

The role of 50 kHz ultrasonic vocalizations as play signals in rats

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THE ROLE OF 50 kHz ULTRASONIC VOCALIZATIONS AS PLAY SIGNALS IN
RATS

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ABSTRACT

The role of 50 kHz ultrasonic vocalizations as play signals was examined, in this thesis by investigating the effects of devocalization on playful interactions in both juveniles and adults. In both studies, the animals played either with a devocalized or an intact partner. In juveniles, it was shown that 50 kHz calls are not essential for playful interactions to remain playful. However, in adults, it appears that the tactical use of 50 kHz calls is necessary to keep playful interactions from becoming aggressive in some contexts. Thus, 50 kHz calls appear to be functioning simply as an expression of affect in juveniles, whereas once sexually mature, the tactical use of ultrasonic vocalizations in male rats becomes more essential in navigating some social situations.

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LIST OF ABBREVIATIONS

CCBN	Canadian Centre for Behavioral Neuroscience
DE	devocalized
FM	frequency modulated
IN	intact
Hz	hertz
kHz	kilohertz
NSERC	Natural Sciences and Engineering Research Council
SE/SEM	standard error of the mean
USVs	Ultrasonic vocalizations
VTA	ventral tegmental area

Chapter 1
General Introduction

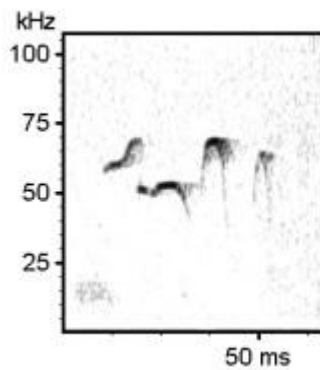
1.1. The role of ultrasonic vocalizations in social interactions

Not all forms of vocal communication in animals are audible to the human ear, which on the acoustic spectrum has a typical range of 20 Hz to 20 kHz (Heffner & Heffner, 2007). On the low end of the acoustic spectrum, there are low pitch sounds with a range from close to 0 to 20 Hz, such as the infrasound calls, produced by elephants (*Elephantidae*). Beyond the human auditory range are high pitch ultrasounds with a range of 20 kHz to 160 kHz, sounds that can be heard by dogs and cats, and are produced by dolphins (*Delphinidae*), bats (*Chiroptera*), and rodents, such as rats (*Rattus*), mice (*Mus*), ground squirrels (*Spermophilus*) and flying squirrels (*Pteromyini*).

Rats produce three distinct types of USVs (Brudzynski, 2009; Knutson, et al, 2002; Portfors, 2007; Wintink, 2001; Wright, et al., 2010). These are 22-kHz, 40-kHz and 50 kHz calls). The two primary calls in adults and juveniles are the 22-kHz and 50 kHz calls (Fig1.1), with the 40-kHz calls being emitted primarily by the pups as isolation calls when separated from their mother and littermates. The 22-kHz calls are usually observed in aversive situations and function as alarm calls (Litvin et al., 2007). These calls are emitted when a rat is facing danger, in situations in which they experience significant anxiety and if they are anticipating threats such as an aggressive opponent or a predator (Brudzynski, 2009). 50 kHz calls have been referred to as appetitive or “happy” calls and are associated with positive and rewarding situations, such as in the anticipation of a sexual encounter, drugs, or when meeting other rats after a period of separation (Brudzynski, 2009; Burgdorf et al., 2008; Knutson, et al, 1998; Knutson et al., 2002;

Portfors, 2007). In rats, the functions of 50 kHz calls, however, have yet to be determined. This thesis examines one possible function for 50 kHz calls as a play signal.

A.



B.

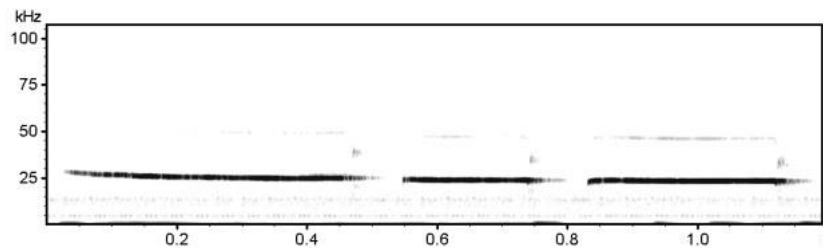


Figure 1.1. Spectrograms that show changes in frequency over time of (A) 50 kHz ultrasonic vocalization (B) 22 kHz ultrasonic vocalization

Several studies have shown that a high rate of 50 kHz calls occur when the rats are experiencing pleasure or are in rewarding social situations (Calcagnetti & Schechter, 1992; Humphreys & Eison, 1981). One such situation that appears to be particularly

rewarding to rats is rough and tumble play, whether that play is with another rat or a human hand (Burgdorf & Panksepp, 2001; Cloutier et al, 2012; Panksepp & Burgdorf, 2000). Further support for the appetitive nature of 50 kHz calls is seen in situations in which the social reward changes over the course of the interaction. For example, in adult sexual encounters when mounting and during ejaculation the frequency of 50 kHz calls increase, but then, following ejaculation, they decrease and, as they do so, the frequency of 22-kHz calls increase (Burgdorf et al., 2008; Burgdorf, et al, 2011; Knutson et al., 2002). Similarly, there is a positive correlation between the frequency of play and the amount of 50 kHz USVs emitted (Knutson et al., 1998), and, as the play wanes, the rats then become increasingly likely to avoid contact, and the frequency of 50 kHz calls decreases and the frequency of 22-kHz calls increases (Burgdorf et al., 2008). The same kind of pattern has been shown for emitting 50 kHz USVs and tickling of rats by the experimenter's hand (Burgdorf & Panksepp, 2001; Panksepp & Burgdorf, 2000).

1.2. Types of ultrasonic vocalizations

During playful interactions, two forms of 50 kHz calls can occur (Burgdorf et al., 2011). In one type, the frequency remains relatively constant throughout the duration of the call, and is referred to as a flat 50 kHz call. In the other type, the frequency rises and falls, often multiple times, during the duration of the call, and is referred to as a frequency modulated (FM) 50 kHz call. In playful contexts, over 90 percent of the calls emitted are typically of the FM subtype. Furthermore, there are 14 different kinds of FM 50 kHz calls, and all of them can occur during playful interactions (Wright et al., 2010).

The diversity of 50 kHz calls occurring during rewarding playful interactions suggests that, in addition to the role of the vocalizations during playful interactions expressing the animal's affective state, they may also be acting as play signals to promote and maintain playful interactions.

1.3. Play signals

The traditional view of play signals is that they are performed, usually prior to making contact, so as to inform the partner that the contact about to occur is playful, thus reducing the risk that the encounter will escalate into aggression (Bateson, 1955; Bekoff, 1975; Fagen, 1981). Play signals have been reported for many species. Some of the best known include the play bow of dogs (Bekoff, 1995) and the open-mouth play face of primates (Van Hooff, 1967). Play signals are frequently emitted prior to grabbing or biting the partner (Bekoff, 1995; Pellis & Pellis, 1997b) and in situations in which there may be ambiguity in the interpretations of actions performed during play, such as when multiple partners are involved or when there is a size asymmetry between the players (Bekoff, 1995; Fagen, 1981; Palagi, 2008; Palagi & Mancini, 2011; Van Leeuwen et al., 2011; Waller & Dunbar, 2005). Most play signals characterized to date involve the visual modality, such as the play bow and the open-mouth play face. In rats, no discernible play-related facial signals have been reported, but locomotor-rotational movements that are associated with play fighting could function as play signals, as has been suggested for other species (Petrù et al., 2008; Wilson & Kleiman, 1974). Indeed, at least one form of jump does appear to be a reasonable candidate as a play signal in rats (Pellis & Pellis,

1983). However, while house mice have as high or higher frequencies of locomotor-rotational movements (van Oortmersen, 1971; Walker & Byers, 1991) as do rats, they also have a much reduced and simplified pattern of playful contact (Pellis & Pasztor, 1999). Moreover, other rodents, such as Syrian hamsters (*Mesocricetus auratus*), have patterns of play fighting that are as complex as those of rats (Pellis & Pellis, 1988), yet never perform locomotor-rotational movements. Based on this evidence from other species, specific locomotor rotational movements neither predict the occurrence of play fighting nor are they essential for play fighting to occur. Alternatively, the rich repertoire of 50 kHz calls may provide rats with a source of auditory play signals. In fact, several examples of possible play signals involving vocalizations in other species have been reported (Biben & Symmes, 1986; Masataka & Kohda, 1988; Rasa, 1984). Kipper and Todt (2002) showed that playful vocalizations in Barbary macaques (*Macaca sylvanus*) are more frequent preceding an attack than following contact and also that different variants of these calls are emitted at different times during the encounter.

A recent study in our laboratory similarly showed that in the playful interactions of juvenile rats, 50 kHz calls were more frequent immediately before contact than immediately after termination of contact. In addition, different FM subtypes were associated with such approach and withdrawal (Himmler et al., 2014). While this provides support for the hypothesis that 50 kHz calls are potentially acting as play signals, the main problem with this study is that in measuring vocalizations in fast-paced, dynamic playful interactions, no particular call can be readily attributed to a specific

member of the playing pair. Thus, while it is reasonable to conclude, given the traditional play signal hypothesis, that the increased frequency of calls happening prior to contact arises from the attacking partner, it cannot be concluded to be so with certainty, as potentially, both rats could be calling. In order to determine which rat is calling, the attacker or the defender, I used devocalized rats in my thesis research. Using devocalized rats also provided a means to study how the absence of vocalizations from one or both partners affects playful interactions. By manipulating whether neither or only one partner in a playing pair could emit 50 kHz calls, I was able to test several hypotheses about how 50 kHz calls could be being used as play signals.

For this thesis, devocalization was produced by bilateral re-sectioning of the recurrent laryngeal nerves (White & Barfield, 1987; Snoeren & Amgo, 2013). An alternative approach could have been to deafen the rats (Siviy & Panksepp, 1987) or mask their ability to hear by flooding the room with white noise (Pellis et al., 1996). However, although deafening can significantly reduce the frequency of close quarter wrestling, the overall frequency of play remains unaltered (Siviy & Panksepp, 1987). Even though deafened, the rats could still emit 50 kHz vocalizations. It has been demonstrated that the production and emission of 50 kHz vocalizations stimulates the release of dopamine in the mesolimbic pathway (Burgdorf et al., 2007), and so the deafened rats were still potentially able to activate the release of dopamine keeping the playful interaction rewarding. Therefore, the first study focuses on determining if the

ability both to hear and produce vocalizations is necessary for the facilitation of playful interactions.

The first set of experiments, which used juvenile pairs of devocalized rats and sham-devocalized rats, indicated that, in the absence of USVs, the frequency of play decreased, and how the rats played, in terms of defense tactics used, changed. Together, these two findings suggest that 50 kHz calls are indeed being used to motivate and coordinate play. However, in these experiments, rats that were familiar with one another were used. Interactions with unfamiliar partners, specifically those that are devocalized, could provide a situation in which 50 kHz calls may be essential in order to motivate and coordinate playful interactions. Furthermore, with the onset of sexual maturity, the play of male rats becomes more aggressive (Takahashi, 1986; Takahashi & Lore, 1983), potentially increasing the importance of signaling to avoid escalation to serious fighting. Therefore, in the second set of experiments, devocalized rats were tested with unfamiliar partners, both as juveniles and as adults.

1.4. The social behaviors studied in this thesis

1.4.1. Play behaviors

Rats are a good model to study play behavior and its relationship to the brain because they are cost effective (Panksepp et al, 1984; Thor & Holloway, 1984; Pellis & Pellis, 1998; Vanderschuren et al., 1997). The most common type of play in rats is rough and tumble play fighting (Bolles & Woods, 1964; Poole & Fish, 1975), which appears as

early as postnatal day 18, increases until it peaks around the age of 30-40 days and then slowly declines, but is not abolished, with the onset of sexual maturity (Thor & Holloway, 1984; Bolles & Woods, 1964; Pellis & Pellis, 1990, 1997). The key distinction between play fighting and serious fighting is the target of attack. In play, the target is typically the nape, which if contacted is nuzzled, whereas in serious fights, attacks are directed at the lower flanks and face (Blanchard et al., 1977; Pellis & Pellis, 1987). At all ages, playful interactions involve competition for the nape (Pellis & Pellis 1987; Siviy & Panksepp, 1987). However, there are age related changes in defense, especially in male rats (Meaney & Stewart, 1981; Panksepp, 1981; Pellis & Pellis, 1987, 1990, 1997; Takahashi & Lore, 1983; Thor & Holloway, 1986). In juveniles, the rotation to supine tactic is the most frequent form of defense whereas in adults, the forms of defense, in which the rat remains standing are more frequent (Pellis & Pellis 1987, 1990). When analyzing playful interactions there are several key defense tactics that are commonly scored.

1.4.1.2. Evasive defense

In an evasive defense, when the attacker contacts the nape of the defender and nuzzles it, indicating a playful attack, the defender will walk, run, leap or swerve away so that it moves its face away from its attacker, but also ends up facing away from the attacker itself as well (Himmler et al., 2013; Pellis et al., 1992b).

