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Prairie Vole Pups Show Potentiated Isolation-Induced Vocalizations Following Isolation From Their Mother, But Not Their Father

ABSTRACT: *Vocalizations can be markers of emotional social communication. Maternal potentiation was originally described as an increased rate of vocalization by isolated rat pups following an interaction with their mothers, but not with other social companions. Here we asked if potentiation in prairie voles, a species with pair-bonding and bi-parental rearing, is parent-specific. We found that isolated, 8–11-day-old voles exhibited potentiation following reunions with the dam, but not the sire. These responses were present whether parents were anesthetized or active during the reunion. There were no significant correlations between parental behaviors during reunions and pup vocalization rates during re-isolation. The absence of potentiation to the sire contrasts to findings in bi-parentally reared rat pups, which do potentiate vocalizations to the sire. We interpret these results to be consistent with the idea that potentiation reflects disruption of mother–infant coregulation and is dependent upon the unique biology of mothering.* Dev Psychobiol 9999: 1–13, 2016.

Keywords: *ultrasonic vocalization; USV; maternal potentiation; prairie vole; Microtus ochrogaster; bi-parental; infant; coregulation; social behavior; nursing; attachment*

INTRODUCTION

Isolation-induced ultrasonic vocalizations (USV) of young rodents have long been studied as indicators of social and emotional behavior (Noirot, 1972; Oswalt & Meier, 1975; Sewell, 1970). Evidence for their link to emotions includes the fact that USV is accompanied by other behaviors often considered emotional when occurring in stressful situations, for example, increased activity, corticosterone secretion, defecation/urination (Bowlby, 1973; Hofer, 1975; Marco et al., 2013; Shapiro & Insel, 1990). Furthermore, anxiolytic agents decrease the rate of USV in isolation, while anxiogenics increase it, reviewed in (Brunelli & Hofer, 2001; Groenink, Verdouw, van Oorschot, & Olivier, 2008). The response to social companions introduced into the isolation can be considered evidence of both social and emotional effects. In general, social stimuli present during isolation decrease the rate of USV and may alter other parameters of the sound including waveform (Brudzynski, 2005; Carden & Hofer, 1992; Scattoni, Crawley, & Ricceri, 2009). The identity of the social companion can greatly affect USV production upon re-isolation, as will be described below. Communicatory functions of USV, which are almost by definition social, have been repeatedly demonstrated: calls by

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pups guide and elicit retrieval by their mothers (Bowers, Perez-Pouchoulen, Edwards, & McCarthy, 2013; Brunelli, Shair, & Hofer, 1994; Ehret, 2005; Sewell, 1970). Although there have been attempts to attribute USV production to thermoregulatory (Blumberg & Alberts, 1990; Blumberg, Efimova, & Alberts, 1992; Blumberg & Sokoloff, 1998) and/or cardiovascular processes (Blumberg, Sokoloff, & Kent, 1999; Kirby & Blumberg, 1998; Kirby, Sokoloff, Perdomo, & Blumberg, 1999), these hypotheses have not been supported by direct tests (Hofer & Shair, 1993; Shair, Brunelli, Masmela, Boone, & Hofer, 2003; Shair & Jasper, 2003), and summarized in (Shair, 2007), except in the case of recovery from deep hypothermia (Hofer & Shair, 1992).

If, as described above, USV subserves emotional and social functions, one might expect that parent/infant interactions would influence USV production through developmental processes/mechanisms. Observations suggest this to be the case in several murid species. In rats, selective breeding for high and low rates of pup USV in isolation resulted in strains with variations of maternal behavior as well. Mothers of the strain with high amounts of USV engaged in lower levels of licking and high-arched nursing (Brunelli et al., 2015). In various vole species, parental behavior and USV rate are also related, albeit in the opposite direction. High levels of parental care shown by species with monogamous social relationships and bi-parental rearing (prairie and pine vole) are associated with higher isolation-elicited USV calling in pups compared with pups raised by polygamous dams (meadow and montane vole) (Blake, 2002, 2012; Rabon, Sawrey, & Webster, 2001; Shapiro & Insel, 1990). Two species of deer mice show patterns similar to voles [compare (Vieira & Brown, 2002) and (Smith, 1972)]. These studies do not, however, distinguish between genetic effects (species or strain) and the level or patterns of parental care. Other work has less chance of confounding the two. For example, in the mandarin vole (monogamous, bi-parental), there is a positive relationship between USV in isolation and the amount of parental care received by pups (Yu et al., 2011). In rats, observations of home cage maternal behavior found a negative relationship between the amounts of licking received and later isolation-induced USV rates, as in the study in selectively bred rats (Wohr & Schwarting, 2008). In mice, embryo transfer demonstrated an effect of maternal behavior on vocalization (Wohr et al., 2008).

