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

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ABSTRACT

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

1. Introduction

Play fighting involves partners competing for some advantage, such as biting a particular body area (Aldis, 1975; Symons, 1978). While superficially similar, play fighting can be distinguished from serious fighting by several criteria (Smith, 1997): (1) a resource, such as a piece of food, is not gained or protected; (2) the contact is restrained, or, at least, there are no combat-induced injuries; (3) there are frequent role reversals between a pair, with partners alternating as to which is the attacker and which is the defender; (4) even if chasing ensues following contact, further affiliation is likely; and (5) the presence of play signals. However, play fighting can escalate into serious fighting because either one partner is excessively forceful in its attempts to gain the advantage or, because the recipient fails to recognize that the action performed by the partner is a playful one (Aldis, 1975; Fagen, 1981; Pellis and Pellis, 1998). In these situations in which play fighting can be ambiguous, play signals, such as the primate play face (van Hooff, 1967) and the canid play bow (Bekoff, 1974), can be critical in alleviating the misunderstanding and so maintain the encounters as playful (Palagi et al., 2016b). For example, dogs are more likely to perform play bows before initiating playful bites (Bekoff, 1995), with mutual signaling by the partners leading to more prolonged play bouts (Cordoni et al., 2016).

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

In rats, play fighting is most common in the juvenile period (Meaney and Stewart, 1981; Thor and Holloway, 1984), but continues into adulthood (Pellis and Pellis, 1990, 1991b), when adult males use it as a means of both reinforcing and testing dominance relationships (Pellis and Pellis, 1991a; Pellis et al., 1993). For practical purposes, the play

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

Except for the possible use of hops (Pellis and Pellis, 1983) and an open mouth facial gesture (Panksepp and Burgdorf, 2003), rats do not have any obvious overtly performed actions that can unambiguously serve as play signals, but they do emit many ultrasonic vocalizations (Burgdorf et al., 2008; Knutson et al., 1998), and some of these may potentially function as play promoting signals (Himmler et al., 2014). These calls are of two types, those around 50-kHz, that are associated with positive affective states such as sex and the administration of psychoactive drugs, and those at 22-kHz, that are associated with negative affective states, such as aggression and drug withdrawal (Brudzynski, 2013; Wöhr and Schwarting, 2013). There remains some ambiguity about whether 22-kHz calls are purely positive and 22-kHz calls exclusively negative. For example, 22-kHz vocalizations are emitted by male rats following ejaculation, suggesting an association with a positive affective state (Bialy et al., 2016); however, once the refractory period is over and the male approaches and mounts the female, the male switches back to emitting 50-kHz calls (Burgdorf et al., 2008). The latter findings suggest a switch from an initial, post-ejaculatory state, when the continued presence of the female may be negative, to one in which her presence is again positively rewarding. Conversely, the usage of 50-kHz calls is not always reflective of a positive state. For example, flat 50-kHz calls can occur in ambiguous situations reflecting at least a partially negative affective state (Wöhr et al., 2017). Thus, there may well be gradations in the usage of 22-kHz and 50-kHz emissions as encounters shift from being positively reinforcing to aversive and back again. This may be particularly evident in cases, such as the one studied in the present paper, in which playful encounters escalate to aggression.

Both 50-kHz and 22-kHz calls have been implicated in mitigating aggression. In the resident-intruder test, an unfamiliar adult male is placed in the home cage of another adult male, and the resident attacks the intruder, directing bites at the lower flanks and dorsum (Blanchard and Blanchard, 1994). In these encounters, the intruders emit 50-kHz calls (Takahashi and Lore, 1983; Thomas et al., 1983), and the likelihood of a biting attack is reduced following the emission of 22-kHz calls by one or both animals (Lehman and Adams, 1977; Lore et al., 1976; Sales, 1972; Sewell, 1967). While the 22-kHz calls are found in a narrow frequency range and have a simple, long, flat profile, the 50-kHz emissions are more variable (Wright et al., 2010). This variability of 50-kHz calls provides the possibility that at least some of these calls may be used for more specific purposes than simply communicating affect (Pellis et al., 2017 (in press); Wöhr et al., 2015).