1.4.1.3. Facing defense

In a facing defense, when the nape is playfully contacted, the defender will turn to face the initiator of the playful attack. From this point, there are two categories of facing defenses the defender can choose and are sometimes referred to as “pins” or close contact wrestling, (i) complete rotation, in which the defender turns from a standing position to fully supine (ii) partial rotation, in which one or both of the defender’s hind paws remain on the ground and the forequarters are turned (Himmler et al., 2013; Pellis & Pellis, 1987)

1.4.2. Aggressive Behaviors

As noted above, in rats, serious fighting involves biting attacks directed at the lower dorsum and flanks and also at the face (Blanchard et al., 1977). In addition, during aggressive behavior, the rats may perform a variety of actions that likely function as aggressive displays (Grant, 1963). Both overt biting and the presence of these agonistic displays can be used to assess how aggressive interactions may be (Smith et al., 1999).

1.4.2.1. Agonistic Displays

At lower levels of intensity of aggression, there are several, overt behavioral markers associated with aggression. These agonistic displays include pilo-erection, arching of the back, lateral displays and tail wagging, in which the tail moves rapidly from side to side (e.g., Barnett & Marples, 1981; Blanchard et al., 1977; Grant, 1963).

1.4.2.2. Agonistic Attacks

At the most extreme form of overt, agonistic behavior is an attack in which the attacker lunges and bites at the defender's rump, lower back and flanks. At the same time, the defender can deliver bites directed at the attacker's face and snout (Blanchard et al., 1977; Pellis & Pellis, 1987).

1.5. Summary: Thesis Objectives

The present studies were designed to enhance our understanding of the role of 50 kHz ultrasonic vocalizations as play signals. The following objectives were addressed in this series of studies:

1. To investigate whether there is any effect on juvenile playful interactions in the absence of vocalizations (using bilateral re-sectioning of the recurrent laryngeal nerves, devocalization).
2. To investigate whether unfamiliar rats are able to coordinate playful interactions with devocalized partners as juveniles and adults and further investigate the role of 50 kHz calls in ambiguous situations in adulthood.

Chapter 2

Are 50 kHz Calls Used as Play Signals in the Playful Interactions of Rats? II. Evidence from the Effects of Devocalization*

*Kisko, T.M., Himmler, B.T., Himmler, S.M., Euston, D.R., Pellis, S.M. (2013). Are 50 kHz calls used as play signals in the playful interactions of rats? II. Evidence from the effects of devocalization. *Submitted to Behavioral Processes April, 2014.*

Abstract

During playful interactions, juvenile rats emit many 50 kHz ultrasonic vocalizations, which are associated with a positive affective state. In addition, these calls may also serve a communicative role - as play signals that promote playful contact. Consistent with this hypothesis, a previous study found that vocalizations are more frequent prior to contact than after contact is terminated. The present study uses devocalized rats to test three predictions arising from the play signals hypothesis. First, if vocalizations are used to facilitate contact, then in pairs of rats of which one is devocalized, the higher frequency of pre-contact calling should only be present when the intact rat is initiating the approach. Second, when both partners in a playing pair are devocalized, the frequency of play should be reduced and the typical pattern of playful wrestling disrupted. Finally, when given a choice to play with a vocal and a non-vocal partner, rats should prefer to play with the one able to vocalize. The second prediction was supported in that the frequency of playful interactions as well as some typical patterns of play was disrupted. Even though the data for the other two predictions did produce the expected findings, they also support the conclusion that, in rats, 50 kHz calls are likely to function to maintain a playful mood and for them to signal to one another during play fighting.

2.2. Introduction

When rats are engaged in social play they emit ultrasonic vocalizations (USVs) (Burgdorf et al., 2008; Knutson et al., 1998). These vocalizations are typically in the 50 kHz range and usually of the frequency modulated (FM) subtypes of 50 kHz USVs (Burgdorf et al., 2011; Wöhr et al., 2008; Wright et al., 2010). In non-playful contexts, calls have been shown to attract approach by other rats (Wöhr & Schwarting, 2009; Wöhr & Schwarting, 2012) and recently, it has been found that these vocalizations are more frequent before playful contact is made than when such contact is terminated (Himmler et al., 2014). These findings suggest that, during playful interactions, FM 50 kHz USVs may be being used as play signals to promote and maintain play. If USVs are acting as play signals, then playful interactions between rats should be disrupted in their absence. Indeed, it has been found that in deafened rats the close quarter wrestling component of play is significantly diminished, although the frequency of play initiation remains unchanged (Siviy & Panksepp, 1987).

Play signals have been reported in many species. Some of the best known include the play bow of dogs (Bekoff, 1995) and the open-mouth play face of primates (Van Hooff, 1967). Play signals are frequently emitted prior to grabbing or biting the partner (Bekoff, 1995; Pellis & Pellis, 1997b) and in situations in which there may be ambiguity in the actions performed during play, such as when multiple partners are involved or when there is a size or dominance asymmetry between the players (Bekoff, 1995; Fagen, 1981; Palagi, 2008; Palagi & Mancini, 2011; Van Leeuwen et al., 2011; Waller &

Dunbar, 2005). Most play signals characterized to date involve the visual modality, such as the play bow and the open-mouth play face mentioned above. However, several examples of possible play signals involving vocalizations have been reported (Biben & Symmes, 1986; Masataka & Kohda, 1988; Rasa & Anne, 1984). Kipper and Todt (2002) showed that playful vocalizations in Barbary macaques (*Macaca sylvanus*) are more frequent preceding an attack than following contact and also that different variants of these calls are emitted at different times during the encounter. As noted above, we previously found a similar pattern with regard to 50 kHz calls in rats when they are playing (Himmler et al., 2014).

In nocturnal animals, such as rats, visual signals are likely not as useful as vocal ones. For example, nocturnal primates have a richer vocal repertoire and a more limited visual repertoire than do diurnal ones (Braune et al., 2005; Zimmermann, 1995). USVs emitted by rodents dissipate easily, allowing communication with a reduced risk of being detected by predators (Brudzynski, 2009), thus making 50 kHz calls low risk signals to communicate play. In addition, in rats, not only are 50 kHz calls divided into flat and FM modulated ones, but also the latter are made up of at least 14 different variants (Wright et al., 2010), providing the potential for different calls or combination of calls serving different communicatory functions.

During playful interactions, rats attack and defend the nape of neck, which if contacted is nuzzled (Pellis & Pellis, 1987; Siviy & Panksepp, 1987). The tactics used to defend against nape contact can either promote bodily contact in the form of wrestling or

decrease the chance of contact by withdrawing from the attacker (Pellis & Pellis, 1987). Bodily contact has been shown to increase the occurrence of 50 kHz USVs (Burgdorf & Panksepp, 2001; Cloutier et al., 2012; Knutson et al., 2002; Panksepp & Burgdorf, 2000), increasing the likelihood that these calls could be used as play signals to encourage further bodily contact with the other animal. The present study uses rats that have been devocalized, and so unable to produce USVs, as to test-three predictions to evaluate whether these calls are used as play signals.

If the 50 kHz calls function as traditionally envisaged play signals – signifying “I want to play with you” (Bekoff, 1975, 1995), then they should be emitted more frequently immediately before contact is made than immediately after terminating contact. As already noted, this is the case (Himmler et al., 2014). However, play signals may be multi-functional, signaling not only approach, but also withdrawal from the partner and, in some cases, being used for self-communication by the performer (Pellis & Pellis, 1997b, 2011; Pellis et al., 2011). In fact, in our previous study, we found that even though one particular type of FM call, the trill, was most often emitted during play, a finding consistent with other studies (Snoeren & Amgo, 2013; Wright et al., 2010), different FM calls were associated with withdrawal compared to approach (Himmler et al., 2014), suggesting a difference when signaling contact to come *versus* termination of contact (Pellis & Pellis, 1997b). Therefore, while consistent with the traditional view of play signals (i.e., signals that promote playful contact), the significantly higher frequency of calls prior to contact may also be consistent with alternate hypotheses.

The main problem with measuring vocalizations in fast-paced, dynamic playful interactions is that any one call cannot readily be attributed to a specific member of the playing pair. Thus, while it is a reasonable inference, given the traditional play signals hypothesis, that the increased frequency of calls present prior to contact arises from the attacking partner, it cannot be concluded to be so with certainty, as both rats could be potentially calling. Nonetheless, if the calling is used to announce imminent contact by the attacker, then the higher frequency of calling prior to contact should arise because the attacker calls more. To test this, calling during play in pairs in which one partner of a playing pair was devocalized was evaluated. The specific prediction was that when the attacker is the non-vocal animal, then there should be no difference in the frequency of calling before contact and immediately after contact is terminated – both should be low. In contrast, when the attacker is able to vocalize, then there should be a significantly higher frequency of calling immediately before contact than immediately after, as previously found (Himmler et al., 2014).

If vocalizations are promoting playful interactions, then in their absence the frequency of playful contact should decrease. Knutson et al. (1998) showed that over the course of a test trial, the frequency of emitting 50 kHz calls is correlated with the waxing and waning of nape contact, but not with wrestling. In seeming opposition to these findings, Siviy & Panksepp (1987) showed that when pairs of rats are deafened, they will initiate playful contact just as often as intact rats, but are significantly less likely to wrestle with one another. Eliminating hearing, however, is not the same as eliminating

vocalizations. Rats that are deaf not only cannot hear vocalizations, but also cannot hear other auditory cues from their partner that are potentially important, such as footsteps and breathing (Beatty & Costello, 1983; Pellis et al., 1996; Pellis et al., 1992b; Thor & Holloway, 1982). Thus, a deaf rat may misjudge the proximity of their partner and reduce the likelihood of engaging in a defensive tactic that promotes wrestling. A more direct way to determine if it is, in fact, the USVs that are responsible for either facilitating playful contact or wrestling is to eliminate the rat's ability to emit USVs. If USVs are critical for facilitating play, then when both play partners are devocalized, there should be a reduction of nape attacks, wrestling or both. To test this, the play of pairs of devocalized rats was compared to the play of sham-treated control rats.

When given the option, rats prefer to socialize with a partner that emits many 50 kHz USVs than one that emits few (Wöhr et al., 2008; Wöhr & Schwarting, 2009), and in playback experiments, rats will spend more time around the speaker emitting 50 kHz vocalizations (Burgdorf et al., 2008; Sadananda et al., 2008). However, the role of vocalizations in attracting play partners has not previously been studied. Given that rats find 50 kHz calls attractive, it may be the case that when given a choice, rats will preferentially play with a partner that can *vocalize*. To test this prediction, quads of rats, with two devocalized and two intact, were placed together in the testing enclosure. If vocal partners are more attractive, then it should be the case that all the rats will prefer to initiate play with a partner that can vocalize and will avoid initiating play with ones that do not.

2.3. Methods and Materials

2.3.1. Subjects

Thirty-two male Long Evans rats were used. The rats were obtained from Charles River Laboratories (St. Constant, Quebec) around 23 days of age and housed in the vivarium at the Canadian Centre for Behavioral Neuroscience. They were housed in quads (which consisted of four rats caged together), with food and water available *ad libitum* and were on a 12-hour light-dark schedule, lights off at 1930. The animals were maintained at a constant temperature of around 21-23°C and were housed in polyethylene cages 46cm×25cm×20cm with processed corncob as bedding. Devocalization and the sham surgeries were performed when the rats were at or between 28-30 days of age, with half of the quads having two members devocalized and half sham-treated. The rats remained in their respective quads after surgeries and testing for play occurred when they were between 35-40 days old, the age when juveniles are most playful (Pellis & Pellis, 1990; Thor & Holloway, 1984).

2.3.2. Apparatus

Testing took place in a 50cmx50cmx50cm clear Plexiglas enclosure which was placed into a sound proof box (inside measurements: 59cm×65.5cm×81.5cm). The inside of the play box was filled with approximately 1-2cm of CareFresh® bedding, as this bedding emits less background noise when walked on by the rats during recording compared to standard corncob bedding. The sound proof box was fitted with sound-attenuating foam (Primeacoustic, Port Coquitlam, British Columbia) and an ultrasonic

microphone (Model 4939, Brüel & Kjaer, Denmark), connected to a Soundconnect™ amplifier (Listen, Inc, Boston, MA) which processed recordings via a multifunction processor (RX6, Tucker-Davis Technologies, Alachua, CA) run through MATLAB, on a desktop in the same room. The receiver was set to record sensitive frequencies ranging between 4-and 100-kHz. All files were then converted to .wav files. A DVD103 Sony Handycam with night-shot capacity was used for filming playful interactions and was placed against a small window opening into the box at a 45° angle, which is the optimal angle from which to analyze playful tactics (Pellis & Pellis, 1987). Off-line analysis of vocalizations was performed using Raven Pro 1.4 software (developed and distributed through Cornell University, Ithaca, NY) for audio files and Avidemux 2.6 software for video files.

