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Supplemental Information

Wolf Howling Is Mediated

by Relationship Quality Rather

Than Underlying Emotional Stress

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Supplemental Results

Power analysis

We conducted a power analysis, which investigated the influence of the number of observations on the attained significance level for the effect of number of positive interactions (SI) on cortisol levels. We simulated the data 10,000 times for different numbers of observations and estimated statistical significance. We found that, on average, sample sizes larger than 100 observations/individual were still not sufficient for statistical significance to be reached (see figure S1). Analyses and simulations were conducted in R version 2.12.

Relationship asymmetry and howling behavior

Due to the potential skewed nature of dyadic relationships we also calculated a measure of relationship asymmetry (see ESM methods) and used this as a predictor of mean howls and mean spontaneous howls. All prior observed relationships detected when using SI as a predictor variable, however, remained consistent (see table S3) and hence we continued to employ SI as our primary social affiliation explanatory variable.

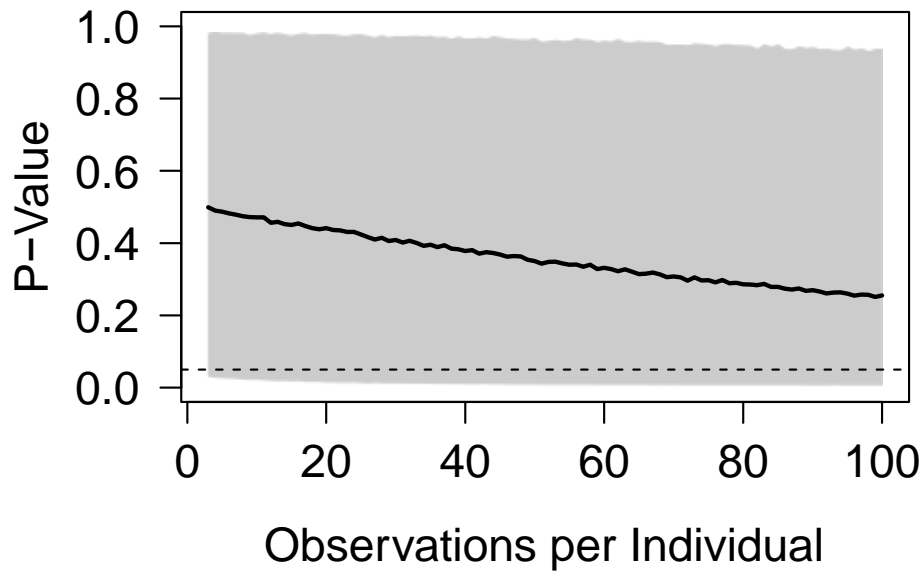


Figure S1. Related to Figure 2

Power analysis plot demonstrating the number of observations required per individual wolf for positive interactions (SI) to significantly affect cortisol levels. Grey shaded area represents 95% confidence intervals, dotted line represents alpha level ($p=0.05$).

Table S1. Related to Table 2

Predictor on howling	β	SE	CI Upper bound	CI Lower bound
Intercept	13.3869	3.737	20.71142	6.06238
SI	2.36	0.81	3.9476	0.7724
Rank Out A	5.296	2.47	10.1372	0.4548
Rank Out b	-7.61	3.55	-0.652	-14.568
Rank Out B	4.95	2.51	9.8696	0.0304
Rank Out C	-4.38	3.25	1.99	-10.75
Rank Out D	1.71	3.17	7.9232	-4.5032
Predictor on spontaneous howling	β	SE	CI Upper boundary	CI Lower boundary
Intercept	1.39	0.33	2.0368	0.7432
SI	0.39	0.088	0.56248	0.21752

Model estimates, SE's and confidence intervals for the influence of predictor variables SI and Rank Out on the mean total number of howls and spontaneous howls