While some 50-kHz calls have a flat frequency profile, the most commonly emitted 50-kHz calls during play are those that are frequency modulated (FM) (Burgdorf et al., 2008), and of the FM 50-kHz calls, trills are the most common (Himmler et al., 2014; Wright et al., 2010; Burke et al., 2016). While such trilling may reflect the positive affective state induced by play (Panksepp and Burgdorf, 2003), some types of FM 50-kHz calls, though less frequently emitted than trills, are statistically associated with particular actions performed during play, such as when evading contact and when nosing a partner's nape (Himmler et al., 2014; Pellis et al., 2017), suggesting that the animals may be signaling in specific ways to influence the behavior of their partner. Even so, among juveniles, the elimination of the ability to hear (Siviy and Panksepp, 1987) or produce (Kisko et al., 2015a, b) ultrasonic calls has only a modest effect on the frequency and form of play (Kisko et al., 2017). However, in the context of the rougher play present in adult rats, the ability to emit ultrasonic calls appears more critical (Kisko et al., 2015b).

Recently, we showed that the risk of aggression among unfamiliar male rats is greatly elevated when one of the pair has been devocalized (Kisko et al., 2015b). When the rats were introduced into the test arena they investigated one another, and then began to play. The play, which could become increasingly rough, in some cases continued until the rats began to exhibit visual threat signals, and then finally deliver a bite. After a bite, the encounter would gradually become gentler until the animals played again (Kisko et al., 2015b, 2017; Pellis et al., 2017). The pairs with a devocalized member initiated just as much play (i.e., nuzzling attacks to the nape), but also more frequently displayed the various signs of aggression (e.g., tail rattling, piloerection). Most strikingly, while pairs from both groups vigorously pushed one another in the mutual upright position, none of the intact pairs escalated to actual biting, whereas all pairs with a devocalized member did so (Kisko et al., 2015b). These findings suggest that fully vocal pairs may be able to use ultrasonic vocalizations in a manner that can diffuse intense moments of the interaction, such as when they vigorously pushing one another in the mutual upright configuration, from escalating to all-out fighting (Kisko et al., 2017). In the present study, we categorized calls and examined their relationship to specific behaviors on a fine (sub-seconds) time-scale, creating plots of the rate of co-occurrence of each vocalization and behavior. These plots provide insight into how calls are used to coordinate adult social interactions. By comparing vocal usage between pairs of adult rats with and without a devocalized partner, we sought to determine which calls were used in which behavioral contexts to mitigate the risk of a playful encounter escalating to aggression. Presumably, these vocal signals should be present in the intact animals and missing when one partner is devocalized.

2. Methods

2.1. Subjects and experimental procedures

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

For testing, the designated pairmates were placed in the test enclosure and videotaped in the dark using cameras with night shot capability for 12 min (for further procedural details see Himmler et al., 2013). Ultrasonic vocalizations were collected using a high frequency microphone (Model 4939, Brüel & Kjaer, Denmark), with sensitivity to frequencies ranging from 4-Hz to100-kHz. The microphone was located in the ceiling of the chamber and was approximately 15 cm above the middle of the play enclosure. The microphone was connected to a Soundconnect amplifier (Listen, Inc, Boston, MA) and recordings were processed via a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, CA) using a self-developed MATLAB acquisition program. All files were then converted to wav files and were analyzed using the RavenPro system (Cornell, MA). To synchronize the video and audio recordings, a device emitting a simultaneous light and sound cue was used (Himmler et al., 2014; Kisko et al., 2015a).

2.2. Behavioral analyses

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

2.3. Vocalization analyses

Acoustic data were analyzed using Raven Pro 1.4 software (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, NY). The Raven Pro software generated spectrograms with a 256-sample Hann window from which the experimenter manually selected 50-kHz vocalizations. The different types of 50-kHz vocalizations were scored according to the criteria established by Wright et al. (2010) (see Fig. 1). The occurrence of these calls was used to compare rates of calling, types of calling and whether different types of calls were associated with particular types of actions. The first four minutes from each test session was used to evaluate overall group differences in vocalizing.