2.3.3. Surgery

Sixteen rats were devocalized and 16 rats were sham devocalized, two from each quad, under isoflurane anesthesia. Devocalization surgery involved bilateral sectioning of the recurrent laryngeal nerves (Snoeren & Amgo, 2013). A 3-cm incision was made on the ventral surface of the neck and the sternohyoideus muscle was separated to expose the trachea. After locating the recurrent laryngeal nerves, they were freed from the surrounding fascia and a 3-mm section was removed bilaterally. The muscle was then loosely sutured in place and the skin was closed with subcutaneous sutures. Sham animals had an incision made on the ventral surface of the neck and then had the skin closed with subcutaneous sutures. Buprenorphine was administered at the time of surgery

(0.05mg/kg *s.c*) and again every 12h for three successive days. Four days were allowed for recovery after surgery. On the fifth day, the rats were tested in the soundproof box to ensure that they were unable to emit ultrasonic vocalizations. This was confirmed – none of the surgically devocalized rats could emit USVs whereas all the intact and sham rats did so.

2.3.4. Testing procedures

Once the success of the devocalization surgery was confirmed, the rats were habituated with their cage mates (i.e., in quads) to the testing enclosure for 30 min a day for three days prior to the experimental tests being conducted. A standardized testing and scoring paradigm was used to evaluate the play (Himmler et al., 2013).

Pairs of rats were tested following 24-hours of social isolation to increase their playfulness when introduced in the experimental enclosure (Panksepp & Beatty, 1980; Pellis & Pellis, 1990). One intact and one devocalized or one sham-treated rat from each quad were chosen as the subjects, then each of these rats were tested once each with the other intact rat and the devocalized or sham-treated rat. Trials were counterbalanced to avoid any order or age effect (Pellis & Pellis, 1990; Pellis et al., 1992a). In the group consisting of devocalized animals after the final trial involving dyads, each quad was given another 24 h of social isolation and then all four quad members were placed together in the test enclosure. Because vocalizations and play behavior were recorded on separate devices, the vocalizations and the video recordings were synchronized by a device emitting a simultaneous light/sound cue. At the beginning of each trial, three

consecutive cues were given and trials were considered to have begun with the final beep (Himmler et al., 2014).

2.3.5. Behavioral analyses

Videotaped sequences were evaluated at normal speed, slow motion and frame-by-frame to record a variety of measures related to playful attack and defense (Himmler et al., 2013). When the snout of one rat was either in contact or moved towards the nape of the other rat, a playful attack was scored. The recipient of a nape attack can either defend itself or can continue with its ongoing behavior and so seemingly not respond to the attack. The probability of defense is calculated as the number of times a rat defends divided by the total number of times it is attacked. When a rat moves to defend against contact with its nape, there are two major categories of tactics: (a) evasion, in which the defender moves away from the attacker and does so by running, leaping, or swerving away and thus ends up facing away from the partner, and (b) facing defense, in which the defender moves its nape away from the attacker by turning to face them and, in doing so, blocks access to its own nape by putting its teeth in between itself and its partner's nape. There are three forms a facing defense can take: (i) complete rotation, in which the defender rolls completely over onto its back, (ii) partial rotation, in which the defender's forequarters are rotated, but maintains contact with the ground with one or both of its hind feet, and (iii) other, in which defensive actions involve rotations or other movements in other dimensions (e.g., rotating vertically in a horizontal plane; (Pellis et al., 1994;

Pellis et al., 1992a)). In addition to specific components of this scoring system being used, other measurements unique to each of the following predictions were tested.

Prediction 1: This involved comparing the vocalizations emitted when an intact rat attacked a devocalized partner to when a devocalized rat attacked an intact partner. For each pair, I only used playful interactions that had an uninterrupted 300ms before contact was made and had an uninterrupted 300ms immediately after contact was terminated. The period prior to contact had to involve the approaching rat clearly moving towards the nape and the termination had to involve the defending animal standing on all four of its paws (Himmler et al, 2013). A minimum of eight such before and after conditions had to be scored for each pair. Based on these criteria, only 11 of the 16 pairs qualified for inclusion for this prediction (intact-devocalized N=6, devocalized-intact N=5). As described in Himmler et al. (2014), this restricted time frame was used to increase the likelihood that the vocalizations recorded were related to the play fight about to occur and to the one just finished. The calls were analyzed manually from the spectrograms (Wright et al., 2010). The experimental design provided independent samples of intact rats attacking devocalized ones and devocalized ones attacking intact rats.

Prediction 2: This involved comparing the social interactions of pairs of devocalized rats with those of pairs of sham-treated rats. Due to a fire alarm going off during their testing session, one of the devocalized-devocalized pairs had to be excluded from the analysis as they froze and did not interact. Thus, this analysis involved 15 pairs (eight pairs of sham-sham and seven pairs of devocalized-devocalized) instead of 16. The frequency of playful

attacks occurring during the 10 min trials, the probability that the recipient of such attacks defended and the probability of different types of defensive tactics occurring when a defense did take place, were scored (Himmler et al., 2013).

Also, during play fighting, rats may launch counterattacks after successfully defending their nape from their partner (Pellis & Pellis, 1990). Successful counterattacks to the nape lead to role reversals, in which the original attacker is put on the defensive (Pellis, Pellis & Foroud, 2005). For play to remain playful, interactions need to be reciprocal with the frequency of role reversals providing a measure of the reciprocity (Pellis, Pellis & Reinhart, 2010). Role reversals were scored for both sham-treated and devocalized pairs. A sequence of attack and defense that led to the original attacker becoming the defender was recorded as a role reversal. For each pair, the percentage of attack-defense sequences that led to a role reversal was calculated and these were used to calculate group means.

As devocalization may have disrupted social behavior more generally and not just play, four additional behaviors that were not related, or were indirectly related to play were also scored. These were: (1) social investigation, which consists of anogenital sniffing and “nosing” the parts of the partner’s pelage that does not involve the nape; (2) social grooming, which consists of licking and nibbling the partner’s pelage; (3) social grooming associated with play, which typically happens when the rats stop competing for access to the nape when in the pin configuration; and (4) startle responses, which consist

of noticeable jumps and hops by one rat when contacted in non-playful contexts. The frequency of these four behaviors was scored over the 10 min trials.

Prediction 3: This involved determining whether playful attacks were directed more often to partners that could vocalize when a choice between partners was available. All eight quads of the devocalized group were used. The playful attacks of each quad member were scored and these were divided into attacks directed at intact partners and attacks directed at devocalized partners. The mean frequency of attacks per 10 min was calculated for each of the possible pairings: intact-intact, intact-devocalized, devocalized-devocalized and devocalized-intact. However, given that in a quad, an intact rat had only one possible intact partner and two possible devocalized partners, and similarly, a devocalized rat had a choice of only one devocalized partner but two intact partners, the rate of partner-directed attacks had to be corrected. The frequency of attacks in the intact-devocalized/devocalized-intact group was halved.

2.3.6. Statistical analyses

The data from prediction 1 were analyzed using a repeated measures 2x2 ANOVA with pair type (intact-devocalized, devocalized-intact) and time (before, after) as the factors of interest. The data from prediction 2 were analyzed using 2-tailed unpaired Student's t-tests comparing the means from the devocalized pairs to the sham-treated pairs groups. The data for prediction 3 were analyzed using a 2x2 ANOVA with attacker (intact, devocalized) and target of attack (intact, devocalized) as the factors of interest. For all data, differences were considered significant for p values $\leq .05$. For

graphical and tabular representation of the data, values are given for group means with the standard error of the mean (SEM).

2.3.7. Inter-observer reliability

The most difficult aspects of behavior to score were the various subcomponents of play and the scoring of the vocalizations. Therefore, a subset of trials of play were re-scored by a second scorer (S. M. H.) and compared to the values from the primary scorer (T. K.). Similarly, a subset of the vocalization were re-scored by a second scorer (B. T. H.) and compared to the values from the primary scorer (T. K.). Using Pearson's r to compare between the raters revealed significantly high, positive correlations for all the play behaviors scored ($r = 0.899$ to 0.993), as well as for the vocalizations ($r = 1.0$). For consistency, all the data shown in the Results are from values derived from the primary scorer.

2.4. Results

Prediction 1: More 50 kHz calls should be emitted prior to an attack than after termination of playful contact when the attacker is intact but not when the attacker is devocalized.

The analysis of variance revealed a significant main effect of time ($F [1, 9] = 6.044$, $p = 0.0362$), but not for the vocal ability of the playful attacker ($p > 0.05$). That is, calling was more frequent before contact was made than after contact was terminated, but there

was no difference in number of calls based on whether the attacker could vocalize or not (Fig. 2.1).

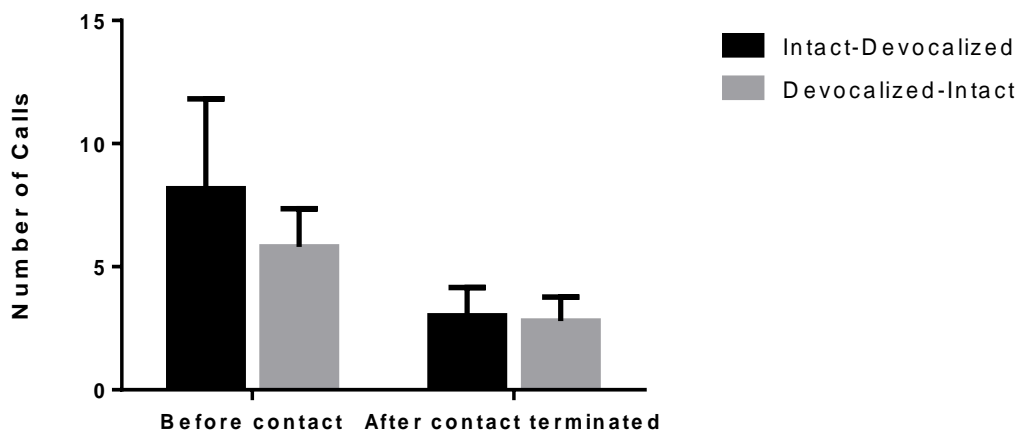


Figure 2.1. Prior to an attack, more calls are emitted no matter whether the attacker is the one able to vocalize or not. Mean and SE are shown for attacks initiated by either an intact animal or a devocalized animal.

Prediction 2: If vocalizations are promoting playful interactions, then, in their absence, the frequency of interactions should decrease or cease and/or their content should change.

Pairs of devocalized rats played significantly less than the sham-treated control rats ($t(13) = 3.002, p < 0.05$) (Fig. 2A) and were significantly more likely to defend themselves ($t(13) = 7.436, p < 0.05$) (Fig. 2B).

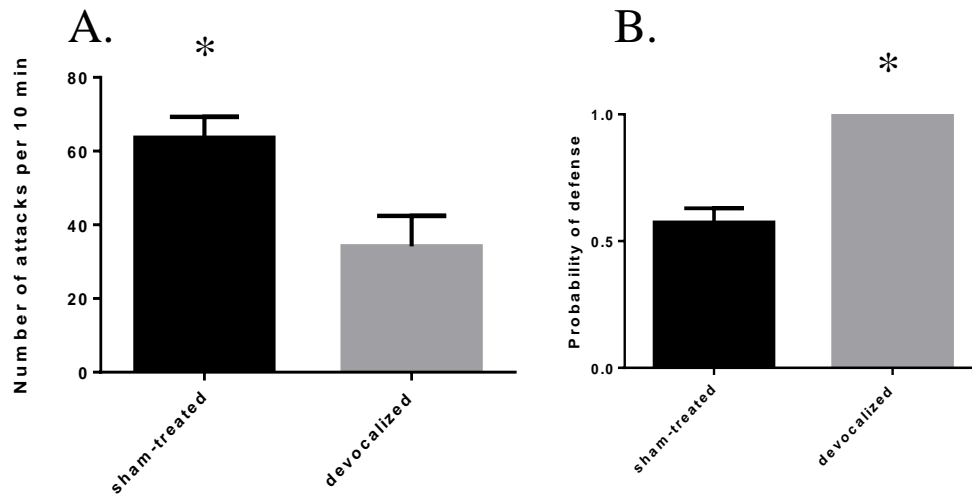


Figure 2.2. Mean and SE are shown for various measurements of play in same condition pairs of sham-treated and devocalized rats: (A) The frequency of playful attacks per pair per 10 minutes, and (B) the probability that a playful attack leads to a defensive action.

With regard to types of defense, there were significant group differences in probability of evasion with devocalized rats evading less than sham-controls ($t(13) = 2.425, p < 0.05$) (Fig. 2.3A) and there was a significant difference in the probability of complete rotations with devocalized rats rolling over significantly more than the sham-controls ($t(13) = 3.021, p < 0.05$) (Fig 2.3B). There was a significant difference in the frequency of occurrence of role reversals ($t(10) = 2.219, p < 0.05$), with play fights by devocalized pairs being less likely to involve role reversals (mean% \pm SD: 16.38 ± 6.11) than the sham-treated pairs (23.43 ± 4.84).