One particular variant of isolation-induced vocalization, maternal potentiation, is hypothesized to be a marker for the earliest social bonds, especially that of infant with mother (Shair, 2014). If so, it will allow investigation into the behavioral and neurobiological mechanisms necessary to acquire critical early-life relationships. Maternal potentiation has been defined as the greatly increased rate of vocalizations that occurs in infants during an isolation that has immediately followed a brief interaction with their mother (but not following a similar interaction with their littermates or other stimuli), first described in rats (Hofer, Brunelli, & Shair, 1994). Subsequently, it has been described by others in a number of mammals: domestic pigs (Colonnello, Iacobucci, & Newberry, 2010), guinea pigs (Hennessy, Miller, & Shair, 2006), *Octodon degus* (Fuchs, Iacobucci, MacKinnon, & Panksepp, 2010), and several strains of mice (Moles, Kieffer, & D'Amato, 2004; Scattoni et al., 2009; Young, Schenk, Yang, Jan, & Jan, 2010), although the details for this phenomenon vary with species (Shair, 2014). We have hypothesized that the evolution of isolation-induced USV production has been shaped by two selection pressures: the likelihood of reestablishing maternal contact vs. detection by a predator (Hofer, 2010; Hofer et al., 1994; Shair, 2007). One basis for this hypothesis is that potentiation, with its very high rates of calling, occurs just after the pup has been in the presence of the dam. Potentiation, therefore, takes place in the situation where it is most probable that the mother will respond first. Potentiated USV also carries communicatory information: dams respond preferentially to potentiated USV in comparison nonpotentiated (Rohitsingh, Smith, & Shair, 2011). But it may be that the potentiation response can not only be evoked by dams, but by sires or other adults involved in the rearing of the young. In rats at least, this appears to be the case as a similar, less robust potentiation response has been observed in rat pups after brief interaction with their sire or another intact male, that the pups have been reared with from birth (but not to a castrated adult male with which they have been reared in the same manner; Brunelli, Masmela, Shair, & Hofer, 1998; Shair, 2007). Rats, however, do not typically demonstrate bi-parental rearing of pups. Studies are needed to determine if there are differential infant vocalization responses to mothers and fathers in species that evolved to be bi-parental in their natural conditions.

The prairie vole (*Microtus ochrogaster*) is a species similar to humans in that males and females form monogamous pair-bonds and both parents are involved in rearing offspring. In fact, except for gestation and lactation, the general finding is that prairie vole fathers are just as involved in pup care as mothers (Ahern, Hammock, & Young, 2011; Gruderadams & Getz, 1985; Lonstein & De Vries, 1999). Furthermore, as noted above, prairie vole pups produce higher levels of USV in isolation in comparison with vole species reared by their dams only. Voles have other advantages for study. Much that is known about the neural bases for affiliative behavior in adults (i.e., pair bonding) emerged from work with prairie voles and closely related species, including pivotal roles for the neuropeptides, oxytocin, and vasopressin (Carter, 1998; Insel & Young, 2001). Such information can guide future work on affiliative behavior in infants of other species. It has also been suggested that prairie voles have a more sensitive autonomic nervous system in comparison with other, less social species, and more like human autonomic responsiveness (Carter & Keverne, 2002; Getz & Carter, 1996; Grippo, Lamb, Carter, & Porges, 2007). For these reasons, we chose prairie voles as subjects in the proposed experiments.

We considered two possible outcomes of this study. First, if prairie vole pups showed USV potentiation at all, they would show it after a reunion with either mother or father. In rats, exposure of pups to paternal stimuli is crucial for pup expression of USV potentiation, even though the pups may have little or no actual physical interaction with their sires (Brunelli et al., 1998; Shair, 2007). As vole fathers are highly involved in the rearing of their pups (for example Ahern et al., 2011), based on this hypothesis, this experience should lead to even more robust paternal potentiation in this species. The studies described in an earlier paragraph, furthermore, suggest that early-life interactions can influence isolation-induced USV rates in voles. Such a result would prompt further research into whether the timing, quantity, and/or quality of parent/pup interactions lead to different levels of USV potentiation.

The alternative hypothesis was that vole pups would potentiate to their mothers but, despite considerable interaction between vole pups and their fathers, they would not potentiate to their fathers. This possibility was suggested by observations that despite being routinely exposed to both parents, children show differential responses to mothers and fathers (unreported observations made during the course of clinical interventions (Welch, 1988; Welch & Chaput, 1988). During intervention sessions, differential behavioral responses to separation from mother versus father were observed: namely that separation from the mother elicited more emotional distress. This led to the idea that strong, mother-specific responses are conditioned by contingent, mutual regulation (coregulation) that begins in utero and continues after birth during breastfeeding and other mother–infant interactions. Beyond this, however, the mother may provide unique sensory experiences (smell, oxytocin in milk) that differentiate her from the father. Interestingly, in an intervention study of adopted orphans, who had not shared the birthing and nurturing experiences with the adoptive mother (Welch et al., 2006), similar clinical observations of emotional distress to maternal, but not paternal, separation were also common, suggesting that early mother-specific conditioning can be re-established to an adoptive mother. Based on these ideas, it was predicted that bi-parentally reared infant voles would not show potentiation in response to separation from the sire, but would to the dam. This result would guide research more directly into differences between stimuli during interactions of pups with mothers and fathers, including nursing and the prenatal period.

