Current Biology

Report

Cooperative Communication with Humans Evolved to Emerge Early in Domestic Dogs

Highlights

- Dog puppies are more attracted to humans than wolf puppies raised by humans
- Dog puppies use human gestures and make eye contact more than wolf puppies
- Both species perform similarly on memory and inhibitory control tasks
- Dogs' early emerging social skills demonstrate domestication's effect on cognition

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In brief

As a result of domestication, dogs develop early emerging social skills that allow for cooperative communication. Salomons et al. compare typically reared dog puppies to wolf puppies with extensive human exposure. The dog puppies outperform the wolf puppies in cooperative-communicative tasks with humans, but not in non-social tasks.

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Cooperative Communication with Humans Evolved to Emerge Early in Domestic Dogs

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SUMMARY

Although we know that dogs evolved from wolves, it remains unclear how domestication affected dog cognition. One hypothesis suggests dog domestication altered social maturation by a process of selecting for an attraction to humans.[1–3] Under this account, dogs became more flexible in using inherited skills to cooperatively communicate with a new social partner that was previously feared and expressed these unusual social skills early in development.[4–6] Here, we comparedog ($n = 44$) and wolf ($n = 37$) puppies, 5–18 weeks old, on a battery of temperament and cognition tasks. We find that dog puppies are more attracted to humans, read human gestures more skillfully, and make more eye contact with humans than wolf puppies. The two species are similarly attracted to familiar objects and perform similarly on non-social measures of memory and inhibitory control. These results are consistent with the idea that domestication enhanced the cooperative-communicative abilities of dogs as selection for attraction to humans altered social maturation.

RESULTS

Domestic dogs (*Canis familiaris*) rely on communicative gestures when cooperating with humans,[7,8] and dogs with more skill in comprehending human gestures are more successful as detection and assistance dogs.[9] This interspecific communication is unusual: dogs are more skilled at using human gestures than mother-reared chimpanzees and other great apes.[4,10,11] Like human children, but unlike mother-reared great apes, dogs can spontaneously use novel and arbitrary gestures (e.g., a physical marker).[12–16] Control conditions reveal that this flexibility is not simply explained by the use of olfactory cues, an attraction to human hands or bodily motion created by a gesture.[7,12,13,15,17–20] Instead, analysis of individual differences suggests the communicative flexibility of dogs is human-like. Dogs and human infants show similar correlated variance in their use of different human gestures—a pattern not observed in other great apes.[11]

The domestication hypothesis (DH) posits that the ability of dogs to understand human gestures (without intensive training) is a product of domestication.[4] Several lines of evidence support this hypothesis.

First, comprehension of human gestures in dogs varies independently from success in other cognitive tasks,[11,15,21,22] and breed differences in these skills are predicted by genetic similarity among breeds and associated with genes expressed in the brain.[22,23] Crucially, a recent study found that the ability to follow human pointing gestures is highly heritable, and over 40% of the variation in this skill is attributable to genetics.[24]

Second, the interspecific communicative abilities of dogs emerge early.[3] Although dogs can become more skillful at using human gestures with age and training,[24,25] the use of human gestures does not require intensive exposure to humans. Around the age of weaning (~7–9 weeks) dog puppies can already use human gestures,[4,6,13] and free-ranging dog puppies

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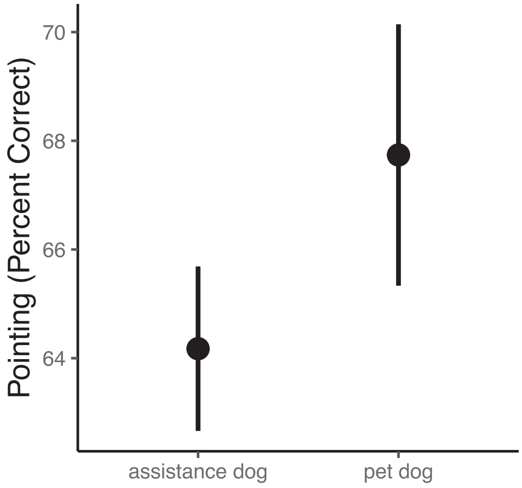

Figure 1. Dog population comparison

Adult Canine Companions for Independence (CCI) dogs tested for their ability to follow a human pointing gesture in a procedure highly similar to the pointing gesture test used with puppies here are not more skilled than a heterogeneous sample of adult pet dogs, t(156.64) = −1.25, p = 0.21. These CCI dogs were tested around 18–24 months of age just before formal training for working as assistance dogs began.[9]

as well as litter-reared assistance dog puppies are successful in using these gestures from their very first experimental trial.[16,26]

Third, experimental foxes selected for an attraction toward humans exhibited dog-like skills at reading human gestures. Experimental fox kits used human gestures at the level of dog puppies. They also used human gestures more than age-matched control foxes (bred irrespective of their response to humans). They showed more skill with two different communicative tasks even though the control foxes were raised with intensive exposure to humans and outperformed the experimental foxes on a non-social task.[27] This work led to the proposal that a similar process occurred during dog domestication and led to the early emergence of dogs' unusual social skills.[3,28]

The canid ancestry hypothesis (CAH) provides an alternative to the DH and suggests instead that dogs inherited their inter-specific communicative abilities from their ancestor with wolves.[4,12,29] The ability of some adult wolves to learn the use of human social gestures can be viewed as support for the CAH,[6,30–34] but to date there is limited evidence that adult wolves, even if hand raised by humans from the first days of life, show spontaneous use of human gestures as seen in dogs.[4,6,30,33,34] Any skill they demonstrate likely requires intensive exposure to humans or explicit training not required for the appearance of these same skills in dogs. However, initial comparative developmental studies with dogs and wolves have yielded conflicting findings. Although one comparison found that dogs but not wolf puppies spontaneously read human gestures,[6] another found the two species both performed similarly.[30] This might suggest the early emerging skill of dogs is inherited from a common ancestor with wolves or that the second comparison was not sensitive enough to detect a significant developmental difference between the species (i.e., this experiment only included a small sample of wolf puppies, N = 6, because the rest were too aggressive to test[30]).

A large-scale comparison of wolf and dog puppies on a battery of cognitive tasks that includes social (especially those requiring the use of human gestures) and non-social problems is therefore urgently needed. The DH predicts that even young dog puppies around the age of weaning, with limited human interaction, will be attracted to humans more than wolves and will outperform wolves in using human gestures but not in the non-social tasks. In contrast, the CAH predicts the amount of exposure to humans will be related to performance on social cognitive tasks in both species (i.e., older dogs should outperform younger dogs and human-raised wolves should outperform the youngest dog puppies still living with their mother and littermates).

Here, we provide this critical test by comparing the temperament and cognition of the largest sample to date of dog and wolf puppies between 5 and 18 weeks old (see Data S1 for subject details). All dog puppies were retrievers bred and raised for assistance work (Figure 1). Most (94%) wolf puppies were only the first (56%) or second (37%) generation bred in captivity. Wolf puppies remained with littermates but received 12 h (24%) or 24 h (76%) human care from 10 to 11 days after birth. This included caregivers remaining available for constant contact, feeding the puppies by hand, and sleeping with them each night up to and throughout the testing period. One of the experimenters who helped raise the wolves was always present during testing. In comparison, the dog puppies received far less contact with humans. All dog puppies remained with their mothers until weaning around 6 weeks of age, and with their littermates until ~8 weeks of age. During this time, they mainly socialized with humans during short routine caretaking tasks. Around 8 weeks of age, puppies were then sent to live with human families. The majority of dog puppies (n = 27) were tested at 7–8 weeks of age, prior to going to live with human families, whereas a subset of puppies (n = 18) were tested between 10–17 weeks of age while living with a human family. As part of their training, dog puppies did not sleep with humans at night.

We first ran a temperament test in which subjects (dog: n = 44; wolf: n = 28) could approach an unfamiliar or familiar human or object to retrieve food. The unfamiliar human was an experimenter whom the puppy had never met, whereas the familiar human had calmly interacted with the puppy for at least 30 min. The unfamiliar object was a novel toy (a plastic bear) and the familiar object was taken from the subject's enclosure (e.g., a plastic bottle, etc.). We measured how often subjects touched each stimulus in two sessions of four 4-trial blocks yielding a total of 32 trials. A linear contrast test on a mixed-effects logistic regression model revealed that compared with a wolf puppy, a dog puppy's odds of touching the unfamiliar human, familiar human, and unfamiliar object were 30.52 (95% confidence interval [CI] 14.48–73.68), 5.36 (95% CI 2.99–10.06), and 1.91 (95% CI 1.16–3.20) times higher respectively (Figure 2).