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

2.4. Detailed vocal-behavioral analysis

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

2.5. Statistical analyses

To evaluate the associations between all the behaviors and call types, a Monte Carlo Shuffling method was used (Burke et al., 2016). This method was used because the total number of behavioral and vocal events differed substantially between different categories, making a simple correlation between behaviors and vocalizations difficult to interpret. For example, there were a very large number of trills which occurred during walking simply because trills were the most common vocalizations and walking was one of the most common behaviors. A statistical analysis that accounted for these differences in event baselines was necessary to determine whether a particular vocalization co-occurred with a particular behavior above chance levels. Therefore, a Monte Carlo technique was used to calculate the probability distribution of each unique combination of vocalizations and behaviors. First, we counted the number of co-occurrences of each vocalization type with each of the coded behavioral categories. Next, for each animal pair, vocalizations were repeatedly shuffled and the number of behavior-vocalization co-occurrences computed. A vocalization was counted as occurring during a particular behavior if the mid-point of the call occurred between the start and stop time of the behavior. To allow for errors in the coding of the behavior times, we expanded the time window for each behavior by 200 ms in each direction. Shuffling was achieved by assigning each vocalization a random time within the duration of the four minute observation period. Hence, the relative frequency of vocalizations was kept the same for each shuffle. This

Table 1
Description of the social behaviors that were scored.

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

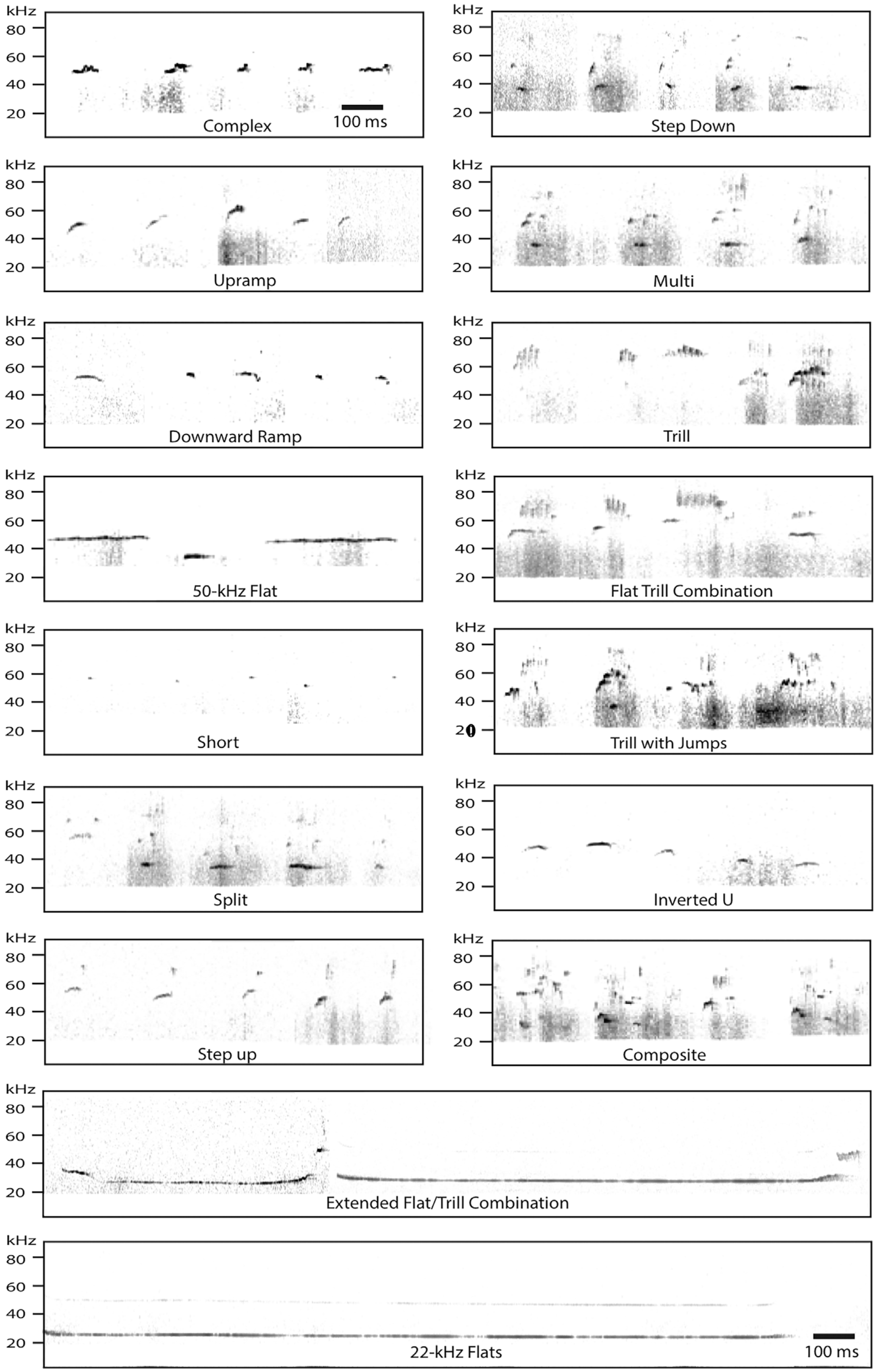

Fig. 1. Examples of the different types of ultrasonic calls (derived from Wright et al., 2010) that are used in the present study.

