Disambiguating stress-buffering and relationship-renewal in reunions between female degus

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Abstract

A full understanding of a species' sociality requires knowledge of their specific social motivations. Following social isolation, animals may show general interest in companionship as a buffer for stress or loneliness, but may also be driven to re-establish expectations and dyadic roles with individuals they had been previously separated from. By deconstructing social behavior across experimental manipulations, it may become possible to disentangle these motivational factors. Physical and vocal interactions were recorded from adult female degu dyads during a series of 20 minute reunion sessions across four experiments. Experiments 1 and 2 found that degu interactions increased following isolation, but also increased after dyads were separated without isolation from other conspecifics. Isolation resulted in more early-session interactions, higher allogrooming before rear-sniffing, and a higher ratio of chitter to non-chitter vocalizations. Experiment 3 showed that a non-social, footshock stressor selectively increased early-session interaction, and Experiment 4 revealed high interaction rates between strangers, with more non-chitter vocalizations and late-session rear-sniffing, and reduced pre-rear-sniff allogrooming. A novel, repeated-k-means clustering approach helped to further specify differences in vocalizations between conditions; e.g., "chaff"-type syllables were more common when relationships were new or potentially being renewed. Results suggest that degus are motivated to establish or re-establish expectations with one-another, and these interactions can differ from those associated with the stress of isolation.

keywords: motivation, ethology, relationship, social, rodents

Introduction

In many animal species, a period of isolation can strongly motivate subsequent social interaction (e.g., humans: [1], rats: [2]). The increased drive to interact after social deprivation is reminiscent of traditional models of motivation, in which the energy for a behavior increases with time since the behavior is last expressed [3,4]. But studies on the motivational effects of social isolation often conflate two factors: the temporal distance between two specific individuals, and the stress associated with being isolated from all individuals. Evidence from a diversity of species suggests that greeting behaviors following a period of separation serves to reestablish relationships, offering a means for individuals to renew trust or expectations [5–8]. It is also known that complete social isolation increases stress (particularly well characterized in murine rodents [9–16]), which can be reduced through interaction ("social buffering", reviewed by [17–22]). Factors related to both stress and to relationship likely play a role in social behaviors that follow isolation, where "relationship" is operationally defined as the sets of expectations, emotional associations, and dyadic roles (like dominance) that two animals have for one another. This raises the question of whether stimulus-specific versus general motivations can be teased apart by a dyad's interaction patterns.

The present experiments test whether interaction patterns between adult, female degus differ following isolation, separation, and stress experimental manipulations. Degus (*Octodon degus*) are an interesting species for studies of social interaction on account of their high sociality [23,24], dynamic group structures [25–28], as well as the parallel research that can be done in both field and laboratory settings. Female degus especially are recognized for their constructive interactions, such as communal nursing, with both related and unrelated, same-sex individuals [26,29–32]. Degus also express a wide range of vocalizations in the human hearing range, and these have been manually classified based on spectral features and social context in which they are used [33]. This allows for a large range and dimensionality of behaviors that can be quantified to compare interactions between different experimental conditions.

We recorded degu behavior during 20 minute "reunion" sessions following manipulations that included social isolation, separating dyads without isolating them from other conspecifics, and exposure to an external stressor. We found several subtle but notable distinctions in how different forms of motivation impact behavior. Both isolation and stress caused more interactions earlier in the reunion sessions, consistent with social buffering hypotheses. After dyads were separated without isolation, rear sniffs were less likely to be preceded by nose-to-body contact, as though information about the others' identity took precedence over the presumably affiliative "nuzzling" or allogrooming gestures. Vocalization behavior was highly variable, but exploratory analyses suggested that "chaff" type vocalizations may be differentially involved in the establishment or resestablishment of social bonds.

Methods

Subjects

Thirty-four female degus aged 6 to 19 months (mean = 10.2 ± 3.6 SD) were used for all experiments. Prior to the start of experiments, degus were housed either in pairs within 14" x 11" x 9.5" polycarbonate cages or, in the case of Experiment 2, in groups of four within 15" x 19" x 8" cages in a vivarium at the biological science facility at the University of Toronto. Degus were kept on a 12:12h light/dark cycle with food and water available ad libitum. All procedures were conducted in accordance to the policies of the biological facilities at the University of Toronto and all protocols were approved by the University of Toronto Animal Care Committee.

Apparatuses

Behavioral recording took place in degus' homecages placed inside a 15''x19''x 24''plexiglass chamber. The roof of the social reunion chamber was embedded with a Sony (Sony; Tokyo, Japan) USB2 camera and Sony lapel microphone. Data were collected using Bandicam multi-media recording software (Bandisoft; Seoul, Korea). Social isolation was peformed by placing each degu into separate, 14" x 11" x 9.5" chambers, either in the experiment room (for 1 and 45 min isolation periods) or the vivarium (for 24 hr isolations). Footshocks used for the "acute stress" condition in

Experiment 3 were delivered in a 18” × 17.5” × 15” conditioning chamber with a metal grid floor consisting of 5 mm thick stainless steel rods spaced 1 cm apart (Colbourne instruments; Holliston, USA).

Protocol.

Habituation: Prior to behavioral testing, degus were acclimated to the recording chamber. Degu pairs were brought to the conditioning room and their homecage was placed into the soundproof recording chamber. The pair spent 1 hour undisturbed in the chamber for each of 3 consecutive days.

Experiment 1: To test the effects of social isolation on interactive behavior, 9 pairs of female cagemate degus (18 degus) were placed in separate cages for either 1 or 45 min in the recording room, or 24 h in the vivarium. The isolation chambers were the same size as the animals' homecages and were transparent and contained clean bedding. Notably, although they were "isolated" in the sense of being unable to touch or interact with other individuals, they could still see and hear other degus through the cage. Members of a dyad were kept on different sides of the room to prevent direct communication between them. Following the social isolation period, the original homecage was placed within the soundproof chamber for 20 minutes, during which time video and audio recordings were collected. The order of conditions was counterbalanced across groups. The basic protocol is illustrated in Figure 1A.

Experiment 2: To test the differences between social isolation and separation-without-isolation, each of 4 groups of 4 cagemate degus (16 degus) were first separated into two cages of only two degus (SEP). Following 24 hours, one member of each of the new cages was placed together in a clean 14” x 11” x 9.5” cage within in the recording chamber for a 20 min reunion session, after which the recorded pair is taken to the vivarium and kept together in that same cage for the subsequent 24 hours. The same is done with the remaining members of the two cages. Following 24 hours as reunited cagemates, the dyad is separated into two individual cages for a 1 minute period, and then returned for a second, 20 minute reunion (CTL). Degus are then returned to

separate cages and remained isolated for 24 hours, after which a final, 20 min reunion session was performed (ISO).

Experiment 3: To test the impact of stress on social interactions, the same degus from Experiment 1 were presented with two additional conditions. In one, cagemate dyads were separated into two different cages in two separate rooms for a 45 minute period. During this time, following a 5 minute adaptation period, 8 sunflower seeds (a familiar, preferred food) were placed one at a time over a 2.5 minute period in each cage (RWD). When the 45 minute period had elapsed, they were placed together in their homecage, in the recording chamber for a 20 minute reunion session. In the other condition, cagemate dyads were separated into two separate rooms and placed in an conditioning chamber with a metal grid. Following a 5 minute baseline period, degus received 10, 1.0 mA footshocks lasting 2 s each over a 2.5 minute period. This shock level and sequence was based on a protocol we used previously to evoke contextual and observational fear learning [34], though to reduce total aversiveness we decreased the number of shocks by one-half. After receiving footshock, degus were placed in separate cages for the remainder of the 45 minutes.

Experiment 4: To further investigate features of interactive behavior associated with novelty and relationship establishment, degus were united with unfamiliar, stranger conspecifics following 24 hours of isolation. Animals used for Experiment 4 were the same as those used in Experiment 2, but had been pair-housed with a cagemate for at least one week after the prior experiment had ended.

Behavioral analysis

Software: Analysis relied on several software packages. Scoring of physical behavior was performed using BORIS (Behavioral Observation Interactive Research Software; Torino, Italy), which allows users to log events during video playback. Scoring of vocalization behavior was performed using Audacity (Audacity v.2.1.2; Pittsburg, USA), as well as Sound Analysis Pro (SAP), a vocalization detecting software developed for segmenting syllables and vocalization structure in birdsong [35]. All other analyses were custom-written in MATLAB, as described below and provided at https://github.com/ninsel/DeguReunionMotivation.

Physical interactions: Each video recording was manually scored by blind raters using BORIS. Ethograms included four general types: *agonistic encounters* (bites, characterized by face-to-body contact of one degu with the other making a startled movement away; mounting, involving one degu placing forepaws on the other while the other made avoidance movements; wrestling, characterized by rapid movements of both bodies and usually accompanied by other aggressive actions; punching/boxing, with degus sometimes rearing on hindlegs; body-slams, in which degus kicked with their hind legs, and any other apparently aggressive encounter), *allogrooming/body sniffing* (one degu's nose touching and/or interacting with the neck or body of the other), *rear-sniffing* (a.k.a. anogenital investigation, where one degu's nose was near and directed toward the base of the tail of the other), and *face-to-face* interactions (one degu's nose near and directed toward the nose or mouth of the other). For each scored behavior, the identity of the animal was also included. Often behaviors overlapped: e.g., one degu may initiate rear-sniffing and the other may then reciprocate before the first behavior ended. In the case of face-to-face, the action was necessarily mutual, but these were still labeled with the identity of the degu that initiated the interaction. One potential problem with behaviors overlapping in time is that the sum of all behaviors performed by one degu added to the sum of all behaviors performed by the other could, in principle, exceed the total time of the session. Most analyses therefore combined interaction time from each degu by considering all time during which *either* one degu *or* the other was engaging in the action (i.e., the union of time, rather than the combined total of both).

Comparisons of physical interactions between experimental conditions were analyzed using the following three tests: 1) Total time dyads spent interacting, and total time engaging in different interaction types. As noted above, interaction time was primarily analyzed as all time that either one or the other degu of a dyad was engaging in an interactive behavior (body-sniffing, face-to-face, etc.). 2) Temporal distribution of interactions across the session, primarily quantified and illustrated by cumulative distributions of interaction over time. Cumulative distributions of interactions were computed with a 2 s bin for each session. 3) Temporal relationships between interaction types, quantified and illustrated by the cross correlograms. Cross correlations were computed with time (x-axis in the cross-correlogram) preceding 0 referring to actions that took place before the onset of a reference action, and times following 0 referring to actions that took

place after the offset of the reference action. Values (y-axis in the cross-corelogram) were normalized by dividing by the number of reference actions that took place, and also normalized by further dividing by the proportion of time during the session that the comparison action took place; in other words, values of "1" in the cross correlation meant there was no observable temporal relationship between two action types. To assess significance, all values from -10 to -1 s (before onset) and from 1 s to 10 s (after offset) were averaged within each session, and a paired t-test was performed to assess whether values were A) consistently higher than 1, and whether values were B) consistently higher than a surrogate dataset in which onset/offset times of the comparison action type were randomly shuffled within a -5 to 5 minute interval. Only those cross correlations that met both criteria at an alpha value of 0.05 were considered significant. In cases where comparisons were being made between conditions, a paired or two-sample t-test was used.

Vocal interactions: Vocalizations were scored by loading audio files into Audacity and using both audio and visual spectrograms to identify vocalization start and end times. To reduce noise, the Audacity "noise reduction" feature was used, which creates a filter based on a selected audio baseline period. Experimenters scored vocalization by loading individual sound clips generated by Audacity into SAP, where spectrograms and summary statistics (amplitude, frequency modulation, duration) were compared with those presented by Long (2008; [33]). Comparisons of vocal behavior between sessions relied on the following tests: 1) total vocalization rates (number of syllables over session time), and rates of specific, manually scored vocalization types, and 2) differences in the profile of vocalization types detected through unsupervised clustering of the syllables (repeated-k-means).