We then tested subjects' (dog: n = 44; wolf: n = 25) memory for where they had recently seen food hidden. Subjects watched from 2 m away as an experimenter hid food in one of two bowls separated by 2 m. Once the food was hidden, the experimenter, sitting centered between the bowls, lowered her head and remained motionless while the subject was released to search for the food treat. A choice was scored when the subject approached either bowl closely enough that the nose passed over the bowl's edge. We measured how many trials it took

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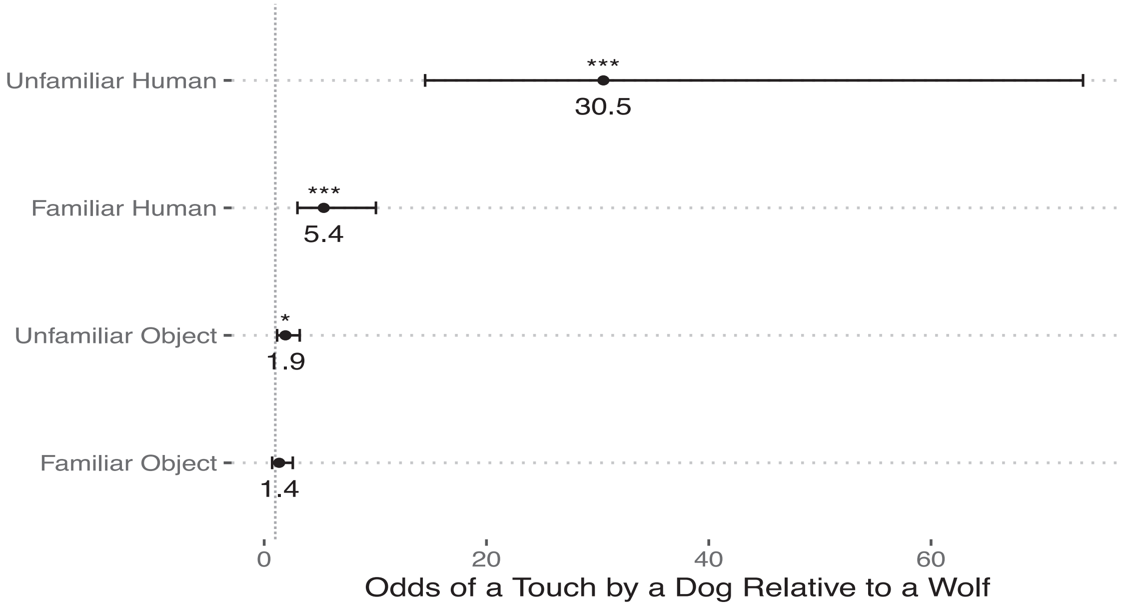

Figure 2. Temperament test

Odds that a dog would approach and touch the stimuli in each condition as compared to a wolf (i.e., a dog's odds of touching the unfamiliar human are 30.5 times higher than those of a wolf). ***p < 0.001, *p < 0.05. Vertical dotted line (odds = 1) signifies the point of no difference between species. Bars signify the 95% confidence interval.
See also Data S1 and S2.

each subject to correctly choose the baited bowl in four out of five consecutive trials. On average, the two species took approximately the same numbers of trials to meet this criterion (dog: M ± SD = 11.26 ± 7 trials; wolf: M ± SD = 11.15 ± 6.4 trials; t(56.63) = 0.06, p = 0.95, Welch independent t test) (Figure 3A).

Subjects (dog: n = 31; wolf: n = 26) were then tested for their understanding of human gestures by using the same method as the memory test, except the experimenter sham baited the bowls so the subject knew food was hidden but did not know where, and then gave a communicative gesture indicating the food's location before releasing the puppy. The experimenter sat centered between the bowls and gestured by either (1) pointing with an extended arm while gazing at the food location or (2) placing a physical marker (small ordinary wooden block) next to the correct location (see Agnetta et al.[12] and Riedel et al.[13] for demonstrations of how both adult dogs and puppies interpret this type of physical marker as communicative). Subjects received six trials with each gesture as well as six trials with each of the two control conditions. The controls tested (1) subject's preference between two competing social cues: the location of a human near one bowl and a pointing gesture toward the other bowl, and (2) their ability to find hidden food with only olfactory information. Using a mixed-effects logistic regression model with random intercepts for each subject, we estimated the probability of choosing the hiding location an experimenter gestured toward for each species by using linear contrasts. The dog puppies were estimated to choose the indicated location 77.99% of the time (95% CI 70.26–84.17, z = 6.11) for the pointing gesture and 78.01% of the time (95% CI 70.73–83.89, z = 6.46) for the marker gesture, whereas wolf puppies were estimated to choose the indicated location 62.08% of the time (95% CI 52.86–70.51, z = 2.55) for the pointing gesture and 57.25% of the time (95% CI 48.59–65.50, z = 1.64) for the marker gesture. The dog puppies as a group performed above chance (50%) for both the pointing gesture (p < .001) and the marker gesture (p < .001) but not the controls (body versus point: below chance, p < .05; odor control: p = 0.61). The wolf puppies as a group performed above chance for the pointing gesture (p = 0.011) but not the marker gesture (p = 0.100) or the controls (body versus point: p = 0.56; odor control: p = 0.07). However, the dog puppies outperformed the wolf puppies when using both the pointing and marker gestures by choosing the indicated location significantly more in each condition (pointing p = 0.006, marker p < .001) (Figure 3B). A linear contrast test on a binomial logistic regression model revealed that compared with a wolf puppy, a dog puppy's odds of choosing the indicated location for the pointing gesture and the marker gesture were 2.08 (95% CI 1.14–3.77) and 2.58 (95% CI 1.42–4.68) times higher, respectively (Figure S1).

As individuals, 17 out of 31 dog puppies and 0 out of 26 wolf puppies performed above chance when combining their performance with both gestures (≥10 out of 12 correct using a significance threshold of p < .05, binomial probability). On their very first trial dog puppies used each gesture significantly above chance (pointing: 28 of 31 correct, p < .001; marker: 27 of 31 correct, p < .001, binomial test), whereas wolf puppies were not above chance on their first trial with either gesture (pointing: 17 of 26, p = 0.16; marker: 15 of 26, p = 0.56). Using a binomial logistic regression, we modeled the effect of trial number on performance and found no evidence of learning within the test in either species (pointing p = 0.15; marker p = 0.28; see Data S2A and S2B]. In the two control conditions both species

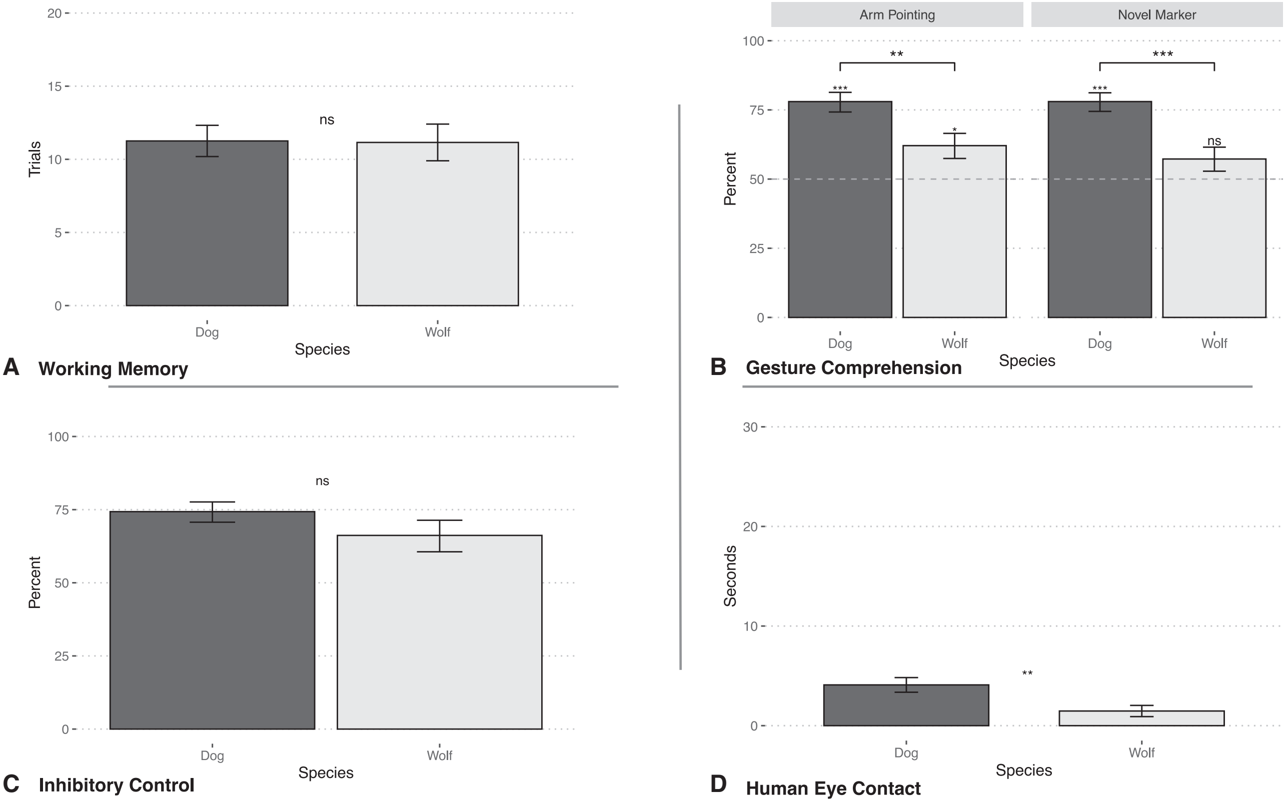

Figure 3. Cognitive tests

(A) Mean number of trials (±SEM) subjects needed to reach criterion in memory task.

(B) Estimated probability (±SE) of subjects choosing the location indicated by the experimenter's gesture, with dashed line at the level of chance (50%).

(C) Estimated probability (±SE) of subjects retrieving the food by navigating directly to the side opening without first touching the clear cylinder in the inhibitory control task.

(D) Mean total number of seconds (±SEM) subjects spent looking at a human face across 4 30 s trials in human eye contact task.

For all, *p < .05, **p < .01, ***p < .001. See also Data S1 and S2.

performed at chance levels, ruling out either an aversion to approaching a hiding location near a human or a reliance on olfactory information. Using linear regression models including age and sex as covariates, species was the most significant predictor variable for performance on both gesture tests, and including the age parameter did not improve either gesture model for either species (Data S2C and S2D). Finally, we found that number of approaches in the temperament test was positively associated with performance in the pointing and marker gesture comprehension tasks but not the controls. However, the relationship between temperament and gesture comprehension did not hold once species was included in the model, suggesting that the relationship was due to the species' difference in temperament and not individual variation (Data S2I and S2J).