The current report describes two experiments in which 8–11-day-old prairie vole pups were tested for USV potentiation (Hofer, Shair, & Brunelli, 2002). USV rate was measured as a pup undergoes a brief isolation in a novel cage (Isolation 1); a reunion with a social stimulus; and a re-isolation in the novel cage (Isolation 2). In the first experiment, the reunion companions were anesthetized to remove variability in behavioral interactions during the reunion. The three experimental groups included reunions with the anesthetized dam, sire, or littermates in a novel cage. We also tested a control group that was handled like the companion groups, but remained alone in the middle period. In rats, interactions with an active, unanesthetized dam produce more robust potentiation than to an anesthetized dam (Hofer, Masmela, Brunelli, & Shair, 1998). Potentiation to an unanesthetized dam also does not require olfactory cues (Shair, Masmela, & Hofer, 1999). Although potentiation to active adult male rats has not been tested, it seemed possible that reunion with unanesthetized vole sires might have a better chance of eliciting potentiation of their pups' USV if we did not find it with an anesthetized sire. Thus, in the second experiment, the reunion companions were unanesthetized and both parties were allowed to interact freely. The two experimental groups included reunions with the dam or sire. The control group was handled like the companion groups, but remained alone with home cage shavings in the middle period. In both experiments, nonvocal behaviors of pups were measured to determine if any vocal changes might be due to generalized changes in arousal level.

MATERIALS AND METHODS

Animals and Husbandry

Prairie voles (*M. ochrogaster)* were bred from founders donated by Larry Young of Emory University. Animals were housed in disposable or washable cages under a 14:10 hr light–dark cycle and given standard enrichment items. Food (Lab Rabbit Diet, PMI Nutrition International) and water were available ad libitum. Cages were provided with alfalfa.

Temperature (~22–23˚C) and humidity (~40–70%) were regulated throughout experimentation. Postparturition, adult pairs were kept together in Innocage® disposable cages (interior dimensions: 141 in^2 floor space, 7″ height) with 1/8″ corn cob bedding (bed-o'cobs). Mated animals were provided with sunflower seeds 3 days a week. Nulliparous females and males were mated between 80 and 360 days postnatal. All litters tested ranged between three and eight pups. Except for normal husbandry, pairs and litters were not disturbed until testing. Eighteen litters and 91 pups were used in Experiment 1 (passive anesthetized reunion). Eleven litters and 39 pups were used in Experiment 2 (active unanesthetized reunion). Half the breeding pairs provided multiple litters for testing in Experiment 1 (see Supplemental Section). In Experiment 2, each pair was used once. All experiments were conducted in the morning during the spring and summer months.

Observations of Parental Behaviors

Home cage observations of litters used in the unanesthetized-reunion adult experiment were performed on eight litters containing pups between 1 and 11 days postnatal. Methods for, as well as results of, these observations can be found in the Supplemental Section, placed there because the results do not illuminate the USV data.

Testing Procedures

On the day of testing between 9 and 11 AM, the dam and sire were removed from the home cage, which was placed on a thermostatically controlled water blanket set to maintain pup temperature at normal nest levels (35–36 ˚C). In all cases, pups were attached to their dams' teats at the time of separation. Prairie vole pups are known to cling tightly to the nipple (Salo, Shapiro, & Dewsbury, 1994), requiring significant force to detach them from the dam. Litters were given 15–20 min to settle down in the warm home cage after this experience. For the first experiment, the dam, sire, and littermates were anesthetized with ketamine and xylazine (100 + 10 mg/kg, respectively) and placed in a holding cage with clean shavings in an auxiliary testing room. For the second experiment, the dam and sire were not anesthetized. They were placed immediately into the cages that would be used for testing, which contained ¼ to ½ inch of home cage shavings. The home cage shavings performed the necessary function of absorbing urine and feces, as well as making the containers less unfamiliar to adults and pups.

Pups were tested in random order. Each pup in its turn was picked up from the home cage and placed into a novel test chamber with the floor marked in a grid of six equal squares. Observation began immediately. The potentiation paradigm used in Experiments 1 and 2 consists of three epochs: a 2-min long initial isolation (Isolation 1), followed by a 2-min reunion/stimulus period (Reunion/Stimulation), and finally a 2-min re-isolation of the pup (Isolation 2). The cage top was open to allow USV detection (see below). After the third stage of testing, the pup's axillary temperature was measured (Physitemp I-18), its weight recorded, and it was returned to the home cage.

Vole pups between postnatal days (PNDs) 8–11 are capable of locomotion and are not confined to the nest. Some of the pups tested could see. Our lab found eye opening to occur around PND 10, similar to previous studies (Solomon, 1991). Because of these factors, reaching into the cage to remove a pup for testing sometimes involved catching a moving pup and often caused the remaining pups to scatter. Preliminary analyses tested whether age influenced the USV results. In most cases it did not and the data are combined across ages and eye condition. Possible exceptions are discussed in the results.