We chose to use an unsupervised clustering method over a supervised algorithm out of interest for a method that was complimentary to, rather than dependent on, manually-labeling. Unsupervised methods have the potential of identifying syllable clusters based on features that human listeners are not as well tuned to, thereby revealing syllable types that we would not have known to look for. The sequence of steps involved in this analysis consisted of the following: 1) First, a set of 31 features was computed for each identified syllable. The first feature was syllable duration, while the remaining thirty were dominant frequency, spectral dispersion, and amplitude across each of ten deciles of time. These values were standardized across all syllables, and then weighted such

that syllable duration counted for 10 times each dominant frequency value, 20 times each spectral dispersion value, and 40 times each amplitude value. The weightings were chosen based on subjective assessment of which features should play the strongest roles in syllable classification, an approach that has precedence in the field [36]. 2) To reduce the dimensionality of the feature space, and also account for correlations between features (e.g., adjacent deciles are likely to be correlated), we converted features to principle components. 3) Describing each syllable by its first 10 principle components, we then entered all recorded syllables across all experiments into a k-means clustering method. This was modified from a similar method used in previous work from our group [34]. K-means clustering was used to subdivide the pool into 2 clusters, 3 clusters, 4, and on up to 30 clusters (10 replicates each, with 20 repetitions each to assess cluster stability). 4) For each syllable cluster, the number of cases in each session was identified. The proportional rate of syllables in one condition could then be compared with another condition by computing an effect size (Cohen's D) and a significance value (p value from a paired t-test when considering the same animals, or two-sample t-test in Experiment 4 when comparing different dyads). In some cases, more than one cluster might show a strong difference, with some being sub-divisions of others. To identify only the *independent* groups of syllables that differed between conditions, only the strongest cluster was considered, then all syllables from that cluster were removed and significance testing between clusters was performed again, across multiple iterations until no significant clusters were identified.

One acknowledged risk of the repeated-k-means analysis is that if syllables are randomly distributed across all sessions, one might expect to observe significant clusters roughly 5% of the time, given an alpha of 0.05. Empirically this was not the case; however, taking this concern into account, identification of clusters was treated as purely exploratory. For partial validation of the results, we performed an empirical assessment of the individual clusters (including consistency of the cluster across the 20 repetitions, as measured by the Rand index, and how the cluster mapped onto manually-labeled vocalizations) and present figures of effect size distributions for qualitative comparisons between experiments. All identified clusters were reported in Results, and to reduce biases from multiple-comparisons across repetitions only the median significance values and effect sizes across the 20 repetitions were reported.

Although behaviors were performed within a soundproof chamber, it was still possible that vocalizations below a certain volume were undetected by out methods. To assess whether this might be the case, vocalization syllables were segmented into 10 ms bins and the peak amplitude was measured relative to the dominant frequency at that amplitude. This was first done across syllables recorded in several, adult female degu "reunion" experiments which used the same methods (24,535 syllables). An initial look at the data revealed that a substantial proportion of syllables (approximately 43%) were not loud enough, or of high enough frequency, to exceed the volume of the ambient noise at 400 Hz (the lower cutoff that was used to compute dominant frequencies). Syllables were therefore separated into two populations: those with amplitude peaks that exceeded a 400 Hz cutoff, and those that did not. Amplitudes and dominant frequencies of those that reached threshold are illustrated in **Supplementary Figure 1A**; of these, only one small grouping of low-amplitude, low-frequency syllables, almost exclusively labeled as chitters, appeared to have a distribution with the lower-end cut-off from detection. Of those that did not stand out from the noise, also most often labeled as chitters, the distribution also appeared to be positively skewed (**Supplementary Figure 1B**). These data suggest that only a minority of vocalizations failed to reach detection threshold, and most of these were likely to be "chitters."

Results

Experiment I: Isolation time

We first sought to confirm that degus become increasingly motivated to interact following increasing intervals of social isolation. Nine, adult female degu cagemate dyads were pre-exposed to a behavioral recording chamber for one hour each day over three days and then isolated from one another in separate cages for 1 min, 45 min, and 24 h (order counterbalanced across dyads; **Figure 1A**). Following each isolation period, dyads were brought back together for 20 minutes in the recording chamber while video and audio data were collected. Consistent with expectations, there was a significant impact of isolation time on the time degus spent physically interacting (repeated-measures ANOVA, $F_{2,16} = 4.93$, $p = 0.021$, **Figure 1B**). The effect of isolation was particularly strong earlier in the session (**Figure 1C**), with the peak differences between conditions observed 3 to 4 minutes into the session (paired t-test of 1 min vs 24 hr $p = 6.8 \times 10^{-6}$ at 194 s; 45

min vs. 24 hr p = 1.3 x 10^{-5} at t = 282 s; 1 min vs 45 min p = 0.0014 at t = 224 s). Interactions were parsed into four types: agonistic (which included mounting), allogrooming/body sniffing, rear-sniffing (i.e., anogenital investigation), and face-to-face. Significant changes in interaction time were observed across conditions for rear-sniffing ($F_{2,16}$ = 5.9, p = 0.012) and face-to-face interactions ($F_{2,16}$ = 6.78, p = 0.0074), with a trend also observed for grooming/body sniffing ($F_{2,16}$ = 3.58, p = 0.052; **Figure 1D**). The time spent in agonistic behavior did not appear to vary across the conditions ($F_{2,16}$ = 0.23, p = 0.80).

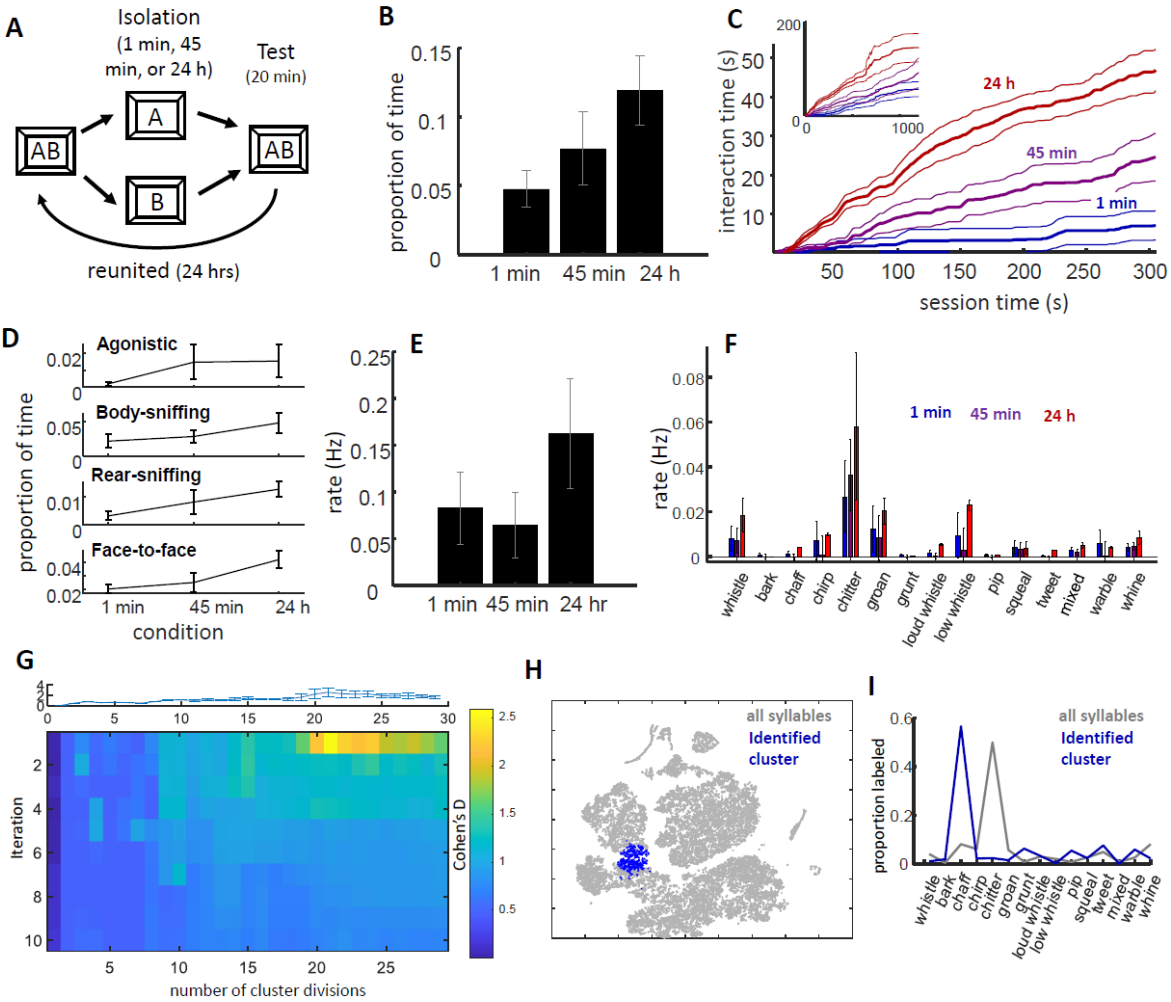


Figure 1. Effects of social isolation time on reunion behavior. **A)** Illustration of experimental design. Degus were separated from cagemates for 1 min, 45 min, or 24 hours then interactions were examined in a testing chamber. After being reunited for 24 hours the process was repeated. **B)** Proportion of time engaged in physical interactions (y axis) increased with the amount of

preceding time of isolation (x-axis). **C)** Cumulative distributions of physical interactions over session time. Higher levels of interaction in the 24 hr condition were seen within the first minute and remained high for the first 5 minutes. Inset shows full session time; although interaction time in the 24 hr condition remained higher, interactions in the 1 and 45 min conditions were more distributed across session time. **D)** Line graphs of interaction time across types of physical interaction; no interaction between type and condition was observed. **E)** Vocalization rates (y-axis) significantly increased across conditions (x-axis). **F)** Vocalization rates were consistently higher in the 24 hr condition across the 15 vocalization categories tested. **G)** Heatmap of identified syllable clusters with the strongest difference between the 24 hr and 1 min conditions, across k-means clusters repeated for 2-30 cluster divisions (columns), iterated after removing syllables from the best cluster (rows). Plot on top is an errorbar showing variance of the first (top-row) across 20 repetitions. The hot-spot in the heat map shows that when syllables were divided into 20 clusters, one cluster, consistent across the 20 repetitions, showed a particularly high effect size and was significantly, across dyads, more highly expressed in the 24 hr condition. **H)** Map of t-SNE projection of vocalization features. The identified cluster on this projection is represented in blue dots. **I)** Proportion of manual label types across syllables across all studies (grey) is plotted alongside the proportion of labels for the identified cluster. Syllables in this cluster were predominately labeled as "chaffs", and almost never as "chitters".

To assess the structure of behavior, we examined whether there were temporal relationships between physical behaviors. Cross-correlograms were generated for each pair of interaction types (e.g., face-to-face and rear-sniffing, rear-sniffing and allogrooming/body sniffing, etc), where values on the negative side of the x-axis (time) represented the occurrence of an action type *before the onset* of another "reference" type, and values on the positive side of the x-axis represented the occurrence of an action type *following the offset* of the reference type. A significant temporal relationship was determined if the cross correlogram values were A) consistently higher than baseline (i.e., systematically higher than 1), and B) consistently higher than a surrogate dataset, in which onset/offset times of the comparison action were randomly shuffled within a -5 to 5 minute interval (see Methods). Significant relationships (described as comparison relative to reference) were identified for: 1) allogrooming/body sniffing preceding rear-sniffing (above baseline: $p = 0.010$, above shuffled: $p = 0.0022$), 2) agonistic preceding and following agonistic (above baseline: $p = 0.024$ and 0.0059 respectively, above shuffled: $p = 0.045$ and 0.0047 respectively), 3) rear-sniffing following allogrooming/body sniffing (above baseline: $p = 0.0024$, above shuffled: p – 0.0014), 4) allogrooming/body sniffing following face-to-face (above baseline: $p = 0.0023$, above shuffled: $p = 0.011$) 5) face-to-face following allogrooming/body sniffing (above baseline: $p = 0.018$, above shuffled: $p = 0.021$). Averaged cross correlograms are presented in **Supplementary Figure 2**, examples provided in subsequent sections.

As with physical interactions, isolation also appeared to increase vocalizing, measured by the frequency of syllables over session time ($F_{2,16} = 4.18$, $p = 0.034$, **Figure 1E**). To unpack this result, vocalization syllables were manually and automatically parsed into types. Manual classification included 32 categories, guided by previous research on degu social vocalizations[33]. Of these, only approximately 14 vocalization types were observed at least once, on average, per session—for the purposes of analysis, all others were included within a 15th "mixed" category. Each vocalization type showed high variability between dyads and sessions, and no significant interaction was observed between condition and vocalization type (2-way ANOVA, effect of condition: $F(2,360) = 3.04$, $p = 0.049$, effect of type: $F(14,360) = 5.22$, $p = 5.3 \times 10^{-9}$; condition x type, $F(28,360) = 0.38$, $p = 1.00$, **Figure 1F**).

Differences between manually-scored syllable categories were sometimes subtle, and there was likely discrimination ambiguity between certain types (illustrated by the overlap of the different classes in feature space, **Supplementary Figure 3**). We therefore also used an unsupervised clustering method to assess whether clusters of syllables with shared features might be differentially expressed between conditions. The repeated-k-means approach is detailed in Methods. Briefly: syllables were divided into clusters using a k-means algorithm (2 to 29 clusters, repeated 20 times, all across 10 iterations of removing the best cluster from the previous) and syllable clusters that made-up a higher proportion of those expressed during one condition compared with another were identified. For the purposes of this investigation, consistency across dyads, was emphasized over magnitude of difference, and proportion of syllable type within a session emphasized over total numbers. The method was considered exploratory and results were interpreted only within the context of manual scoring.