shuffling was done 10,000 times and the total number of co-occurrences of each vocalization type with each type of behavior was tabulated. Based on the distribution of these random-shuffle counts, we assigned a z-score to the actual number of occurrences. The higher the z-score, the less likely that specific combination of vocalization and behavior could have occurred by chance (i.e. a z-value of 1.96 gives a p-value of < 0.05 and a z-value of 2.58 gives a p-value of < 0.01). Shuffling was performed separately for each animal pairing and the z-scores averaged across play pairings to generate the final, average z-score values.

To compare differences across the two groups (i.e., vocal–vocal pairs versus vocal-devocalized pairs), independent samples t-tests were used, and for comparisons of the same individuals within groups, pair-

Table 2
The means and standard deviations for duration and call frequency are compared for two versions of the flat/trill combination call. The "average frequency," derived by averaging the maximum and minimum frequencies in the call, is essentially the mid-point between the trill and flat component.

	Original Flat/trill call	Extended version	t-test (2-tailed)
Average Duration (ms)	74.10 + 16.34	321.00 + 130.57	t(7) = 5.114, p < 0.001
Average Frequency (kHz)	49.90 + 12.63	43.03 + 0.97	t(7) = −4.03, p = 0.005
Flat Frequency	34.99 + 5.70	28.05 + 3.24	t(7) = −3.37, p = 0.012

samples *t*-tests were used. Differences were judged to be significant if $p < 0.05$. For example, this approach was used to evaluate whether there was an overall significant difference between the two groups in the number of vocalizations and whether particular calls were used differently at specific moments in the encounters.

3. Results

3.1. Vocalization analysis

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

We observed a novel type of flat/trill combination call, characterized by an extended flat component. As shown in Table 2, these calls differed significantly in both duration (s) and mean frequency (kHz) compared to the originally categorized flat/trill combination, as described in Wright et al. (2010). Characteristically, the flat component of the extended flat/trill combination has a frequency that is close to that of 22-kHz calls. To further test whether the extended flat/trill combination call may be a separate type of call, the frequency and duration of all calls with a flat component (22-kHz flats, 50-kHz flats, flat/trill and extended flat/trill combination) was plotted (Fig. 2). Three clusters are evident. One cluster includes the 50-kHz flat calls and the originally described flat/trill combo and has a mean frequency close to 50-kHz. A second cluster of these same two call types has a mean frequency just above 30 kHz. The third cluster is the "22-kHz flat" calls, which in our study actually have a mean frequency just below 20 kHz. The extended flat/trill combination calls seem to be a transitional group between the 30 kHz flat/trill group and the 22-kHz group, increasing in duration and lowering in frequency compared to the 30 kHz flat/trill cluster. The current findings cannot differentiate whether the extended flat/trill combo is truly a unique call or represents a graduated change from the 50-kHz flats to the 22-kHz flats, but either way, the variations may reflect different functional uses during interactions. As such, they were coded separately in our behavioral-vocalization analysis.

3.2. Vocal-behavioral analysis

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

3.3. Mutual uprights

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

A number of major differences were revealed for actors and recipients in the vocal-devocalized pairs (Fig. 4). Delivering a bite, is strongly associated with the extended flat/trill combination ($z = 3.4$), whereas being bitten is strongly associated with 50-kHz flat calls ($z = 3.6$). Attacking the nape playfully is strongly associated with split calls ($z = 3.3$), whereas receiving such a nape attack is strongly associated with flat/trill combinations ($z = 6.0$). Being pinned is strongly associated with down ramps ($z = 7.9$), whereas the strongest association with pinning is with the 22-kHz flat ($z = 3.3$). Finally, approaching a partner is associated with trills ($z = 6.3$), whereas being approached is associated with inverted u calls ($z = 3.3$). The complementary patterns of calling between partners suggest that both animals may need to reciprocate specific calls to facilitate social interactions.