Experiment 1 (Reunions With Anesthetized Dam, Sire or Littermates). All tests took place in floor marked, empty testing chambers (polycarbonate terrarium, 7″ × 11″ × 6″) within an incubator kept between (27–31˚C). After the first 2-min isolation, the pup was gently picked up, and placed in a novel test chamber identical to the first, which contained an anesthetized adult (dam or sire), 2–3 anesthetized littermates, or nothing. After a second 2-min period, the pup was picked up and placed back into the original test chamber for a final two-min re-isolation (Isolation 2). Previous lab experience found that pups quickly attached to the teats of their anesthetized dam during the reunion. To avoid having to struggle to detach pup from teat, adhesive tape was placed over the teats of the dam to prevent nipple attachment. A similar area of the sire was covered to ensure that response to the tape did not determine results. At the start of the reunion, the pup was placed in contact with the adult's flank, and observed for 2 min. In the control condition (pick-up), the pup was treated exactly the same except that there was no dam, sire or littermates in the novel cage in the reunion/stimulus period.

Experiment 2 (Reunions With the Unanesthetized Dam or Sire). Procedures from Experiment 1 were repeated with a few exceptions designed in order to reduce the stress/agitation of active adults. During the isolation periods, pups were tested in novel cages (polycarbonate terraria, 7″ × 11″ × 6″) within an incubator, identical to Experiment 1. It was not appropriate, however, to keep adult animals in a warm incubator for long durations. The reunion/stimulus period, therefore, took place outside the incubator at room temperature (~22˚C). Additionally in the reunion/stimulus period, pups were carried in gloved hands to a separate testing room where the dam and sire were kept throughout the experiment in separate testing chambers. Due to the jumping ability of adult voles, the chamber used was 9½ ×7 × 12 inch made of Plexiglas. The floor of the chamber was covered with approximately ½ cup of home cage shavings. The top was open. Interactions between the active parent and pup were recorded during the two minute epoch along with ultrasonic vocalizations. In the control condition (home cage shavings), pups were handled identically to pups in the companion group but remained alone in home cage shavings during the reunion/stimulus period.

Nipple lines were not taped so that pups and dams were free to interact. In all instances, pups attached to the teat of the awake dam. In a few supplemental trials, adhesive tape was placed on the dam in an attempt to prevent nipple attachment (see Supplemental Section, not included in main analysis).

USV Recording

The total number of USVs was recorded during each 2-min period. Ultrasonic vocalizations were transduced into the audible range using a bat detector (Pettersson Elektronik D 240×, Uppsala, Sweden) with its microphone suspended approximately 10 cm above the test container floor. The detector was used in the heterodyne mode, tuned to 40 kHz; a setting that excludes counting any adult vocalizations (~20 kHz) in Experiment 2. The experimenter wearing earphones counted USV pulses by pressing the button of a silent electronic counter. Periodic inter-rater reliability tests are performed in the laboratory to ensure that counting of USV is more than 90% reliable, as previously reported (Hofer & Shair, 1978).

Behavioral Observations

The number squares entered, wall climbs (raising the head above shoulder level and at least one leg from the floor), self-grooming, and 360 degree turns inside a square were noted. No vole pups were observed to defecate or urinate during any test. In order to provide an indicator of the overall activity level of each pup, a composite score was used as the principal means of behavioral analysis. The score was calculated as the sum of all activity behaviors noted (squares entered, rises, turns-in-square, and self-grooming).

During reunions with both anesthetized and unanesthetized companions, we noted the percentage of time spent in contact and whether a pup was able to nipple attach. For the 2-min reunion/stimulus period of Experiment 2, parental behaviors were also observed. These behaviors included time spent in contact with the pup, as well as stepping on, sniffing, licking and carrying the pup (usually by the nape of the neck).

A second experimenter recorded all observations. Ambient temperature was monitored throughout the test with an air sensor positioned 7 cm above the cage floor. At the end of each experiment, pups were sacrificed by exposure to CO_2 in an uncrowded container and breeding pairs were returned to the colony room. All procedures were approved by the Institutional Animal Care and Use Committees of New York State Psychiatric Institute and Columbia University College of Physicians and Surgeons, as well as complying with the American Psychological Association's ethical standards in the treatment of animal subjects.

Statistical Analyses

Data recorded during testing were entered into a database/statistics program (SYSTAT) for analysis. The litter was the unit of analysis, that is, when more than one pup was exposed to the same stimulus in a litter, a litter mean was used. Paired *t*-tests were used to analyze a priori hypotheses, for example, whether or not potentiation occurred. For other analyses, 2-Way (Reunion/Stimulus Condition × Epoch) repeated measures analyses of variance (ANOVAs) were performed with epoch (Isolation 1, reunion/stimulus, Isolation 2) as the repeated measure. Significant findings were analyzed further using 1-Way ANOVAs as post hoc tests (Reunion/Stimulus Condition or Epoch as appropriate). As the ANOVA test is known to be highly robust in cases of nonnormal distribution (McDonald, 2015), ANOVA results are presented throughout. Comparisons among variables were Pearson Product-Moment correlations using data from individual pups, not litter means. Distribution normality was checked by Shapiro–Wilk Test of Normality (SAS, Cary, NC). Nonparametric tests (Kruskal–Wallis ANOVA and Spearman's r) were performed on any data not found to be normally distributed. The results of the nonparametric tests produced comparable results to the original parametric tests in all instances.