Table S2. Related to Table 2

Model		AICc		
Full Model		443.2		
SI		451.9		
Rank Out		503.3		

Predictor on cortisol levels	B	SE	CI boundary Upper	CI boundary Lower
Intercept	999.8	321.4	1629.744	369.856
SI	-146.1	112.7	74.792	-366.992
Rank Out A	-985.4	382.1	-236.484	-1734.316
Rank Out b	-656.2	504.9	333.404	-1645.804
Rank Out B	-244.9	385	509.7	-999.5
Rank Out C	-962.7	468.1	-45.224	-1880.176
Rank Out D	-927.8	457.5	-31.1	-1824.5

Predictor on SI	B	SE	CI boundary Upper	CI boundary Lower
Intercept	0.08	0.18	0.4328	-0.768288
Rank Out A	-0.06	0.25	0.43	-0.9028
Rank Out b	-0.39	0.32	0.2372	-0.854912
Rank Out B	-0.1	0.25	0.39	-0.8644
Rank Out C	-0.19	0.3	0.398	-0.97008
Rank Out D	-0.02	0.3	0.568	-1.13328

AICc values when removing predictor variables SI and rank out from the full model on cortisol difference and model estimates, SE's and confidence intervals for the influence of predictor variables SI and rank out on the difference in cortisol levels and rank out on the number of positive interactions (SI).

Table S3. Related to Figure 2

Howls	Chi Square	P
Full model vs. Model without factor		
Asymmetry	4.81	0.028
Rank Out	11.7	0.038
Spontaneous howls	Chi Square	P
Full model vs. Model without factor		
Asymmetry	5.39	0.02

Likelihood ratio tests comparing final reduced models with and without the predictor variable for response variable: howling and spontaneous howling

Table S4. Definitions of the Behaviors Collected to Define the Dominance Ranks and the Affiliative Relationships, Related to the Experimental Procedures

Affiliative interactions

Behaviour	Definition
Enthusiastic approach	To go or run forward within 2 m to another subject, maybe pointing the ears forward and wag the tail
Greeting	To interact in a friendly manner, ears back, much tail wagging and licking the mouth. They orient to each others faces to lick, sniff it.
Inspection	To interact friendly, stand next to each other, rub on side by side, smell at other one put heads together and may lick, sniff and so on
Genital sniffing	To sniff the genital area of another wolf also often seen during the breeding season
Lie friendly	To lie on the back, wag the tail, maybe kick with the foreleg against another wolf with open mouth
Grooming	To excitedly nip, lick or scratch the fur or skin occasionally the neck or rub under chin to press against, then rub across another wolf's chest
Playing	To run around another one, kick it, jump on them maybe snap or bite without enough pressure to cause injury at it, further perform an invited chase
Play invitation	To invite another wolf to play, by running in front of them with the head and chest often lowered sidewise and an ached back, often with tail wagging
Wrestling	If at least two wolves play together by lying on each other and biting or chewing the others mouth or the tail or leg, also the other wolf is wrestling
Hide and Seek	To invite chase with dodging behind obstacles. This is likely to occur with frequent change of directions around bushes, trees, rocks and the like, ultimately resulting in the wolves meeting face to face

Agonistic and dominance interactions

<i>Behavior</i>	Definition
Dominant approach	To go forward within 3 m to another subject with the tail perpendicularly or above the plane of the back and the ears erects and pointed forward and head held high
Stand tall	Straighten up to full height and appearing as large as possible may include raised hackles, ears erect and tail perpendicularly or above the back
Stand over	To stand over opponent's body, or place the forepaws on the opponent and stand tall over them
T – position top	Wolf, which stands at the top of the “T” formation. It was approached by another one which stand close to its shoulder and putts the head on its shoulder
Mark	To urinate with the hind leg lifted up in the air mostly near or on bushes or on a tree
Mark over	To deliberately mark beside or on top of the urine mark of another wolf
Passive submission	To lie on the back demonstrate the stomach and the tail between the legs. The ears directed backwards and close to the head and raises a hind leg for inguinal presentation
Active submission	The wolf has its tail tucked between hind legs sometimes wag it while he is in a crouched position and may attempt to paw and to lick the side of aggressor’s muzzle and mostly pees
Muzzle bite	To grab the muzzle of another wolf which can be either soft or with enough pressure to make the grabbed wolf whimper
Ride up	To mount another one from behind or from side
Growl	To make a low, guttural, menacing sound sometimes with showing the teeth
Attack	A running or jumping approach toward another one with tail, ears and sometimes hackles up, often bites at the neck or muzzle, forcing it on ground and holding it there
Snapping	To snap into the air with the teeth being visible
Bite	To move quickly forward and bites by closing the jaws and the teeth on

another, possibly accompanied by showing the teeth and eventually growling and barking

