary to sustain reciprocal playful exchanges may be compromised, and in adults, the absence of mutual signaling may impair the ability to sustain the coordination needed to prevent playful encounters from escalating to aggression.

Further supporting the importance of reciprocal exchange, we also observed differences in vocal usage when one partner was devocalized. Strikingly, the frequency and distribution of call types in pairs when only one partner could vocalize were no different from pairs where both could vocalize. This suggests that the vocal partner must have compensated for the absence of vocalizations from its devocalized partner. We also observed specific changes in vocal usage, including an increase use of low-frequency flat calls during pins when one partner was devocalized. These disruptions in normal vocal

usage further support the view that reciprocal vocalizations are integral to normal social interactions.

Our study highlights a new type of call, the extended flat trill, which has a long flat component very similar to the 22 kHz call, albeit with a slightly higher frequency, and a trill component in the 50 kHz range. This call has also recently been discovered by another group who call it a “22 kHz trill” (Hernandez, 2017). In their experiments, it was emitted by sexually naïve, but not experienced, male rats when exposed to an estrous female. In our experiment, the extended flat trill is used in many dyadic interactions, especially by the active partner (i.e., the rat doing the sniffing, pinning, napping, or biting). Our parametric analysis (Figure 2) suggests that these calls lie between Wright et al.’s (2010) flat/trill combination and the 22 kHz flat calls. We have insufficient data at this point to say whether these calls lie along a continuum or, in fact, are simply extreme examples of the two existing call categories. Interestingly, our data suggest that Wright et al.’s original flat/trill combination calls themselves may consist of two separate categories, a 50 kHz group and another with a mean frequency around 32 kHz. Future functional studies will be needed to clarify exactly how calls should be categorized so as to capture their actual usage.

Here, we have used devocalization as a method to discover which vocalizations can be attributed to each individual in a dyadic exchange. The gross similarity between call profiles between vocal-vocal and vocal-devocal pairs (Figure 3A versus 3B) shows that this method has promise, but the differences show the method is imperfect. Obviously, if reciprocal exchange of vocalizations plays a functional role, devocalizing one partner will affect the way calls are used by its partner. Having rats that can both

vocalize is needed, but recorded with methods that can track their individual vocalizations. Such data could be collected with the use of multiple microphone arrays (Neunuebel et al., 2015), but the ability to discriminate between callers may fail once the rats are engaged in close-quarter wrestling. Another strategy would be to attach small backpacks to the rats that could carry miniaturized equipment capable of recording vocalizations (Anisimov et al., 2014), but again, these may interfere with rats rolling on their backs when they wrestle (Pellis & Pellis, 1987). These and other techniques need to be explored so as to track the vocal interchanges between partners to compliment the video record of their movements (Kisko et al., 2017), and so be able to fully unravel the code for the vocalizations uttered during play.

#### **4.6. Acknowledgements**

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## **Chapter 5**

### **Discussion**

## 5.1. General Discussion

### 5.1.1. Summary of Thesis Objectives

1. Are vocalizations tied to specific behaviours? We have shown that specific types of rat 50-kHz vocalizations are strongly associated with specific behaviours during anticipation of play. Vocalizations frequently co-occurred with active behaviours, such as running, walking and jumping. Less active behaviours, such as exploratory sniffing and rearing were strongly predictive of a lack of any vocalizations. There was also a high correlation among the usage of trill calls and active behaviours. Our results show that there is far more detail in both the specificity of calls and in their temporal coupling to behaviour than previously believed.
2. How are calls used during juvenile play? Even though the play of the devocalized juveniles was increased to normal levels when playing with an intact partner, some aspects of how they play remained atypical (Kisko et al., 2015b). The present analysis confirmed that not only did these devocalized rats initiate more playful attacks, but they also engage in defensive actions that are more likely to lead to wrestling and pinning. The lack 50-kHz flat calls by the devocalized partner may potentially be responsible for the behavioural difference between groups.
3. What calls are used to de-escalate adult aggressive encounters? Both the frequency and distribution of calls in dyads with a devocalized animal were no different to intact dyads, thus suggesting that the vocal partner must have

compensated in some way for this absence. Further, both the levels of aggression and correlations between vocalizations and behaviour differed between groups. The pairs with a devocalized animal consistently escalated to aggressive bites while the intact pairs did not (Kisko et al., 2015b). Our results show that low frequency flat type calls, especially during mutual upright behaviours, may be critical to de-escalating these interactions.

