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Genetics of behavior in the silver fox

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Abstract The silver fox provides a rich resource for investigating the genetics of behavior, with strains developed by intensely selective breeding that display markedly different behavioral phenotypes. Until recently, however, the tools for conducting molecular genetic investigations in this species were very limited. In this review, the history of development of this resource and the tools to exploit it are described. Although the focus is on the genetics of domestication in the silver fox, there is a broader context. In particular, one expectation of the silver fox research is that it will be synergistic with studies in other species, including humans, to yield a more comprehensive understanding of the molecular mechanisms and evolution of a wider range of social cognitive behaviors.

Introduction

Domestication as a special form of evolution (Clutton-Brock 1992, 1995, 1999; Darwin 1868; Diamond 2002; Price 1999) offers valuable insight into how genomic variation contributes to complex differences in phenotypes, including both morphological (Boyko et al. 2010; VonHoldt et al. 2010) and behavioral traits (Hahn 1990; Hahn and Wright 1998; VonHoldt et al. 2010). Indeed, the behavioral changes associated with the domestication of mammals offer one of the most compelling demonstrations of the influence of genes on behavior. The overall behavior of each such domesticated species differs dramatically from that of its wild counterpart, and because these "tame" behavioral phenotypes "breed true" in all the domesticated mammals, domestication clearly represents an evolutionary process involving the genotypic adaptation of animals to the captive environment (Price 1999; Price and King 1968).

The first domesticated species was the dog (*Canis familiaris*) which diverged from its progenitor, the grey wolf (*Canis lupus*) at least 14,000 years ago (Acland and Ostrander 2003; Crockford 2000; Davis and Valla 1978; Galibert et al. 2011; Leonard et al. 2002; Morey 2010; Nobis 1979; Pang et al. 2009; Sablin and Khlopachev 2002; Savolainen et al. 2002; Tchernov and Valla 1997; Turnbull and Reed 1974; Verginelli et al. 2005; Wayne and Ostrander 1999), and possibly much earlier (Germonpré et al. 2009; Ovodov et al. 2011; Vilà et al. 1997), and had clearly been developed into morphologically (and presumably behaviorally) distinct strains prior to 2000 BCE (Carter et al. 1900) and probably prior to 6000 BCE (Pickeral 2008). Domestication enabled the dog to read human intent in a manner generating mutual trust and permitting playful interactions with humans (Gacsi et al. 2004, 2005, 2009; Hare et al. 2002; Miklósi 2009; Miklósi and Topál 2005; Topal et al. 2005). Comparison of the dog and wolf genomes has identified regions bearing signals of selection and harboring genes potentially involved in such canine domesticated behavior (VonHoldt et al. 2010).

The red fox (*Vulpes vulpes*), and the other fox-like and dog-like carnivores that form the *Canidae* family, diverged

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from a common ancestor some 10–12 million years ago (Bardeleben et al. 2005; Wayne 1993; Wayne et al. 1997). Because comparative cytogenetic, molecular genetic, and phylogenetic analyses have clarified the evolution, divergence, and conserved homologies of these canids (Graphodatsky et al. 2008; Switonski et al. 2009; Yang et al. 1999), genomic tools developed in one such species can be applied to address questions in other members of the family, particularly the fox (Kukekova et al. 2004, 2007, 2009). The genome of the dog currently has the most richly developed set of tools among the *Canidae*, including, most prominently, an assembled genome sequence (Lindblad-Toh et al. 2005) which is, so far, lacking for all other members of this family. The canine genome sequence thus offers an important and valuable resource for investigations in other canid species. For example, a meiotic linkage map of the silver fox, constructed using dog-derived microsatellite markers (Kukekova et al. 2004, 2007) and aligned against the dog genome, identified highly conserved marker order between orthologous regions of dog and fox chromosomes and enabled linkage mapping in fox pedigrees (Kukekova et al. 2011a).