Comparing the 24 h to 1 min isolation conditions, only one cluster stood out as showing a systematic (across dyad) difference (**Figure 1G & H**). This group of syllables was most precisely isolated when the pool was divided into 20 clusters (median p across repetitions = 0.013, median Cohen's D = 2.7; consistency of cluster across iterations was also high: average adjusted random index = 0.96, where 1 is perfect cluster overlap). This syllable type was often manually scored as a "chaff" (**Figure 1I**) and was distinguished by relatively high average dominant frequencies

around 6000 Hz, slightly lower-than-average entropy or frequency dispersion (i.e., more "scratchy") and slightly higher than average amplitude. These vocalizations were not more temporally associated with specific interactive behaviors than average vocalizations, although prior work has suggested that chaffs may be more agonistic in nature [33].

Experiment II: Isolation vs. Separation

The second experiment examined whether dyads interact differently when motivated by prior social isolation, as compared with prior separation from one-another without being isolated from other conspecifics ("general" versus "stimulus specific" social motivators). This was tested by comparing interactions in reunited dyads following three manipulations (illustrated in **Figure 2A**): 1) Cages of four female degus were split into two cages for 24 hours, thereby causing separation of two dyads (SEP); 2) all individuals were socially isolated for 24 hours (ISO); and 3) all individuals were isolated for only a 1 min interval (CTL).

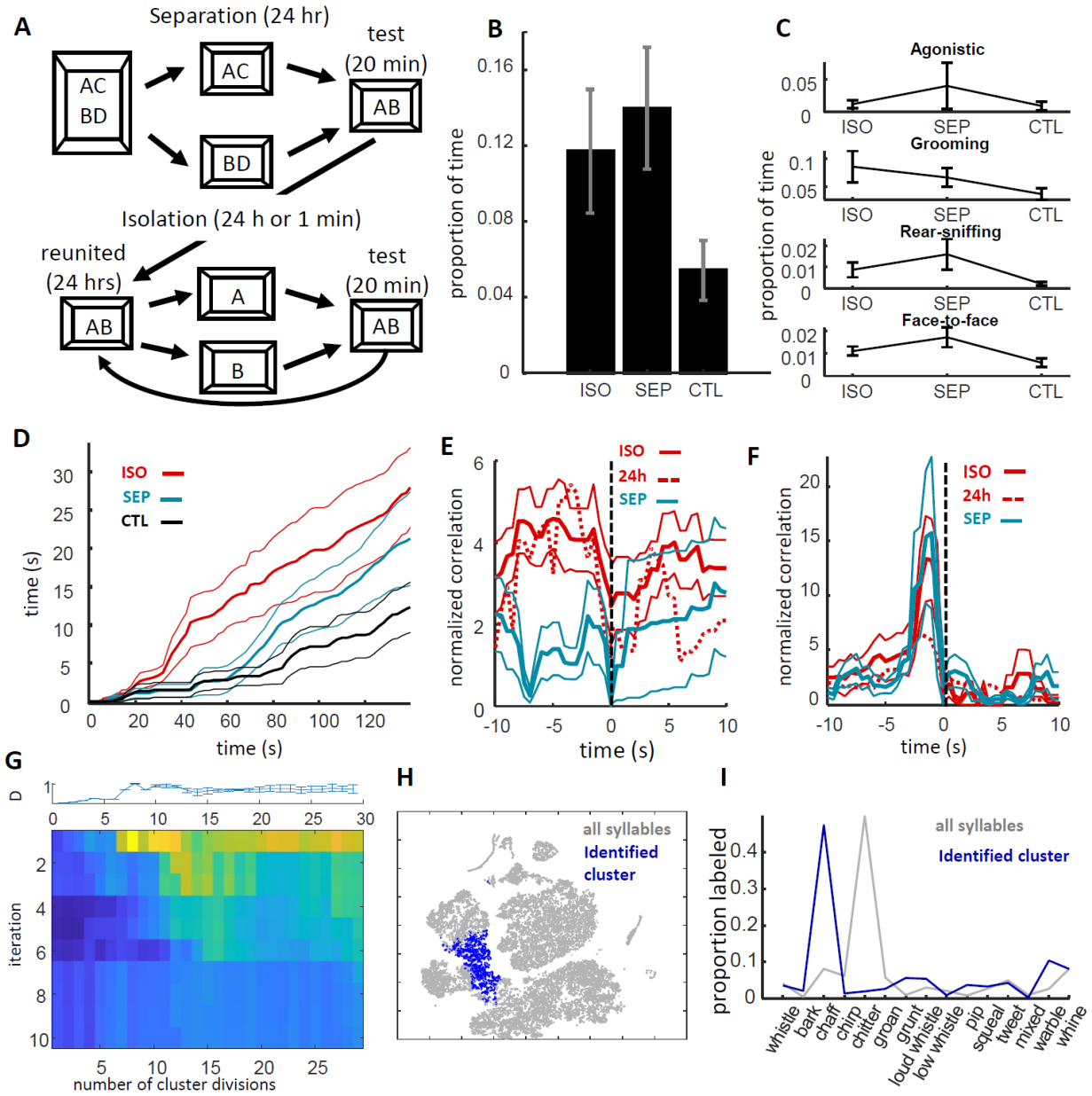


Figure 2. Comparison of isolation versus separation on reunion behavior. **A)** Illustration of experimental design. Cages of four were split into two cages for 24 hours, separated dyads were then united for reunion SEP tests. Subsequently these same dyads were isolated for 24 hours and then 1 min (or vice-versa) for ISO and CTL tests respectively. **B)** Proportion of time degus engaged in physical interactions was at least as high in SEP as ISO, but lower in CTL. **C)** Interaction time for different types of physical interactions. There were no systematic differences between SEP and ISO, though patterns hint at higher rear-sniffing and lower grooming in SEP relative to ISO. **D)** Cumulative distribution of physical interactions over session time. Although interactions in SEP were high over the 20 min, following isolation degus were much more likely to interact during the first minute of the reunion session. **E)** Cross correlogram of levels of allogrooming/body-sniffing (traces) relative to rear-sniffing. Negative time values are prior to rear-sniffing initiation, positive

are after termination of the behavior. Both ISO and the 24h condition from Experiment 1 showed higher allogrooming before rear-sniffing compared with SEP. **F)** Cross correlogram of the levels of face-to-face interactions relative to allogrooming/body sniffing. A significant increase in face-to-face contact before allogrooming was observed for all conditions. **G)** Heatmap of identified syllable clusters with the strongest difference between ISO and SEP, with increasing cluster divisions across columns and iterations after removing syllables from the best cluster down rows. Plot on top is an error bar showing the variance the first (top-row) across 20 repetitions. **H)** Map of t-SNE projection of vocalization features. One cluster was identified of syllables that differentiated between SEP and ISO conditions (blue points). **I)** Proportion of manual labeled types across all syllables (gray trace) and for only those syllables in the identified cluster (blue trace) show that, as with the cluster identified in Experiment 1, these were chaff-type syllables.

Degus interacted more in SEP than they did in CTL (one-way ANOVA effect of condition $F_{2,14} = 4.93$, $p = 0.024$; SEP vs. CTL, paired t-test, $p = 0.0056$; **Figure 2B**), and there was no detectable difference between ISO and SEP ($p = 0.45$). Nor were significant differences were detected between ISO and SEP in any of the individual behaviors (agonistic, body-sniffing, rear-sniffing, face-to-face; paired t-tests, $p > 0.05$). Importantly, while there were no overall differences between these two conditions over the full 20 min period, differences were observed in their interaction levels during the start of the session; at 60 s, ISO degus had interacted on average 20% of the time, while SEP degus had interacted an average of only about 5% (paired t-test, $p = 0.043$, Cowen's d = 1.16). The difference in interaction levels between the conditions was most evident between 40 to 70 s (**Figure 2C**). There was a corresponding statistical trend for steeper initial slopes in cumulative interaction behavior in the ISO condition compared with more evenly paced interactive behavior in the SEP condition (paired t-test on the latency to half-amplitude, $p = 0.074$). In summary, although interactive behavior between SEP and ISO conditions did not show a net difference over the 20 min period, interactions tended to take place earlier following isolation (ISO), while being more evenly distributed over the session following only separation (SEP).

By comparing the temporal relationships between physical interactions in ISO and SEP we found a significant decrease in the proportion of rear-sniffing cases preceded by allogrooming/body-sniffing (paired t-test, $p = 0.0066$). Importantly, patterns in ISO replicated those observed in the 24 h condition of Experiment 1 (**Figure 2D**). In other words, while there were no apparent systematic differences in the total levels of face-to-body contact between conditions, degus who had been previously isolated were more prone to engage in allogrooming ("nuzzling") immediately

prior to rear-sniffing. This may reflect a tendency toward affiliation in animals that had been previously deprived of social stimulation. All other temporal relationships were highly consistent between ISO and SEP, and consistent with those observed in Experiment 1 (e.g., face-to-face contact prior to allogrooming/body sniffing, **Figure 2E**).

As was observed in Experiment 1, vocalization rates were highly variable between sessions and dyads. There were no significant differences across the three conditions (repeated-measures ANOVA, $F_{2,14} = 2.27$, $p = 0.14$); however, it seems likely that lower vocalizing in CTL would be observed with more statistical power (paired t-test between ISO and CTL, $p = 0.055$, Cohen's $D = 0.78$). Given the low power and high variance, it is also unsurprising that we observed no interaction between condition and vocalization type (2-way ANOVA, effect of condition: $F(2,300) = 3.94$, $p = 0.020$, effect of type: $F(14,300) = 19.07$, $p = 7.03 \times 10^{-34}$, condition x type: $F(28,300) = 1.25$, $p = 0.19$; **Supplementary Figure 4**). More generally, however, there appeared to be a higher ratio of non-chitters to chitters in SEP compared with ISO (less than half the number of non-chitters to chitters in ISO, and around twice the number of non-chitters to chitters in SEP; a non-parametric, Wilcoxon rank-sum test was used because ratios did not distribute normally, $p = 0.0080$). This was notable in that a similar but stronger pattern was observed in strangers (Experiment 4, below).

Our repeated-k-means clustering method revealed only one cluster with different proportional expression in SEP versus ISO conditions (**Figure 2F & G**). The syllable type was most strongly revealed when all syllables were subdivided into 8 clusters (median $p = 0.038$, Cohen's $D = 1.01$ across 20 repetitions). Unexpectedly, while the cluster was highly overlapping with the one identified in Experiment 1, and typically labeled as "chaffs", these syllables were expressed more in SEP than ISO (**Figure 2H**). The syllables of the cluster identified in Experiment 1, contained within this larger grouping, were themselves also more common in SEP than ISO ($p = 0.037$). One interpretation for the higher incidence of chaff-type vocalizations in the 24 h isolation condition of Experiment 1, and higher levels of these same vocalizations after 24 hours of separation in Experiment 2, is that in both cases cagemates were being reunited after the first time they had been separated for a full day, and chaffs were part of a set of interactions involved in re-establishing their relationship. Comparing ISO and CTL, several non-overlapping clusters reached criteria, but

the effect magnitudes were very low relative to the other clusters identified and did not lend themselves to straightforward interpretation. Two of these clusters showed higher proportionate activity in ISO but were distributed across several manually-labeled categories (e.g., chaffs, groans, chirps, tweets, whines; median $p = 0.023$ and $p = 0.027$), another, of manually labeled tweets and chaffs, was found to be proportionally more common in CTL (median $p = 0.027$).

Experiment III: Acute Stressor vs. Reward

One factor that might have played a role in the differences between isolation and separation is the stress associated with isolation [11–16]. To better understand the ways in which stress modulates female social behavior, we examined behavior following a short period of exposure to an acute, unpredictable stressor (a series of 10, uncued footshocks spaced over 2.5 minutes within a 45 min isolation period; SHK), as compared against the effects of 45 minute isolation in a neutral environment (NTL), and exposure to an unpredictable reward (un-cued delivery of sunflower seeds over a 2.5 minute period within a 45 min isolation period; RWD; **Figure 3A**).