### ***5.1.2. Comparisons Across Experiments***

This thesis has the unique opportunity to compare the same animals across all three experimental settings. This allows us to make direct comparisons between the animals in different social contexts, affective states and at different ages. First, similarities across conditions can reveal consistent patterns of general use of particular calls, such as the usage of trill and trill with jump calls during runs and jumps. Second, changes across conditions can reveal how some calls may have specific uses, such as the role of flat calls by juveniles to facilitate normal play and by adults to avoid aggression. Thirdly, comparisons between pairs in which both partners can vocalize versus those in which only one can do so, reveals that some aspects of communication using USVs involves reciprocation of calling by the partners, such as with the flat/trill combination calls and mutual uprights and nape attacks. Overall, the ability to associate different calls and behaviours across conditions gives us important information confirming the communicatory roles of USVs and begins to reveal the “language” the animals are using.

#### ***5.1.2.1. Trill Calls and Active Behaviours***

First and foremost, using anticipation of play, juvenile play, and adult interactions, this thesis has shown a link between specific categories of 50-kHz USVs and specific



behaviours. The association between trill type calls and active behaviours remained consistent across all three experiments. When anticipating play, juvenile animals used trill type calls more often when performing active behaviours in contrast to sedentary ones. This association remained consistent in juvenile play; the most active actions, such as pinning, nape attacks, and chasing, all had high associations with trill type calls. In contrast, trilling was negatively associated with sniffing and passive pins, more sedentary play behaviours. Among adults also, trill calls were associated with active behaviours such as nape attacks, chasing, and active pins, although in adulthood, these active behaviours were also significantly associated with flat type calls.

The tight linkage that appears between the trill calls and active behaviours, as discussed in Chapter 2, is not related to a breathing by-product and, therefore, must have another purpose. Trill type calls have been hypothesized as a potential form of “laughter” for rats (Panksepp and Burgdorf 2000). However, our results do not agree with this hypothesis for two reasons. First, the high usage of trill calls while alone in anticipation of play indicates that these calls are not analogous to laughter, as there would be little purpose for the animals to perform them alone. Second, the high production of trills in adult dyads with a devocalized animal, all of which escalated to biting, indicates trills are not unequivocally positive as human laughter. Therefore, trill calls likely serve an alternate function in social interactions in rats.

It has also been hypothesized that trills could function as mood enhancing signals used to elevate and modulate the level of play. Indeed, our juvenile and adult data supports this. The groups with a devocalized animal had roughly the same number of calls as the intact groups in both juveniles and adults. This means that the vocal animal is

compensating for the number of vocalizations by producing almost twice the amount, than when paired with another vocal counterpart. We did not specifically examine whether the compensation is specific to 50 kHz calls, but since these are by far the most frequent call types, this is likely to be the case. In juveniles the devocalized animals were more likely to initiate the interactions, indicating that the other animal may be promoting the initiation by producing those extra calls.

The vocal-devocal pairs also afford us the opportunity to expand the vocal-behaviour analysis by identifying which member of the pair is vocalizing in specific behavioural contexts. The similar vocal-behavioural associations seen between the juvenile and adult rats indicate potential patterns of vocal usages that are not changed due to age or context. In both cases being chased is associated with splits and trill with jumps while being chased is associated solely with trill with jumps. Further, both receiving and instigating a nape attack is associated with trill calls. Finally, simply approaching another animal is also associated with trills. This comparison gives us more information about the trill type calls. Because they are the only vocalizations which are consistent across context and age, they are likely a more engrained vocalization, and likely communicate general positive affect.

#### *5.1.2.2 Flat Calls and Social Coordination*

One hypothesis about 50-kHz USVs is that the flat calls in this range serve as contact calls, used to establish the location of a partner. In support of this hypothesis are the findings that these calls are emitted by animals left in the enclosure after the removal of a partner (Wöhr et al., 2008). Our anticipation of play study is not consistent with this hypothesis, as once the animals learned to anticipate the imminent arrival of a play

partner and increased their overall rate of calling, they rarely emitted 50-kHz flat calls. In contrast, our juvenile play study shows high frequencies of 50-kHz flats when the intact dyads were actively interacting. The difference between groups indicates that this call is more likely being used to communicate, as it is only present during social interactions.