The silver fox is a naturally occurring melanistic variant of the red fox. Its characteristic coat color is an autosomal recessive mendelian trait caused by a 1.2-kb deletion mutation in the agouti gene (ASIP) that removes exon 2 of the coding sequence and, presumably, expression of the agouti protein (Våge et al. 1997). Farm-bred strains of silver fox were first derived from natural fox populations in 1895 on Prince Edward Island, St. Lawrence Bay, Canada, by Charles Dalton, Robert Oulton, and Robert Rayner (Rubtsov 1998). Additional color-phase variants were subsequently identified and adopted by the fur-farming industry (Balcom 1916; Nes et al. 1988; Rubtsov 1998; Våge et al. 1997; see <http://www.furcommission.com/farming/colors.htm>). Mitochondrial DNA analysis of modern American and Russian (Novosibirsk population) farm-bred foxes revealed an Eastern Canadian origin of the most common mitochondrial haplotypes observed in these populations (Statham et al. 2011a, b). Despite captive breeding for over a century, however, these foxes for the most part retained a characteristic fear-aggressive response toward humans and could not be said to have become truly domesticated, until the deliberate effort to recapitulate canine domestication by selectively breeding silver foxes for behavior beginning in the mid-20th century (Belyaev 1969, 1979; Trut 1999, 2001; Trut et al. 2009). This effort resulted in the newest addition to the range of truly domesticated species, and a unique opportunity to examine the molecular determinants of the behavioral changes that occur during domestication.

Tame foxes, unlike their aggressive counterstrain, and dogs, unlike wolves, have evolved social cognitive mechanisms that more closely resemble human social cognitive skills than do those of chimpanzees, enabling them to interact with humans (Hare and Tomasello 2005a, b, c, 2006; Hare et al. 2007). Critically, these tame foxes were not directly selected for such social skills but for reduced fear-based aggression toward humans; the social communicative skills subsequently emerged spontaneously. This supports the emotional reactivity hypothesis, i.e., that evolution of human-like temperament (social tolerance) was the necessary prerequisite for significant social cognitive evolution in humans, dogs, and foxes (Hare et al. 2005).

The combination of opportunities for genetic mapping of behavioral loci in experimental informative pedigrees, selection sweep identification in populations under intense selection, and comparative expression analysis for identification of loci and genes for behavior makes the silver fox a powerful model, intermediate between rodents and primates in biological complexity, for gaining insight into the genetics of social behaviors. Such insights can be particularly valuable in the context of knowledge gained in other species. For example, in a genomic comparison between dogs and wolves, VonHoldt et al. (2010) identified a number of selective sweeps associated with the emergence of dogs as a species. In this purely genomic approach, it was not possible to assign phenotypes to the loci identified. Exploiting the known conserved syntenies between the fox and dog genomes, Kukekova et al (2011a) have shown that the most significant of these regions is strongly homologous to and overlaps a highly significant behavioral locus identified in our fox pedigrees. A synergistic analysis of fox pedigrees segregating behavioral phenotypes and genomic regions bearing signals of positive selection in the domestication of dogs from wolves presents a promising strategic approach to the identification of behavioral loci in canids.

History of the farm fox experiment

Beginning in the mid-20th century, a program designed to recapitulate canine domestication was implemented in the silver fox. Belyaev et al. at the Institute of Cytology and Genetics of the Russian Academy of Sciences, Novosibirsk, Russia (Belyaev 1969, 1979; Trut 1999, 2001; Trut et al. 2009) hypothesized that selection of farm foxes for less fearful and less aggressive behavior would yield a strain of domesticated fox (reviewed in Trut 1999, 2001; Trut et al. 2009). Beginning in 1959, a selective breeding program, based solely on behavioral criteria, was instituted that generated a population of foxes that respond to humans in a friendly or tame manner just like domestic dogs: by 1 month postnatal they become eager to establish human contact, whimpering to attract attention and sniffing and

licking at humans, just as canine puppies do. These tame foxes exhibit highly social behavior with both other members of their own species and humans, in a playful, friendly manner (Trut 1999, 2001). To ensure that tameness results from genetic selection, foxes are not trained and are allowed only brief "time dosage" contacts with humans.