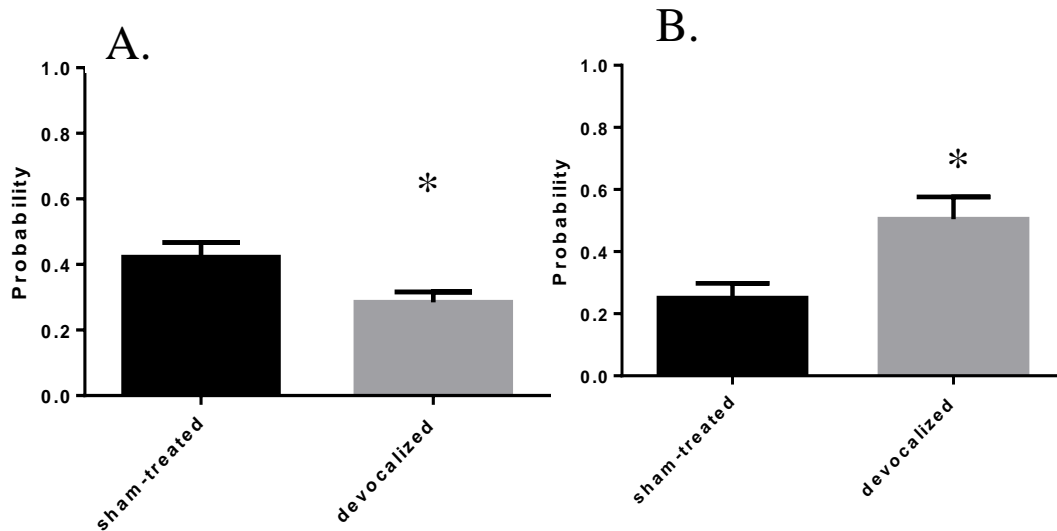


Figure 2.3. Mean and SE are shown for the probability of types of defensive tactics used: (A) the percentage of defenses that involved evasion, and (B) the percentage of facing defense involving complete rotation.

There were no group differences in social investigation and allogrooming, including allogrooming at the termination of competition for playful nape contact, but the devocalized pairs were significantly more likely to show a startle response when contacted by their partner (Table 2.1).

Non-social play behaviors

<i>Behavior</i> (#/10min)	<i>Sham pairs</i>	<i>Devocalized pairs</i>	<i>t-tests</i>
<i>Social investigation</i>	9.00 ± 1.07	11.43 ± 2.89	<i>t</i> = 1.4718 <i>p</i> = 0.16
<i>Social grooming</i>	4.38 ± 0.65	5.71 ± 1.44	<i>t</i> = 1.9007 <i>p</i> = 0.07
<i>Play ending with social grooming</i>	0.68 ± 0.12	0.68 ± 0.13	<i>t</i> = 0.5915

<i>Startle in response to non-playful contact</i>			<i>p = 0.56</i>
	0	1.86 ± 0.46	t = 4.347
			p < 0.001

Table 2.1. Mean (\pm SE) values for the different measures of non-play social behavior in pairs of sham-treated and devocalized rats.

Prediction 3: When given the option to play with a mute versus a vocal partner, rats should choose to initiate play with rats capable of vocalizing.

The analysis of variance revealed no significant differences ($p > 0.05$). Neither intact nor devocalized animals showed a significant difference in their preference for devocalized or intact play partners. (Fig. 2.4).

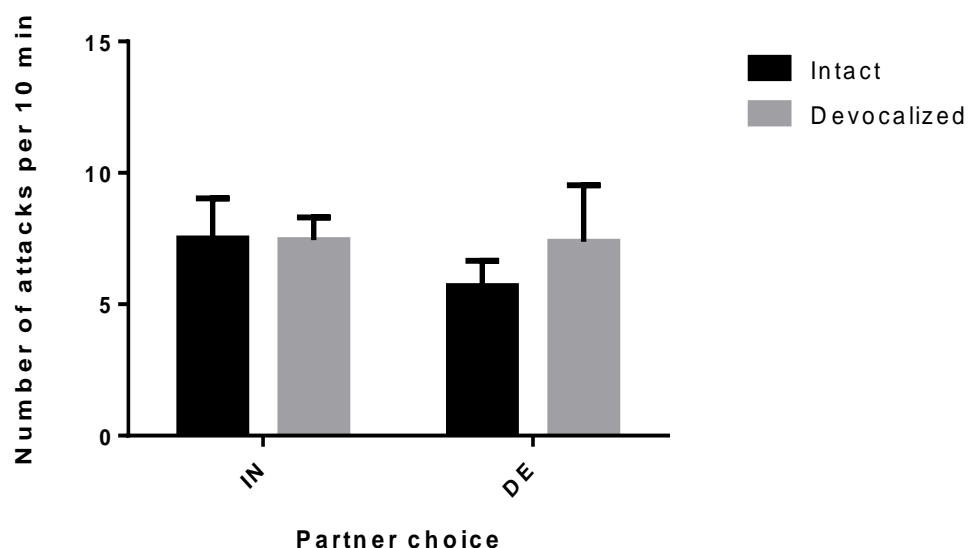


Figure 2.4. Mean and SE are shown for the frequency of playful attacks, involving multiple partners, initiated on either a devocalized or intact an intact partner.

2.5. Discussion

The aim of this study was to test the hypothesis that 50 kHz ultrasonic vocalizations function as play signals, acting to promote and maintain playful interactions in juvenile rats. Even though only one of the three predictions was supported, the data as a whole suggest that USVs are functioning as play signals. How they do so, however, may be more complex than traditional theories of play signals suggest.

Visual and vocal play signals (e.g., Bekoff, 1995; Kipper & Todt, 2002) are more likely to be emitted prior to the initiation of an attack than following an attack, suggesting they are emitted to signal to one's partner that playful contact is about to be made (Bekoff, 1975). Similarly, rats emit 50 kHz calls more frequently prior to playful contact

than following termination of playful contact (Himmler et al., 2014), the same pattern shown in the present study. However, in the present study, it was found that such pre-contact calling was just as frequent when a devocalized rat was the attacker as when an intact was. Thus, prediction 1, that the 50 kHz calls are used, as are other play signals, to telegraph imminent contact by the attacker, was not supported. It is still possible that these vocalizations are acting as play signals, but in this case, as enticements by one animal to solicit attack from another. Similar enticements have been reported in the visual domain. For example, rolling over, somersaulting or pirouetting in front of a potential partner have all been shown to increase the likelihood of the partner to launch a playful attack (LeResche, 1976; Palagi, 2008; Pellis et al., 2014). Therefore, the present results support the possibility that the 50 kHz calls may have multiple functions (Himmler et al., 2014), as indicated by studies of other play signals (Pellis & Pellis, 1996, 2011; Pellis et al., 2011).

Direct support for the hypothesis that 50 kHz calls are used to facilitate play fighting in rats is provided by the results from prediction 2. While most of the non-social behavior of devocalized pairs of rats did not differ from the controls, the devocalized rats launched fewer playful attacks compared to the sham-controls, were more likely to respond to an attack, and when they did defend, were less likely to evade and more likely to maintain contact by rotating to supine (i.e., complete rotation tactic). That is, the frequency and some aspects of the patterns of play were changed when both partners failed to emit 50 kHz calls. The increased defensive responsiveness may not be a unique

change restricted to when they played, as they were also more likely to show a startle response when contacted by the partner in a non-playful manner. It seems likely that this hyper-defensiveness arises from the absence of vocalizations that would normally alert the rat that another rat is approaching. This hyper-defensiveness, however, did not lead to an increased avoidance of the play partner - quite the opposite - play was more likely to involve close-quarter wrestling.

The reduced frequency of launching playful attacks and the increased probability of using complete rotations for defense seem to suggest opposing influences. Typically, playful attacks and playful defense can vary independently of one another (Pellis & Pellis, 1991; Reinhart, McIntyre & Pellis, 2004), with an increased frequency of launching attacks signifying an increased motivation to play (Thor & Holloway, 1983, 1985). With regard to tactics of defense, increased evasion often indicates a reduced motivation to play (Varlinskaya et al., 1999), whereas an increased use of complete rotations suggest an increased motivation to play (Pellis, Field, Smith & Pellis, 1997). Thus, the decreased frequency of launching attacks and the increased likelihood of using complete rotations, indicate a decrease and an increase in playful motivation. However, another change in the play of devocalized rats may clarify this seeming discordance. High rates of playful attack are associated with high rates of counterattack (Pellis & Pellis, 1990), indicating that both are correlated with an increased motivation to play. Since successful counterattacks lead to role reversals (Pellis et al., 2005), the significant reduction of role reversals in the devocalized pairs suggests a reduced motivation for play

in the devocalized pairs. This leaves the increased use of complete rotation and reduced use of evasion by the devocalized rats requiring a different explanation.

The absence of calls may make it more difficult for the rats to rapidly gain, lose and regain contact that is so typical of play fighting (Poole & Fish, 1976). Without the calls, the rats may shift to adopting tactics that maintain contact. In this way, at least some of the calls may function as contact calls. This is supported by the higher frequency of calls by the vocal partner preceding attack by the devocalized partner (see data from prediction 1). Indeed, from casual observation when scoring the videotaped sequences, it did appear that attack and defense were slower and the rats, once in contact, seemingly more lethargic. These observations need to be confirmed by detailed analysis, but overall, the changed pattern of play in the devocalized rats-suggest that the USV calls may have multiple functions.

Given the familiarity with the test enclosure and of the play partner therein, it is possible that in the absence of a choice, the available rat suffices. In this way, dyads of intact and devocalized rats played together. However, when given a choice, if 50 kHz calls are play signals, then a vocalizing partner should be more attractive than a non-vocalizing partner (prediction 3). Contrary to this predicted pattern, the data showed that the non-vocal rats were just as attractive as ones that can vocalize. While these findings suggest that 50 kHz calls are neither necessary to signal contact by the attacker nor necessary to entice contact by the receiver in playful interactions, there is an important *caveat* that must be borne in mind. The quads of rats had been living together for about

10 days prior to testing, with two members of each quad being mute for most of that time. Therefore, the subjects were familiar with one another and so may have compensated for the lack of vocalizations from some of their cage mates. Given that rats have been shown to be attracted to 50 kHz calls (Willadsen et al., 2014; Wöhr et al., 2008; Wöhr & Schwarting, 2009; Wöhr & Schwarting, 2012), a more convincing test would be to use juveniles that were unfamiliar with one another. In this case, an unfamiliar vocal rat may be more attractive as a play partner than an unfamiliar mute one. Nevertheless, the present findings clearly show that the devocalized rats are not aversive targets to other rats, as they are just as likely to be selected as play mates as intact rats. This supports the conclusion that the changes in the frequency and pattern of play in the devocalized rats result from the effects of the absence of USV calls on the motivation to play and some possible essential signaling between pair mates.

Thus, while not all predictions were supported, the data from predictions 1 and 2 both provide evidence that 50 kHz calls are used in a manner that promotes the occurrence of play. However, only some of the calling may serve the traditional “I am about to attack you” function of play signals (Bekoff, 1975). Given the complex array of 50 kHz calls (Wright et al., 2010), the contextually patterned use of the calls during play fighting (Himmler et al., 2014) and the results from predictions 1 and 2 from the present study, it seems likely that these calls have multiple functions. These may include signaling imminent attack, soliciting attack, signaling withdrawal, maintaining contact,

coordinating reciprocal exchanges and simply ensuring the maintenance of a positive mood within which play is likely to occur and continue.

The findings from the present study appear to be consistent with other studies associating 50 kHz calls with both communication and play. Rats are attracted to 50 kHz calls, but in these demonstrations, the rat is typically separated from other rats, with the cue for the presence of a potential partner only offered by the occurrence of 50kHz calls, calls that are associated with positive affective states (Burgdorf et al., 2008; Knutson et al., 1998; Knutson et al., 2002; Willadsen et al., 2014; Wöhr et al., 2008; Wöhr & Schwarting, 2009). So, while these calls are shown to be attractive, that does not necessarily mean that the calls are emitted so as to signal to the other rat. Indeed, a recent study found that, while low-dose injections of ethanol in juvenile rats increases the attractiveness of a conspecific peer across a partition, the increases in emitted 50 kHz calls is not correlated with a physical approach towards the barrier (Willey & Spear, 2013). But rats also emit more 50 kHz calls when they are anticipating play (Knutson et al., 1998) and emit more calls when engaging in high frequencies of play (Burgdorf et al., 2008). The results from the present study add to these findings linking 50 kHz calls and play by providing support for the hypothesis that 50 kHz USVs are emitted to promote and maintain playful interactions in juvenile rats.

2.5.1. The importance of 50 kHz calls in maintaining play

Our data show that 50 kHz calls are necessary to facilitate play, perhaps by maintaining a positive affective state, and also show that they are not necessarily used to telegraph playful contact as do traditionally conceived play signals (Bekoff, 1975). However, these calls may still serve a valuable communicatory function in some circumstances. Three hypotheses seem to be particularly worth considering. First, the use of 50 kHz calls as play signals may be most critical in situations of ambiguity. Ambiguity may arise when the partner behaves in an unexpected way or if the play escalates to a more aggressive form. In the juvenile period, rats are just as motivated to play with a novel partner as with a familiar partner (Ikemoto & Panksepp, 1992; Panksepp, 1981; Panksepp & Beatty, 1980). With the onset of puberty, especially for males, when dominance relationships develop (Pellis & Pellis, 1992; Takahashi & Lore, 1983), play can become rougher, especially when unfamiliar males play together (Smith et al., 1999). In such situations, vocal signals may be important in avoiding escalation to serious fighting. When an older or more dominant animal attempts to solicit play from a younger or subordinate animal, the initiator often exaggerates the frequency or form of play signals (Bekoff, 1974; Cheney, 1978; Palagi, 2008). As 22-kHz calls have been shown to be correlated with submissive behavior (Portavella et al., 1993; Sales, 1972), likely functioning to inhibit further attacks (Lore et al., 1976; Sales, 1972), 50 kHz calls may be used among older rats to ensure that playful interactions remain playful. Our finding that devocalized pairs of rats playing together tend to be hyper-defensive is consistent with the possibility that vocalizations may attenuate moments of ambiguity in

playful interactions. Such ambiguity may be exacerbated among adults in which the risk of escalation to serious fighting is greater.