We measured subjects' (dog: n = 39; wolf: n = 21) inhibitory control by using a task in which an experimenter placed food inside a transparent cylinder as subjects watched. Subjects were released to retrieve the food by reaching their muzzle into one of the open ends of the cylinder. Because the cylinder was oriented perpendicularly to them, subjects needed to walk around to an open side to access the food. First, they were familiarized with this navigation on an opaque cylinder; then the cover was removed, but they still needed to walk around to a side opening despite now being able to see the food directly in front of them through the transparent cylinder wall. In ten test trials, subjects' responses were coded as correct when food was obtained by making a clear route around to one of the open ends, or incorrect when the subject first attempted to obtain the food by touching the transparent wall of the cylinder. A mixed-effects logistic regression model with random intercepts for each subject showed that the two species did not differ in their performance in the inhibition task (p = 0.19; dog estimate: 74.01% of trials correct, 95% CI 70.71–77.62, z = 5.86; wolf estimate: 66.19% of trials correct, 95% CI 60.59–71.37, z = 2.78) (Figure 3C).

As a secondary social measure, a subset of subjects (dog: n = 34; wolf: n = 15) were assessed for their propensity to make eye contact with a human experimenter during an "unsolvable" task. Subjects watched as food was placed into a container that they had previously opened and successfully obtained food from; however, it was then secured so they could no longeropen it. In 4 trials of 30 s each, we measured the amount of time subjects spent making eye contact with the experimenter after encountering the box they could no longer open on their own. Dog puppies made significantly more eye contact with the experimenter than wolf puppies did (dog: M ± SD = 4.09 ± 4.29 s; wolf: M ± SD = 1.47 ± 2.18 s, t(45.9) = 2.83, p = 0.007,

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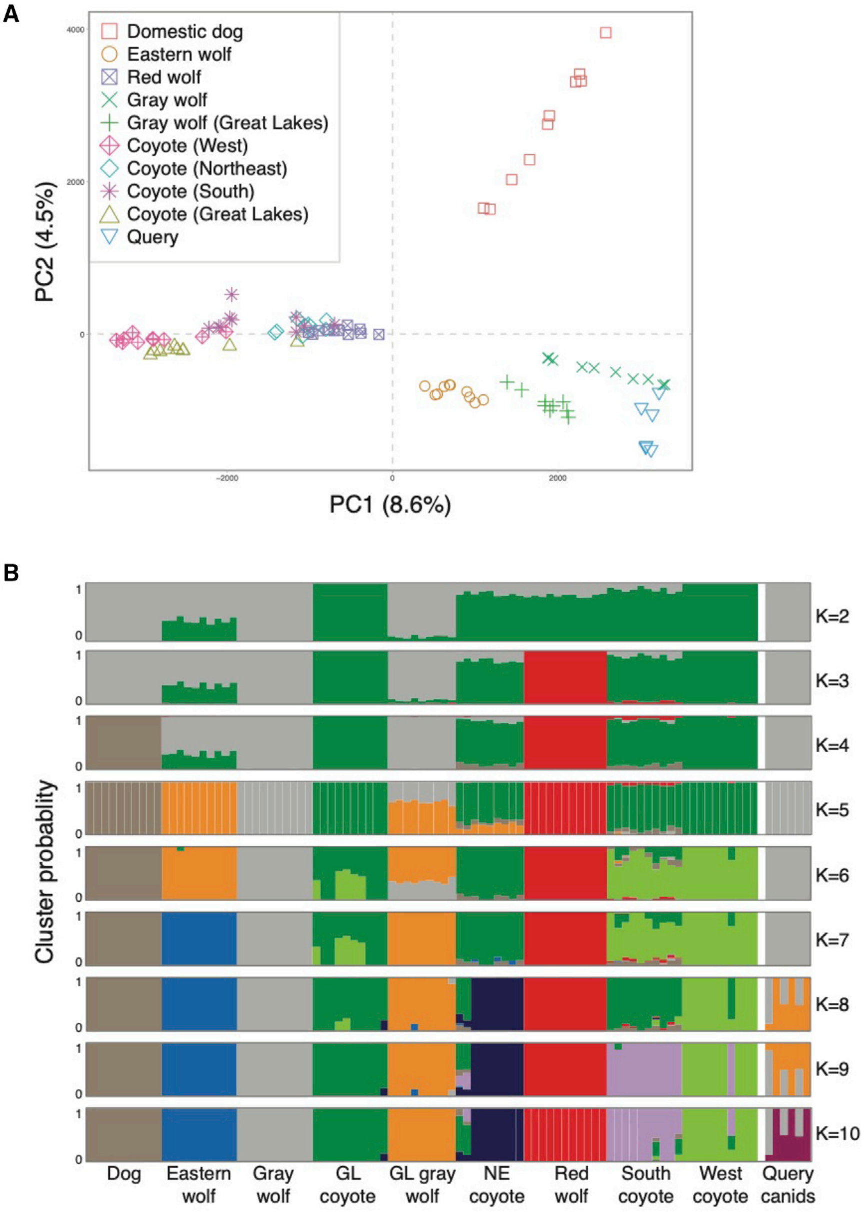

Figure 4. Genetic ancestry analysis

(A and B) Cluster analysis of six query wolves from this study with respect to 89 reference canids from North America (see Table S3 for details) with (A) principle component analysis (PCA) (percent variation are provided) and (B) cluster probability derived from a maximum likelihood *ADMIXTURE* analysis of 108,353 statistically unlinked SNPs. (Abbreviations are as follows: GL, Great Lakes; NE, northeast).

Welch independent t test) and a linear model again showed that species was a significant predictor of eye contact duration (Figure 3D and Data S2H).

Sex, age, and trial number were considered as covariates for each of the temperament and cognitive tests using linear regression model comparison, and species was always the most highly significant predictor variable on tasks where a significant species difference was found (Data S2C–S2H). Including age as a covariate did improve the models for all tests except the gesture comprehension tests (arm pointing and marker), further reinforcing that this ability is early emerging in puppies (Data S2C–S2H).

An important factor in any dog-wolf comparison is the certainty of genetic ancestry of the participating wolves, especially when working with a captive population that might have ambiguous origin records or pedigree documentation. The concerns primarily regard the genetic ancestry or admixture proportions of the wolves in question and potential effects of inbreeding. We therefore genotyped a subset of wolves in our cognitive sample for single nucleotide polymorphisms (SNPs) distributed across the genome by using RADseq (see method details) to assess their genetic ancestry. Each of the query wolves had observed heterozygosities that were within the range of all reference wild canids analyzed (H_O range, query = 0.092–0.152, wild canids = 0.079–0.155) (Table S1). Using two different methods of evaluating population structure, the six study wolves showed negligible admixture with dogs and instead clustered generally with gray wolves, more specifically with gray wolves from the Great Lakes region (Figure 4). Furthermore, inferred ancestry analyses with respect to two possible reference populations (gray wolf and dog) indicated high-content gray wolf ancestry across the study wolves (range: 0.944–0.982, Table S2).

DISCUSSION

Our results support the predictions of the domestication hypothesis. Dog but not wolf puppies are attracted to humans and show early emerging skills for reading human gestures, even though the wolf puppies received more intense human socialization. Dog puppies' odds of using each human gesture correctly were more than twice those of wolf puppies. Half of the dog puppies were successful at the individual level, whereas no wolf puppy was. Dog puppies also spontaneously used both gestures on their first test trial and there was no evidence for increasing success within test sessions or in older puppies. The youngest dogs also spontaneously used cooperative-communicative gestures despite having far less human exposure than the wolf puppies.

Success with the arbitrary marker rules out that dogs only use gestures because of an attraction to human hands, because in this condition the human placed their hand next to both of the possible hiding locations. The chance-level performance of

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wolves on the marker gesture rules out an aversion to the block influencing their choices. Chance performance in the body position control rules out an aversion to approaching humans explaining the wolf puppy's performance. Wolves did not avoid the location adjacent to the pointing experimenter. The olfactory control rules out the possibility that subjects found the food rewards by using olfactory cues.

Performance on the non-social tasks also suggests that compared with wolves, dog puppies are specialized for cooperative communication with humans.[11,35,36] Both species performed similarly on the memory and inhibition tasks, suggesting that species differences in early ontogeny are predominantly in the social domain (with any species differences in these non-social skills likely appearing later in development[37]). Our supplemental social task further suggests that dog puppies are prepared to communicate with humans given that they made more eye contact than the wolf puppies, though further development might then interact with species differences in persistence or enhance the use of human gaze.[38,39] Results from the temperament tests replicate similar findings[40–43] and support the idea that it is an unusual interest in humans that motivates the early emerging social skills of dogs. Dogs' odds were 30 times higher than wolves' to approach a stranger and 5 times higher to approach a familiar caretaker. This approach behavior was linked to proficiency in using human gestures in a similar way, as has previously been observed in experimentally domesticated foxes.[27] Given that the wolves in this population exhibit minimal signatures of admixture with dogs or reduced heterozygosity, we also argue that this population of wolves is particularly appropriate for dog-wolf studies and these genetic factors will need to be considered in future work.