In a parallel experiment, a further strain of foxes was established by breeding for aggressive responses to humans (Trut 1999, 2001). This was undertaken because under farm conditions, even "unselected" foxes are subject to some degree of less deliberate selection for relatively tame behavior. The observation that even unselected farm-raised foxes exhibit some adaptive behavioral changes is supported by observations that free-living foxes with coat colors typical of farm-raised strains, and thus probably descended from escaped farm-bred foxes, exhibit reduced avoidance of humans compared to red foxes (Keeler 1974; Keeler et al. 1968). The tame and aggressive fox strains differ in many distinct and specific behavioral traits: their position and posture within their cage when approached by a human; the noises that they make; the carriage of their ears and tail; and their willingness and desire to be touched as opposed to their eagerness to attack and bite.

The genetic basis of these fox behavioral phenotypes has been clearly and carefully demonstrated (Trut 1980a), with extensive and accurate documentation, including not only scrupulously maintained pedigree data and records for these behavioral phenotypes, but also comprehensive data from biochemical, morphological, and other studies as reported in a large body of literature (see Trut 2001 for review). The heritability of these traits, as opposed to a significant epigenetic or maternal environmental influence, was established in studies in the late 1970s involving cross-upbringing of domesticated pups by nondomesticated mothers and vice versa as well as cross-transplantation of blastocysts (Trut 1980a, b; Trut et al. 2004a, b). To drive the selective breeding program, a scoring system for assessment of tame and aggressive behavioral phenotypes was established at ICG to enable selection of foxes for behavior. Soon after the beginning of the program, however, this required two separate scoring systems: one for the "tame" and one for the "aggressive" population because although the original farm fox population showed a continuous variation in behavior, very quickly the phenotypes in the selected tame and aggressive populations no longer overlapped (Trut 1980a, 1999; Vasileva and Trut 1990).

Furthermore, even though these strictly defined behavioral criteria were the sole basis for selection, other differences emerged. The tame foxes' eyelids and external ear canals opened earlier; their sensitive period for socialization lasted longer than in unselected foxes (60 days versus less than 45); and play activity, restricted in unselected foxes to the juvenile period, persisted into adulthood (Belyaev et al. 1985; Plyusnina et al. 1991; Trut 2001). Significant differences between tame and control foxes emerged for a range of hormones, neurotransmitters, and steroids. In comparison to controls, tame foxes have lower plasma cortisol and ACTH levels, lower density of 5-HT1A serotonin receptors in the hypothalamus, and higher levels of serotonin and tryptophan hydroxylase in the midbrain and hypothalamus (Gulevich et al. 2004; Oskina and Tinnikov 1992; Popova et al. 1997; Trut 2001). Differences in mRNA expression in amygdala, frontal cortex, and hypothalamus were noted between tame and control foxes, particularly in several heme-related genes (Lindberg et al. 2005, 2007). Perhaps most remarkably, the tame foxes display an ability to read human cues, just as domestic dogs do (Hare et al. 2005), and tame foxes developed a novel and distinctive repertoire of vocalizations toward humans that were significantly different from those of aggressive foxes (Gogoleva et al. 2009, 2011).

Resources for mapping fox behavior

During the historic period of development of these behaviorally defined, selectively bred, farm-raised strains of silver fox, the range of genomic tools available for the species was extremely limited. Classical genetic approaches to genome mapping had derived a rudimentary map of the fox genome by 1998, with a well-defined fox karyotype and sparsely populated linkage groups (for review see Rubtsov 1998). In order to begin an attack on the molecular genetics of the behavior in these foxes, however, further resources were essential, including a set of suitable pedigrees for mapping, an adequate set of suitable molecular markers, and a robust method for measuring behavior in resegregating pedigrees.

Accordingly, a program was instituted at ICG to cross-breed foxes from the tame and aggressive strains, producing an F1 population, and then generating informative backcross and intercross pedigrees. Simultaneously, the canine genome sequence and linkage map were exploited to identify a set of microsatellite markers shared between the dog and fox (Kukekova et al. 2004).