A second hypothesis for the role of 50 kHz calls is that they are not used as play signals, but rather, as contact calls, which facilitate locating partners. Contact calls are used by many troop living primates to coordinate their movements when visual access is obscured. In squirrel monkeys (*Saimiri*), the juveniles emit calls when playing that attract the attention of the adults, who likely use these as cues to monitor the environment for potential predators (Stevenson & Poole, 1982). Similarly, lion-tailed macaques (*Macaca silenus*) live in dense arboreal forests in which visual signals are not particularly useful, rather, vocalizations are used to locate and communicate with other troop members (Hohmann & Herzog, 1985). As noted above, rats are nocturnal animals and do most of their playing in the dark, in which visual signals are likely not very useful. In this regard, 50 kHz vocalizations in the wild may be useful to monitor each other's location when groups of rats are playing. In the enclosure sizes typically used in laboratory settings, contact calls may be of less functional value. Previous studies, however, have demonstrated that, when rats are separated from their cage mates, the rats remaining in the home cage increase their rate of emitting 50 kHz calls, suggesting that they are trying to re-establish contact with their partner (Wöhr et al., 2008). Willadsen et al. (2014) also provided further evidence for the attractive nature of the 50 kHz calls by showing that adult females are more likely to display high levels of social approach behavior in response to males emitting 50 kHz USV calls, but not to time- and amplitude- matched

white noise. These findings suggest that these vocalizations serve important roles in establishing and maintaining social proximity.

A third possible hypothesis for the role of 50 kHz vocalizations is not as a specific play signal, but rather, as a self-regulating signal, the performance of which maintains the playful mood. In the study by Siviy and Panksepp (1987) in which rats were deafened, it was shown that there was no decrease in the amount of play by the pairs of deaf rats. Although the animals were no longer able to hear, they could still vocalize and thus the rhythmic muscular contraction patterns produced when emitting these vocalizations may have still provided proprioceptive feedback to the emitter and so maintained the mood altering effect of producing 50 kHz calls. In other words, the execution of motor patterns used in vocalization may, in and of itself, promote dopamine release. Indeed, the ventral tegmental area (VTA) is activated and dopamine released following the emission of 50 kHz vocalizations (Brudzynski, 2007). A recent study of prenatal exposure to ethanol found that this led to reduced activity of the VTA when the animals reach 4 weeks of age (Choong & Shen, 2004), the age at which play is most frequent (Pellis & Pellis, 1990), thus potentially reducing the rewarding qualities of play and vocalizing. The rats in the present study were devocalized during this apparently critical period when vocalizations become able to stimulate the release of dopamine from the VTA, potentially affecting the rat's ability to self-regulate their affective state through these vocalizations. Similarly, some forms of birdsong appear to be performed solely to influence the performer's affective state, as the production of the vocalizations act to release rewarding

neurotransmitters (Riters, 2011). Thus, while unheard vocalizations in deaf rats may still provide feedback from proprioceptive means as the throat vibrates, this would not be possible for the devocalized rats. Consequently, deafened, but vocal rats, may be better able to sustain the positive affective state to promote play. Using playback of recorded calls may be one possible way to test out this hypothesis.

2.6. Conclusion

That playful interactions do not continue at the same rate in pairs of devocalized rats compared to controls suggests that 50 kHz USVs are necessary as play signals to facilitate play by maintaining playful interactions.

2.7. Acknowledgements:

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Chapter 3

Are 50 kHz Calls used as Play Signals in the Playful Interactions of Rats? III. The Effects of Devocalization on Play with Unfamiliar Partners as Juveniles and as Adults

Abstract

When playing, rats emit 50 kHz calls which may function as play signals. A previous study using devocalized rats provided limited support for the hypothesis that 50 kHz calls are functioning to promote and maintain playful interactions. However, in that study, all pairs were cage mates and familiar with one other, and this may have reduced the importance of their signaling during play. Therefore, in the present study, the play of rats that were unfamiliar to one another, and in which one partner is devocalized, was examined. Four hypotheses by which 50 kHz calls could act to facilitate play were tested: that they could (1) maintain the playful mood of the partner (2) act as contact calls (3) attract partners, and (4) be used tactically to prevent play fighting from escalating to serious fighting. Tested in juveniles, the first three hypotheses were not supported, suggesting that for younger animals, 50 kHz calls are not important in facilitating playful interactions as play signals. The fourth hypothesis, which was tested in adult animals unfamiliar with each other, was supported. Pairs in which one partner was devocalized were more likely to engage in aggression than in pairs in which both animals could vocalize. These findings provide support for the tactical use of the vocalizations in, at least, some contexts.

3.2. Introduction

Play fighting involves competition for some advantage over the partner, such as contacting a particular body target (Aldis, 1975). Because such competition can resemble serious fighting, it has been hypothesized that play signals are used to ensure that this competition does not escalate from playful to serious fighting (Bekoff, 1975; Fagen, 1981). This can be achieved by emitting a play signal before playful contact is made (Bekoff, 1995; Pellis & Pellis, 1997a). Play signals appear to be particularly important when there is potential ambiguity during play, such as when multiple partners are involved or when there is a dominance asymmetry between the partners (e.g., Bekoff, 1995; Fagen, 1981; Palagi, 2008; Palagi & Mancini, 2011; Van Leeuwen et al., 2011; Waller & Dunbar, 2005). While visual signals such as the play-face in primates (Van Hooff, 1967) and the play bow in dogs (Bekoff, 1995), are the ones that are most well-known, examples of play signals occurring in other sensory modalities, such as vocalizations, (Biben & Symmes, 1986; Masataka & Khoda, 1988; Rasa & Anne, 1984; Kipper & Todt, 2002) and chemical cues (e.g., Wilson, 1973), have been reported.

Juvenile rats engage in frequent play fighting with conspecifics (Bolles & Woods, 1964; Thor & Holloway, 1984; Panksepp, 1981) and emit ultrasonic vocalizations as they do so (Knutson et al., 1998). These vocalizations are mostly in the 50 kHz frequency that is associated with a positive affective state (Burgdorf et al., 2008; Knutson et al., 1998). Recent findings suggest that rats are potentially using some of these vocalizations as play signals (Kisko et al., 2014; Himmler et al., 2014). It was found that 50 kHz vocalizations

occur more frequently immediately before playful contact is made than immediately after contact is terminated (Himmler et al., 2014). This is consistent with the view that play signals are used to alert the partner that contact is imminent (Bekoff, 1975, 1995). However when pairs of rats with one devocalized partner and one intact partner were examined, it was found that the same pattern was present whether it was the intact or the devocalized rat that was initiating the attack (Kisko et al., 2014). Given that vocalizations are just as frequent by the rat about to receive an attack as by the rat launching an attack, this suggests that such vocalizations have more than one potential play signaling function. Four possible functions are explored in the present paper.

One possible function is suggested by the reduced amount of play performed by pairs of devocalized rats compared to pairs of intact rats (Kisko et al., 2014). Emitting 50 kHz USVs triggers the release of dopamine (Brudzynski, 2007) and playback of 50 kHz vocalizations stimulates the areas of the brain involved in positive affect and reward (Sadananda et al., 2008). Similarly, it has been shown that some forms of birdsong are self-rewarding (Ritters, 2011). Therefore, it is possible that 50 kHz calls function to maintain the playful mood of the potential play partner, the performer, or both, as has been suggested for some visually mediated play signals (Pellis & Pellis, 2011; Pellis et al., 2011). Interestingly, Siviy and Panksepp (1987) showed that, in the absence of hearing rats had significantly lower frequencies of pinning (i.e., close-quarter wrestling with one rat on top of another, supine rat). If some of the 50 kHz vocalizations are used as emotional self-regulators then the absence of hearing one's own, or a partner's

vocalizations, could explain this decrease in playful motivation and so the reduction in the frequency of pins. That both devocalization and loss of hearing lead to a reduction in markers that indicate playfulness, supports the possibility that the 50 kHz calls help maintain a rat's playful mood. However, in the previous experiment using devocalized rats (Kisko et al., 2014), there was a confound in that the pairs of devocalized rats were living together for over a week and so the effects of lack of vocalizations could have produced a chronic depression for playful motivation rather than an acute failure to motivate play. Given that juvenile rats readily play with unfamiliar juveniles (Varlinskaya et al., 1999; Panksepp, 1981), in the present study, devocalized juvenile rats were introduced to unfamiliar intact playmates. If vocalizations are necessary to promote a playful mood in the play partner, then an intact rat should play less with an unfamiliar devocalized rat compared to an unfamiliar intact partner (prediction 1).

A second hypothesis is derived from the finding that devocalized rats are more likely to use contact promoting supine defensive tactics and less likely to use evasive tactics that lead to a loss of contact (Kisko et al., 2014). Rats have brief bouts of wrestling contact interspersed with rapid withdrawal, chasing and renewed contact (Pellis & Pellis, 1983, 1987; Poole & Fish, 1975, 1976). Increasing the frequency of rotating to supine diminishes approach and withdrawal and increases the time that the animals remain in physical contact. The increase in supine defense by devocalized rats may reflect a reluctance to move too far away from a devocalized partner (Kisko et al., 2014), which suggests that some of the 50 kHz calls function as contact calls. In several primate

species contact calls are used to localize group members (Schrader & Todt, 1993; Caine & Stevens, 1990; Boinski, 1991). Such calling in rats, would facilitate the animals being able to locate one another, quickly, after withdrawal. This possibility is supported by the findings that, in non-playful contexts such calls attract approach by other rats (Wöhr et al., 2009; Wöhr & Schwarting, 2013). However, there was a potential confound in the previous study showing altered patterns of defense in devocalized rats (Kisko et al., 2014). The devocalized rats were tested with other devocalized rats, making it unclear whether the change in the use of defensive tactics was due to a reduction in the rats' ability to locate one another or because devocalization simply changed their pattern of attack or defense. Therefore, in the present study, a focal intact rat was tested with an unfamiliar devocalized and unfamiliar vocal partner. If 50 kHz calls are acting as a contact call to localize the play partner then the intact focal rat should be less likely to use tactics of evasion and more likely to use the supine defense tactic when paired with a devocalized rat than with an intact rat. Conversely, if the changed pattern of defense in devocalized rats previously found were due to the need to maintain contact with a non-vocal partner, then with a vocal partner, the use of defense tactics should not differ to those of a vocal rat playing with another vocal rat (prediction 2).

A third possible function is derived from the findings that when given the option, rats prefer to socialize with partners that emit high frequencies of 50 kHz calls (Wöhr et al., 2008; Wöhr & Schwarting, 2009), and that in playback experiments, rats will spend more time around the speaker emitting 50 kHz calls (Burgdorf et al., 2008; Sadananda et al.,

2008). This would suggest that one way 50 kHz calls can promote play is by simply attracting approach by potential partners, rather than being used to locate partners as in the previous hypothesis. However, our previous findings, that in quads of two devocalized and two vocal animals there was no difference in preference for partner (Kisko et al., 2014), did not support this hypothesis. However, there was a confound in the previous study, in that for about 10 days prior to testing, all members of the quad were cagemates and had been living together. Familiarity could have mitigated the effect of the differential attractiveness of the vocalizations. Therefore, in the present study, three rats (one devocalized, two intact) that had not been living together were tested together. If 50 kHz calls promote play by attracting social partners, then it was predicted that the focal intact rat should initiate more play with the intact than the devocalized partner (prediction 3).

The finding that devocalized rats can play and that their play does not escalate to serious fighting (Kisko et al., 2014), suggests another possible hypothesis for the role of 50 kHz calls. Juvenile rats are highly motivated to engage in play (Holloway & Sutter, 2004; Varlinskaya et al., 1999) and they just as readily play with unfamiliar (Panksepp, 1981) as familiar (Pellis & Pellis, 1990) partners. Even in test paradigms in which an unfamiliar male peer is introduced into the home cage of the test subject, encounters are playful, with the resident only exhibiting aggression with the onset of sexual maturity (Takahashi, 1986). The play of juveniles may be such that the 50 kHz calls are limited to facilitating functions (see previous hypotheses) rather than being critical for tactical

deployment to reduce the risk of escalation. Conversely, this conclusion leads to our fourth hypothesis, that this tactical use of vocalizations may be important in adulthood, where playful interactions are present (Pellis & Pellis, 1990) but tend to be rougher (Foroud & Pellis, 2003). Indeed, when adult male rats are confronted by an unfamiliar male in a neutral arena, they play, but do so more roughly than with familiar partners in their home cage. The effect of this rough play is to provide a means with which to establish a dominance relationship, which once established, leads to gentler play. However, when play fails to establish a dominance relationship, the encounter can escalate to serious fighting (Smith et al., 1999). The 50 kHz vocalizations, or other ultrasonic calls, may be used tactically in such a situation to reduce the risk of escalation. If this is the case, then in pairs of unfamiliar adult male rats in which one is devocalized, the interaction should be more likely to become aggressive than in unfamiliar pairs in which both can vocalize (prediction 4).