Pin To grab another one at the neck or at the muzzle, forcing it down to the ground and holding it there

Flee To walk or run with tail tucked and body ducked away from other wolf

Crouch To lower the head, bent the legs, the back often arched and the tail between looks hunched and smaller

Mob Two or more wolves crowding around another wolf chase bite or wrestling with them in harassing manner. The mobbed animal is mostly submissive.

Fight A general term for high intensity, aggressive, often damaging encounters

Supplemental Experimental Procedures

Subjects

All wolves (n=9) that participated in this study originated from North America and were born in captivity. Three wolves (2 males, 1 female) from two different litters were born at Herberstein Zoo, Styria, Austria in May 2008. Six additional wolves from four different litters were raised in May 2009. Two brothers were obtained from Basel Zoo, Switzerland; the other four animals (one brother-sister pair, 1 unrelated male, 1 unrelated female) were born at the Triple D Farm, Montana, USA. All of them were hand-raised in peer groups at the Wolf Science Center (www.wolfscience.at) after being separated from their mothers in their first 10 days. They were bottle-fed and later hand-fed by humans and had continuous access to humans in the first 5 months of their life. During this period, the animals were kept in a 1000 m² outside enclosure with access to an indoor room where the hand-raisers spent the nights with them. When the second generation was five months old, they were introduced to the pack of the 1.5 year-old wolves of the first generation. Later 2 packs were formed out of the 9 wolves: pack 1 consisted of the three older animals and two younger ones (4 males, 1 female), whereas pack 2 consisted of the remaining two younger males and two younger females.

The packs lived in two large 8000 m² enclosures where they were kept during this study. The enclosures were equipped with trees, bushes, logs and shelters. Water for drinking was permanently available. The wolves received a diet of meat, fruits, milk products and dry food throughout the study period. During the first months of their lives, they were fed several times per day, which was slowly reduced to being fed major meals twice or three times per week according to their natural rhythm.

All animals received intensive obedience training including sitting and rolling-over on a daily basis using a clicker (operant conditioning with a secondary reinforcer). This training assures that the wolves are cooperative and attentive towards humans and also allows veterinary checks without sedating the animals. Part of this training was also taking saliva samples by placing swabs into the wolves' mouths until they are soaked with saliva. The wolves participate in training and various behavioural tests several times a week, where they are also rewarded with food. A testing room (6 x 10m) and an outside testing enclosure next to the living enclosures allows for training and testing the animals in visual isolation from the packs. All wolves are separated from their pack on a daily basis. Participation in all training and testing sessions is voluntary and all wolves compete with each other to have access to the testing room to interact

with the experimenters. Moreover, from the age of 6 weeks on, each animal has been taken out for walks on a 10-meter-long leash in the nearby forest on a weekly basis as part of their enrichment. All animals participate in these walks voluntarily by offering to leave the enclosure when called by name.

Collection and analyses of observational data

Focal animal sampling

In order to define the social relationships within each pack, we collected data on the social behaviour of all individuals from January to April 2011 by focal animal sampling [S1] using a handheld computer (HP ipaq) with the software program Pocket Observer (The Observer XT 10.0, Noldus Information Technology, BV, Wageningen, The Netherlands). For definitions of the social interactions see table S4. Focal samples were 10 minutes long and each individual was sampled only once per day. Samples were randomly distributed over light hours and were only collected during times when all members of the packs were present and no disturbance occurred (e.g. pack visits, visitors in the park). If an animal was out of sight, the entire focal observation was repeated (N=1).