Together, these results support the idea that, as human foragers became more sedentary, a population(s) of wolves was selected for an attraction to humans (and their food waste[44]). Before this selection, any human-wolf interaction was constrained by the flight response of wolves. Once attraction replaced fear, inherited social skills were applied toward humans in a new way and early in development (other species selected similarly might or might not show similar abilities depending on the social skills inherited from their progenitor species, e.g., ferrets[45]). Future research is needed to understand whether a second wave of selection occurred, which might help explain variation in social skills across groups of domestic dogs.[23,24,46] Given the uncertainty regarding the wolf population(s) that dogs diverge from, future work might also need to extend comparisons to diverse wolf populations.[47,48]

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.06.051.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, investigation, all authors; Writing – Original Draft, B.H. and H.S.; Writing – Reviewing and editing, all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
Raw data	This paper	https://doi.org/10.5061/dryad.2547d7wqm
Experimental Models: Organisms/Strains		
Grey wolf	Wildlife Science Center	www.wildlifesciencecenter.org
Domestic Dog	Canine Companions for Independence	www.canine.org
Domestic dog	Paws 4 People	www.paws4people.org
Domestic dog	Ears Eyes Nose & Paws	www.eenp.org
Biological samples		
Grey wolf whole blood	Wildlife Science Center	www.wildlifesciencecenter.org
Oligonucleotides		
Sample 91 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGA GCA GGA ATG CA	Integrated DNA Technologies	N/A
Sample 91 Bottom RADseq adaptor: /5Phos/TTC CTG CTC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1403 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGC CGT GAG ATG CA	Integrated DNA Technologies	N/A
Sample 1403 Bottom RADseq adaptor: /5Phos/TCT CAC GGC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1408 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGG CCA CAT ATG CA	Integrated DNA Technologies	N/A
Sample 1408 Bottom RADseq adaptor: /5Phos/TAT GTG GCC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1409 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGT CCG TCT ATG CA	Integrated DNA Technologies	N/A
Sample 1409 Bottom RADseq adaptor: /5Phos/TAG ACG GAC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1601 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGA CAC TGA CTG CA	Integrated DNA Technologies	N/A
Sample 1601 Bottom RADseq adaptor: /5Phos/GTC AGT GTC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1605 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGC CGA CAA CTG CA	Integrated DNA Technologies	N/A
Sample 1605 Bottom RADseq adaptor: /5Phos/GTT GTC GGC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1703 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGA CAT TGG CTG CA	Integrated DNA Technologies	N/A
Sample 1703 Bottom RADseq adaptor: /5Phos/GCC AAT GTC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
Sample 1705 Top RADseq adaptor: /5Biosg/GTA CGT CCT GCA GGA ACG CTT ATG CA	Integrated DNA Technologies	N/A
Sample 1705 Bottom RADseq adaptor: /5Phos/TAA GCG TTC CTG CAG GAC GTA C	Integrated DNA Technologies	N/A
NEBNext Index 11 Primer for Illumina: 5′-CAA GCA GAA GAC GGC ATA CGA GAT GGC TAC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG ATC-s-T-3′	New England Biolabs	E7321
Critical commercial assays		
SbfI-HF restriction enzyme	New England Biolabs	NEB R3642
Adaptor Ligation Module	New England Biolabs	NEB M0202
Agencourt AMPure XP DNA beads	Beckman Coulter	A63881

(*Continued on next page*)

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dynabeads M-280 Streptavidin	Invitrogen	11205D
Qubit dsDNA HS assay kit	ThermoFisher	Q32851
NEBNext Ultra II DNA Library Prep Kit	New England Biolabs	E7645
Software and Algorithms		
Analysis code	This paper	https://doi.org/10.5061/dryad.2547d7wqm
STACKS v2	Catchen et al.[49] and Rochette et al.[50]	https://catchenlab.life.illinois.edu/stacks/
Stampy v1.0.21	Lunter and Goodson[51]	https://www.well.ox.ac.uk/research/research-groups/lunter-group/lunter-group/stampy
Samtools v1.9	Li et al.[52]	http://www.htslib.org/
PLINK v1.90b3i	Chang et al.[53]	https://www.cog-genomics.org/plink2/
detectRUNS	Marras et al.[54]	https://cran.r-project.org/web/packages/detectRUNS/vignettes/detectRUNS.vignette.html
flashPCA	Abraham et al.[55]	https://github.com/gabraham/flashpca
ADMIXTURE	Alexander et al.[56]	https://dalexander.github.io/admixture/publications.html
STRUCTURE v2.3.4	Pritchard et al.[57]	https://web.stanford.edu/group/pritchardlab/structure.html
ELAI	Guan[58]	https://www.haplotype.org/software.html
Deposited data		
RADseq aligned (BAM) files	This paper	NCBI SRA BioProject PRJNA699018
Dog reference genome NCBI build 3.0, CanFam3.1 (GCF_000002285.3)	NCBI	https://www.ncbi.nlm.nih.gov/assembly/GCF_000002285.3/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources can be directed to the lead contact Hannah Salomons (hannah.salomons@duke.edu) or the P.I. Brian Hare (b.hare@duke.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data and the code to analyze them are available in DRYAD (https://doi.org/10.5061/dryad.2547d7wqm).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

A total of 44 dog puppies and 37 wolf puppies participated in cognitive and temperament testing. See Data S1A for the species, sex, population, testing year, rearing experience, and age at each test for each subject. All dog puppies came from lines of dogs being bred and socialized for work as assistance dogs (see MacLean and Hare[9] for details). Assistance dogs in training are not particularly skilled at using human gestures relative to other populations of dogs. MacLean and Hare[9] found that a large sample of adult dogs from Canine Companion for Independence (CCI) performed comparably in their use of a human pointing gesture in comparison to a heterogenous population of adult pet dogs (see Figure 1). Although this made it less likely we might see a difference between the dog and wolf puppies we compared, we decided to test assistance dogs as our comparison group because they offer ease of access to large numbers of same-aged puppies, and relatively high quality information available regarding their breeding and rearing histories. This means all dogs were Labradors, golden retrievers, or Labrador golden crosses with known pedigrees.

The majority of dog puppies were tested at Canine Companion for Independence (www.cci.org) in Santa Rosa, CA. The other puppies were tested at the Duke Canine Cognition Center (DCCC) in Durham, NC. The puppies tested at CCI (n = 27) were all born in Northern California, either at the Canine Early Development Center on CCI's campus (n = 6) or in the homes of volunteer breeder caretakers (n = 21). Puppies stayed in a whelping pool with their mother for the first three weeks, and then moved to a larger

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pen with their mothers until they weaned at ∼6 weeks of age. During this time, puppies had relatively limited interaction with human caretakers (< 2 h total per day), who mainly moved pups while cleaning the pool and pen, cut toenails, took daily weights, and provided food and water for the mother. After weaning, puppies continued to live and sleep in a large floor pen with littermates, either in the whelping center or inside a volunteer home, and humans provided kibble three times per day (see Bray et al., 2020[16] for details). Although puppies had multiple hours of exposure to humans each day, their living set-up (i.e., floor pens) allowed for less prolonged interaction with humans as the wolf puppies. Furthermore, unlike the wolf puppies, dog puppies only ever slept with conspecifics.

Additionally, a few CCI puppies were tested at the DCCC (n = 5). These puppies had the same breeding pedigree and rearing experience as those tested at CCI and were then sent to live at Duke Puppy Kindergarten (DPK) when they were ∼10 weeks old. At the DPK, the puppies lived together and spent their days in an indoor playroom, outdoor playpen, and on brief outings around campus. They had human caregivers with them all day and were occasionally visited by adult CCI dogs for play sessions. They slept separately in kennels without humans at night.

Finally, a minority of dogs were recruited from two local assistance dog nonprofits that breed and raise dogs near Duke University: Paws 4 People (P4P) (www.paws4people.org) (n = 8), and Ears Eyes Nose & Paws (EENP) (www.eenp.org) (n = 5). Similarly to the CCI puppies, these dogs were raised in human homes with their mothers and litter mates until ∼8 weeks. They were then weaned and reared with their littermates in the homes of the staff until our testing was completed. These staff raisers brought the puppies to DCCC for testing.

Dog puppies were selected for testing solely on the basis of their age and availability. We attempted to test 44 dog puppies and were able to test all 44 (although we aborted testing after the first temperament session for one puppy, CCI31, due to low motivation).

All wolves were born and raised at the Wildlife Science Center in Stacy, Minnesota (WSC) (www.wildlifesciencecenter.org). All the wolf puppies tested were either the first (n = 21), second (n = 14), or third (n = 2) generation to be born in captivity, and known to have wild North American ancestry (see below for genetic analyses). The wolves were selected for testing based on their availability, age, and willingness to participate in tasks involving human interactions. The wolves were taken off their mothers at 10–11 days of age (not for the purpose of this study) and raised by humans, alongside their littermates and adult dogs. Twenty-eight wolf pups had human and adult dog contact 24 h per day 7 days a week until all testing was completed: at least one human raiser and at least one adult dog were always inside their enclosure, even at nighttime, when the pups often slept next to or on top of the human raiser. The other 9 wolf puppies were in constant human and adult dog contact during the day (11–12 h per day), but at night were only with other same age wolf pups, until testing was complete. All wolf pups were bottle fed by their human raisers until at least 8 weeks of age, with some raw ground meat introduced by hand during this period as a supplement. After 8 weeks, they continued to eat raw meat (fresh ground and/or carcasses) provided by their human caretakers and were occasionally offered bottles for comfort as needed.

We attempted to test a total of 49 wolf puppies and were able to collect data from 37. We were unable to collect any data with the other 12 wolf puppies because they were too nervous around unfamiliar humans (i.e., experimenters), even though they had been heavily exposed to humans: all 12 were hand raised after 10–11 days with their mothers, with 3 being raised with approximately 12 h per day of human and dog contact, and 9 being raised with approximately 24 h contact. There was always a human raiser and a familiar adult dog present during testing, but even so, they would hide, pace, or refuse to eat when an unfamiliar human was in the testing room. Testing of these 12 subjects was abandoned after failing in multiple attempts to habituate them to the presence of a novel experimenter, as they were unwilling to approach the testing area, make choices, or search for food.

Not all subjects participated in all tests, largely due to time constraints and logistics of collecting data over multiple years at two field sites located in different regions of the United States during defined data collection periods that matched the required stages of development being tested. This was particularly difficult in the case of the wolves, which have one whelping period in spring as opposed to dog puppies, which are born year-round. We completed the entire battery of five tests (i.e., temperament, working memory, inhibition, gesture comprehension, and human eye contact test) with 8 wolf pups and 28 dog puppies. Nine additional wolf pups completed all tests except the human eye contact test (which we only began implementing with the wolves in 2017).