3.2. Methods and Materials

3.3.1 Subjects:

Twenty-four male Long Evans rats were used. The rats were obtained from Charles River Laboratories (St. Constant, Quebec) around 23 days of age and housed in the vivarium at the Canadian Centre for Behavioral Neuroscience (CCBN). As juveniles, they were housed in quads and as adults they were housed in pairs. Food and water were available *ad libitum* and the animals were on a 12-hour light-dark schedule, lights off at

1930. The rats were maintained at a constant temperature of around 21-23°C and were housed in polyethylene cages 46cm×25cm×20cm with processed corncob as bedding. Devocalization surgeries were performed when the rats were between 28-30 days of age, with three of the quads having two members devocalized and 2 intact cage mates. The rats remained in their respective quads after surgeries. Testing for play occurred when they were between 35-40 days old, within the age range at which juveniles are most playful (Pellis and Pellis, 1990; Thor & Holloway, 1984). The rats were tested again for play behavior between 85-90 days of age.

3.3.2. Apparatus:

Testing took place in a 50cmx50cmx50cm clear Plexiglas enclosure which was placed into a sound proof box (inside measurements: 59cm×65.5cm×81.5cm). The inside of the play box was filled with approximately 1-2cm of CareFresh® bedding, as this bedding emits less background noise when walked on by the rats during recording compared to standard corncob bedding. The sound proof box was fitted with sound-attenuating foam (Primeacoustic, Port Coquitlam, British Columbia) and an ultrasonic microphone placed approximately 44cm from the bottom of the play box (Model 4939, Brüel & Kjaer, Denmark), connected to a Soundconnect™ amplifier (Listen, Inc, Boston, MA). Vocalizations were recorded at a sample rate of 195,313 Hz using 16-bit resolution via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, FL) which recorded frequency ranges from 4-100 kHz and was controlled via custom software written in Matlab (the Mathworks, Natick, MA). All audio files were converted

to .wav files. A DVD103 Sony Handycam with night-shot capacity was used for filming playful interactions and was placed against a small window opening into the box at a 45° angle, which is the optimal angle from which to analyze playful tactics (Pellis & Pellis, 1987). Off-line analysis of vocalizations is performed using Raven Pro 1.4 software (developed and distributed through Cornell University, Ithaca, NY) for audio files and Avidemux 2.6 software for video files.

3.3.3. Surgery:

Six rats were devocalized, two each from three quads, under isofluorane anesthesia. Devocalization surgery involved bilateral sectioning of the recurrent laryngeal nerves (Snoeren & Amgo, 2013). A 3 cm incision was made on the ventral surface of the neck and the sternohyoideus muscle was separated to expose the trachea. After locating the recurrent laryngeal nerves, they were freed from the surrounding fascia and a 3 mm section was removed bilaterally. The muscle was then loosely sutured in place and the skin was closed with subcutaneous sutures. Sham animals had an incision made on the ventral surface of the neck and then had the skin closed with subcutaneous sutures. Buprenorphine was administered at the time of surgery (0.05mg/kg *s.c*) and again every 12h for three successive days. Four days were allowed for recovery after surgery. On the fifth day, the rats were tested in the soundproof box to ensure that they were unable to emit ultrasonic vocalizations. This was confirmed – none of the surgically devocalized rats emitted USVs.

3.3.4. Testing Procedures:

Once the success of the devocalization was confirmed, the rats were habituated with their cage mates (i.e., in quads) to the testing enclosure for 30 min a day for three days prior to the experimental test being conducted. A standardized testing and scoring paradigm was used to evaluate the play (Himmler et al., 2013). Because vocalizations and play behavior were recorded on separate devices, the two types of recordings needed to be synchronized. To do this, a device emitting a synchronized light flashes and audible beeps was used. At the beginning of each trial, three consecutive flash/beep signals were given and trials were considered to have begun with the final beep (Himmler, et al., 2014)

Pairs of rats were tested following 24-hours of social isolation to increase their playfulness when introduced in the experimental enclosure (Panksepp & Beatty, 1980; Pellis & Pellis, 1990). For testing as juveniles, two intact rats from each of the three quads containing only intact rats were tested twice, once with a devocalized-unfamiliar partner from another quad and once with an intact-unfamiliar partner from another quad. Trials were counterbalanced to avoid any order or age effect (Pellis & Pellis, 1990). After the final trial involving dyads, each quad was given another 24 h of social isolation and then a rat from each quad was placed in the test enclosure with a devocalized rat and an intact rat. All three of these rats were unfamiliar with one another.

Once the testing for the juvenile age was completed, each quad was divided into pairs, with each pair being composed of two intact partners or two devocalized partners. When they reached the appropriate age in early adulthood (see above), they were once again habituated to the test enclosure, and then given 24 h isolation prior to testing (see Himmler

et al., 2013). One intact rat from each of the six intact pairs was tested twice, once with an unfamiliar intact partner and once with an unfamiliar devocalized partner. Again, this testing was counterbalanced to avoid an order effect.

3.3.5. Behavioral analyses:

Videotaped sequences were evaluated at normal speed, slow motion and frame-by-frame to manually code a variety of measures related to playful attack and defense (Himmler et al., 2013). When the snout of one rat was either in contact or moved towards the nape of the other rat, a playful attack was scored. The recipient of a nape attack can either defend itself against such contact or continue with its ongoing behavior and so seemingly not respond to the attack. The probability of defense is calculated as the number of times a rat defends divided by the total number of times it is attacked. When a rat moves to defend against contact with its nape, there are two major categories of tactics: (a) evasion, in which the defender moves away from the attacker and does so by running, leaping, or swerving away and thus ends up facing away from the partner, and (b) facing defense, in which the defender moves its nape away from the attacker by turning to face them and, in doing so, blocks access to its own nape by putting its teeth in between itself and its partner's nape. There are three forms a facing defense can take: (i) complete rotation, in which the defender rolls completely over onto its back, (ii) partial rotation, in which the defender's forequarters are rotated, but maintains contact with the ground with one or both of its hind feet, and (iii) other, in which defensive actions involve rotations or other movements in other dimensions (e.g., rotating vertically in a horizontal plane).

To determine whether the trials involving unfamiliar adults led to aggression, two measurements were used. First, interactions in which the initiating attack was to the nape (play) versus the rump or face (agonistic) (Blanchard et al., 1977; Pellis & Pellis, 1987) were scored for each pair. Second, the occurrence of behavioral markers that are associated with aggression, such as pilo-erection, lateral displays, tail wagging and biting that occurred during the trial as a whole, were scored (Smith et al., 1999). For the latter, the numerical frequency of any and of all these markers occurring during the trial was combined into an overall index of aggressiveness.

3.3.6. Statistical analyses

The data from prediction one, two, three and four were analyzed using a 2-tailed paired Student's t-test, comparing the means of attacks on the unfamiliar devocalized to the attacks on the unfamiliar intact from the experimental vocal animals. A 2-tailed unpaired Student's t-test was also used for prediction two to compare the means from the attacks on the experimental vocal animal from the unfamiliar devocalized animals to the unfamiliar intact animals. For all data, differences were considered significant for p values ≤ 0.05 . For graphical and tabular representation of the data, values are given for group means with the standard error of the mean (SEM).

3.4. Results

Prediction 1: Devocalization will reduce the frequency of play.

If vocalizations promote a playful mood, an intact rat should initiate less play when paired with a devocalized rat than with an intact partner. Contrary to this prediction, there

were significantly more playful attacks launched against devocalized partners than intact ones ($t(5) = 2.789, p < 0.05$) (Fig 3.1A). Consistent with the previous studies findings devocalized rats were significantly more likely to defend themselves when attacked than were the intact rats ($t(5) = 2.591, p < 0.05$) (Fig 3.1B)

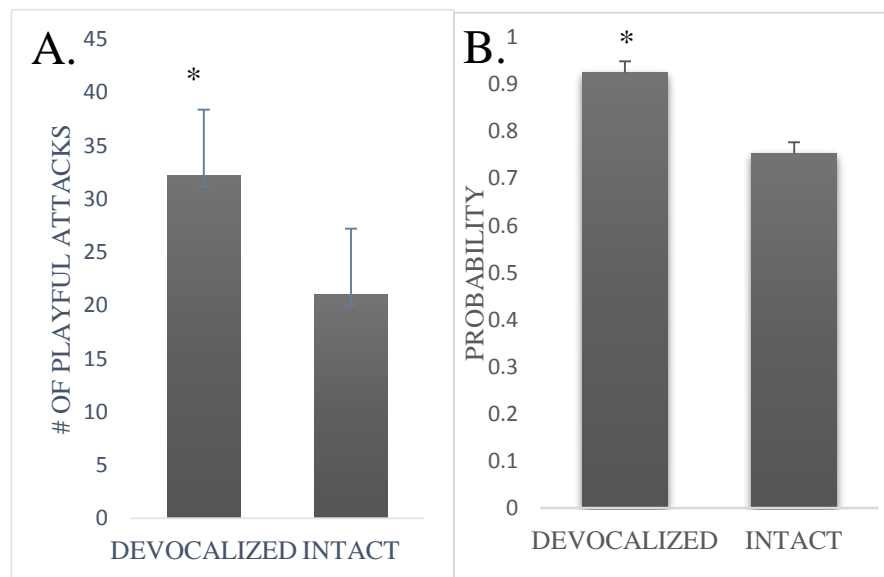


Figure 3.1. The mean frequency of playful attacks launched in the 10 min trials by the intact, focal partner when playing in dyads with either an unfamiliar devocalized partner or an unfamiliar, intact partner (A). The probability that a playful nape attack is responded to defensively is shown for the devocalized and intact partners of the focal intact rats (B). The mean and SE is shown for each group in this figure and all subsequent ones.

Prediction 2: Devocalization will increase the tendency to maintain physical contact.

If 50 kHz calls are acting as contact calls, providing critical information about the location of a play partner, then devocalizing one member of a pair should make the other member more prone to maintain physical contact. This would allow the partner to

compensate for the missing proximity cues by using touch. Conversely, the devocalized rats should not differ from the intact partners of the intact focal rats. The data did not support these predictions. Whether attacked by the devocalized or intact partner, the intact focal rat did not differ significantly ($p > 0.05$) in the likelihood of evading (mean \pm SE: 0.47 ± 0.04 (with devocalized partner) versus 0.43 ± 0.07 (with intact partner)) or rotating to supine (0.07 ± 0.04 (with devocalized partner) versus 0.20 ± 0.06 (with intact partner))

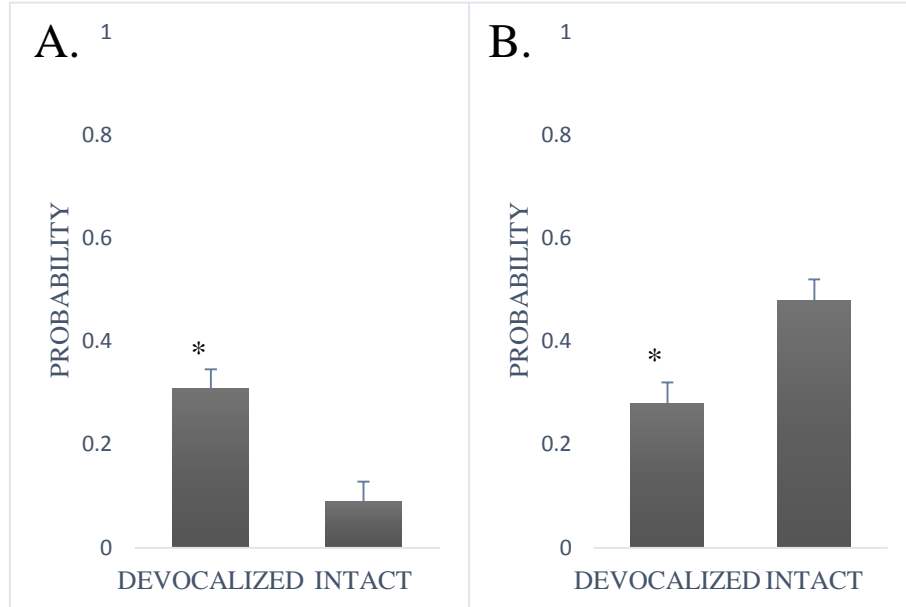


Figure 3.2. The probability of using the complete rotation tactic (A) and evasive defense (B) is shown for the devocalized and intact partners of the focal intact rats.

In contrast, the devocalized and intact partners of the intact focal rats differed in their patterns of defense. The devocalized rats were significantly more likely to use the supine defense tactic ($t(5) = 3.310, p < 0.05$) (Fig. 3.2A) and significantly less likely to use evasive tactics ($t(5) = 2.820, p < 0.05$) (Fig. 3.2B).

Prediction 3: Rats should play preferentially with vocalizing versus silent partners.

If 50 kHz calls promote play by attracting social partners then the focal intact rat should initiate more play with the intact than the devocalized partner when both partners are simultaneously available. Contrary to this prediction, when young rats were placed in triads there was no significant difference ($p > 0.05$) in the degree to which the focal animal directed playful attacks toward vocal over devocalized rats (Fig 3.3).