That reciprocity of mutual calling may be important in coordinating actions is supported by the surprising absence of the use of the extended flat/trill combination and 22-kHz flat calls during the mutual upright position for the vocal-devocalized pairs (Figs. 3 B and 4). In contrast, these calls were strongly associated with the mutual upright position in the vocal–vocal pairs (Fig. 3A and Table 3). These data suggest that low-frequency flat calls, presumably used to mitigate aggression, only occur when both partners can participate in a two-way dialog.

4. Discussion

The most striking behavioral effect of devocalizing one partner in a pair of unfamiliar adult rats was that these pairs escalated to aggression and biting while intact pairs did not. Our findings suggest two mechanisms by which the coordinated use of vocalizations may help mitigate the risk of aggression. The first is the use of low-frequency flat calls (22-kHz and extended flat/trill), especially during mutual upright behaviors. While these calls were strongly associated with mutual

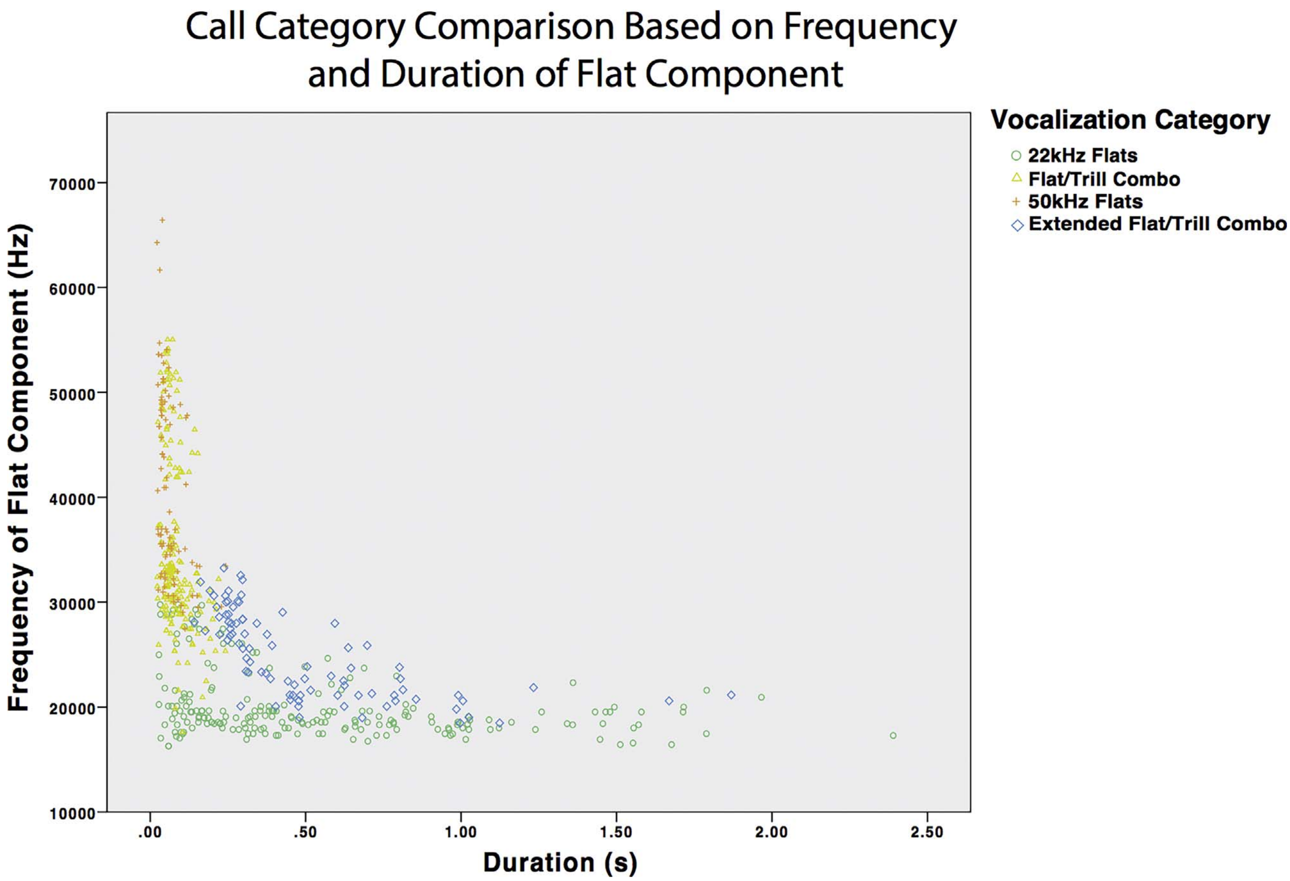

Fig. 2. This scatter-plot shows the distribution of calls containing flat components, plotted according to their frequency (kHz) and duration (s). The 50-kHz flat calls and the flat/trill combination calls, originally described by Wright et al. (2010), are clustered together to the left of the graph, having a narrow distribution of durations but covering a wide range of frequencies. The 22-kHz flat calls, in contrast, have a narrow frequency range around 20 kHz but are highly variable in duration. The newly described extended flat/trill combination call is intermediate, having a wider frequency range than the 22-kHz calls and wider range for duration than the other 50-kHz calls.