RESULTS

Experiment 1: Vocal Responses

Prairie vole pups showed USV potentiation following reunion with the mother, but did not following reunion with the father (see Fig. 1). That is, compared to the initial isolation, there was a significant increase in vocalization rate upon re-isolation after a reunion with the dam ($t[17] = 4.12$, $p < .01$), but not after reunion with the sire ($t[17] = .72$, *not significant* [*ns*]). No significant increases in vocalization were found following brief reunions with littermates ($t[14] = -.5$, *ns*) or after handling, in the pick up control group (t $[15] = .22$, *ns*). None of the experimental groups differed significantly in USV rates during the first isolation ($F[3, 63] = .15$, $p = .93$), consistent with the

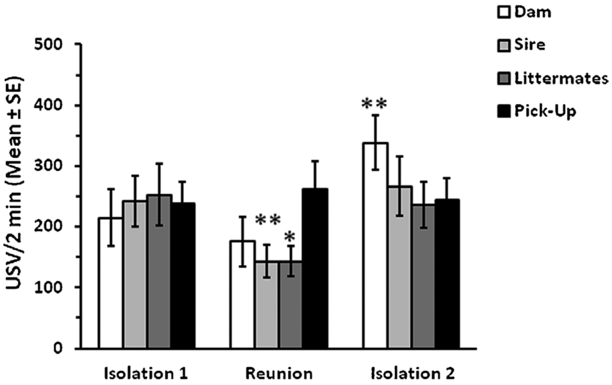

FIGURE 1 Vocalizations in the passive companion experiment. The rate of ultrasonic vocalization (USV) during the initial isolation, the reunion/stimulus condition, and the second isolation. Pups were tested in an incubator (27–31°C). Each observation period was 2 min. Values are USV rate means calculated from litter means and error bars represent standard error of the means. Asterisks denote a significant change from Isolation 1; $^{*}p < .05$, $^{**}p < .01$.

fact that all animals had received the same experience up to that point.

Consistent USV quieting (decreasing USV from first isolation to reunion) was observed in response to contact with sires and with littermates ($t[17] = -3.44$, $p < .01$ and $t[14] = -2.5$, $p = .03$, respectively). Only a trend toward USV quieting occurred during brief reunions with anesthetized dams ($t[17] = -1.58$, $p = .13$). In a direct comparison of contact quieting responses to dams and to sires, an ANOVA showed a similar trend ($F[1, 34] = 2.54$, $p = .12$). The control group, which remained isolated in the reunion/stimulus period, did not alter USV rate in that epoch ($t[15] = .73$, *ns*).

Experiment 1: Nonvocal Behaviors

Using a composite score of the sum of nonvocal behaviors (see Methods), we found no significant overall effect of companion condition on activity ($F[3,63] = .94$), but there was a significant difference due to epoch ($F[2,126] = 13.55$, $p = .00$). The pups were less active during the reunion/stimulus period. Companion condition and epoch showed an almost significant interaction effect ($F[6,126] = 2.10$, $p = .058$), which post hoc 1-way ANOVAs demonstrated to be due to differences in the reunion/stimulus period. During the reunion/stimulus period, pick up control pups were more active than those reunited with a companion ($F[3, 63] = 3.22$, $p = .03$), probably because those pups remained in contact with their companions, and as a result moved less about the cage. Pups were in contact 90 ± 4% of the time with the dam; 81 ± 6% with the sire; and 80 ± 6% with littermates, not significant differences ($F[2, 48] = 1.05$). No differences in activity were found between groups in either the first or second isolations ($F[3, 63) = .22$ *and* $F[3,63] = .52$, respectively). Nor was there a companion condition effect on the change in scores from isolations 1 to 2 ($F[3, 63] = .35$, $p = .79$; see Tab. 1). (For changes broken down by individual behaviors, see Supplementary Tab. S3.)

Correlations were performed to determine if, in voles, nonvocal activity level and USV rate interacted, which could suggest a connection between general arousal level and vocalization. There was a modest correlation between nonvocal behavior and USV in the first isolation ($r = -.24$, $p = .03$ [*see Supplementary Tab. S1*]). As nonvocal behaviors increased, USV rate showed a slight decrease; however, this explains only 6% of the variance. There were no significant correlations between nonvocal behavior and USV rate within Isolation 2. Examining the only group that clearly demonstrated potentiation, the dam group, a negative correlation between activity score and USV in Isolation 2 did not reach significance ($r = -.35$, $p = .10$).

Experiment 2: Vocal Responses

In the period following a reunion with their active dam, pups showed potentiation of their USV responses to isolation ($t[10] = 6.01$, $p < .01$), see Figure 2. However, pups did not show a similar potentiation of USV responses after reunion with their active sire; instead a modest, but significant reduction in vocalization occurred ($t[11] = -3.68$, $p < .01$). A similar reduction occurred in the home cage shavings control pups during the stimulus period ($t[9] = -2.37$, $p = .04$). None of the experimental groups differed significantly in USV rates during the first isolation (F[2, 30] = 1.39, $p = .27$).