In both species we were able to balance for the number of males and females tested. We also made sure that the population of wolves we tested were not younger than the dogs tested. This ruled out explaining any performance by the dog puppies that was significantly better than the wolves as a result of the dogs being more mature developmentally. For all tests, the mean age of the wolves was older than that of the dogs. We also sampled from a wider range of ages in the dogs than the wolves, with the one exception being in the inhibition test where two 5-week-old wolves were included.

For the genetic analysis eight individuals with banked blood samples were chosen for sequencing; these individuals were selected to represent litters with unique parentage (i.e., no full siblings were sequenced).

This research was approved by the Duke University IACUC #A105-17-04.

METHOD DETAILS

Setup of Testing Area

Dog puppies were tested in a quiet testing room, at either CCI or DCCC as described above. Before the first testing session, all the pups tested at CCI were unfamiliar with the room. All EENP and P4P pups tested at DCCC were also unfamiliar with the room prior to testing. The 5 CCI puppies raised in DPK and tested at DCCC were familiar with the room before testing. All wolves were tested in a familiar room at WSC, adjacent to their outdoor living area, in which they had spent significant time. Although background environmental sounds (such as dogs barking or facility trucks driving on gravel) could be heard from within the room, these were sounds that

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the wolf pups heard all day throughout their lives and rarely reacted to during testing; if the wolf puppies seemed distracted by ambient noise, a pause was taken between trials. All dog and wolf puppies, regardless of prior familiarity with the room, were given a few minutes to explore and acclimate to the room before testing began. Although testing room familiarity was different between species, dogs have performed similarly on these types of tests in both familiar and unfamiliar test spaces (10). The unfamiliarity of the testing spaces for most dog puppies is conservative given that it potentially works against the experimental hypothesis (i.e., if anything, dog puppies might perform less skillfully in new surroundings).

The standardized testing area (Figure S2) was delineated by using chalk (WSC), tape (CCI HQ), or testing mats (DCCC). The setup for this study consisted of two 2 m lines on the floor arranged in the shape of a capital "T," which intersect at position C. Positions L and R were 1 m to the right and left of position C, and positions R' and L' were 20 cm to the right and left of position C. At CCI HQ, the testing area was fenced in within the larger room by a puppy pen. At WSC and DCCC, the room was partitioned into a smaller area by use of a temporary wall (DCCC) or wire fencing (WSC). All testing sessions were recorded by two or three handheld video cameras (Sony Handycam HDR-CX405 or similar), mounted on tripods and positioned outside the testing area at locations which best captured the behaviors of the subject and experimenter for each test.

Temperament Test

General Procedures:

Subjects were presented with the opportunity to retrieve a piece of food nearby a familiar or unfamiliar human or object. For the dog puppies, the familiar human was someone to whom the subject had at least 30 min of prior exposure. For the puppies tested at CCI, the person who would play the "familiar human" role became familiar with the subjects on the morning of their testing day. The familiar human entered the pen where the subjects lived with their littermates and engaged in whatever way the subjects showed interest (sitting with and talking to, petting, playing, etc.) for 30 min. For the puppies tested at DCCC, the familiar person was one of their regular caretakers. For the wolf pups, the familiar human was always a hand-raiser. For both species, the unfamiliar human was a stranger the subject had never seen before. The familiar object for both species was a toy that the subject had prior exposure to and had been observed interacting with in their living area, whereas the unfamiliar object was a remote-controlled plastic bear the subjects had never seen before. Each stimulus was presented in four 4-trial blocks for a total of 16 trials during each session, counterbalanced for order across individuals in four different session orders (familiar/unfamiliar first, and human/object first). The experimenter (E) presented the food and the stimuli, and the handler (H) positioned the subject and released the subject at the appropriate times during trials. Prior to the stimuli presentation, E always baited the platform (overturned bowl) with a piece of food at location C. In each 4-trial block, the stimuli were always placed at positions R, L, R' and L' (Figure S2) in that order.

Warm-up Trials

Warm up trials were done prior to completing any test trials to introduce the set-up to the subject. H centered the subject at the start line (S) and gently held the subject in place. E approached the subject to present the food reward in her hand and said "look!," allowing the puppy to see and sniff the food briefly. E walked backward (facing the puppy with food visible in hand) to set the reward on top of the platform at location C, then exited the testing area (or walked to the back of the room if the testing area was not fenced) and stood still and silent, facing the wall. Once E was positioned, she said "okay!," signaling H to let go of the subject. The subject had 30 s to approach the platform and retrieve the food reward. If the subject did not approach the platform within 30 s, the trial was repeated. The subject was required to successfully retrieve the reward on two consecutive trials in order to advance to testing. If the subject did not show any interest in approaching the food at first, a warm-up trial was done with the food placed directly in front of the subject, then halfway between the subject and position C, and then the standard warm up trials were completed at position C.

Session 1: Stationary Stimuli

Familiar and Unfamiliar Object Trials

Object trials were conducted in the same way as the warm-up trials, with the addition of the object presentation. The trial began as described for the warm-ups, but after E placed the food on the platform, E then retrieved the appropriate object for the trial (familiar or unfamiliar) from a table within reach but outside the testing area. E approached the subject again with the object in hand, bent down, and presented it a few inches from the subject's face, and said "look!." E walked backward, facing the puppy with the object visible in hand, to place the item at the appropriate position (R, L, R', or L'). E then exited the testing area and stood still and silent, facing the wall. Once E was positioned, she said "okay!," signaling H to let go of the subject. The subject had 30 s to approach the platform and retrieve the food reward and/or interact with the object if desired. During the trial, H sat still in place, watched the subject without interacting. She said "Food" aloud in a neutral tone if the subject ate the food, and "Touch" if the subject touched the object with its nose, mouth, or front paws. This allowed E, who could not see the subject during the trial, to record these responses on the data sheet, and was helpful for reference in the videos (although we only used whether or not the subject touched the stimulus in the final analysis). Regardless of the subject's behavior, the trial ended after 30 s, and the subject was not given the food reward if she had not retrieved it.

Familiar and Unfamiliar Human Trials

The human trials were exactly the same as the object trials, except that the stimulus was a human instead. The familiar and unfamiliar people carried out the role of E. After E placed the food on the platform, E then knelt at the appropriate position (R, L, R', or L'), facing toward C, and looked down with her hands flat on her thighs. Once E was positioned, E said "okay!," signaling H to let go of the subject. E remained in position for the duration of the trial, which proceeded in the same way as the object trials described above.

Session 2: Moving Stimuli

Warm Up Trials

Refamiliarization trials, identical to the warm-ups of Session 1, were conducted if there was more than a 30-min break between Session 1 and Session 2. Most subjects completed these sessions consecutively and refamiliarization was not required.

Familiar and Unfamiliar Object Trials

Session 2 trials were identical to Session 1 except that the stimuli moved as described below before the dog was released to make a choice:

Familiar Toy: After placing the object at the appropriate position (R, L, R', or L'), E stood behind the object and, keeping her eyes looking down at the ground, pulled on a string attached to the object to lift it off the ground to about knee height, then quickly lowered it back to its place on the floor. E lifted and lowered the toy this way three times before exiting the testing area.

Unfamiliar Toy: After placing the object (a plastic bear) at the appropriate position (R, L, R' or L'), the bear moved three times. For some of the initial subjects E stood behind the bear looking down while the bear turned 360 degrees via remote control but for most subjects E simply moved the bear up and down using a string.

Familiar and Unfamiliar Human Trials

Session 2 trials were identical to Session 1, except that the familiar or unfamiliar human playing the role of E moved her upper body after kneeling at the appropriate position (R, L, R' or L'). E bent her upper body down as much as she was able, so that her torso approached her thighs and her forehead nearly touched the ground, placing both hands on the ground underneath her shoulders, and then pushed herself back up. She performed this unusual movement a total of three times before resuming the same still position as in Session 1.

Abort Criteria

If the subject exhibited any signs of significant stress (including excessive whining, barking, escape behavior, or defecation), a break was taken. If time allowed, another attempt occurred later the same day. After a break, the subject repeated the warm-up trials before resuming.

Working Memory

Warm-Ups - Visible Placement

At the beginning of the session, the subject was required to pass a warm-up criterion prior to completing any test trials. Warm-up trials were conducted to assure that the subjects were motivated to search for the reward and to prevent side biases. Warm-up trials consisted of two phases: (1) one-bowl centered visible placement (2) one-bowl alternating visible placement.

Phase 1—One Bowl Centered

H first centered the subject at the start line (S) and gently held the subject in place. E approached the subject to present the food reward in her hand and said "look!," allowing the puppy to see and sniff the food briefly. E walked backward, facing the puppy with food visible in hand, to gently set the reward into the food bowl at location C without making a sound. After baiting, E knelt behind the bowl and rested her hands flat on her thighs, looking straight down at her lap. The subject was then allowed to approach the bowl and obtain the reward. If the subject did not approach the bowl within 15 s, the trial was repeated. This phase of warm-ups familiarized the subject with the set-up and assured that the subject was motivated to find the reward. To pass the warm-up criteria the subject was required to successfully retrieve the reward from the bowl on two consecutive trials within a maximum of six trials.

Phase 2—One Bowl Alternating

Phase 2 warm-ups were identical to phase 1, except that the bowl's position was counterbalanced between the R or L positions. If the subject did not approach the bowl in 15 s, the trial was repeated. Repeating these trials served as a correction procedure for spontaneous side biases and ensured that subjects gained experience finding the reward in both locations. To pass the warm-up criterion the subject was required to successfully retrieve the reward from the bowl on two consecutive trials (one on each side) within a maximum of six trials.