upright interactions in vocal–vocal pairs, they were seldom used in the vocal-devocal pairs. The second mechanism to avoid aggression may be the mutual exchange of vocalizations. The vocalizations associated with several dyadic interactions are asymmetric (see Fig. 4). For example, a rat attacking the nape of its partner often makes a split call while the rat receiving the nape attack is more likely to emit a flat/trill combination call. Similar asymmetric vocalizations were observed for pins and approaches. These observations are consistent with the view that the reciprocal exchange of vocalizations is important for normal social interactions.

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

Flat calls appear to play an important role in adult interactions that could potentially become aggressive. Our data help refine our understanding of previous findings about the role of these calls. Adult aggressive encounters between adult rats are known to be associated with increased rates of 22-kHz calling (Thomas et al., 1983; Panksepp et al., 2004; Burgdorf et al., 2008). Panksepp et al. (2004) have shown that 22 calls are emitted only when one rat exhibits submissive behavior and occur almost exclusively after a bite has occurred. One interpretation of these data is that 22-kHz calls signal the expectation of an unpleasant encounter (Brudzynski, 2009). Our data agree with the alternate interpretation that the low-frequency flat calls are employed as a mechanism to mitigate aggression (Lehman and Adams, 1977; Lore et al., 1976; Sales, 1972; Sewell, 1967). We found that 22-kHz calls were associated with mutual upright interactions than resolved amicably, suggesting they play a role in negotiating a non-aggressive end to such encounters. We also observed that both flat 22 kHz calls and extended flat/trill combinations (in the 20 kHz range) were associated with a wider range of behaviors in the vocal–vocal pairs than in the vocal-devocalized pairs (Fig. 3 and Table 3). This raises the possibility that the failure to integrate calls with low-frequency flat components, or overreliance on one version, the extended flat/trill combination, may have put the vocal-devocal pairs at greater risk of escalating their encounters. Finally, in our vocal-devocal pairs, the use of extended flat/trill calls by the biting rat, usually the dominant, suggest that these calls serve as a warning signal to the non-dominant rat to keep away. This contrasts with early reports that 22-kHz calls are emitted as a submission signal (Lore et al., 1976; Sales, 1972; Sewell, 1967).