No significant contact quieting was observed during reunion with either active dam or sire ($t[6] = -.98$, *ns*; $t[6] = -.79$, *ns*; respectively). In home cage shavings control pups, there was also no significant change in USV during the reunion/stimulation period compared to the initial isolation ($t[9] = 1.48$, $p = .17$).

Table 1. Nonvocal Behaviors During Isolation Epochs

	Isolation 1 (Mean ± SE)	Isolation 2 (Mean ± SE)	Δ Iso2–Iso1	*p*-Value
Experiment 1: passive companion				
Dam	35 ± 6	44 ± 6	+9	*NS*
Sire	36 ± 7	38 ± 9	+2	*NS*
Littermates	30 ± 7	31 ± 7	+1	*NS*
Pick-up	37 ± 7	35 ± 7	−2	*NS*
Experiment 2: active companion				
Dam	23 ± 6	26 ± 7	+4	*NS*
Sire	35 ± 8	14 ± 6	−21	*<.01*
HC shavings[a]	20 ± 7	6 ± 2	−14	*<.05*

Values represent the mean composite score for each companion condition. The composite score is the sum of squares entered, rises, turns-in-square, and self-grooming (see Methods). A two-way ANOVA (Companion Condition × Epoch) with repeated measures on epoch was performed (see Results) followed by post hoc one-way ANOVAS (Bonferroni-corrected).

[a] Home cage shavings.

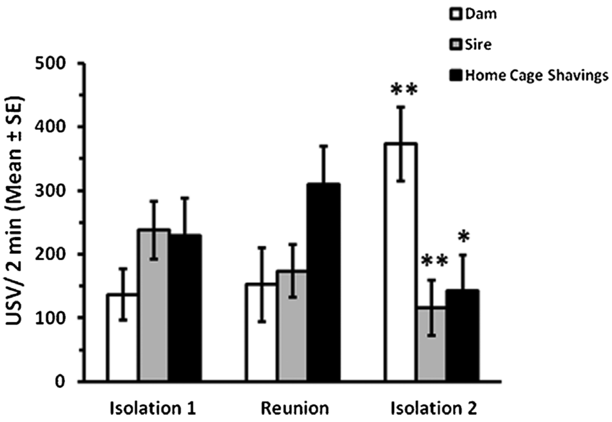

FIGURE 2 Vocalizations in the active companion experiment. The rate of ultrasonic vocalization (USV) during the initial isolation, the reunion/stimulus condition, and the second isolation. Pups were tested in an incubator (27–31°C) during the isolations. During the second period (Reunion), pups were tested at room temperature. Each observation period was 2 min. Values are USV rate means calculated from litter means and error bars represent standard error of the means. Asterisks denote a significant change from Isolation 1; $^{*}p < .05$, $^{**}p < .01$.

Experiment 2: Nonvocal Behaviors

Nonvocal behaviors were analyzed using the same composite activity score as described for Experiment 1 (see Supplementary Tab. S4 for changes in individual behaviors). As above, there was no significant overall effect of companion condition on activity ($F[2,29] = 2.05$, $p = .15$), but in this case, there were significant effects of both epoch and the interaction of companion condition and epoch ($F[2, 58] = 3.59$, $p = .03$, *and* $F[4, 58] = 21.90$, $p = .00$, respectively). As the pups' behavior during the reunion/stimulus period was likely to be affected by the actions of the adults, we reran the statistics without that epoch. The results were much the same [*Companion condition* $F[2, 29] = 2.29$, $p = .12$; *Epoch* $F[1, 29] = 7.90$, $p = .01$; *Companion* × *Epoch* $F[2, 29] = 7.79$, $p = .00$). Post hoc 1-way ANOVAs demonstrated that pups in all three groups did not differ significantly in the first isolation ($F[2, 30] = 1.40$, $p = .26$). Pups given a reunion with the dam maintained that level of activity in both isolations ($F[1,10] = 2.07$, $p = .18$). Pups in the other conditions both became less active during the second isolation (*Sire* $F[1, 10] = 21.25$, $p = .00$; *Home cage shavings* $F[1, 10] = 5.10$, $p = .05$). Similar to reunions with anesthetized parents, pups were in contact with active dams (99%) or sires (89%) most of the time and did not differ significantly between the two.

Observations of parental behavior *during the 2-min reunion/stimulus period* yielded no significant sex effects on any parental behavior except licking. The mean number of licks from the dams (7.6) was significantly higher than licks from sires (5.6) ($F[2, 28] = 3.42$, $p = .05$). Despite this, no significant correlations were found between any behaviors of parents during reunions and the resulting vocalization rates of pups during re-isolation (Isolation 2).

Examining all pups tested in isolation, there was no significant correlation between nonvocal behavior composite scores and USV totals in the first isolation ($r = .20$, *ns*; *see Supplementary* Tab. S1). In Isolation 2, however, in pups that had been previously tested with unanesthetized companions, a significant correlation was found between USV and nonvocal behavior ($r = .54$, $p < .001$). The correlation of USV to nonvocal behavior was strong and positive in both groups exposed to a parent, independent of whether the interaction produced USV potentiation: dam group $r = .55$, $p = .05$; sire group $r = .50$, $p = .05$. There was no such relationship in control pups exposed to home cage shavings $r = .10$, ns.