Working Memory Test Trials

Bowls were placed at both positions R and L, and E was provided a list denoting which side to place the food on for each trial. Side placement was counterbalanced with the constraint that the reward could not be on the same side in more than two consecutive trials. H centered the subject at the start line (S) and gently held the subject in place. E approached the subject to present the food reward in her hand and said "look!," allowing the subject to see and sniff the food briefly. E walked backward, facing the puppy with food visible in hand, to gently set the reward into the food bowl at the appropriate location (R or L) without making a sound. After baiting, E walked back to the center (location E), knelt, and rested her hands flat on her thighs, looking straight down at her lap. The subject was then allowed to approach the bowl and obtain the reward. If the subject did not approach the bowl within 15 s, the trial was repeated. If the subject chose the baited bowl, the subject was allowed to have the reward and the next trial was administered. If the subject chose the incorrect bowl, E said "wrong" in a monotone voice and the subject was not rewarded nor allowed to see where the food was located. If the subject did not choose any bowl within 15 s, the trial was repeated. A "choice" was defined as the subject's nose passing over the edge of a bowl. Subjects were required to choose the baited bowl first in four out of five consecutive trials, within a maximum of 20 trials, to advance to the Gesture Comprehension test. The number of trials taken to either reach criteria or max-out in the first non-aborted testing session was recorded as the subject's score for this task—a lower score indicates better performance. However, subjects that failed to meet this criterion within 20 trials in their first session were tested in another session

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after a break of at least 30 min, if time allowed. This allowed for another opportunity to meet criterion and advance to the Gesture Comprehension tests.

Abort Criteria

If the subject did not make a choice within 15 s on two trials in a row or exhibited any signs of significant stress (including excessive whining, barking, escape behavior, or defecation), a break was taken. In most cases where a break was needed, another attempt occurred later the same day. After a break, the subject started over from the beginning of the Phase 1 warm up trials, regardless of how many trials had been completed before the break.

Gesture Comprehension

General Procedures

There were four different tasks in this session: arm pointing, novel marker, body versus point, and odor control. Six trials of each task were conducted. The order of the tasks was the same for all subjects. Bowls were placed at both positions R and L, and E was provided a list denoting which side to place the food on for each trial. Side placement was counterbalanced with the constraint that the reward could not be on the same side in more than two consecutive trials. H first centered the subject at the start line (S) and gently held the subject in place. E approached the subject to present the food reward in her hand and said “look!,” allowing the puppy to see and sniff the food briefly. E then closed her fingers around the food and rotated her wrist so that the back of her hand faced the subject, occluding the food. With the reward occluded, E then walked backward to the bowl at position R, bent down and either placed or pretended to place (as appropriate for the trial) the food in the bowl, then walked across to and did the same at the bowl at position L, making identical hand movements and sounds at each bowl. E then knelt behind the center position (Location E) and performed the designated gesture for the task. She then said “okay!,” signaling H to let go of the subject. Throughout the trial, H (who did not know the location of the hidden food) sat still in place.

A choice was defined as the subject touching the bowl or the subject’s nose passing over the edge of the bowl. If the subject correctly chose the baited bowl, the subject was allowed to eat the food and E gave verbal praise. If the subject chose the incorrect bowl, E said “wrong” in a monotone voice and the subject was not rewarded nor allowed to see where the food was located. If the subject did not make a choice within 15 s, the trial was repeated.

Arm Pointing

After baiting, E knelt at location E, bent forward to eye level with the subject, and pointed with her proximal arm to the baited bowl, index finger extended and head turned toward the baited bowl. E then turned her head to look at the subject, said “look!,” and turned her head back toward the baited bowl gaze alternating this way three times, all while maintaining the pointing arm’s position. E then said “Okay!” and maintained the pointing gesture and gazed toward the baited bowl until the trial ended.

Arbitrary Marker

After baiting, E knelt at location E, picked up a small blue wooden block (5 cm x 5 cm x 5 cm) in her right hand, and held it out in front of her to show the subject while saying “look!.” From the kneeling position, E reached over to set the block next to the bowl at position R, and either left it there if that bowl was the baited one in the trial, or picked it back up. She then reached over to either touch her empty hand to the spot next to position L if she had left the block at position R, or set the block next to the bowl at position L. This procedure ensured that both bowls had equal attention from the experimenter and proximity to her hand. E then returned her hands to rest on her thighs, said “Okay!,” and remained kneeling and looking straight down until the trial ended.

Body versus Point Control

This gesture was identical to that in the arm pointing task, except that E knelt directly behind the *unbaited* bowl (behind location R or L) instead of in the center (location E). This created a choice between the bowl which E was closest to in proximity, and the bowl toward which E was pointing. This controlled for the possibility that if wolves performed poorly on the arm pointing task, it could be explained simply by avoidance of the human. If wolves were actively avoiding the human, they would choose the baited bowl on this task.

Odor Control

No gesture was administered - after baiting, E knelt down in the center (location E) resting her hands on her thighs, said “Okay!,” and remained kneeling and looking straight down until the trial ended. This created a situation in which subjects had no social information to use to find the food, testing for the possibility that they could use solely olfactory information to locate the food.

Abort Criteria

If the subject did not make a choice within 15 s on two trials in a row, or exhibited any signs of significant stress including whining, escape behavior, or excessive defecation, a break was taken. In most cases, especially in the middle of a test trial block, another attempt occurred on the same day. In this case, two Working Memory trials were conducted as warm-ups (one on each side). The subject was required to successfully find the reward on both sides to resume test trials. In the case where the session resumed on a subsequent day, another complete Working Memory session was conducted as warm-ups, and subjects were required to reach criterion prior to resuming testing. In some cases, due to time constraints (i.e., field seasons being over), another attempt was not possible.

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Inhibitory Control

Apparatus

A cylinder was constructed by taping clear flexible plastic around two solid plastic rings (1 ft wide x 11 in diameter). A tube of black opaque stretchy fabric was made to fit as a cover. The cylinder and fabric cover both had a strip of Velcro at the bottom, which could attach the cylinder to a strip of Velcro on a square wooden base (2 ft x 2 ft) to keep the apparatus still if bumped by the subject.

Warm-up Trials - Opaque Cylinder

H centered the subject at the near start line (S') and gently held the subject in place. E knelt at location E, behind the cylinder which had the opaque cover on and was positioned at location C. E reached forward (over the cylinder) to present the food reward in her right hand and said "look!," allowing the subject to see and sniff the food briefly. E then placed the food inside the cylinder, entering it from the right side (the open side facing position R). E then placed her hands on her thighs, looked down, and said "Okay!," signaling H to let go of the subject. The subject was permitted 15 s to retrieve the reward. E recorded whether the subject first touched or bumped the front of the cylinder in an attempt to retrieve the reward, or went directly around to the side opening. If the subject did not retrieve the reward within 15 s, the trial was repeated. Subjects were required to correctly retrieve the reward, by going directly to the side opening without touching or bumping the front of the cylinder, in 4 out of a window of 5 consecutive trials before advancing to test trials.

Test Trials—Transparent Cylinder

The test procedure was identical to the warm-up trials, except that the fabric cover was removed so that the cylinder was transparent. As in warm-up trials, E recorded whether the subject first touched or bumped into the front of the cylinder in an attempt to get to the visible reward (incorrect), or went directly around to the side opening to retrieve the reward without bumping into the cylinder (correct). The subject was allowed to retrieve the reward on all trials regardless of the accuracy of their first attempt. Ten trials were conducted.

Abort Criteria

The session was aborted if the subject exhibited significant signs of stress (including excessive whining, barking, escape behavior, or defecation), did not meet the criterion within 25 warm-up trials to advance to the test trials, or did not retrieve the reward within 15 s on a total of 10 warm-up trials.

Human Eye Contact

Apparatus

A clear container with a clear lid that snapped closed (Rubbermaid Brilliance 1.3 cup container or similar) was super-glued to a wooden base (2 ft x 2 ft) so that it could not be picked up by the subject.

Warm-up Trials - Solvable

H centered the subject at the near start line (S') and gently held the subject in place. E knelt at location E, behind the open clear container with the lid off, which was positioned at location C. E reached forward (over the container) to present the food reward and said "look!," allowing the subject to see and sniff the food briefly. E then placed the food inside the container, and positioned the lid loosely on top such that it could easily be knocked off. E then placed her hands on her thighs, looked down, and said "Okay!," signaling H to let go of the subject. The subject was allowed to knock off the lid and retrieve the reward, at which point E provided verbal praise and the trial ended. If at any point during the trial the subject somehow knocked the lid into a position which made the reward unreachable, E quietly intervened to make the reward accessible again. If the subject did not successfully retrieve the reward within 30 s, the trial was repeated. The subject was required to successfully complete 4 warm-up trials before advancing to the test. The first of these started with the lid leaning up against the container, the second with the lid covering about half of the opening of the container, and the third and fourth with the lid covering three-quarters of the opening of the container.

Test Trials—Unsolvable

Test trials were identical to warm up trials except that E sealed the lid onto the container so that it could not be removed by the subject. The subject was given 30 s to attempt to access the reward. E and H remained seated in place and continuously looked at the subject (rotating head and body if necessary) during this period. E and H both held silent stopwatches, and used the start/stop buttons in count-up mode to measure the total time that the subject looked at each of their faces. After each test trial, E praised the subject, opened the container, and allowed the subject to retrieve the contents. Four test trials were conducted, and the sum of the time spent looking at E and H's faces across the four trials was used as the measure for analysis.

Abort Criteria

The session was aborted if the subject exhibited signs of significant stress (including excessive whining, barking, escape behavior, or defecation), or if the subject did not obtain the reward within 12 attempts during the warm-up trials.

QUANTIFICATION AND STATISTICAL ANALYSIS

Temperament and Cognitive Measures

All trials were videotaped from an angle (or multiple angles) which captured the subject's response as well as the experimenter (E). Subject responses were usually live-coded by E after each trial; in a minority of cases E was unable to live-code (e.g., stopwatch malfunction) and designated the trial to be coded from video. During live coding and upon reviewing the video for temperament and gesture comprehension, responses were seen as unambiguous and reliability coding was deemed unnecessary. All of the wolf and 20% of dog trials in the Human Eye-Contact test, and 20% of all trials in the Inhibitory Control test, were independently coded for reliability from video. The subset of trials coded from video were chosen pseudo-randomly (ensuring that at least one subject from each of the testing populations was included for each test). Reliability was excellent for both the Human Eye-Contact test (Pearson's $r(16) = 0.92$, $p < .001$) and the Inhibitory Control test (Cohen's $\kappa = 0.956$, $N = 150$, $p < .001$).