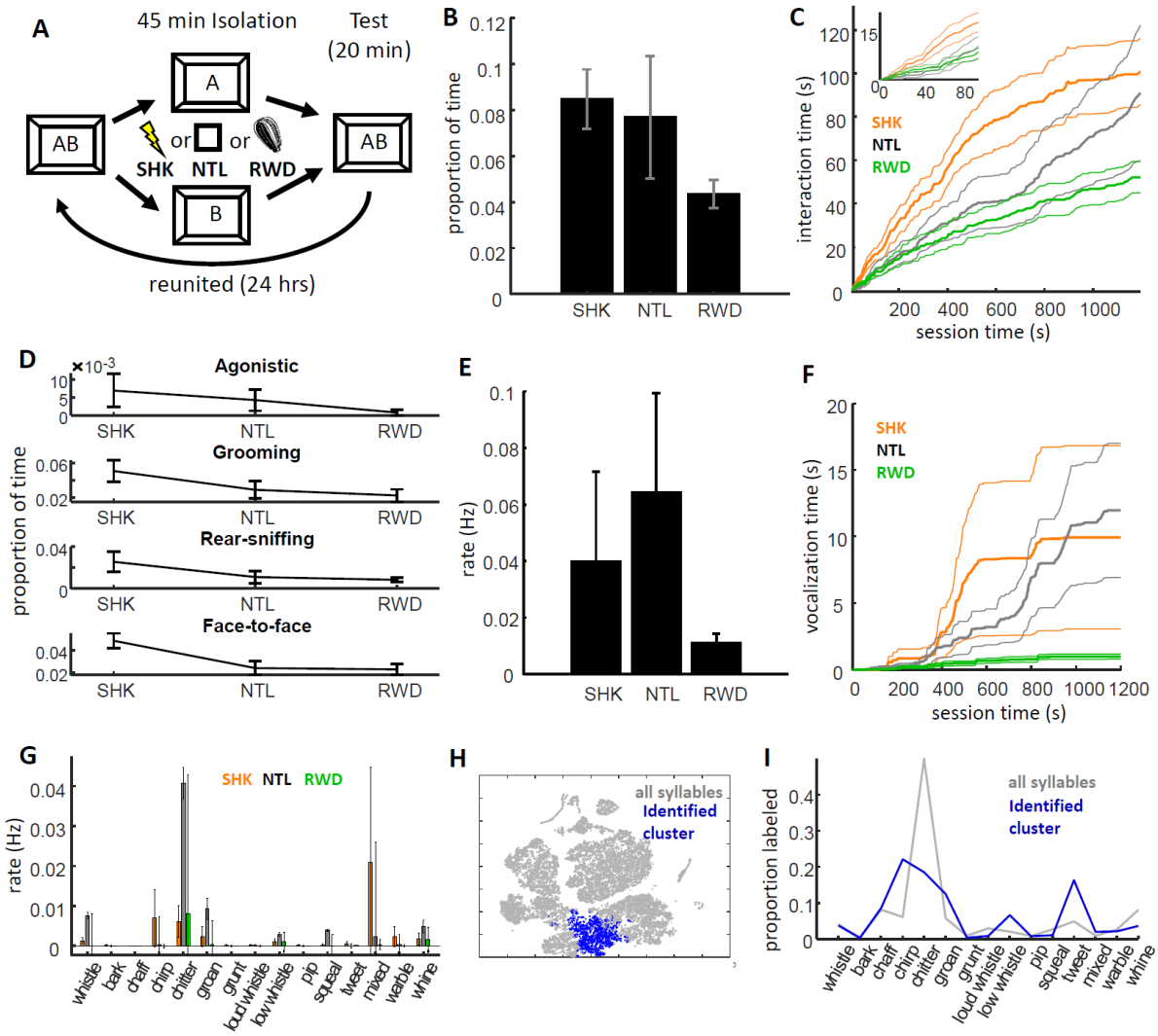


Figure 3. Effects of exposure to an acute stressor on reunion behavior. **A)** Experimental design. Degus were isolated for a 45 min period during which they were exposed to unexpected footshock (SHK), unexpected reward (sunflower seeds; RWD), or neither (NTL). **B)** Proportion of time dyads spent interacting in each condition. **C)** Cumulative distribution of time spent interacting across the session. Footshock stressor increased social interaction earlier during the testing, though this behaviore leveled off in the latter half. **D)** Four line graphs depict the proportion of time degus spend interacting 10 min into the testing session across each of the three conditions. There was a general pattern for higher interaction across behavior types in SHK at the 10 min mark of the reunion. **E)** Vocalization rates (syllables per second) across conditions. Vocalizations rates were highly variable between dyads. **F)** Cumulative time spent vocalizing (y-axis) is plotted against session time (x-axis). Although there was high variance between dyads, the general pattern was for vocalizations to begin after several minutes of physical interactions, and to subsequently approximately follow the temporal patterns of physical interactions. **G)** Vocalization rates are plotted across each of 15 syllable categories. High variance made interpretation difficult, but generally rates of chitters seemed particularly high in NTL relative to SHK and RWD. **H)** Two dimensional t-SNE projection of syllable features, including those for all syllables (gray points)

and those of the cluster of syllables found to be expressed more frequently in SHK. **I)** Proportion of syllables manually labeled across different category types, including all syllables (gray trace) and those syllables within the identified cluster (blue trace). The data suggest that chirp- and tweet-type vocalizations may be more common in degus recently exposed to a footshock stressor.

Physical interaction was affected by a preceding stressor primarily with respect to how the actions were distributed over time. When taking into account the entire 20 minutes, differences between SHK, NTL, and RWD conditions were hinted at by a statistical trend (repeated measures ANOVA, $F_{2,16} = 3.93$, $p = 0.074$; **Figure 3B**), although these numbers were largely diluted by the high variance of the neutral 45 min condition, as a clear difference was observed in interaction levels between SHK and RWD ($p = 0.009$). Looking only at the first half of the session, however, it was clear that the stressor did increase initial interactive behavior (maximum differences observed between SHK and NTL at 353 s, paired t-test, $p = 7.2 \times 10^{-4}$; maximum between SHK and RWD at 255 s, $p = 0.0013$; **Figure 3C**). Paralleling differences between ISO and SEP in Experiment 2, this was accompanied by a statistical trend for steeper initial slopes in the cumulative interaction time (paired t-test on the latency to half-amplitude, $p = 0.064$). There was no evidence that differences between conditions differed between interaction types (2-way ANOVA, effect of condition: $F(2, 96) = 3.8$, $p = 0.026$, effect of type: $F(3,96) = 10.13$, $p = 7.4 \times 10^{-6}$, condition x type: $F(6,96) = 0.48$, $p = 0.82$). although during the first half session, SHK showed much higher levels of both allogrooming/body sniffing and face-to-face contact compared with both NTL (paired t-test, grooming: $p = 0.0033$, face to face: $p = 0.011$) and RWD (grooming: $p = 0.011$, face-to-face: $p = 0.0092$; **Figure 3D**). No differences in the temporal relationships between physical interactions were found between any condition (alpha = 0.05), in all three cases levels of allogrooming-before-rear-sniffing appeared to be intermediate between those observed in ISO/24 hr and SEP conditions.

Similar to Experiments 1 and 2, vocalizations were highly variable across sessions, and no differences were observed between SHK, NTL, and RWD (repeated measures ANOVA on vocalization time: $F(2,16) = 1.24$, $p = 0.32$; **Figure 3E & 3F**). Nor was there a clear differential effect of condition on different types of manually-scored vocalizations (two-way ANOVA, effect of condition: $F(2,315) = 1.85$, $p = 0.16$, effect of type: $F(14,315) = 2.06$, $p = 0.014$, condition x

type: $F(28,315) = 1.1$, $p = 0.34$; **Figure 3G**). Repeated-k-means revealed one cluster of syllables differentially increased in SHK relative to NTL (median p across iterations = 0.015, **Figure 3H**) and an almost completely overlapping cluster that increased in SHK relative to RWD (median p = 0.033), while no clusters met criterion when comparing NTL and RW (alpha = 0.05). These syllables were relatively short in duration (89±14 ms) and were often manually labeled as tweets or chirps (**Figure 3I**).

Experiment IV: Stranger vs. Cagemate

Results from Experiments 1 & 2 were consistent with the possibility that reunion behavior was partly driven by relationship renewal. This included the comparable levels of social interaction after separation as after isolation, as well as the increase in chaff-type vocalizations after the first, post-24 h reunion in both Experiments 1 (following isolation) and 2 (following separation). To further investigate behaviors associated with relationship formation, we compared behavioral patterns in stranger dyads united after 24 hours of isolation (STR) with the the 24 hour cagemate isolation data from Experiments 1 and 2 (CAG1, CAG2; **Figure 4A**).

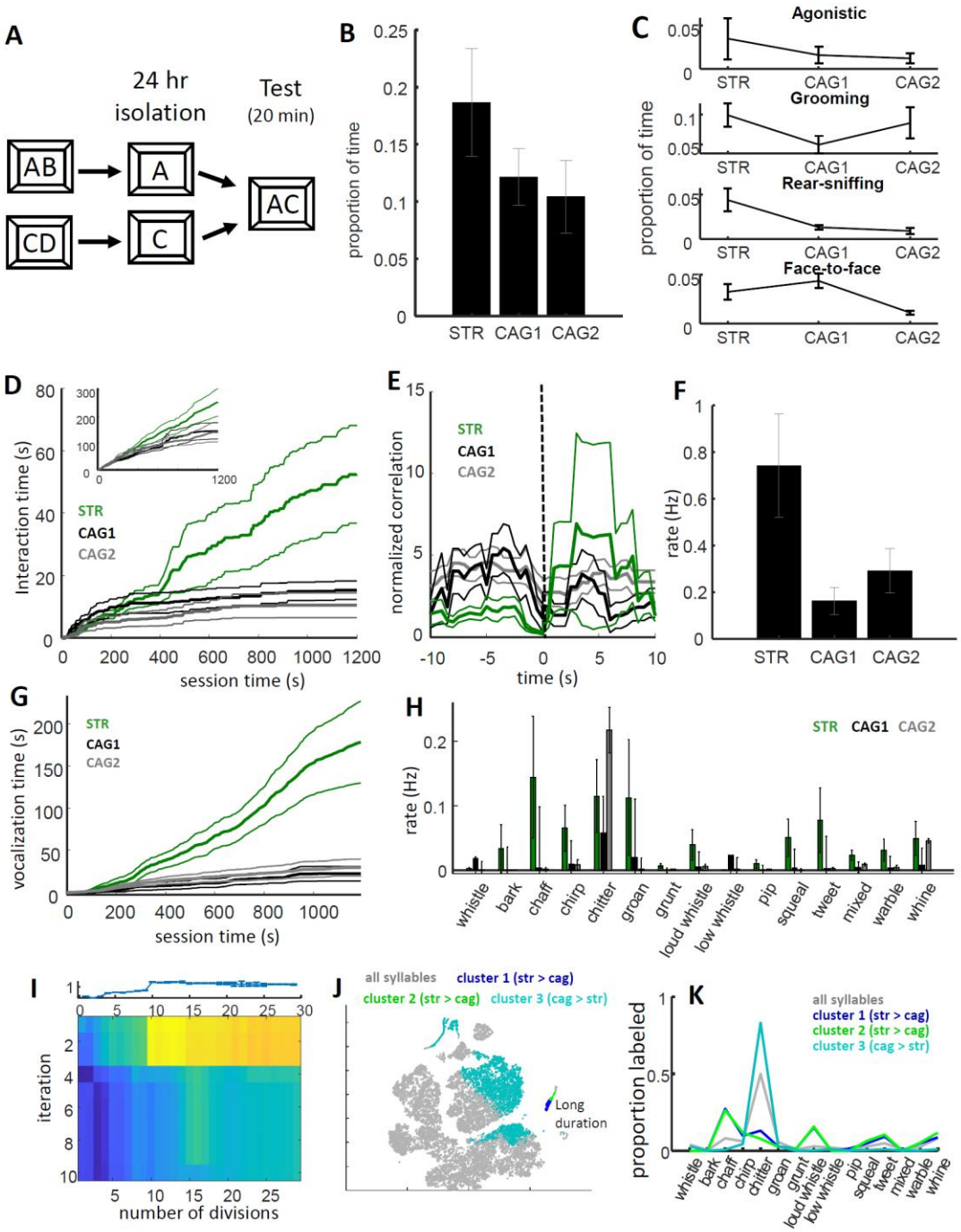


Figure 4. Comparison of social behavior between stranger and cagemate dyads. **A)** Illustration of protocol. Cagemates were isolated for 24 hour and then brough together with an unfamiliar conspecific. **B)** Proportion of time degus spent interacting. There was a statistical trend for stranger pairs to be more interactive than cagemates. **C)** Proportion of time dyads of each group engaged in different types of physical interaction. Although there was a pattern for higher levels of interaction between strangers across all types, a strong difference was only observed for rear-sniffing. **D)** Cumulative time dyads in each group spent engaged in rear-sniffing behavior.