Based on the data we have available, however, how the 50-kHz flat calls are used remains difficult to decipher. What we know about the 50-kHz flat calls is that they are strongly associated with nape attacks in the juvenile animals, but only in the intact pairs. Juvenile vocal animals, when paired with a devocalized animal, only made this call when approached, not during nape attacks. In adulthood the intact pairs did not use this call significantly; however, when interacting with a devocalized partner, 50-kHz flats were emitted when bitten, sniffed, and sniffing the other animal. What then is the purpose of these flat calls? These calls could be used as a signal about imminent contact, as in both juveniles and adults they are emitted preceding the onset of an interaction, such as being sniffed and approached. These calls seem to be playing a role in social coordination between animal pair mates, but not in terms of locating partners at a distance as previously argued (Wöhr et al., 2007), but rather to coordinate close quarter maneuvers. The strong association between being bitten and 50-kHz flats also implicates this call with aggressive signaling. Indeed, flat types of 50-kHz USVs have been previously linked to socially ambivalent or aggressive situations in adult rats (Burgdorf et al., 2008). It is possible that the affiliative uses of 50-kHz calls may be distinguished from the aggressive uses by the relative frequency of the calls, with more aggressive uses involving lowering the frequency towards 30k-Hz. Although these issues remain to be empirically resolved,

we can assume that the flat 50-kHz calls (i.e., those that range between 30-60-kHz) communicate more than simply “who’s there?”

### *5.1.2.3. Reciprocal calling*

A potential mechanism by which vocalizations could foster communication among conspecifics is by reciprocal exchange. As mentioned previously, calls associated with several dyadic interactions appear to be asymmetric and hence, potentially complementary (i.e., one type of call by one partner is matched with a different call by the other animal). For example being bitten is associated with flat type calls, while biting is associated with extended flat/trill combination. It is also possible that rats facilitate play via the rapid reciprocal exchange of the same call. Indeed many species use mimicry of play signals to facilitate play and so increase the duration of the interactions (e.g., Davila et al., 2008; Mancini et al., 2013; Palagi et al., 2015; Scopa & Palagi, 2016). That is, the temporal coordination of either similar or complimentary ultrasonic calls among rats may facilitate the coordination of movements that ensure the continuation of positive interactions. This hypothesis is consistent with comparisons between intact dyads and those with a devocalized animal in both juveniles and adults.

As already noted the frequency and distribution of call types in pairs when only one partner could vocalize were no different from pairs where both could vocalize across both groups. This suggests that the vocal partner must have compensated for the absence of vocalizations from its devocalized partner. However, some of the associations between particular calls and particular actions that were present in intact pairs were abolished in pairs in which only one partner could vocalize. For example, the intact juvenile pairs consistently used flat and step down calls during nape attacks, whereas pairs with a

devocalized animal did not. This suggests that the tight coupling arising between particular calls and particular actions arose in the intact pairs because both partners contributed to the calling. The loss of such reciprocal calling in the pairs with only one vocal partner could account for the changes in behaviour, such as the greater risk of escalation to serious aggression in the adults.

## **5.2. Conclusions**

The methodology pioneered in this thesis for charting the association between particular USVs and particular behavioural actions has several potential implications. The first is the creation of a baseline of rat vocal and behavioural associations, similar to that of a language. The more that this methodology is used the more concrete these associations will become. Eventually we will be able to know that when animals perform a specific behaviour, like a nape attack, they will most likely make a particular call, such as the trill call. We can then use this baseline to compare across strains, between the sexes and detect developmental changes over the lifespan. Knowledge of these associations will give researchers an important insight into what behaviours are significant to the animals and, therefore, be better positioned to design more sensitive tasks to evaluate specific behaviours.

The second implication of this methodology is that better evaluations of medically relevant problems requiring the use of animal models will be possible, as using this technique contributes a more sensitive measure of behavioural and social interactions. This could be extremely useful for pharmaceutical testing. For example, current behavioural assessments using animal models for the assessment of antidepressant drugs is limited to non-social tasks, such as the forced swim test (e.g., failure to attempt to

escape). Yet, in people, one of the most common symptoms of depression is a lack of interest in socializing, a symptom not readily detectable in current animal models. The methodology developed in this thesis provides a detailed framework within which to detect depression in a social setting. Using a drug intervention, we can assess the affect the drug makes on both social and reward tasks, which are associated with an elevated frequency of emitting USVs. If the profile of the pharmaceutically treated rats looks similar to non-depressed controls we can be more confident that this intervention has the potential to work in people.

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