Age-Related Effects in Experiments 1 and 2

Potentiation to the passive dam occurred at all ages from 8–11 days old, but the magnitude of potentiation increased for older aged pups ($r = .59$, $p = .01$) following reunion with the anesthetized dam. There was no trend for increased potentiation in older pups after interacting with active dams ($r = .06$, $p = .85$), suggesting "active" potentiation had fully developed by this age. In both cases, Spearman's rank order correlations were done and supported the same conclusions. This is similar to results seen in rats, where "active" potentiation developed first and "passive" potentiation responses then increased from 11 to 13 days postnatal (Hofer et al., 1998). See Supplementary Table S2 for relationships between pup temperature/weight and vocal/nonvocal outcome measures.

DISCUSSION

The first goal of the present study was to determine if vole pups show increased ("potentiated") USV responses to isolation following a single brief interaction with their dam. Our results demonstrate that 8–11-day-old vole pups do show potentiated USV responses following interactions with either their anesthetized or active dam. Therefore, maternal potentiation of USV responses to isolation in voles is similar to that seen in rats, pigs, guinea pigs, degus, and mice.

The second goal of this study was to determine if vole pups also potentiated USV responses following separation from the sire. We found that in contrast to the strong USV potentiation responses of vole pups to their dams, they did not potentiate to the sire, whether the male was anesthetized or awake. This was true despite the fact that the awake sire sniffed, stepped on, and carried the pup during the reunion at the same rate as the dam and had demonstrated frequent paternal nurturing behaviors in the home cage during the pup's development, as has been reported previously (Ahern et al., 2011; Gruderadams & Getz, 1985; Lonstein & De Vries, 1999; Solomon, 1993; Thomas & Birney, 1979). The most obvious difference in care provided by dams versus sires is that dams nurse their pups and direct specific nursing-associated behaviors toward them. While attachment to the teat during the reunion portion of the test is not necessary for the expression of potentiation to the anesthetized dam in voles, nor to either anesthetized or awake dams in rats, the role of suckling in the developmental acquisition of potentiation remains unknown.

One factor that makes both isolation-induced USV and the potentiated vocal response special is that neither is necessarily part of a generalized increase in all arousal behaviors. In the potentiation test, for example, nonvocal behaviors by rat pups like locomotion and self-grooming do not increase from the first to the second isolation; they often decrease, whether or not the mother is active or anesthetized (Hofer et al., 1998; Hofer, Masmela, Brunelli, & Shair, 1999). Nonvocal behaviors show the same changes after reunions with littermates, for example, as they do to the dam (Brunelli et al., 1998; Hofer, Brunelli, Masmela, & Shair, 1996; Hofer et al., 1999). In fact, dopamine agonists reduce the amount of potentiation while increasing physical activity (Muller, Moore, Myers, & Shair, 2009). For guinea pig pups, however, the vocal and nonvocal behaviors tend to alter together. After a reunion with the dam, neither vocalizations nor activity decrease during re-isolation. After interactions with a littermate, an unfamiliar lactating dam, or an empty cage, both types of behavior decrease dramatically (Hennessy et al., 2006). Pigs show a similar pattern (Colonnello, Iacobucci, & Newberry, 2009). For voles, in the original isolation, there is little evidence that physical activity and USV are linked (a weak correlation in Experiment 1; no significant correlation in Experiment 2). Reunions with anesthetized dams, furthermore, produce the same pattern as rats during the re-isolation (Experiment 1). Vocal rates increase; nonvocal behavioral rates do not and do not depend on the identity of the reunion companion. The pattern of responses in Experiment 2 is more complex and shares some similarities to pigs and guinea pigs, which were also tested with active, unanesthetized mothers. Both vocal and nonvocal behaviors depend on the identity of the reunion companion. The rat mother, of course, produces an increase in USV in the subsequent isolation, unlike a mere absence of decrease produced by the other two species. Like guinea pigs and pigs, however, vole pups do not increase in nonvocal behaviors (Isolations 1–2) following interactions with the mother, but there are significant decreases in activity following exposure to the sire and home cage shavings. Thus, while we can say that maternal potentiation in vole pups is not due to a generalized arousal, the converse is not necessarily true. That is, *lack of vocal potentiation* by voles in other reunion/stimulus conditions may be due to an overall decreased level of activity (which may also be true for guinea pigs and pigs).

As the goals of this research were specific to potentiation and because potentiation has been defined as an increase in the number of vocalizations emitted in a given period of time, we did not measure other USV characteristics. The fact that vole pups do not increase the number of isolation-induced USV in any condition except following reunions with their dams does not mean that other vocal parameters are also unchanged whether or not potentiation occurs. We have shown in rats that maternal potentiation also causes increases in the bout size in which USV are emitted, as well as increased amplitude of each vocalization (and a trend to longer duration; Myers et al., 2004). Other researchers studying USV have measured a wide variety of vocal parameters including characterizing wave forms and demonstrated changes in these characteristics in response to genetic differences, social settings, and environmental challenges (Brudzynski, 2005; Chabout, Sarkar, Dunson, & Jarvis, 2015; Holy & Guo, 2005; Scattoni et al., 2009; Yu et al., 2011). It remains to be determined in vole pups if changes in other vocal parameters vary in systematic ways with changes in the rate of USV emitted.