Although rear-sniffing was common across all groups during the first several minutes, this behavior decreased among cagemates (CAG1 and CAG2, black and gray traces) but continued at high levels in strangers (STR, green trace). Inset shows cumulative interaction time across all interactive behaviors. **E)** Cross correlogram of levels of allogrooming/body-sniffing (traces) relative to rear-sniffing (see Figure 2E). Similar to SEP in Experiment 2, the STR group showed a lower incidence of allogrooming immediately before rear-sniffing compared with 24 h isolated cagemates. **F)** Vocalization rates across groups. More vocalizations were observed in STR. G) Cumulative distributions of vocalization time across groups. After a quiet first few minutes, vocalizations were common in STR throughout the session. **H)** Vocalization rates across all 15, manually-labeled syllable categories examined in the present study. Higher vocalizing in strangers was observed across many types, although this was not as clear for chitters (the most common type of syllable observed between cagemates). **I)** Heatmap of identified syllable clusters with the strongest differences between STR and CAG groups (see Figures 1G and 2G). In contrast with prior experiments, the STR versus CAG difference showed multiple independent clusters with differential, proportional activity between the groups, as illustrated by the hot colors observed in each of the 3 top rows. **J)** t-SNE projection of vocalization features (see Figures 1H and 2H). Syllables from each of the three significant clusters are plotted (blue, green, and turquoise). The first two clusters are made-up of particularly long-duration syllables that cluster in a small island; these are most likely combinations of multiple overlapping syllables, notably far more common in stranger dyads than cagemates. **K)** Proportion of syllables manually labeled across different category types (as in Figures 1I, 2I, and 3I). Labeling of the long-duration clusters (blue and green) was variable, most likely because they comprised multiple overlapping syllables between the two individuals. The turquoise trace represented a group of syllables that was proportionally (relative to all syllables) higher in cagemates. These were typically labeled as chitters.

Stranger dyads showed high levels of physical interaction. A statistical trend suggested that interaction levels were even higher between strangers than between cagemates (two-sample t-test, n = 8 stranger dyads, 17 pooled cagemate dyads, p = 0.082; **Figure 4B**). The effect was largely driven by higher levels of rear-sniffing (anogenital investigation) between strangers (two-sample t-test, p = 0.0017; **Figure 4C**). Over the first several minutes of the session interaction levels between STR were on par with those of CAG1 and CAG2, but while those of cagemates began to decrease and level off, those between strangers continued to remain here. This was particularly evident for rear-sniffing (**Figure 4D**).

Temporal relationships were also different between STR and CAG groups. Paralleling the effects of separation relative to isolation (Experiment 2), strangers were less likely to engage in putative allogrooming immediately before rear sniffing (two-sample t-test, p = 0.0075, **Figure 4E**). This

result is consistent with the idea that the allogrooming/body-sniffing taking place before anogenital investigation represents an affiliative gesture between animals that had been previously isolated.

Vocalization rates were very high between strangers, exceeding those between cagemates following 24 hours of isolation (two-sample t-test pooling cagemate groups, $p = 0.0054$, **Figure 4F**). Higher levels of vocalizing between strangers did not appear to be uniform across syllable types (2-way ANOVA, effect of condition $F(2,330) = 11.5$, $p = 1.5 \times 10\text{-}5$, effect of type: $F(14,330) = 5.33$, $p = 3.6 \times 10\text{-}9$, interaction between condition and type: $F(28,330) = 1.95$, $p = 3.5 \times 10\text{-}3$). Among manually-scored vocalizations, increases were particularly pronounced for chaffs (two-sample t-test, pooling cagemate groups, $p = 0.016$), chirps ($p = 0.016$), grunts ($p = 0.0085$), loud whistles ($p = 0.026$), pips ($p = 0.037$), squeals ($p = 0.0084$), tweets ($p = 0.017$), the mixed category ($p = 0.0054$), and warbles ($p = 0.015$). Very little evidence of difference was observed for chitters, whistles, and whines ($p > 0.25$). More generally, STR exhibited higher non-chitter to chitter ratios (paralleling SEP and ISO differences in Experiment 2: Wilcoxon rank sum test, $p = 0.035$). These results were consistent with results of the repeated-k-means method. Three clusters of syllables were identified as being differentially expressed between STR and CAG, but the first two were part of a small cluster of very-long duration (>1 s) syllables that were likely, in fact, combinations of several syllables, and might have combined vocalizations from both individuals (these were often labeled as chaffs, loud whistles, tweets, and whines). The final cluster was comprised of chitters, found to be differentially expressed (relative to all syllables) in CAG. This is consistent with the idea that chitters represented affiliative interactions more prevalent between familiar individuals.

Discussion

The present study was designed to disentangle factors contributing to social motivation after isolation through detailed inspection of social behaviors during reunions. A general summary of observations is illustrated in **Figure 5**. Experiment 1 found that adult female degus become more motivated to interact following longer periods of social isolation. This is a novel result. Although it conforms to observations that have been made in other non-solitary species, including humans

[1] and young adult male rats [2], work in some species has counterintuitively found reduced interactive behavior during reunion (e.g., lion tamarins: [37], and in some cases, marmosets: [38]). Experiment 2 found that separating degus of a dyad without isolating them from other conspecifics also elicited high interaction levels. To our knowledge, a direct comparison of the effects of isolation versus separation-without-isolation has not been made in non-human animals, although reunions of non-isolated individuals are well studied in other ethology domains, e.g., in the analysis of greeting behavior (discussed below). Differences between reunions following isolation and separation-without-isolation were nuanced but consistent, with interactions following isolation distributed earlier in the sessions, and showing a higher levels of allogrooming-to-rear-sniffing transitions. These, along with the higher levels of non-chitter vocalizations (and particularly the increase in chaff-type syllables) have implications for specific behaviors associate with relationship renewal compared with stress buffering. Experiments 3 and 4 helped confirm that some of these differences are likely related to stress and relationship-establishment respectively: exposure to a footshock stressor (Experiment 3), like isolation, elicited more early-session reunion interactions, and interactions between strangers contained low levels of pre- rear-sniff allogrooming. The apparent nuzzling before rear-sniffing, first identified in Experiment 1 (24 h vs 1 min) but replicated in Experiment 2 (24 h ISO vs 1 min CTL), was highest between cagemates after 24 h isolation, lowest in both strangers and dyads separated without isolation, and apparently at intermediate levels in 45 min isolated animals independent of the stress manipulation performed. The importance of these patterns for understanding the processes of relationship renewal and stress buffering are discussed below.

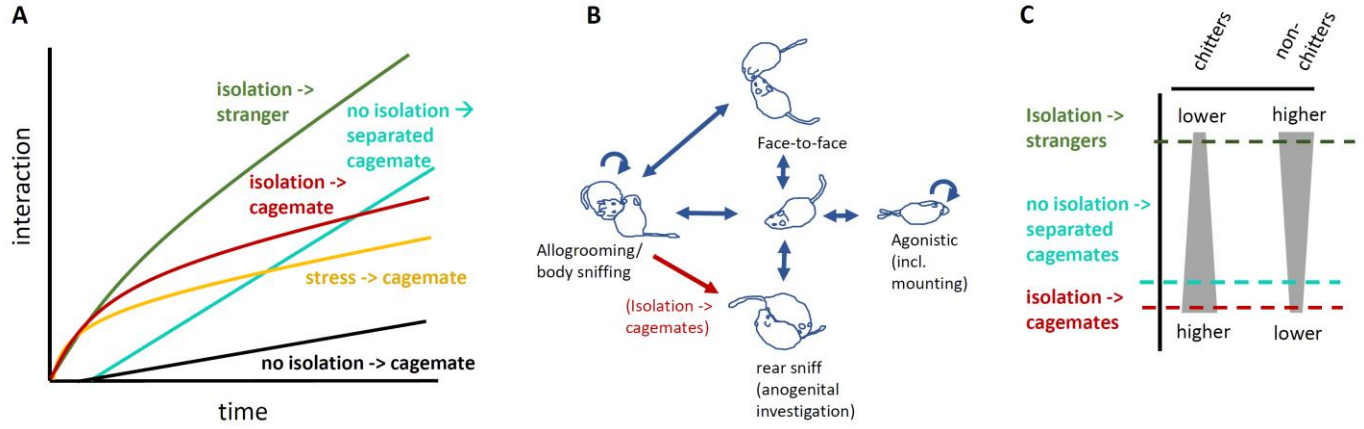


Figure 5. Overview of observed differences between conditions. **A)** Amount of interaction over time (stylized illustration based on data). Both social isolation and footshock stress resulted in higher early-session interaction, while reunions with strangers and previously separated cagemates led to increased interaction over a more prolonged time period. **B)** Illustration of significant transitions between interaction types. An increase in allogrooming before rear-sniffing was primarily observed only in cagemates after isolation. **C)** General differences in vocalizing between groups. Strangers showed highest levels of vocalizations but these were primarily across non-chitters, while the chitter syllable rate in cagemates was very high. Non-isolated, previously separated cagemates showed slightly more resemblance to strangers.

Several qualities about degus made this species uniquely suited for studying the dual motivations of stress buffering and relationship-renewal after isolation. Degus are highly social, particularly sensitive to isolation (studied primarily in pups [23]), and there is converging evidence that females in particular are predisposed to make new relationships. The high sociality and gregariousness of this species makes sense in the context of their behavioral ecology: female degus has been found to increase with higher numbers of individuals within a group [39], but there is a high turnover of group membership over time [28] suggesting potential survival benefits to prosocial interactions with new individuals. Female degus are also known to share burrows with multiple, often genetically unrelated peers [25–27,29,30,32,40] (in some cases even cohabitating with unrelated species [40]). As a point of contrast, prairie voles reside with only familiar pair mates or family members [41] and tend to great strangers aggressively [42]. When the two species were directly compared using a partner preference test, prairie voles have been found to spend almost all of their time near or huddling with a familiar partner [43], while degus spend their time near and huddling with partners and strangers, exhibiting no average preference for either [24]. (Mice, meanwhile, tend not huddle with either partners or strangers, and spend much of their time in a non-social neutral chamber [43].) In the present study we confirm that degus interact heavily with strangers, but also extend this social motivation to cagemates after a period of separation without isolation. These interactions were distributed across many minutes, suggesting they went beyond an ephemeral “greeting” and involved a prolonged period of re-establishing expectations. In the case of strangers, there was also a prolonged period of anogenital investigation, perhaps ensuring subsequent social recognition. One other domain that distinguished new and renewing relationships from stress buffering conditions was in vocal communication.

An intriguing feature of degus is their large repertoire of audible vocalizations [23], and the present study used both manually-scored and automatically-defined syllable categories to identify how vocalizations differed between conditions. Vocalizations were unexpectedly variable between sessions and dyads, far more than physical interactions. Also unexpectedly, while physical interactions began within 10 to 20 s of the reunion, vocal communication was not detected until later, within the first 1 to 2 min (e.g., **Figures 3F & 4G**). It seems unlikely that these patterns were only due to shifts from quieter, undetectable vocalizations, although the amplitude distributions of low-frequency syllables did suggest that some fell outside of the detection range (**Supplementary Figure 1B**). Instead, it may be that vocal communication is a "secondary" quality to their social behavior, used only when they feel the need to modulate others' behavior while patterning physical investigations and affiliative gestures. This would be consistent with the much higher levels of vocal communication observed in strangers, a situation in which individuals may feel more need to avoid misunderstandings. Consistent with this idea, when rats are devocalized by surgical removal of the larynx, their play behavior more readily escalates into fights [44]. In the present study, chaff-type vocalizations were found to be proportionally higher in relationship establishment/re-establishment conditions across three of the experiments. Long [33] reported chaffs as "[O]ften used when the subject is some distance away from a conspecific, but typically directed toward them. Used by males (7%) and females (93%) of all ages, usually during physical separation of observable conspecific (87.3%), but also when meeting an unfamiliar degu (4.5%)." They further specify the effect of chaffs as being approach, and the likely function as encouraging approach. The present work, which found chaffs to be more common in strangers than cagemates, suggests there more context about the state of an animal's relationship or recent experiences may be useful for understanding this call.

In studies of natural social behavior, two factors than can impact conclusions are individual differences and specific decisions about ethograms used. Individuals differ in their social tendencies (i.e., "personalities"), and these differences may be further compounded for dyad combinations. Due to the relatively low sample sizes of the present experiments (8 or 9 dyads each), it is likely that effects that do not generalize across most dyads also failed to reach statistical significance and therefore were not reported. An example of this can be found in the high variane of agonistic behaviors between strangers (**Figure 4C**). This is consistent with observations made

in a prior study, which found that over a 3 h observation period, only 3 in 10 pairs of adult female stranger degus showed high levels of agonistic behavior [24]. The same may also be true of agonistic interactions in SEP compared with ISO, or in allogrooming versus rear-sniffing in these conditions (**Figure 2C)**. The lower sample sizes may miss behaviors that vary between dyads, but can also be valuable for highlighting the "average behavioral patterns" common across most dyads. In addition to sample sizes, the ethogram we used may also have influenced our specific results. Selecting an ethogram for vocalizations was straightforward, as we were able to follow the extensive examinations of Long [33], and then complemented these with an automated clustering algorithm (our repeated-k-means approach). For physical interactions, behaviors were lumped into four types: agonistic (which included mounting as well as more aggressive behaviors like boxing, hindleg kicks, pushes, bites, etc), non-aggressive nose-to-body contact (allogrooming/body-sniffing), anogenital investigation (rear-sniffing), and face-to-face (which included both nose-nose and nose-mouth contact). It may be that visual inspection of face-to-body contact was insufficient for distinguishing between an affiliative behavior, allogrooming, known to play a role in consolation [45], and an information-seeking behavior, body sniffing. Ultimately, increasing sample sizes to reveal variable effects of the manipulations, and modifying our ethogram to tease-apart issues like grooming versus sniffing, would not have changed the overall conclusions that degus interact heavily with separated cagemates and these differ from stress-related interactions.