We collected 8 samples per animals over the entire observation period. All data were collected by the same observer (FM). Observations were extracted from the handheld computer using The Observer XT 10.5™ program (Noldus Information Technology).

Dominance relationships

The dominance ranks for individuals in each pack were calculated based on the outcomes of their agonistic and dominance interactions (see Supplementary Table S4) with other pack members using their David's scores [S2]. The David's score for each member, i , of a pack is calculated with the formula:

$$DS = w + w_2 - l - l_2$$

where w represents the sum of i 's P_{ij} values, w_2 represents the summed w values (weighted by the appropriate P_{ij} values) of those individuals with which i interacted, l represents the sum of i 's P_{ji} values and l_2 represents the summed l values (weighted by the appropriate P_{ji} values) of those individuals with which i interacted. P_{ij} is the proportion of wins by individual i in his interactions with another individual j , that is the number of times that i defeats j divided by the total number

of interactions between i and j . The proportion of losses by i in interaction with j is $P_{ji}=1- P_{ij}$. The David's Score was calculated for each animal in every pack and the rank position number was assigned accordingly.

Affiliative relationships

To describe the relationship between pack members we used the number of affiliative interactions (see table 4) observed between all dyads within each pack. To control for the fact that we had data from two different packs that might differ in their overall affiliative behaviours we computed a standardized sociality measure (see [S3, S4]). Specifically we calculated the rate/min of affiliative interactions between dyad A+B and divided this by the average rate/min of positive interactions at the pack level (See [S4]). The higher the sociality index, the stronger the relationship between the individuals.

Relationship asymmetry

Furthermore, with an additional analysis we also controlled for the potential asymmetry in relationship status between individuals. We followed Fraser [S5] by calculating relationship asymmetry as affiliation from A to B / (aff. from A to B + from B to A) and relating this measure to the amount of howling by A when B left the pack.

Collection and analyses of experimental data

Experimental set-up

During the walks (test condition), always one animal was taken out for a 45-minute walk into the nearby forest. The matched controls were conducted either one day before or after the scheduled walk at the same hour of the day. During the control sessions, the same individual as scheduled for the respective walk was put into the testing house or testing enclosure visually separated from the rest of the pack for the same amount of time. Test condition and matched control were conducted each 3 times per individual, that is each individual left the pack 6 times. The order of individuals leaving was predetermined, randomized and spread out over the study period (January to April 2011).

During the experimental and control sessions, the remaining pack was filmed with a digital camera (Sony Handycam Hdr Cx 520) and all howls were recorded using a Sehneiser ME-66 field microphone connected to a Marantz PMD-661 portable recorder during the first 20 minutes

of the absence of the wolf. The experimenter recorded the identity of the howling wolf on the digital camera by dictating the names.

Twenty minutes after the respective wolf was taken out from the pack, saliva samples were collected from all of the remaining animals (see below).

Experimental procedure

Separating an individual from the remaining pack belongs to the daily routines of the Wolf Science Center. In order to minimize stress and aggression due to separation and pack reunion, we invented a very strict protocol involving two people familiar to the animals and trained on this separation procedure. Next to each living enclosure there is a tunnelling system (a system of smaller pens that are connected by sliding doors that can be operated from the outside). The wolf to be separated can enter the first compartment of the tunnel system when an experimenter standing in the airlock (a safety compartment attached to the door of the enclosure where humans can enter) opens the sliding door for the individual she/he has called by name. Once the subject entered the tunnelling system, the sliding door is closed and the animal is rewarded with food through the fence. In the meanwhile, a second experimenter occupies the rest of the pack, while standing approx. 10 meters to the left or right of the sliding door, by calling all the other animals by their names and throwing food rewards over the fence to keep them away from the door.

From the tunnelling system, the subject can be released either into the airlock by another sliding door and can be taken for a walk (test condition) or it can be shifted through further compartments to the testing house or testing enclosure (control condition). Both procedures are quick and are part of the daily routine of the wolves living at the WSC.