Prediction 4: In adults, devocalization should increase aggression

If 50 kHz calls are used tactically in ambiguous situations to reduce the risk of escalation to aggression then in pairs of unfamiliar adults in which one is devocalized the interaction should be more likely to become aggressive. Counting playful interactions (indicated by an attack focused on the nape of the neck), the frequency of attacks in pairs containing a devocalized partner was higher than in pairs in which both partners were

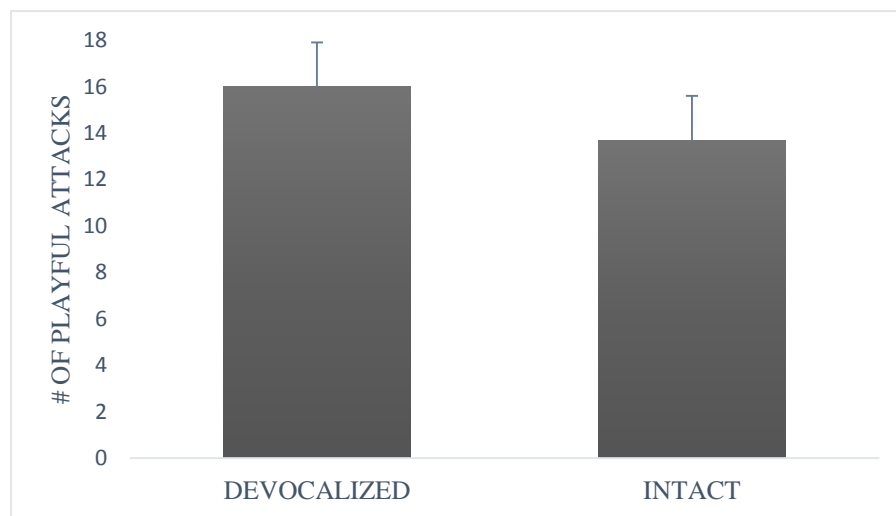


Figure 3.3. The mean frequency of playful attacks launched in the 10 min trials by the intact focal partner when playing in triads containing both an unfamiliar devocalized partner and an unfamiliar intact partner

vocal (Figure 3.4A), but the difference was not significant ($p < 0.05$). However, for aggressive attacks (i.e., those directed towards the face or hind-quarters) the frequency of agonistic attacks being initiated was significantly higher in the pairs containing one devocalized partner ($t(5) = 4.421, p < 0.01$; Figure 3.4B). Overall there was a significantly higher frequency of aggression-related behaviors in pairs of rats in which one partner was devocalized ($t(5) = 7.70, p < 0.001$; Figure 3.4C).

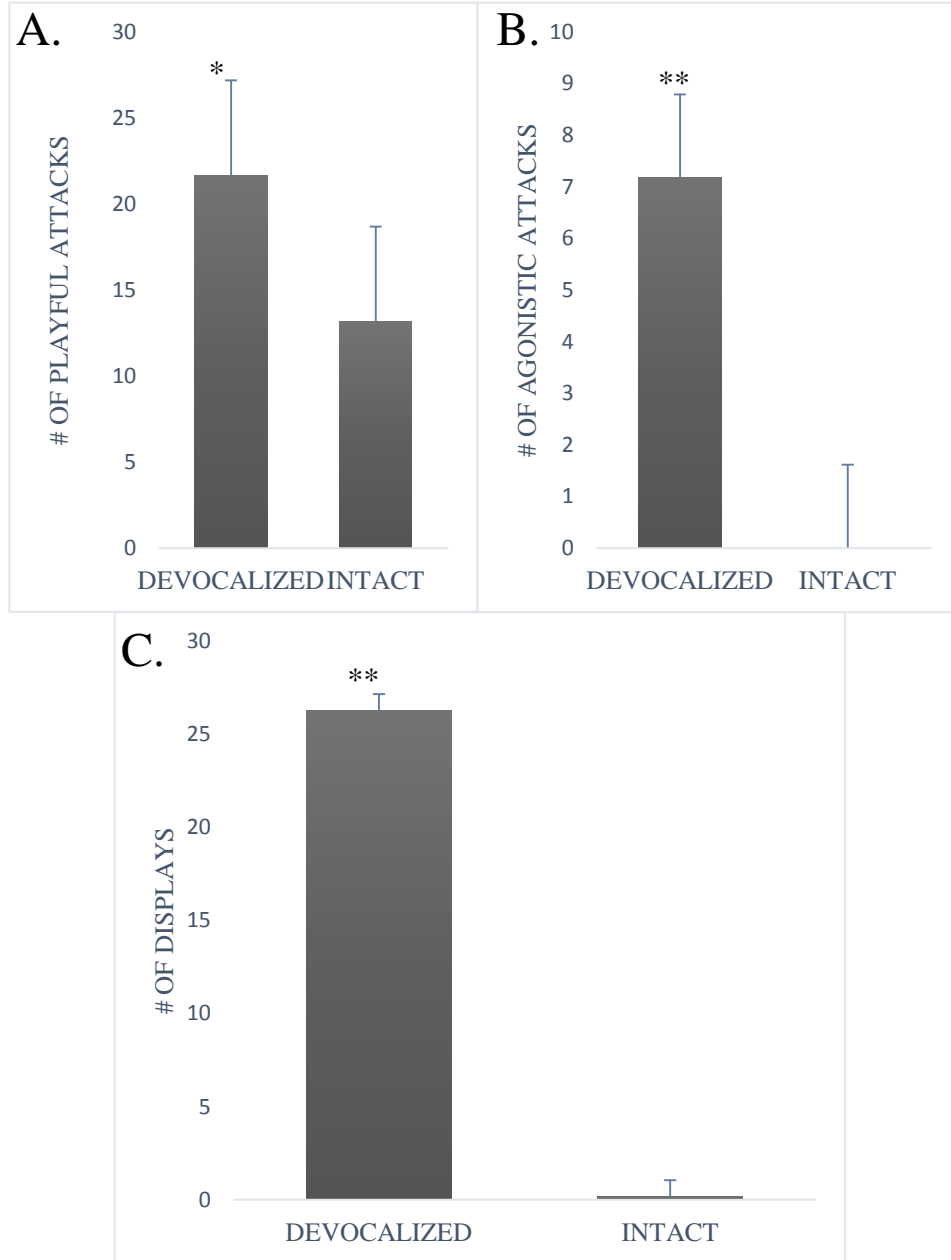


Figure 3.4. Data are shown for pairs of adult males in a neutral test enclosure that are unfamiliar with one another. The frequency of playful attacks (A), agonistic attacks (B) and the performance of agonistic displays (C) in the 10 min trials are shown for pairs in which one partner was devocalized compared to when both partners could vocalize.

3.5. Discussion

The aim of this study was to test four hypotheses for how 50 kHz vocalizations may function as play facilitating signals. Only one of the four hypotheses was supported by the results. Playful interactions in adult male rats are less likely to escalate to serious aggression if both partners can vocalize, suggesting that the vocalizations are used tactically to mitigate the risk of such escalation. Our data fail to support the hypotheses that 50 kHz calls facilitate play by enhancing mood or enticing approach. In fact, in the present results, juvenile intact rats made more playful attacks when paired with a devocalized partner than an intact one. Further, our data do not support the idea that 50 kHz calls are used as contact calls (i.e., to reassure a partner about one's presence). We had predicted that the lack of such a contact call would cause rats to seek physical contact more. Specifically, we predicted that a defending rat would be more likely to choose a defensive strategy leading to maintained contact if playing with a devocalized partner. In fact, defensive strategies were no different when a rat was attacked by an intact or devocalized partner. However, some of the unexpected findings from these tests point to ways in which these calls may contribute to playful interactions and to the development of the brain mechanisms associated with social reward.

In pairs of devocalized, juvenile rats the frequency of launching playful attacks is decreased by 50 percent (Kisko et al., 2014), suggesting that vocalizations are necessary in order to maintain a playful mood. However, in the present study using unfamiliar juvenile rats, it was found that when a devocalized partner was paired with a vocal partner, the

number of playful attacks initiated by the intact partner actually increased. Thus, prediction 1, that in pairs of rats that are unfamiliar with one another, the intact rat should initiate less play when the partner is devocalized as compared to when vocal, was not supported. Given that both emitting (Brudzynski, 2007) and hearing (Sadananda et al., 2008) 50 kHz calls activates the brain systems associated with reward, it is possible that the lone vocal partner may produce sufficient calls to overcome the absence of calling from the devocalized partner. In this way, both partners remain motivated to engage in play. If this is so, then it is possible that the vocal rat compensates for the lack of calling from the devocalized partner by increasing its own calling rate. Consistent with this possibility is the finding that the frequency of calling when an intact rat attacks a devocalized partner is the same as when it attacks an intact one (Kisko et al., 2014), and that when confronted with an anesthetized partner, an awake rat increases the frequency of 50 kHz calls that it emits (Blanchard et al., 1993).

Owren and Rendall (1997, 2001) hypothesize that the primary function of vocalizations in animals is not to convey information about the emotional state of the caller, but instead, to influence the emotional state of the receiver. Indeed, several studies have shown that the physiological and cognitive state of an animal can be altered by hearing species-specific vocalizations (Kuroka & Nakamura, 2010; Gil-da-Costa et al., 2004). In the present study, the lack of vocalizations in one rat would therefore not dampen playful motivation, because the partner can influence the devocalized rats' emotional state through their own calls. These calls may increase motivation in the receiving rat via modulation of

dopaminergic pathways (Burdorf et al., 2007), thus making them more playful. These affective influences of vocalizations produced by at least one partner may account for why the playful motivation of an intact partner is not eroded by playing with a devocalized one, but this does not account for why the focal, intact rat initiated more playful attacks with the devocalized partner.

One potential explanation for this is that devocalized rats are more likely to defend themselves and are more likely to use tactics that maintain physical contact with the partner (Kisko et al., 2014; present study), and so they are more attractive play partners. As both playing (Trezza et al., 2010) as well as producing and hearing 50 kHz calls (Brudzynski, 2007; Sadananda et al., 2008) enhances activity in reward-related brain areas, it is possible that the increased opportunity for physical contact afforded by the devocalized rat compensates for, or even exceeds, the reward of interacting with intact partners. Thus, the changed defense by the devocalized partner and the increase in emission of calling by the intact partner, leads to levels of play in pairs of rats containing an intact and a devocalized rat that are higher than in pairs of intact rats (present study) and in pairs of devocalized rats (Kisko et al., 2014). The reaching of threshold levels of stimulation may also account for the absence of partner preferences when rats are given multiple partners, including devocalized ones, with whom to play. When multiple partners are available playful motivation is increased (Pellis & McKenna, 1992) and so the overall activation of reward systems may reach a level in which play can be directed indiscriminately to both vocal and non-vocal partners.

Given that devocalized rats use contact promoting patterns of playful defense more frequently whether attacked by familiar intact or devocalized partners (Kisko et al., 2014) or unfamiliar intact rats (present study), the question arises as to why devocalization induces this change in defense. A common outcome for playful interactions is that one rat ends up standing over its supine partner (i.e., pinning) (Panksepp, 1981), and a frequent way in which that configuration is achieved is for the defending rat to rotate to supine when its nape is contacted (Pellis & Pellis, 1987). In deafened animals, pinning is reduced (Siviy & Panksepp, 1987) suggesting that vocalizations facilitate supine defenses. The present data suggest just the opposite. Rats playing with devocalized partners were no more likely to use a supine defense than when attacked by an intact, vocal partner. Further, rats unable to vocalize were more likely to adopt a supine defense. These opposing findings suggest that the inability to vocalize and inability to hear must be changing how the rats defend themselves via different mechanisms. Septal and amygdala lesions in rats alter patterns of agonistic defense, increase freezing (immobility) and enhance reactivity to dorsal stimulation (Blanchard et al., 1979), and in cats, rhinencephalic lesions, which included the amygdala, produced a marked tendency to increase physical contact (Schreiner & Kling, 1953). These lesion studies indicate that brain areas connected with the brain's reward systems can alter patterns of social contact. In rats, the ventral tegmental area's (VTA's) dopaminergic connections develop within the first four weeks after birth (Choong & Shen, 2004), overlapping with the age at which the rats in the present study received devocalization surgeries. The absence of vocalizations could interfere with the

development of reward pathways, and thus, indirectly affect the motivation to play and what behaviors are performed during play. Indeed, damage to the amygdala reduces the frequency of play (Meaney et al., 1981) and alters the patterns of defense used during play (Pellis et al., 2010). In sum, the abnormalities in defense exhibited by devocalized animals may be due to developmental abnormalities in the wiring necessary to play in the typical manner.

By being more likely to defend itself and more likely to defend in a way to promote physical contact, the devocalized rats may be actively compensating for the lack of reward they would normally receive by emitting calls. Physical contact is one mechanism for increasing reinforcement. Numerous studies have shown that rats find tickling by a human hand to be a highly rewarding, prompting them to emit high frequencies of 50 kHz calls (Hori et al., 2013; Panksepp, 2007; Panksepp & Burgdorf, 2000). Thus, by being more likely to defend in a way that promotes physical contact, the devocalized rats may be boosting the rewarding properties of play, and by doing so also increasing their attractiveness as play partners. The latter would account for the increased frequency of playful attacks directed at devocalized partners. In sum, the changes in play arising in juvenile rats from the devocalization may be due to compensation for the inability to self-stimulate via vocal feedback or, alternatively, to developmental changes in reward pathways.