The term "social relationship" is often not used in rodent research, although has been defined and applied toward explaining animal behavior [46,47]. While the term is found only sparsely in the rodent literature, social relationships are studied in multiple forms, including dominance (e.g., mice: [48] rats: [49], marmots: [50], voles: [51], hamsters: [52,53], degus: [54]), pair bonds (e.g., voles: [55]), and same-sex peer affiliation (voles: [42]). Here we operationally define social relationships as the set of expectations, affiliative bonds, and intra-dyadic "roles" (e.g., dominance roles) that two individuals have for one-another. Research across a range of species has examined the importance of greetings after a separation for expectations and bonds, although different species may show different emphases. As temporal distance between two individuals increases, their uncertainty about one-another probably does as well, so greetings may be a means by which that learning can take place to mitigate that uncertainty. In some cases this means a relief of tension from potential agonistic or competitiveness interactions (e.g., male baboons [7], black and white

colobus monkeys [56], and spider monkeys [5,57]) while in others this may mean a reaffirmation of affiliative and cooperative bonds (e.g., “high risk” greetings in spotted hyaenas [8] and male guinae baboons [6]). Greetings are less well studied in rodents, but there are a number of exceptions. In marmots, greetings seem to progress behaviorally in ways that arguably go beyond simple social recognition [58], and squirrel greetings vary considerably depending on dominance relationships [59]. Consistent with the present study, greetings between degus in the wild show strong differences depending on whether individuals are from the same or different group areas [40]. Where degu co-residents tending to show face-to-face and mounting behaviors, degus from different areas sometimes exhibiting fighting behaviors and chases. While the present study does not explore the consequences of greeting behavior in previously-separated degus, it does demonstrate that degus are highly motivated to interact with previously separated individuals over a time period of tens of minutes, even in the absence of other stressors, including isolation. It seems reasonable to infer that the consequences of these interactions include re-establishment of expectations, bonds, and dyadic roles.

Reunions between cagemates after isolation tended to show high levels of interaction earlier in the session, more vocalizations with a high chitter to non-chitter ratio, and an increase in pre- rear-sniff allogrooming. We hypothesized that some of these qualities may be due to the stress caused by isolation, although exposure to an external, footshock stressor replicated only the increase in early-session interactions. These early-session interactions may be driven by a well established phenomenon where social interaction can reduce elevated stress levels (“social buffering of stress”). Stress effects of isolation are well studied in many animal species, perhaps most extensively in murine rodents [9–16,60], and social buffering of stress is also well established across a range of species [17–22] (including even cows and horses [61,62]). Why didn’t the stress condition also include more chitters and pre- rear-sniff allogrooming? There are several possibilities. With respect to vocalizations, vocalization levels were highly variable following footshock stress, and it may be that degus may be less inclined to make sound if there is a potential external threat in the environment. Another factor may be differences in the levels, duration, or controllability of stress between the two conditions, all of which can differentially impact physiology and behavior [63]. But it also seems very likely that isolation stress has unique features that engage the neuroendocrine system—including nonapeptides like oxytocin as well as estrogens

and androgens—differently from footshock. Work in Syrian hamsters has shown that social isolation can cause changes in reproductive success, independent from the impact on glucocorticoids [64]. In deer mice, isolation can impact the distribution of estrogen receptors [65]. The neuroendocrine systems responsible for encoding and modulating social reward, including those associated with allogrooming, may be impacted in unique ways by isolation (perhaps involving, for example, the VTA to raphe connection: [66]), which may differentially motivate the pre-rear-sniff nuzzles observed here. Given the sensitivity of degus to social isolation, this species may prove to be valuable for future investigations on the physiological impact of isolation stress.

In summary, the present results provide new information on behaviors associated with relationship renewal and buffering of isolation stress in a highly social, gregarious rodent. These findings are a valuable lead into understanding how animals in general, and female degus in particular, establish and re-establish expectations about one-another after a period of time, as well as how they overcome the loneliness of isolation during reunion.

References

1. Cacioppo JT, Hawkley LC, Norman GJ, Berntson GG. Social isolation: Social isolation. Annals of the New York Academy of Sciences. 2011;1231: 17–22. doi:10.1111/j.1749-6632.2011.06028.x

2. Niesink RJ, van Ree JM. Short-term isolation increases social interactions of male rats: a parametric analysis. Physiol Behav. 1982;29: 819–825. doi:10.1016/0031-9384(82)90331-6

3. Lorenz TK. Ober die Bildung des Instinktbegriffes. : 12.

4. Hogan JA. Energy models of motivation: A reconsideration. Applied Animal Behaviour Science. 1997;53: 89–105. doi:10.1016/S0168-1591(96)01153-7

5. Dias PAD, Rodríguez Luna E, Canales Espinosa D. The functions of the “Greeting Ceremony” among male mantled howlers (Alouatta palliata) on Agaltepec Island, Mexico. American Journal of Primatology. 2008;70: 621–628. doi:10.1002/ajp.20535

6. Whitham JC, Maestripieri D. Primate Rituals: The Function of Greetings between Male Guinea Baboons. Ethology. 2003;109: 847–859. doi:10.1046/j.0179-1613.2003.00922.x

7. Smuts BB, Watanabe JM. Social relationships and ritualized greetings in adult male baboons (Papio cynocephalus anubis). International Journal of Primatology. 1990;11: 147–172. doi:10.1007/BF02192786

8. Smith JE, Powning KS, Dawes SE, Estrada JR, Hopper AL, Piotrowski SL, et al. Greetings promote cooperation and reinforce social bonds among spotted hyaenas. Animal Behaviour. 2011;81: 401–415. doi:10.1016/j.anbehav.2010.11.007

9. Panksepp J, Herman B, Conner R, Bishop P, Scott JP. The biology of social attachments: opiates alleviate separation distress. Biol Psychiatry. 1978;13: 607–618.

10. Gamallo A, Villanua A, Trancho G, Fraile A. Stress adaptation and adrenal activity in isolated and crowded rats. Physiology & Behavior. 1986;36: 217–221. doi:10.1016/0031-9384(86)90006-5

11. Ieraci A, Mallei A, Popoli M. Social Isolation Stress Induces Anxious-Depressive-Like Behavior and Alterations of Neuroplasticity-Related Genes in Adult Male Mice. Neural Plasticity. 2016;2016: 1–13. doi:10.1155/2016/6212983

12. Berry A, Bellisario V, Capoccia S, Tirassa P, Calza A, Alleva E, et al. Social deprivation stress is a triggering factor for the emergence of anxiety- and depression-like behaviours and leads to reduced brain BDNF levels in C57BL/6J mice. Psychoneuroendocrinology. 2012;37: 762–772. doi:10.1016/j.psyneuen.2011.09.007

13. Djordjevic J, Djordjevic A, Adzic M, Radojcic MB. Effects of Chronic Social Isolation on Wistar Rat Behavior and Brain Plasticity Markers. Neuropsychobiology. 2012;66: 112–119. doi:10.1159/000338605

816 14. Pibiri F, Nelson M, Guidotti A, Costa E, Pinna G. Decreased corticolimbic allopregnanolone
817 expression during social isolation enhances contextual fear: A model relevant for posttraumatic
818 stress disorder. Proc Natl Acad Sci USA. 2008;105: 5567–5572. doi:10.1073/pnas.0801853105

819 15. Wallace DL, Han M-H, Graham DL, Green TA, Vialou V, Iñiguez SD, et al. CREB regulation of nucleus
820 accumbens excitability mediates social isolation-induced behavioral deficits. Nat Neurosci.
821 2009;12: 200–209. doi:10.1038/nn.2257

822 16. Thorsell A, Slawecki CJ, El Khoury A, Mathe AA, Ehlers CL. The effects of social isolation on
823 neuropeptide Y levels, exploratory and anxiety-related behaviors in rats. Pharmacol Biochem
824 Behav. 2006;83: 28–34. doi:10.1016/j.pbb.2005.12.005

825 17. Kikusui T, Winslow JT, Mori Y. Social buffering: relief from stress and anxiety. Phil Trans R Soc B.
826 2006;361: 2215–2228. doi:10.1098/rstb.2006.1941

827 18. Hennessy MB, Kaiser S, Sachser N. Social buffering of the stress response: diversity, mechanisms,
828 and functions. Front Neuroendocrinol. 2009;30: 470–482. doi:10.1016/j.yfrne.2009.06.001

829 19. Kiyokawa Y, Hennessy MB. Comparative studies of social buffering: A consideration of approaches,
830 terminology, and pitfalls. Neurosci Biobehav Rev. 2018;86: 131–141.
831 doi:10.1016/j.neubiorev.2017.12.005

832 20. Lieberwirth C, Wang Z. The neurobiology of pair bond formation, bond disruption, and social
833 buffering. Curr Opin Neurobiol. 2016;40: 8–13. doi:10.1016/j.conb.2016.05.006

834 21. DeVries AC, Glasper ER, Detillion CE. Social modulation of stress responses. Physiol Behav.
835 2003;79: 399–407. doi:10.1016/s0031-9384(03)00152-5

836 22. Hostinar CE, Sullivan RM, Gunnar MR. Psychobiological mechanisms underlying the social buffering
837 of the hypothalamic-pituitary-adrenocortical axis: a review of animal models and human studies
838 across development. Psychol Bull. 2014;140: 256–282. doi:10.1037/a0032671

839 23. Colonnello V, Iacobucci P, Fuchs T, Newberry RC, Panksepp J. Octodon degus. A useful animal
840 model for social-affective neuroscience research: basic description of separation distress, social
841 attachments and play. Neurosci Biobehav Rev. 2011;35: 1854–1863.
842 doi:10.1016/j.neubiorev.2011.03.014

843 24. Insel N, Shambaugh KL, Beery AK. Female degus show high sociality but no preference for familiar
844 peers. Behav Processes. 2020;174: 104102. doi:10.1016/j.beproc.2020.104102

845 25. Davis GT, Vásquez RA, Poulin E, Oda E, Bazán-León EA, Ebensperger LA, et al. *Octodon degus* kin
846 and social structure. JMAMMAL. 2016;97: 361–372. doi:10.1093/jmammal/gyv182

847 26. Ebensperger LuisA, Hurtado M, Soto-Gamboa M, Lacey EileenA, Chang AnnT. Communal nesting
848 and kinship in degus (Octodon degus). Naturwissenschaften. 2004;91. doi:10.1007/s00114-004-
849 0545-5

27. Quirici V, Faugeron S, Hayes LD, Ebensperger LA. Absence of kin structure in a population of the group-living rodent Octodon degus. Behavioral Ecology. 2011;22: 248–254. doi:10.1093/beheco/arq196

28. Ebensperger LA, Chesh AS, Castro RA, Tolhuysen LO, Quirici V, Burger JR, et al. Instability Rules Social Groups in the Communal Breeder Rodent *Octodon degus*. Ethology. 2009;115: 540–554. doi:10.1111/j.1439-0310.2009.01635.x

29. Ebensperger L, Veloso C, Wallem P. Do female degus communally nest and nurse their pups? Journal of Ethology. 2002;20: 143–146. doi:10.1007/s10164-002-0063-x

30. Ebensperger LA, Sobrero R, Quirici V, Castro RA, Tolhuysen LO, Vargas F, et al. Ecological drivers of group living in two populations of the communally rearing rodent, Octodon degus. Behav Ecol Sociobiol (Print). 2012;66: 261–274. doi:10.1007/s00265-011-1274-3

31. Ebensperger LA, Villegas Á, Abades S, Hayes LD. Mean ecological conditions modulate the effects of group living and communal rearing on offspring production and survival. Behavioral Ecology. 2014;25: 862–870. doi:10.1093/beheco/aru061

32. Hayes LD, Chesh AS, Castro RA, Tolhuysen LO, Burger JR, Bhattacharjee J, et al. Fitness consequences of group living in the degu Octodon degus, a plural breeder rodent with communal care. Animal Behaviour. 2009;78: 131–139. doi:10.1016/j.anbehav.2009.03.022