In the test condition, the animals were offered a collar where they put their head in voluntarily for a piece of food, and then got a 10-meter-long leash fixed on their collar. Equipped in this way, they left the airlock with their trainer and started toward the nearby forest. During the first 100 meters, they were still in sight of the remaining pack, but then they disappeared behind the bushes. In the control condition, the subject was let into the testing house or testing enclosure where it participated in other behavioural tests out of sight of its pack mates.

Audio-Video analysis

The video recordings were used to identify who emitted the howls and to count the number of howls performed by each individual. Two behavioural variables were used to characterize the amount of howling of the wolves:

- ❖ Total number of howls: all howls emitted by a single individual during a test or control session independent from whether it was howling alone or as part of a chorus. If an individual stopped howling for at least 1 second, a new howl was counted.
- ❖ Number of spontaneous howls: howls that an individual started with no other animal howling for at least 3 seconds beforehand. The 3-second cut-off point was chosen based on a study by [S6]. For example every time that a chorus started from silence, a spontaneous vocalisation was counted for the animal that started the chorus. Accordingly, the number of spontaneous vocalizations is a subset of calls counted in the total number of howls.

Collection and analyses of saliva samples

The trainers of the wolves collected saliva samples of all of the remaining animals of a pack 20 minutes after a wolf had been taken out for a walk and, for reference, during the matched control trials, 20 minutes after the animals were moved to a testing area. This sampling time was chosen on the basis of a study that indicates that blood-cortisol levels peak 20 minutes after a dog encounters a stressor [S7]. Salivary cortisol metabolite concentrations were measured using an enzyme immunoassay developed by [S8], which was validated for dogs [S9]. To collect the samples, the wolves were called out one-by-one into the tunnelling system. Salivation was stimulated by showing pieces of cheese to the wolves (see [S10] for similar methods) while a surgical hydrocellulose swab (Sorbetto produced by Salivette) was placed in the wolf's buccal cavity. Since the animals had been trained on this procedure, they easily allowed the sponge to be inserted into their mouth and moved around until soaked. They were also motivated to do so by the cheese pieces kept in the second hand of the experimenter in front of their mouth. The animals were rewarded with cheese after every 10-15 seconds. Collecting enough saliva from an animal took between 1 and 3 minutes. This method was deemed appropriate because it is known that in dogs there is no confounding of results due to handling stress if the sample is collected within four minutes [S11] or due to using cheese during sampling [S12].

Immediately after sample collection, the swabs were transferred into a plastic tube (Sarstedt) and were stored at -20°C until they were subsequently analyzed. Salivary cortisol metabolite concentrations were measured using an enzyme immunoassay developed by Palme and Möstl [S8], which had been validated for dogs [S9].

Additional statistical information

When investigating the influence of multiple fixed explanatory factors we used Akaike's Information Criterion corrected for small samples (AICc), to select the best fitting, most parsimonious model. Whilst AICc calculation and likelihood ratio testing involves the mixing of hypothesis testing with and without probability value calculation it represents an appropriate method to initially assess the relative contribution of potentially influential variables on model fit and avoids potential model instability and erroneous p value approximation created by the inclusion of multiple fixed and random effects with small relative sample sizes [S13]. Lower AICc values indicate improved support for each model [S14, S15], with terms considered to improve the fit if they inflated the AICc value by more than two units [S16]. We then sequentially removed the variable with the lowest non-inflating AICc and re-ran the model excluding this variable and again compared AICc values (see AICc II in tables S2 and S3). To assess the significance of the reduced models we compared these final models based on AICc values with a null model including only the intercept and the random factors using a likelihood ratio test. Each explanatory variable was then investigated by comparing the minimal "full" model with a reduced model excluding the variable of interest again using a likelihood ratio test [S17]. In the case of mean spontaneous vocalisations and the Sociality Index we derived from number of positive interactions, distributions deviated significantly from normal and hence we applied square root and log transformations respectively. To probe whether experimental condition (e.g. separation into testing chamber vs. walking separation) had a significant impact on the cortisol level of subjects we performed a Linear Mixed Effects Model with subject and trial nested as random effects.

Models were implemented in R v. 2.12 and alpha values were set at < 0.05 .

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