Paternal potentiation may not occur in the prairie vole for a variety of reasons. One possibility is that paternal potentiation is elicited in some species only under conditions that differ from those that elicit maternal potentiation. Even for maternal potentiation, different parameters are needed for it to be elicited in different species, such as the presence of littermates during reunion for the CD-1 strain of mice (Scattoni et al., 2008). This possibility remains to be tested for paternal potentiation. Another possibility is that potentiation does not naturally develop from all patterns of parental behaviors within a species. There was little variability in parental behavior among the litters we observed (Supplemental Tab. S5). Under more naturalistic conditions, more variability is likely to occur and a relationship between early-life interactions and potentiation might be revealed. The possibility that potentiation occurs only to the mother, except in conditions where cues from the father are generalized from the mother, is discussed below. Looking at other species in which maternal potentiation occurs is not helpful, because paternal potentiation has not been tested in any of them yet. Looking at the differences between the postnatal experience of the two species for which paternal potentiation has been tested, voles and rats, there appears to be no obvious reason for the difference in paternal potentiation beyond the amount of experience with the father. Pups of both species are altricial, housed in nests, and can be fostered to other dams without problem (for example McGuire, 1988). Both species engage in communal nursing (Getz & McGuire, 1997; Hayes, 2000; Schultz & Lore, 1993). Rat pups will demonstrate potentiation to any adult rat female (Brunelli et al., 1998), as do degus (Colonnello, Iacobucci, Fuchs, Newberry, & Panksepp, 2011), but this is not true for all species. Guinea pigs increase vocalization only in response to their own dam (Hennessy et al., 2006). Potentiation tests with unfamiliar mothers have not yet been performed with prairie voles. To the best of our knowledge, no one has yet performed preference tests of vole pups for their own dams and sires versus other dams and sires. If, as we have hypothesized (Shair, 2014), potentiation is a marker for filial attachment, other assessments of attachment-like behavior, such as preference tests, are needed in multiple species to investigate the association of potentiation within a variety of infant/mother and infant/father relationships.

One possible explanation for the different paternal potentiation patterns of vole and rat pups may involve pup experience and learning in the home cage in the days before testing. The rat sire is less likely to display parental behaviors, including contact with pups (Lonstein & De Vries, 2000), thus, providing fewer opportunities for rat pups to learn distinguishing properties of dam and sire. In fact, rat pups potentiate USV to their fathers without having experienced any physical contact at all. Exposure to the sire's odor (and possibly auditory cues) in the home cage during development is sufficient (Shair, 2007). Vole pups, on the other hand, have frequent and independent interactions with both mother and father (for example Ahern et al., 2011). As a consequence, voles might be expected to show different responses to each parent during potentiation tests. A future experiment should involve rearing rat pups in a setting that creates increased interaction with the sire. If the above hypothesis is correct, paternal potentiation should be extinguished. Conversely, the hypothesis would also be supported if vole pups reared only with dams, but in the presence of the sire's odor, later potentiated to that sire. Of course, even if this hypothesis is true, it will not explain everything. For example, we know that rat pups do not show potentiation to castrated adult males they have been reared with (Brunelli et al., 1998; Shair, 2007). There must be something extra (presumably odor) during early life that elicits differential reactions of rat pups to intact and castrated males. Furthermore, infant titi monkeys, a species in which fathers often engage in greater caregiving behavior than the mother (depending on the mother's age, experience, and hormonal status (Jarcho, Mendoza, & Bales, 2012)), show greater vocal and cortisol responses when separated from such fathers. Reunions of isolated infants with the father also reduced vocalizations more than reunions with the mother (Hoffman, Mendoza, Hennessy, & Mason, 1995).

Finding maternal but not paternal USV potentiation after a brief reunion is consistent with the hypothesis that potentiation reflects a form of dysregulation resulting from the unexpected interruption of "coregulation" of physiological and behavioral systems inherent, and specific to, mother–infant interactions. When the mother–pup reunion is allowed to continue for at least 15 min, pups do not express USV potentiation (Shair et al., 2015), presumably because separations after prolonged periods of coregulation are less disruptive to the infant.

Consistent with work by others showing that vagal signaling plays an important role in regulating affective behavior (Goehler, Lyte, & Gaykema, 2007; Klarer et al., 2014; Luheshi et al., 2000), we speculate that afferent activity from the gut may be an integral component of experiencing a positive state of coregulation and its loss in separation. Supporting this, we found in an earlier study that cutting the vagus nerve below the diaphragm prevented expression of maternal potentiation of infant rat USV (Shair, Smith, & Welch, 2012). We hypothesize that the afferent and efferent vagal activity which is critical for pups to express potentiation is conditioned by previous associations between mother/infant coregulation and suckling and, possibly, other aspects of infant care that are specific to mother/pup interactions. In this view, vole pups do not potentiate to their sire because the extensive sire/infant interactions during development do not include the requisite associations required for conditioning of gut signaling.

NOTES

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