Statistical analyses were done using R v.3.5.2. For all analyses, species was treated as a categorical variable, and age and trial number were treated as continuous variables. Bowl choice (baited/unbaited) in the gesture comprehension tests, as well as response (touch/no touch) in the temperament and inhibitory control tests, were treated as binary variables. Looking time in the human eye contact test and the number of trials needed to reach criterion in the working memory test were both treated as continuous variables.

A mixed-effects logistic regression model with random intercepts for each subject was used to estimate the effect of species for each condition in the temperament test. This model also accounted for age and interaction type. Welch's unequal variance t tests were performed to compare species performance on the Working Memory and Human Eye-Contact tests. We used a mixed-effects logistic regression model with random intercepts for each subject to estimate the probabilities of choosing the indicated location for each species in the two Gesture Comprehension tasks and their two controls. To understand how well individual wolves and dogs performed on the gesture comprehension tests, we counted the number of individuals who achieved significant performance at the $p < .05$ level, defined by non-parametric binomial probability test as 10 out of 12 correct. We also used a mixed-effects logistic regression model with random intercepts for each subject to estimate the probabilities of navigating directly to the side opening without first touching the clear cylinder for each species in the Inhibitory Control test.

Binomial logistic regression models, with species as a predictor variable, were performed for both the Gesture Comprehension tests, as well as the Inhibitory Control test, to determine whether species had a significant effect on performance. Linear regression models, with species as a predictor variable, were performed for the working memory test and the human eye contact test, to determine whether species had a significant effect on performance. To determine whether age, sex, trial number, or temperament had a significant effect on performance, these predictor variables were added to the models, and models were compared using AIC and R^2.

In addition to the main findings reported in the Results, supplemental analyses yielded the following results. Using a linear contrast test on the mixed-effects logistic regression model revealed that compared with a wolf puppy, a dog puppy's odds of touching the unfamiliar human, familiar human, and unfamiliar object, were 30.52 (95% CI 14.48-73.68, $z = -8.374$, $p < .001$), 5.36 (95% CI 2.99-10.06, $z = -5.593$, $p < .001$), and 1.91 (95% CI 1.16-3.20, $z = -2.57$, $p = 0.01$) times higher respectively. The dog puppies' odds of touching the familiar object were not significantly different than the wolves' (odds ratio = 1.35, 95% CI 0.72-2.57, $z = -0.94$, $p = 0.34$) (Figure 2). The effect of trial number was nonsignificant using a likelihood ratio test (Chisq = 17.036, df = 15, $p = 0.3167$). The interaction between species and trial type (stationary versus moving stimulus) was nonsignificant using a likelihood ratio test (Chisq = 0.4826, df = 1, $p = 0.4872$). Similarly, the interaction between species and age was nonsignificant using a likelihood ratio test (Chisq = 1.89, df = 1, $p = 0.1692$).

A Welch's two sample t test comparing performance of dog puppies and wolf pups on the working memory task demonstrated that dogs (M = 11.26 trials, SD = 7) and wolves (M = 11.15, SD = 6.4) were not significantly different in the number of trials it took to reach criteria (correct response on 4 out of 5 consecutive trials), $t(56.63) = 0.06$, $p = 0.95$ (Figure 3A).

The mixed-effects logistic regression model showed that dog puppies were estimated to choose the indicated location 77.99% of the time (95% CI 70.26 – 84.17, $z = 6.11$) for the pointing gesture. Wolf puppies were estimated to choose the indicated location 62.08% of the time (95% CI 52.86 – 70.51, $z = 2.55$) for the pointing gesture. The dog puppies as a group performed above chance (50%) for the pointing gesture ($p < .001$), as did the wolf puppies as a group ($p = 0.011$). The dog puppies chose the indicated location significantly more than the wolf puppies did on the arm pointing gesture ($p = 0.006$) and the marker gesture ($p < .001$) (Figure 3B). Using a linear contrast test on a binomial logistic regression model revealed that compared with a wolf puppy, a dog puppy's odds of choosing the indicated location for the pointing gesture were 2.08 (95% CI 1.14 – 3.77) times higher (Figure S1). We also modeled the effect of trial number on performance using a binomial logistic regression and found no significant relationship ($p = 0.15$) (Data S2A). Finally, we used a Welch's two sample t test to compare the number of "no-choices" (see Methods) that occurred over the course of obtaining the 6 complete arm-pointing trials, and found no significant difference between the dog (M = 0.55, SD = 1.12) and wolf (M = 0.69, SD = 1.44) puppies, $t(46.88) = -0.42$, $p = 0.68$. This indicates that their differing performance on this task cannot be accounted for by differing motivation to participate in the trials.

The mixed-effects logistic regression model showed that dog puppies were estimated to choose the indicated location 78.01% of the time (95% CI 70.73 – 83.89, $z = 6.46$) for the marker gesture. Wolf puppies were estimated to choose the indicated location 57.25% of the time (95% CI 48.59 – 65.50, $z = 1.64$) for the marker gesture. The dog puppies as a group performed above chance (50%) for the marker gesture ($p < .001$), but the wolf puppies as a group did not ($p = 0.10$). The dog puppies chose the indicated location significantly more than the wolf puppies did on the marker gesture ($p < .001$) (Figure 3B). Using a linear contrast test on a binomial logistic regression model revealed that compared with a wolf puppy, a dog puppy's odds of choosing the indicated location for the marker gesture were 2.58 (95% CI 1.42 – 4.68) times higher (Figure S1). We also modeled the effect of trial number on performance using a binomial logistic regression and found no significant relationship ($p = 0.28$) (Data S2B). Finally, we used a Welch's two sample t test to compare the number of "no-choices" (see method details) that occurred over the course of obtaining the 6 complete marker trials, and found no significant difference between the dog (M = 0.16, SD = 0.58) and wolf (M = 0.53, SD = 1.14) puppies, $t(35.74) = -1.53$, $p = 0.13$. There is therefore no evidence for differing motivation between the two species as an explanation for their differing performance on this task.

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For the body versus point condition, the mixed-effects logistic regression model showed that dog puppies were estimated to choose the location indicated by the point 33.95% of the time (95% CI 23.62 – 46.08, $z = -2.56$). Wolf puppies were estimated to choose the location indicated by the point 45.99% of the time (95% CI 33.14 – 59.38, $z = -0.58$). The dog puppies performed significantly below chance ($p < .05$), while the wolf puppies did not perform significantly differently from chance ($p = 0.56$). The performance was also not significantly different between the two species ($p = 0.18$). Using a linear contrast test on a binomial logistic regression model revealed that relative to a wolf puppy, a dog puppy's odds of choosing the indicated location were not significantly different than a wolf puppy's (odds ratio = 0.67, 95% CI = 0.38 – 1.16, $p = 0.24$) (Figure S1).

For the odor control, the mixed-effects logistic regression model showed that dog puppies were estimated to choose the baited location 51.89% of the time (95% CI 44.70 – 59.00, $z = 0.51$). Wolf puppies were estimated to choose the baited location 42.67% of the time (95% CI 35.00 –50.70, $z = -1.78$). Neither dog puppies ($p = 0.61$) nor wolf puppies ($p = 0.07$) performed significantly different from chance. The performance was also not significantly different between the two species ($p = 0.09$). Using a linear contrast test on a binomial logistic regression model revealed that compared with a wolf puppy, a dog puppy's odds of choosing the indicated location were not significantly different than a wolf puppy's (odds ratio = 1.45, 95% CI = 0.84– 2.51, $p = 0.32$) (Figure S1).

We used a Welch's two sample t test to compare the ages of the participants, and found that there was no significant difference in average age between the dog puppies ($M = 10.87$ weeks, $SD = 3.18$) and the wolf pups ($M = 11.38$ weeks, $SD = 1.70$) who participated in the social cues test, ($t(47.31) = -0.78$, $p = 0.44$).

We explored the impact of the individual's performance on the temperament tests on their performance on the gesture comprehension tasks using linear regression models. We found that the overall combined score on the temperament tests was a significant predictor of performance on the arm pointing task and marker task, with a higher temperament score (i.e., more attraction to objects and humans) slightly increasing the likelihood of a correct response (Data S2I and S2J). However, when added to models which included species as a predictor variable, the temperament score was no longer significant, suggesting that this influence was due to the species difference in temperament rather than capturing the impact of individual variance (Data S2I and S2J). Temperament scores were not significant predictor variables for the performance on the two control tasks for gesture comprehension.

The temperament test included both a familiar and unfamiliar object test. The dogs and wolves did not differ significantly in their approach behavior toward the familiar object—only the unfamiliar object (a small plastic bear w/ eyes). The physical marker we used in the gesture test was a familiar object—a nondescript small wooden block (i.e., intentionally chosen to be very uninteresting). It was not a strange, salient, attractive, or alarming object typically used in neophobia tasks (e.g., like the teddy bear with big eyes used in the current study or see Morretti et al.[43]). Our wolf puppies were very accustomed to human artifacts, like a wooden block, around the area where they were raised. In addition, if the wolf or dog puppies were neophobic (fearful) toward the marker they would have avoided approaching the hiding location to which it was adjacent. The wolf puppies approached the marker location at chance levels in their very first trial (15 of 26 puppies approached the marker on trial one) and when considering all 6 test trials (as a group, wolf puppies approached the marker a mean of 57% of trials). Unlike Moretti et al. (2015), we did not observe the wolf puppies running in fear (tail tucked), vocalizing toward or behaving in any fearful manner toward the marker. Finally, our linear regression models analyzed the effect of performance in the temperament tests on the use of each human gesture but found no effect of temperament on the use of the marker gesture. So overall, the behavior the wolf puppies showed toward the marker is not consistent with a neophobic response to a strange or unfamiliar object. Instead, it is the typical response toward a familiar object.