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

Another potential mechanism by which vocalizations could act to

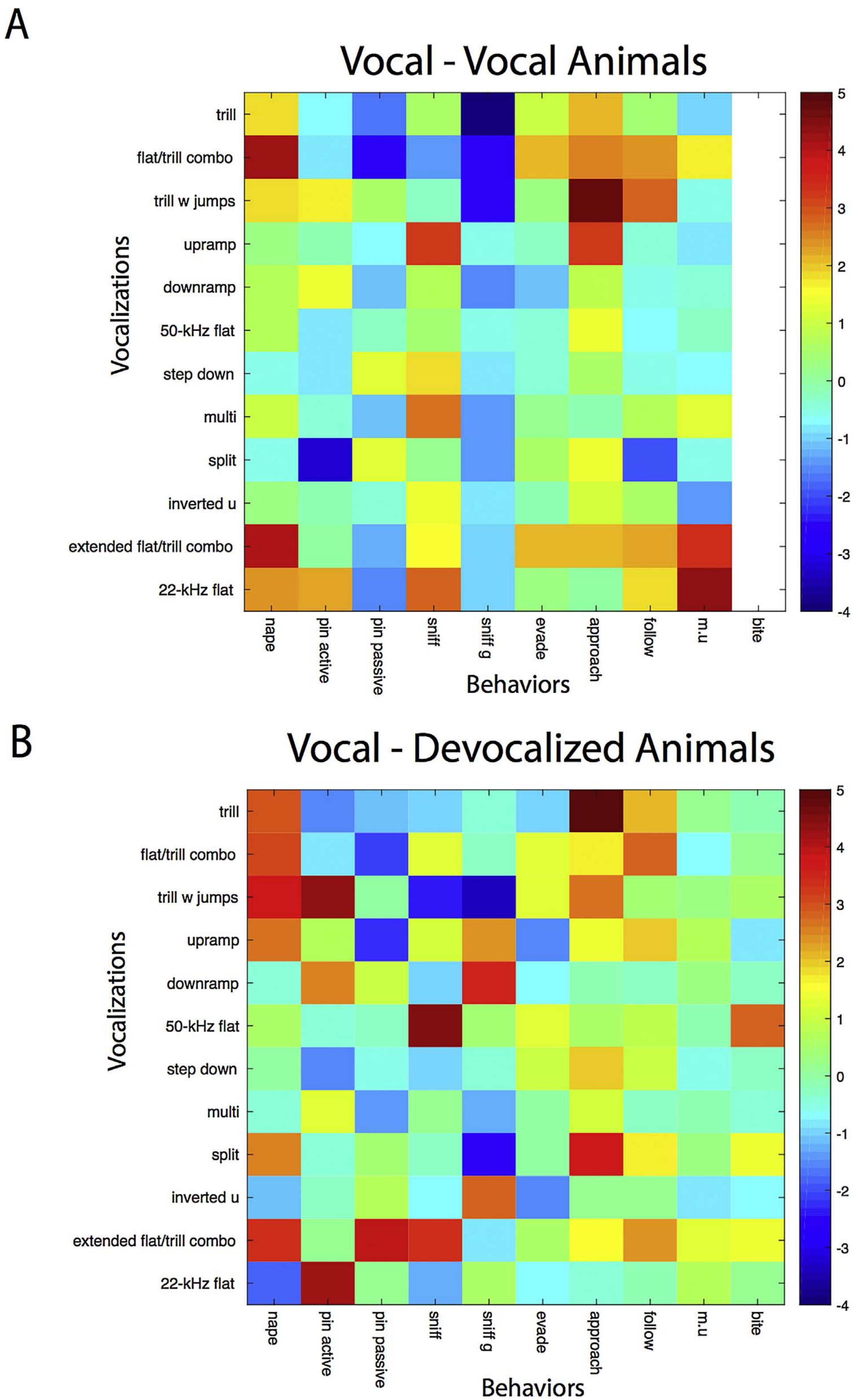

Fig. 3. The matrix shows the strength of association, as a z-score, for each combination of behavior (x-axis) and vocalization category (y-axis). Deep red indicates the strongest association and deep blue the weakest. The pattern for the vocal–vocal pairs is shown in A and the pattern for the vocal–devocalized pairs is shown in B. No data is shown for bites within the vocal–vocal group because these animals exhibited this behavior. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

offset escalation from play to aggression is via reciprocal exchange. We have already mentioned the finding that calls associated with several dyadic interactions appear to be asymmetric (e.g., different calls for initiating and receiving a nape attack) and hence, potentially complementary. It is also possible that rats facilitate play via the reciprocal exchange of the same call. For example, during a mutual upright, both rats may emit low-frequency flat calls to facilitate play. The plausibility of this scenario is enhanced by the finding that devocalizing one animals causes a dramatic reduction in all vocalizations during mutual upright interactions. Consistent with this idea, many species use rapid mimicry of play signals to increase the duration of play fights (e.g., Davila Ross et al., 2008; Mancini et al., 2013; Palagi et al., 2015; Scopa and Palagi, 2016). That is, the temporal coordination of either similar or complimentary ultrasonic calls among rats engaged in play may

Table 3
Comparison of the significant positive associations ($p < 0.01$; $z > 2.58$) present in the two groups of dyads. The numbers in parentheses shows the z-scores for the association.

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

facilitate the coordination of movements that ensure the continuation of play. This explanation is consistent with findings from the play fighting in juvenile devocalized rats. Among juvenile rats, about 30% of play fights involve role reversals (Himmler et al., 2016), and this reciprocity is essential to maintain play as playful (Dugatkin and Bekoff, 2003; Palagi et al., 2016a; Pellis et al., 2010). In the absence of either partner being able to vocalize, such reciprocation is halved (Kisko et al., 2015a). That is, in the absence of calls, the coordination necessary to sustain reciprocal playful exchanges may be compromised, and in adults, the absence of mutual signaling may impair the ability to sustain the coordination needed to prevent playful encounters from escalating to aggression.