33. Long CV. Vocalisations of the degu Octodon degus, a social caviomorph rodent. Bioacoustics. 2007;16: 223–244.

34. Lidhar NK, Insel N, Dong JY, Takehara-Nishiuchi K. Observational fear learning in degus is correlated with temporal vocalization patterns. Behav Brain Res. 2017;332: 362–371. doi:10.1016/j.bbr.2017.06.011

35. Tchernichovski O, Nottebohm F, Ho CE, Pesaran B, Mitra PP. A procedure for an automated measurement of song similarity. Animal Behaviour. 2000;59: 1167–1176. doi:10.1006/anbe.1999.1416

36. Coffey KR, Marx RG, Neumaier JF. DeepSqueak: a deep learning-based system for detection and analysis of ultrasonic vocalizations. Neuropsychopharmacol. 2019;44: 859–868. doi:10.1038/s41386-018-0303-6

37. Shepherd RE, French JA. Comparative analysis of sociality in lion tamarins (Leontopithecus rosalia) and marmosets (Callithrix kuhli): Responses to separation from long-term pairmates. Journal of Comparative Psychology. 1999;113: 24–32. doi:10.1037/0735-7036.113.1.24

38. Taylor JH, Mustoe AC, Hochfelder B, French JA. Reunion behavior after social separation is associated with enhanced HPA recovery in young marmoset monkeys. Psychoneuroendocrinology. 2015;57: 93–101. doi:10.1016/j.psyneuen.2015.03.019

39. Ebensperger LA, Correa LA, León C, Ramírez-Estrada J, Abades S, Villegas Á, et al. The modulating role of group stability on fitness effects of group size is different in females and males of a

886 communally rearing rodent. Dantzer B, editor. J Anim Ecol. 2016;85: 1502–1515.
887 doi:10.1111/1365-2656.12566

888 40. Fulk GW. Notes on the Activity, Reproduction, and Social Behavior of Octodon degus. Journal of
889 Mammalogy. 1976;57: 495–505. doi:10.2307/1379298

890 41. Getz LL, McGuire B, Pizzuto T, Hofmann JE, Frase B. Social Organization of the Prairie Vole
891 (Microtus ochrogaster). Journal of Mammalogy. 1993;74: 44–58. doi:10.2307/1381904

892 42. Lee NS, Goodwin NL, Freitas KE, Beery AK. Affiliation, Aggression, and Selectivity of Peer
893 Relationships in Meadow and Prairie Voles. Front Behav Neurosci. 2019;13: 52.
894 doi:10.3389/fnbeh.2019.00052

895 43. Beery AK, Christensen JD, Lee NS, Blandino KL. Specificity in Sociality: Mice and Prairie Voles
896 Exhibit Different Patterns of Peer Affiliation. Front Behav Neurosci. 2018;12: 50.
897 doi:10.3389/fnbeh.2018.00050

898 44. Burke CJ, Kisko TM, Pellis SM, Euston DR. Avoiding escalation from play to aggression in adult male
899 rats: The role of ultrasonic calls. Behav Processes. 2017;144: 72–81.
900 doi:10.1016/j.beproc.2017.09.014

901 45. Burkett JP, Andari E, Johnson ZV, Curry DC, de Waal FBM, Young LJ. Oxytocin-dependent
902 consolation behavior in rodents. Science. 2016;351: 375–378. doi:10.1126/science.aac4785

903 46. Hinde RA. Interactions, Relationships and Social Structure. Man. 1976;11: 1. doi:10.2307/2800384

904 47. Barrett L, Henzi SP. Constraints on Relationship Formation among Female Primates. Behaviour.
905 2002;139: 263–289.

906 48. Wang F, Kessels HW, Hu H. The mouse that roared: neural mechanisms of social hierarchy. Trends
907 Neurosci. 2014;37: 674–682. doi:10.1016/j.tins.2014.07.005

908 49. Blanchard DC, Blanchard RJ. Behavioral correlates of chronic dominance-subordination
909 relationships of male rats in a seminatural situation. Neuroscience & Biobehavioral Reviews.
910 1990;14: 455–462. doi:10.1016/S0149-7634(05)80068-5

911 50. Huang B, Wey TW, Blumstein DT. Correlates and Consequences of Dominance in a Social Rodent:
912 Marmot Dominance. Ethology. 2011;117: 573–585. doi:10.1111/j.1439-0310.2011.01909.x

913 51. Lanctot RB, Best LB. COMPARISON OF METHODS FOR DETERMINING DOMINANCE RANK IN MALE
914 AND FEMALE PRAIRIE VOLES (*MICROTUS OCHROGASTER*). Journal of Mammalogy. 2000;81: 734–
915 745. doi:10.1644/1545-1542(2000)081<0734:COMFDD>2.3.CO;2

916 52. Drickamer LC, Vandenbergh JG. Predictors of social dominance in the adult female golden hamster
917 (Mesocricetus auratus). Animal Behaviour. 1973;21: 564–570. doi:10.1016/S0003-3472(73)80017-
918 X

919 53. Drickamer LC, Vandenbergh JG, Colby DR. Predictors of dominance in the male golden hamster
920 (Mesocricetus auratus). Animal Behaviour. 1973;21: 557–563. doi:10.1016/S0003-3472(73)80016-
921 8

922 54. Correa LA, Frugone MJ, Soto-Gamboa M. Social dominance and behavioral consequences of
923 intrauterine position in female groups of the social rodent Octodon degus. Physiology & Behavior.
924 2013;119: 161–167. doi:10.1016/j.physbeh.2013.06.002

925 55. Young LJ, Wang Z. The neurobiology of pair bonding. Nat Neurosci. 2004;7: 1048–1054.
926 doi:10.1038/nn1327

927 56. Kutsukake N, Suetsugu N, Hasegawa T. Pattern, Distribution, and Function of Greeting Behavior
928 Among Black-and-White Colobus. International Journal of Primatology. 2006;27: 1271–1291.
929 doi:10.1007/s10764-006-9072-x

930 57. Aureli F, Schaffner CM. Aggression and conflict management at fusion in spider monkeys. Biol Lett.
931 2007;3: 147–149. doi:10.1098/rsbl.2007.0041

932 58. Barash DP. The social behaviour of the hoary marmot (Marmota caligata). Animal Behaviour.
933 1974;22: 256–261. doi:10.1016/S0003-3472(74)80077-1

934 59. Fisler GF. Agonistic Signals and Hierarchy Changes of Antelope Squirrels. Journal of Mammalogy.
935 1976;57: 94–102. doi:10.2307/1379514

936 60. Hofer MA, Shair HN. Isolation distress in two-week-old rats: influence of home cage, social
937 companions, and prior experience with littermates. Dev Psychobiol. 1987;20: 465–476.
938 doi:10.1002/dev.420200410

939 61. Coe CL, Franklin D, Smith ER, Levine S. Hormonal responses accompanying fear and agitation in the
940 squirrel monkey. Physiology & Behavior. 1982;29: 1051–1057. doi:10.1016/0031-9384(82)90297-9

941 62. Boissy A, Le Neindre P. Behavioral, cardiac and cortisol responses to brief peer separation and
942 reunion in cattle. Physiol Behav. 1997;61: 693–699. doi:10.1016/s0031-9384(96)00521-5

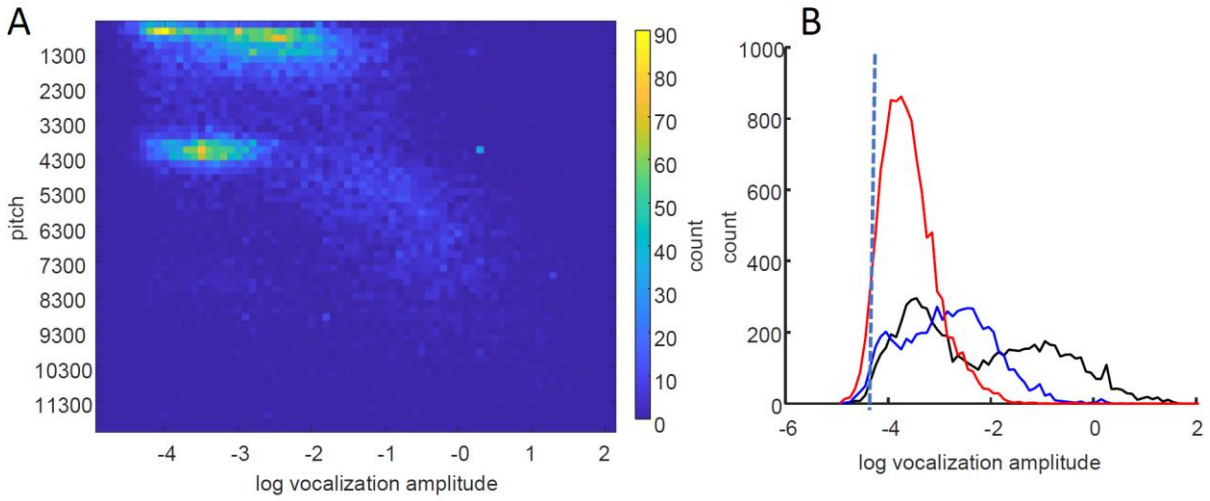
943 63. Sapolsky RM. Why zebras don’t get ulcers / Robert M. Sapolsky. 3rd ed. New York: Times Books;
944 2004.

945 64. Chelini MOM, Palme R, Otta E. Social stress and reproductive success in the female Syrian hamster:
946 endocrine and behavioral correlates. Physiol Behav. 2011;104: 948–954.
947 doi:10.1016/j.physbeh.2011.06.006

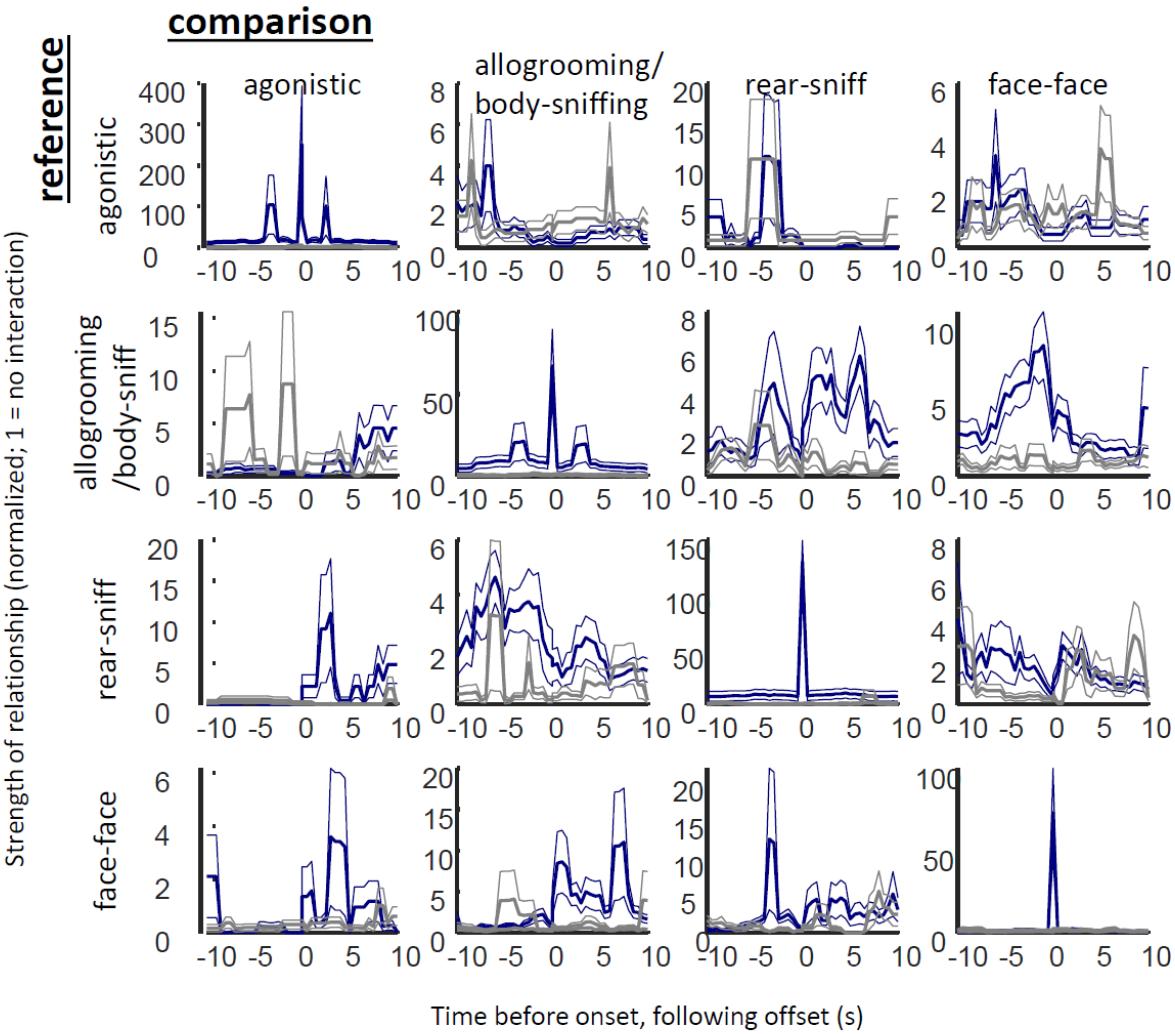
948 65. Ruscio MG, King SB, Kinley-Cooper S-K, McKendrick G. Social environment affects central
949 distribution of estrogen receptor-α in Peromyscus californicus. Gen Comp Endocrinol. 2018;269:
950 81–87. doi:10.1016/j.ygcen.2018.08.018