While vocalizations are not critical in juvenile play, they appear to play an important role in helping unfamiliar adults avoid aggressive encounters. As already noted, pairs of

unfamiliar, adult male rats encountering one another in a neutral arena play more roughly with one another than with cage mates (Smith et al., 1999), and the present findings show that, in such a context, if one partner is devocalized, the encounters are more likely to escalate to serious aggression. However, it does not seem that it is the lack of vocalizing *per se* that is critical. As part of the general animal husbandry protocols at CCBN, all rats are checked regularly, both by the animal care staff and the experimenter, to make sure that they are not in ill health. The adult pairs of devocalized rats living together were never seen to engage in serious fighting when in their home cages and none exhibited any evidence of fighting-induced damage (e.g., lesions on the skin of the rump or face – see Blanchard & Blanchard, 1977; Blanchard et al., 1985). Apparently, rats interacting with their home-cage mates can use other cues (e.g., visual, chemical) to establish a non-aggressive relationship with one another. It is when adult male rats are placed in a situation in which they are interacting with an unfamiliar male that the role of ultrasonic vocalizations becomes critical in attenuating the risk that playful encounters will escalate into aggressive ones. It is possible that during these interactions, in which dominance is being contested and established, ultrasonic vocalizations are being used in a tactical way to attenuate the risk of further escalating the encounter. For example, 22-kHz calls are correlated with submissive behavior (Portavella et al., 1993; Sales, 1972), likely functioning to inhibit further attacks (Lore et al., 1976; Sales, 1972). Similarly, 50 kHz calls are also emitted during aggressive encounters (Burgdorf et al., 2008) and in the

resident-intruder paradigm, it seems likely that it is the intruder that emits these calls and so inhibits attack (Takahashi et al., 1983).

3.6. Conclusion

The present findings, along with those from a previous study (Kisko et al., 2014), show that emitting ultrasonic vocalizations by juvenile rats is not essential for promoting a playful mood (hypothesis 1), locating a partner (hypothesis 2) or attracting play partners (hypothesis 3). The association of 50 kHz calls with a positive affective state (Burgdorf et al., 2008; Knutson et al., 1998) may indirectly account for both acute and chronic changes in patterns of play in devocalized rats, as these animals modify their play to compensate for changes in the rewarding properties of playful interactions. However, for juvenile rats, it does not seem likely that such calls function as play signals, that is, signals that are critical for promoting play (Bekoff, 1975) and ensuring that playful contact does not escalate to aggression (Bekoff, 1995). Such a function is more likely in adulthood, as, in their absence, playful interactions are at greater risk of escalating into serious fights. But even in adults, the importance of this vocal signaling seems to be critical only in ambiguous situations (hypothesis 4), such as when unfamiliar rats encounter one another, not in situations in which rats familiar with one another live together. Together, these findings suggest that ultrasonic vocalizations can be used as tactical or appeasement signals to influence the behavior of social partners, but are used as such only in some contexts.

3.7. Acknowledgements

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Chapter 4
General Discussion

The purpose of this thesis investigated the role of 50 kHz ultrasonic vocalizations as play signals facilitating and promoting playful interactions. The main focus of the research was on the effects of devocalization on the playful interactions of both juveniles and adults. Taken together, 50 kHz vocalizations appear to be critical in adulthood, where they are used in a way that prevents playful interactions from escalating into serious fights. In juveniles, however, 50 kHz calls do not appear to be functioning as play signals to promote or maintain playful interactions. In juveniles, emitting 50 kHz calls appears to instead, be related to the positive affective state of the performer, and preventing such vocalizations can interfere with the regulation of play by changing the rewards gained from playing.

Several studies have shown that, in rewarding social situations, rats emit 50 kHz calls (Knutson et al., 1998; Burgdorf et al., 2008, Portfors, 2007; Brudzynski 2009; Brudzynski & Pniak, 2002; Wright et al., 2010). The production and reception of 50 kHz calls are found to have links to the brain's reward centers (Burgdorf et al., 2007) and if these areas are damaged, there is a subsequent decrease in vocalizations and motivation for rewarding behaviors. In recent studies, it has been shown that playback of 50 kHz calls activates parts of the nucleus accumbens, as well as the frontal and motor cortices (Sadananda, et al., 2008; Wöhr & Schwarting, 2009). These areas are associated with emotion, meaning that the 50 kHz calls could be capable of inducing or changing the emotional state of the receiver and, potentially, the producer. This provides support for 50 kHz calls functioning as an indicator of the affective state of the animal. The data from

the present study appears to be consistent with the affective hypothesis, and more specifically, USVs may be functioning as an emotional and self-regulating signal, the performance of which maintains the playful mood.

In a study by Siviy and Panksepp (1987) in which rats were deafened, it was shown that there was no decrease in the amount of play by the pairs of deaf rats. Although the animals were no longer able to hear, they could still vocalize and thus the rhythmic, muscular contraction patterns produced when emitting these vocalizations may have still provided proprioceptive feedback to the emitter and so maintained the mood altering effect of producing 50 kHz calls. In other words, the execution of motor patterns used in vocalization may, in and of itself, promote dopamine release. Indeed, the ventral tegmental area (VTA) is activated and dopamine is released following the emission of 50 kHz vocalizations (Burgdorf et al., 2007; Brudzunski, 2007). A study on prenatal exposure to ethanol found that ethanol exposure led to reduced activity of the VTA when the animals reached four weeks of age (Choong & Shen, 2004), the age at which play is most frequent (Pellis & Pellis, 1990). Thus, the decrease in the release of dopamine could be potentially reducing the rewarding qualities of play and vocalizing. In the present study, the rats were devocalized during this apparently critical period when vocalizations become able to stimulate the release of dopamine from the VTA, potentially affecting the rat's ability to self-regulate their affective state through vocalizations. Similarly, some forms of birdsong appear to be performed in part to influence the performer's affective state, as the production of the vocalizations act to release rewarding neurotransmitters

(Riters, 2011). Thus, while unheard vocalizations in deaf rats may still provide feedback from proprioceptive means as the throat vibrates, this would not be possible for the devocalized rats. Consequently, deafened, but vocal rats, may be better able to sustain the positive affective state to promote play.

Vocalizations may also be acting, to influence the affective state of the receiver. Several studies done in primates have shown that vocalizations can induce affective responses in listeners that establish the conditions for learning about the environment (Kuraoka & Nakamura, 2010; Gil-da-Costa et al., 2004; Rendall et al., 2009). Thus, vocalizations could become associated with the affective consequences, especially when paired with an emotionally salient act (Rendall & Owren, 2010). For example, in some primates, the subordinate members of the troop might come to associate a particular call with a positive emotional response, such as grooming, that follows approach and calling (Rendall & Owren, 2010). As juveniles, rats may be experiencing a similar form of learning in associating the 50 kHz calls with positive emotional responses, such as playful nape attacks, or grooming in social interactions. In this way, the vocalizations may also be acting as an auditory signal to indicate a pleasurable situation will occur. Further investigation is needed to test this hypothesis. Possibly, playback of recorded calls with devocalized juveniles may be one way to test this hypothesis.

Auditory signals may have particular relevance to nocturnal animals because visual signals are not always helpful in the dark. This may be especially true, in primates that live in dense forested areas or rodents, such as rats and mice, which must navigate

through clumps of vegetation and other obstacles. Vocalizations are a common adaptation for nocturnal primates as they permit an individual to locate other members of their troop without revealing themselves to potential predators (Wright, 1989; Rasoloharijaona et al., 2006). Since our results of 50 kHz in rats being used as contact calls failed to reveal differences in pairs in which the devocalized animal was paired with an intact one, in the second study, it is unlikely that these vocalizations are acting as contact calls to localize the play partner. It may be possible though, for this study, that the testing enclosure was not large enough for the rats to require vocalization signals in order to locate a partner. Visual, olfactory and tactile cues may have sufficed in these situations. It may also be that the domestication of rats has eliminated a need for contact calls and in more wild-type rats or ones living in large colonies together, these calls may be prevalent and necessary.

In this study, the experiments strongly suggested that, when given the option of multiple play partners, the devocalized ones were not aversive partners. This indicates that 50 kHz vocalizations are not being used as play signals to attract partners. Several studies have failed to find evidence that 50 kHz calls are attractive to other rats. For example, playback of 50 kHz calls during mating interactions appears to have no effect on the attractiveness of the partner, either male or female (Thomas et al., 1981, 1982; Snoeren & Amgo, 2013) and even supports the self-regulation hypothesis, previously mentioned in that, the absence of female vocalizations appears to effect their own behaviour and not the males' behavior (White & Barfield, 1987; White et al., 1993).

Conversely, 50 kHz calls have been demonstrated to induce approach and exploration using playback studies in adult rats (Wöhr & Schwarting, 2007; Sadananda et al., 2008;), pups (Wöhr & Schwarting, 2008) and in mice (Wöhr et al., 2011). While these studies appear to support the hypothesis that rats are attracted to 50 kHz calls, in these demonstrations, the rat is typically separated from other rats, with the cue for the presence of a potential partner only offered by the occurrence of 50kHz calls, calls that are associated with positive affective states (Burgdorf et al., 2008; Knutson et al., 1998; Knutson et al., 2002; Willadsen et al., 2014; Wöhr et al., 2008; Wöhr & Schwarting, 2009). So, while these calls have been shown to be attractive, that does not necessarily mean that the calls are emitted so as to signal to the other rat. Indeed, a recent study found that, while low-dose injections of ethanol in rats increases the attractiveness of a conspecific peer across a partition, the increase in emitted 50 kHz calls is not correlated with a physical approach towards the barrier. Rather, it appears, that the stress from being restrained from the partner is increasing the physical approach of the barrier (Willey & Spear, 2013).

Indeed, the most significant finding, in this thesis, for the effects of devocalization on playful interactions were observed in adults, where the absence of 50 kHz calls from one partner during playful interactions was more likely to lead to aggression than if both partners were able to vocalize. However, this appears to occur only when the animals are unfamiliar with each other as there was no aggression seen between familiar cage mates in which one of the rats was devocalized. Therefore, it

appears that 50 kHz calls are not necessary in all playful interactions between adults. Possibly, other cues, such as visual or olfactory ones, are used with familiar partners. In unfamiliar adults, the ability to vocalize may provide a necessary tactical signal in order to keep the playful interaction from escalating into a serious fight. In a neutral play arena, two unfamiliar rats will compete for the dominant role and play will become rougher as a means by which to establish the dominance (Pellis & Pellis, 1987). Once dominance has been established, the submissive rat will produce 22-kHz calls (Portavella et al., 1993; Sales, 1972; Assini et al., 2013), possibly functioning to inhibit any further attacks (Lore et al., 1976; Sales, 1972). By the same token, 50 kHz calls are produced by the intruder in resident-intruder paradigms (Burgdorf et al., 2008), suggesting that when confronting an unfamiliar animal, it is the 50 kHz calls that are being used to appease the resident and inhibit any further attacks (Takahashi et al., 1983). Appeasement signals are often used to reduce aggression and maintain friendly relationships among group members (Bradbury & Vehrencamp, 1998). Before rats reach sexual maturity, appeasement signals are unnecessary in playful situations in which unfamiliar rats are introduced. As was demonstrated in the present study, juveniles appear to play whether the partner is familiar or not and so no interactions became aggressive. When rats are socially isolated as juveniles and do not receive any play, they are unable to behave appropriately in social situations as adults, and when placed into a playful interaction with a dominant male they will not behave as a submissive rats do (Von Fritag, 2002). It may be that, as juveniles, engaging in playful interactions is a way to practice and refine the use of ultrasonic

vocalizations. If the animal receives no play or they are devocalized, the subsequent effects, such as abnormal submissive behaviors, may be a result of the failure to practice and refine appeasement signals. Thus, when faced with an ambiguous situation, in which, these signals may be essential, the animals without this practice and learning experience will not behave appropriately. In situations such as the one in this study, in which an unfamiliar devocalized rat is paired with an unfamiliar vocal rat, the devocalized rats are unable to coordinate or appease the partner and thus, the situation turns aggressive.

4.1. Conclusion

The findings from this thesis suggest that 50 kHz ultrasonic vocalizations, in juvenile rats, are not acting to promote a playful mood, localize partners, attract partners, nor most critically inform the partner of imminent contact. In other words, they do not serve any of the traditional roles ascribed to play signals. Rather, the results indicate that the association of 50 kHz calls with a positive affective state (Burgdorf et al., 2008; Knutson et al., 1998) may indirectly account for both acute and chronic changes in patterns of play in devocalized rats, as these animals modify their play to compensate for changes in the rewarding properties of playful interactions. Once rats are sexually mature, whatever affective value the vocalizations may have, emitting vocalizations appear to be necessary to prevent playful interactions from escalating into serious fighting, at least among unfamiliar males. These findings indicate that, at least in some cases, USVs are being used as play signals by adults. Which of the many types of calls are used for such

signaling and when in the encounters such signals are tactically deployed remains to be determined.

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