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

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

Here, we have used devocalization as a method to discover which vocalizations can be attributed to each individual in a dyadic exchange. The gross similarity between call profiles between vocal–vocal and vocal-devocal pairs (Fig. 3A versus B) shows that this method has promise, but the differences show the method is imperfect. Obviously, if reciprocal exchange of vocalizations plays a functional role, devocalizing one partner will affect the way calls are used by its partner. Having rats that can both vocalize is needed, but recorded with methods that can track their individual vocalizations. Such data could be collected with the use of multiple microphone arrays (Neunuebel

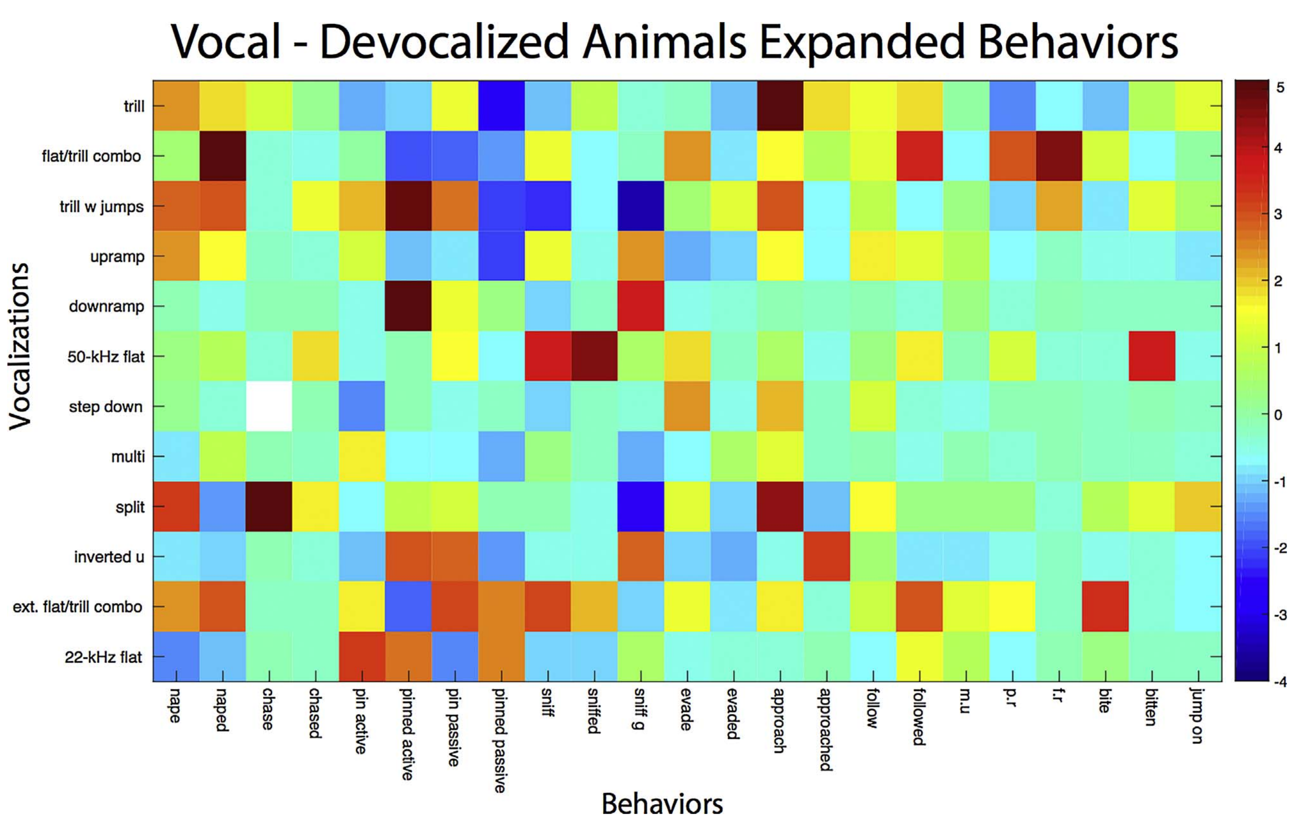

Fig. 4. The matrix in this figure is similar to the one for the vocal-devocalized pairs in Fig. 3, except that this matrix shows whether the recipient or the performer of the behaviors is emitting the calls. The association between chase and step-down was omitted because these events occurred at frequencies that were too low to allow reliable statistical analysis.

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

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