951 66. Matthews GA, Nieh EH, Vander Weele CM, Halbert SA, Pradhan RV, Yosafat AS, et al. Dorsal Raphe
952 Dopamine Neurons Represent the Experience of Social Isolation. Cell. 2016;164: 617–631.
953 doi:10.1016/j.cell.2015.12.040

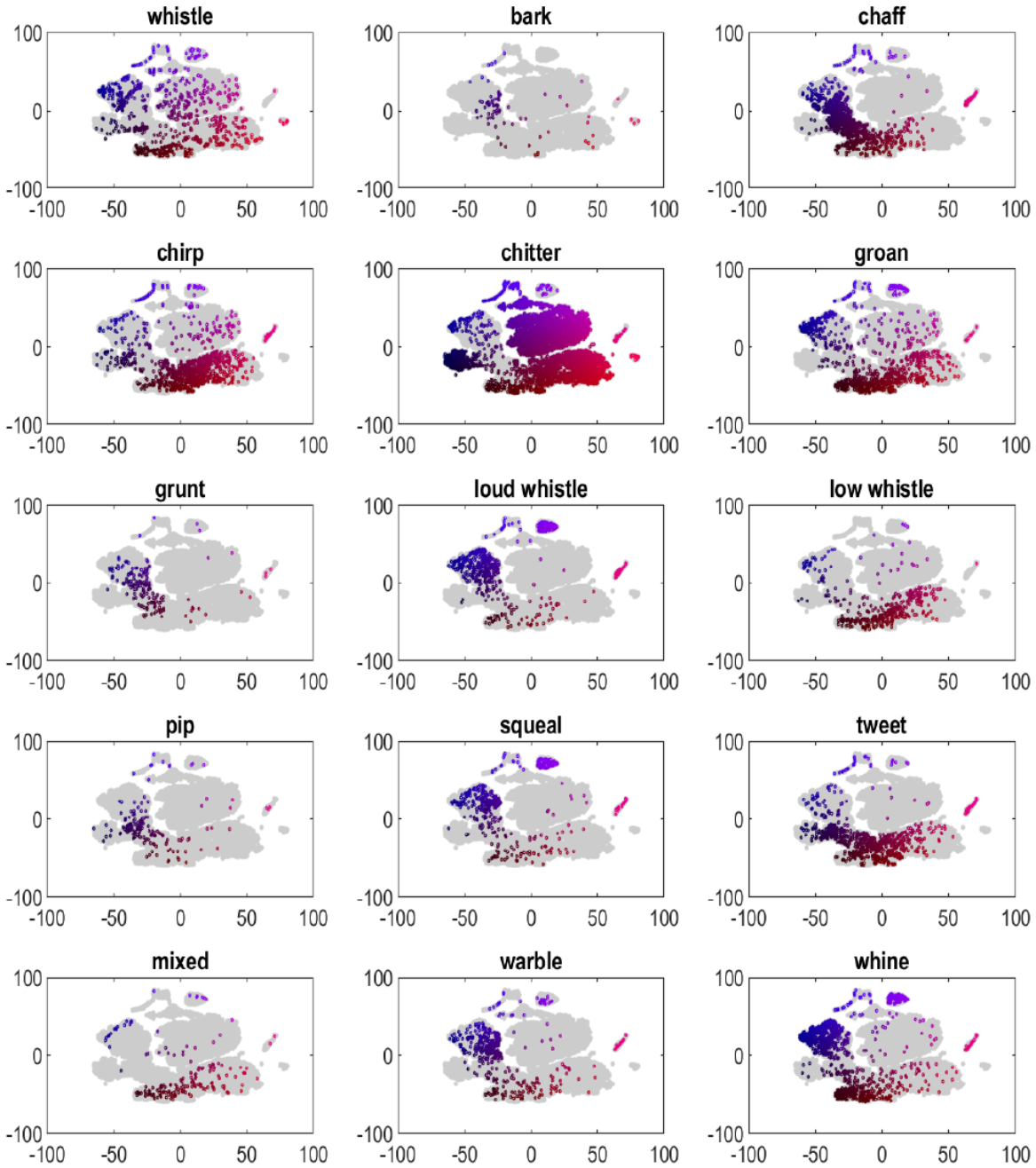
Supplementary Materials



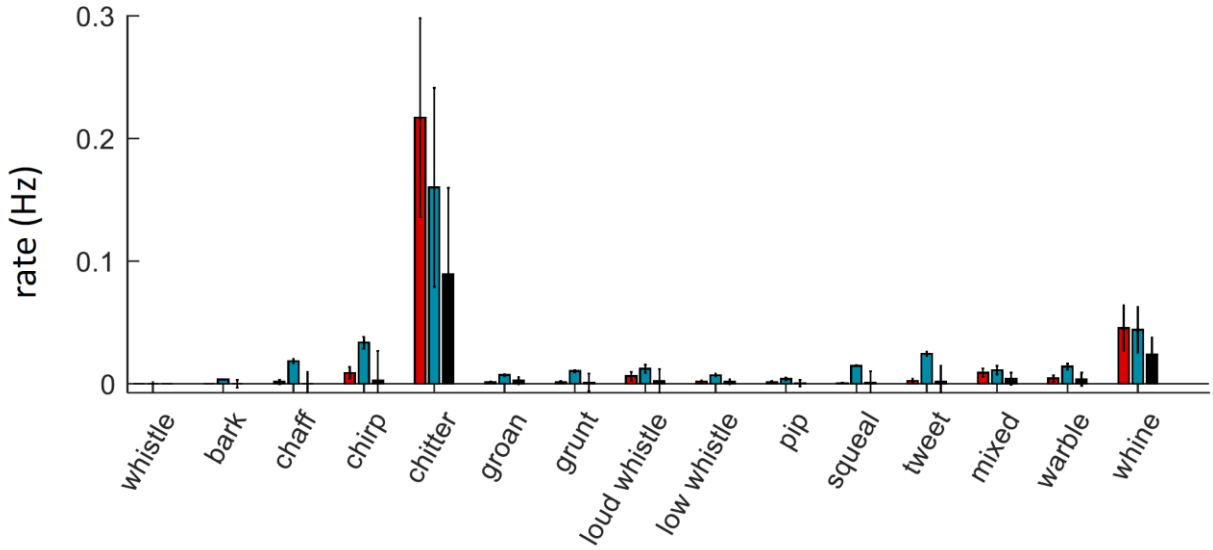
Supplementary Figure 1. Distribution of maximum amplitude and dominant pitch across all syllables. A) Heatmap of 3D histogram of syllable amplitudes versus dominant pitch. Syllables fell into multiple clusters, the cluster of particularly low amplitudes and pitch were almost exclusively chitters. B) Histogram of clusters that did not have identifiable amplitude peaks above 400 Hz (red trace) and both low pitch (blue trace) and high-pitch (black trace) syllables that did. Black dotted line shows approximates the amplitude threshold for detecting syllables, although this would be more true for the < 400 Hz syllables that fell within the ambient noise. The distribution of these syllables was asymmetric, suggesting that some syllables fell below detection threshold.



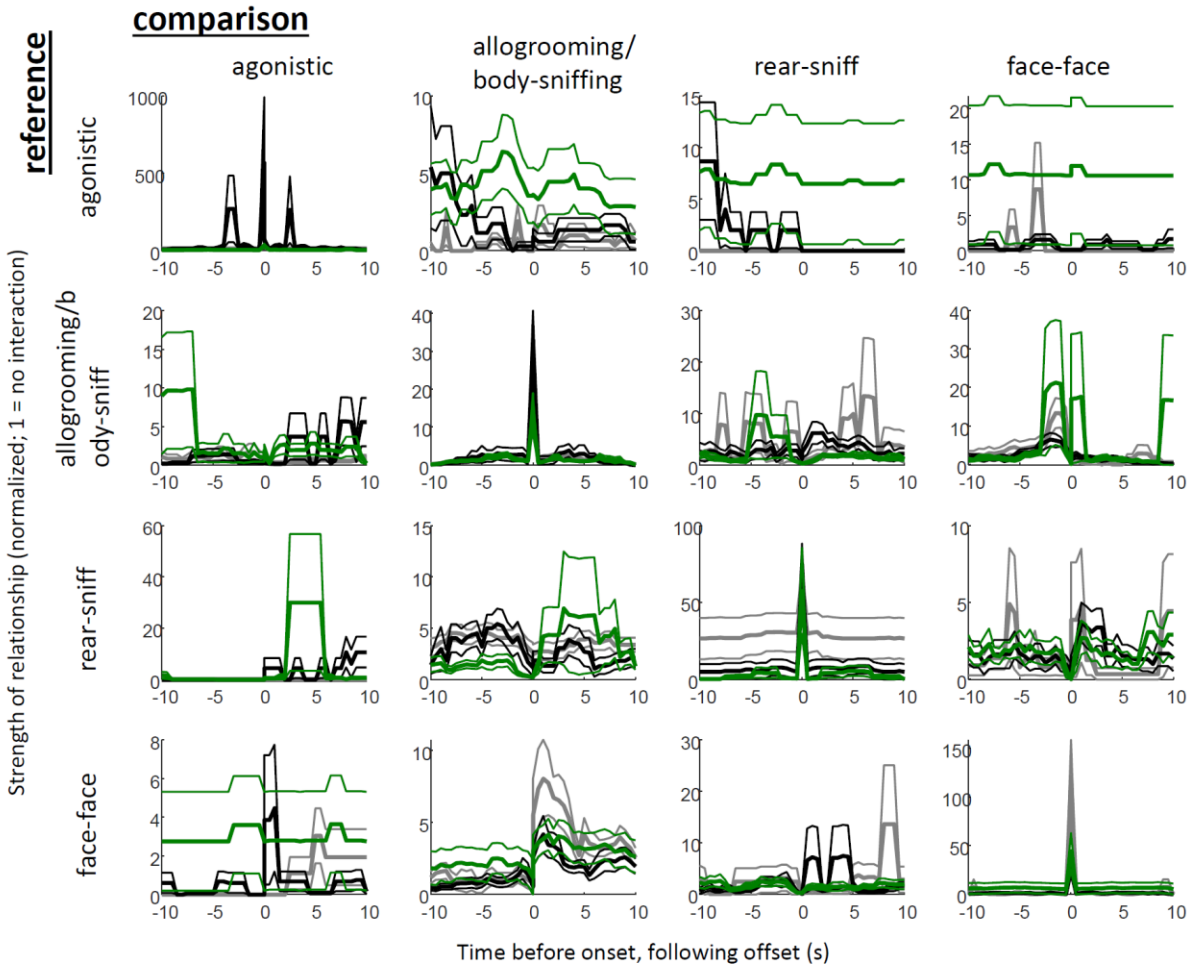
Supplementary Figure 2. Temporal relationships between physical behavior types. Each plot (blue traces) is a cross correlogram of normalized proportion of time degus spent using a comparison behavior (columns) relative to a reference behavior (rows). Times before zero are times before the reference behavior starts and times after zero are after the reference behavior ends. Gray traces show the cross correlograms of a surrogate dataset in which times of the comparison behavior are randomly jittered.



Supplementary Figure 3. Mapping of labeled syllables to a two-dimensional (t-SNE) projection. Although different vocalization types were distributed differently over multi-dimensional space, there was also a degree of overlap, inspiring the use of automated clustering methods. Axes are in arbitrary units.



Supplementary Figure 4. Rates of different vocalization types in the ISO (red), SEP (teal), and CTL conditions (black) conditions. On average, the ratio of non-chitter to chitter vocalizations was higher in SEP.



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993

994 **Supplementary Figure 4**. Temporal relationships between interactions in STR (green), CTL1 (black), and
995 CTL2 (grey) conditions of Experiment 4, organized as in Supplementary Figure 2. The only significant
996 difference was observed in the higher levels of allogrooming/body-sniffing that preceded rear sniffing in
997 both CAG1 and CAG2 conditions. Though not statistically significant, figure also shows apparently higher
998 grooming/body-sniffing before and after agonistic interactions in strangers; this may have been due to
999 either bites to the body, or overly-vigorous (painful) grooming labeled as agonistic in one or a few
1000 stranger dyads.

1001