The mixed-effects logistic regression model with random intercepts for each subject showed that dog puppies were estimated to successfully navigate to the side opening without first touching the clear cylinder 74.01% of the time (95% CI 70.71 – 77.62, $z = 5.86$). Wolf puppies were estimated to do so 66.19% of the time (95% CI 60.59 – 71.37, $z = 2.78$). The performance was also not significantly different between the two species ($p = 0.19$) (Figure 3C). A linear regression model also showed that species was not a significant variable in predicting the outcome of this test (Data S2G).

A Welch's two sample t test comparing behavior of dog puppies and wolf pups during an unsolvable task demonstrated that dogs made eye contact with the human experimenter for significantly more time ($M = 4.09$ s, $SD = 4.29$) than did the wolves ($M = 1.47$ s, $SD = 2.18$), $t(45.90) = 2.83$, $p = 0.007$ (Figure 3D). A linear model showed that species is a significant predictor of the number of seconds of eye contact made by the animal (Data S2H).

Genetic Comparison

RAD Sequencing and SNP Variant Discovery

We prepared high molecular weight genomic DNA from eight canids (presumed gray wolves) for restriction-site associated DNA sequencing (RADseq) following a modified protocol.[59] DNA was quantified by Qubit 2.0 fluorometry system and each sample was subsequently adjusted to 5 ng/μL. We digested DNA with the SbfI restriction enzyme with subsequent library preparation using the NEBnext Ultra II DNA Library Prep Kit for paired-end (2x150 nt) sequencing on an Illumina NovaSeq6000 platform at the Princeton University's Lewis Sigler Genomics Institute core facility. Prior to sequencing, we ligated a unique 8-bp barcoded with a biotinylated adaptor to each unique DNA sample to allow for pooled sequencing. Indexed libraries were pooled and randomly sheared to 300-400bp in a Covaris LE220 with a subsequent step to enrich for adaptor ligated fragments using a Dynabeads M-280 streptavidin binding assay. We used Agencourt AMPure XP magnetic beads for all size selection (250-400 bp) and purification steps. We used STACKS v2[49,50] to retain reads with the Sbf1 cut site, rescue reads with at most a 2bp mismatch, retain reads with a quality score ≥ 10, remove remnant sequences, clip low quality nucleotides, and remove PCR duplicates, which were then aligned to the reference dog genome CanFam3.1 (GCF_000002285.3[60]) using stampy v1.0.21.[51] We excluded samples that did not have at least 100,000

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reads to map. We also excluded multiply mapping reads and those with MAPQ < 96. We used Samtools v1.9[52] for SAM to BAM conversion and the depth function to estimate average sequence coverage for each query sorted BAM file. All mapped data were deposited on NCBI's Short Read Archive (BioProject PRJNA699018).

For SNP discovery, we increased the significance threshold in the gstacks module and implementated the marukilow model (flags–vt-alpha and–gt-alpha, p = 0.01) for variant discovery in the STACKS populations module. We included 89 publicly available canid samples previously mapped to the same reference genome assembly as ancestry reference genomes (Table S5). We filtered data to retain sites with a MAF > 3% (flag–maf 0.03) and allowed up to 20% missingness per locus (flag–geno 0.2) for the initial filtering step in PLINK v1.90b3i.[53] We estimated per sample missingness to determine if any individual sample needed to be further excluded with the–missing flag in PLINK. We excluded any sample with > 50% missing data from all downstream analyses. Observed heterozygosity (HO) estimates were obtained using the PLINK flag–hardy. For demographic analyses, we additionally applied a linkage disequilibrium (LD) filter to remove SNPs that were statistically linked using demographic analyses of genetic structure and diversity estimates, a "statistically unlinked" dataset of SNPs was constructed through pruning (with the PLINK argument–indep-pairwise 50 5 0.5).

We additionally estimated the individual inbreeding coefficients derived from the runs of homozygosity (FROH) using the statistically unlinked SNP set with the R v3.6.0 function detectRUNS as:

$$F_{ROH} = \frac{\sum L_{ROH}}{L_{genome}}$$

where LROH is the sum length of all ROHs detected in an individual and Lgenome is the length of the genome that is used.[54] We required a minimum of 10 SNPs in a track of at least 10,000 bases, with a maximum gap of 106 bases between SNPs, and allowed for a maximum of a single opposite or missing genotype in the track. The total length of the genome was 2,326,123,084 bp.

Of the eight canids in our query, two canids (ID 091 and 1601) were excluded from all analyses due to low coverage (< 100,000 reads), and the remaining six canids had an average of 17.9-fold sequence coverage (Table S3). For the 95 canids, we discovered 2,113,073 SNP variants prior to population-level filtering. We retained 152,124 SNPs after filtering for MAF and missingness, with a final set of 108,353 statistically unlinked SNPs for downstream analysis. We found that each of the query canines had observed heterozygosities that were within the range of all reference wild canids analyzed in this study (H_O range, query = 0.092-0.152, wild canids = 0.079-0.155) (Table S1). We estimated inbreeding coefficients from ROH and found that the six query canines had on average higher coefficients than wild canids (FROH range: query = 0.701-0.820, wild canids = 0.010-0.764), but within the range observed for dogs (0.187-0.850), albeit at the high end of the range (Table S3). However, the query canine's high coefficients were due to fewer ROH tracks than the reference canids analyzed (N ROH range: query = 2613-4125, wild canids = 162-4437, dog = 1715-4281).

Population Structure Analysis

We used two analytical methods with the statistically unlinked SNPs to initially assess possible group membership to the reference samples: a principal component analysis (PCA) with the program flashPCA[55] and a maximum-likelihood method to estimate the probability that each of the eight query canids belong to any genomic reference group in ADMIXTURE.[56] We assessed nine genetic clusters (K = 2-10) and used the cross-validation error (–cv) to evaluate likelihoods of each partition with respect to the others, whereby the K with the lowest cv error is expected to represent the best fit partition. However, there is a general caution that the most likely K value excludes inference of biological and demographic information from other partitions.[61] Hence, we included an assessment of each partition.

We additionally estimated the posterior-probability of belonging to a genetic cluster (K) in a Bayesian framework with the program STRUCTURE v2.3.4.[57] Each query canine was assessed independent of each other in relation to the reference groups at K = 9 with the parameter flags MAPDISTANCES, POPFLAG, and USEPOPINFO and the rest at default parameter settings. We used 10,000 BURNIN and 20,000 MCMC reps after burnin.

Inference of Wolf and Dog Ancestry

We inferred the genomic ancestry of the query canids with respect to two possible reference populations, gray wolves (including Great Lakes gray wolves) and dogs. To maintain high density of loci, we analyzed the full SNP set (unfiltered for LD) in the program ELAI.[58] This algorithm infers the most likely ancestry proportion at each locus from a two-layer hidden Markov model, and loci were excluded if it was lacking in any of the populations analyzed. As per recommendations, we used the following parameter settings: -C was set to 2 and -c to 10. Further, we estimated four time points since admixture (-mg 5, 10, 15, and 20 generations ago) given the uncertainty in the precise timing and duration of potential admixture. We also completed inference in triplicate for each parameter setting with 30 EM steps, and averaged over these replicates.

The six query canines align with the wolf (eastern and gray) and domestic dog end of PC1 (8.6% variation) and separate from domestic dog along PC2 (4.5% variation; Figure 4A). Their spatial coordinates are most similar to that of gray wolves from Wyoming and the Great Lakes (Michigan, Minnesota, Wisconsin). The maximum likelihood cluster method supported the finding that the query canids are most similar across their genome to that of gray wolves across all partitions (Figure 4B). The statistically best-fit partition (K = 2; Table S4) identified two groups of canids, coyote and essentially all non-coyote derived groups (dogs, gray wolves), with partial assignments of lineages known to have admixture or incomplete lineage sorting (e.g., eastern wolf, red wolf, Great Lakes gray wolves, several coyote populations;[62–65]). Due to the known complex demographic history of several reference canid groups, we

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assessed the cluster probabilities for each of the partitions. We found that the query canids on average remained with high probability gray wolf membership (Q: K2-7 = 0.999) (Table S1). At K = 8 and K = 9, three canids were still identified as gray wolf partition members (Q = 0.999), whereas the other three individuals were partially assigned to two gray wolf groups (Q, Wyoming = 0.626, Great Lakes = 0.374). Only at K = 10 did a new undefined genetic cluster appear with high average query probably membership (Q = 0.686), significantly reduced Great Lakes gray wolf cluster membership (Q = 0.000), with the remaining membership to Wyoming gray wolves (Q = 0.314).

We assigned the six query canines to nine reference groups using a Bayesian posterior-probability assignment test. We found that all reference individuals were assigned to their population of origin or the population to which we already are aware of complex demographic history (i.e., coyotes[63,64]) (Table S5). All six query canines were assigned to gray wolf, with an average posterior probability of 0.420 and 0.346 to Wyoming and Great Lakes gray wolves, respectively (Table S5). The remaining of the probabilities include Q = 0.06-0.07 to Great Lakes coyotes and eastern wolves, followed by domestic dog (Q = 0.052) and southern coyotes (Q = 0.048).

We inferred genome ancestry in the six query canines with respect to either gray wolf or domestic dog reference groups. All query canines were found to carry high content (> 90%) gray wolf ancestry across the genome (range: 0.944-0.982), with more variance in gray wolf ancestry found on the X chromosome in relation to the autosomes (autosomes range: 0.948-0.985; X range: 0.770-0.952) (Table S2).