Of 700 canine microsatellite markers tested, 400 ($\sim$60%) worked robustly on fox DNA. Thirty-five of the latter markers were sequenced to determine whether the appropriate microsatellite repeats, as expected from canine marker information, were present in the fox PCR products; in all cases, sequencing confirmed the correct result. Thirty of the 400 markers were then tested to evaluate their polymorphism information content (PIC) in the fox genome. PIC was higher than 0.7 for six markers (20%),

between 0.5 and 0.7 for 12 (40%), between 0.5 and 0.3 for 9 (30%), and lower than 0.3 for only three markers (10%). The number of fox alleles varied among the microsatellites, with a mean allele number of 5.1 for the 30 analyzed microsatellites. Of these markers, 92% tested in the tame population and 88% in the aggressive population were in Hardy–Weinberg equilibrium. Population inbreeding coefficients (F_{IS}), calculated from data for 25 polymorphic markers and for the same animals used for estimation of microsatellite PIC value, yielded mean values of 0.038 for 22 foxes from the tame population and 0.030 for ten animals from the aggressive population. Genotyping results on fox DNA samples analyzed by Marshfield Laboratories Mammalian Genotyping Service (MGS) confirmed the above results and showed that ~55% of the canine marker set used by the MGS worked reliably on fox DNA (Kukekova et al. 2004).

To construct a meiotic linkage map of the fox genome, 37 third-generation silver fox pedigrees, bred at ICG, were selected for genotyping and map construction. Thirty-four of these pedigrees were developed by breeding foxes from the tame and aggressive strains and then backcrossing the F1 progeny to the tame strain: one pedigree was produced by crossing parents from tame and aggressive strains, and two pedigrees were selected from the tame strain. Samples from grandparents were available for all but ten pedigrees. Altogether, 286 individual foxes (180 animals in the third generation) in these 37 pedigrees were genotyped for a total of 320 markers either identified from the Marshfield canine microsatellite set, adapted from the canine integrated map, and/or identified directly from the canine genome sequence. As 318 of these markers could be uniquely identified in the 7.6 × genome sequence of the dog, the resulting fox linkage map is thus directly anchored to the dog genome sequence, enabling detailed comparisons to be made between corresponding chromosomal fragments of the two species and indirect comparisons between fox and human chromosomes. The resulting sex-averaged map covers 16 fox autosomes and the X chromosome, with an average intermarker distance of 7.5 cM. The total map length corresponds to 1,480.2 cM. From comparison of sex-averaged meiotic linkage maps of the fox and dog genomes, suppression of recombination in pericentromeric regions of the metacentric fox chromosomes was apparent, relative to the corresponding segments of acrocentric dog chromosomes. Alignment of the fox meiotic map against the 7.6 × canine genome sequence revealed high conservation of marker order between homologous regions of the two species and provided a robust method for predicting the chromosomal location of the fox orthologs of genes identified in the canine or human genome sequences (Kukekova et al. 2007). A second generation of the fox meiotic linkage map was then developed using an extended set of fox third-generation pedigrees, including a total of 632 progeny in informative generations, and adding an additional 65 microsatellite markers adapted from the dog genome (Kukekova et al. 2011a).

To develop a more detailed gene-specific comparative map between the dog and fox genomes, 40 canine-derived, gene-containing, bacterial artificial chromosome (BAC) clones were mapped to the red fox genome by fluorescence in situ hybridization (FISH). Each BAC clone was uniquely assigned by FISH to a single fox chromosome. The FISH-mapped locations in the fox genome of 38 of these genes agreed with their chromosomal positions predicted from comparative cytogenetic data and alignment of the fox meiotic linkage map to the dog genome; only two genes were located to positions inconsistent with such prediction. These results clearly demonstrated the utility of FISH mapping for construction of a whole-genome gene-specific map of the red fox, and reinforced the robust predictability of the chromosomal location of the fox orthologs of genes identified in the canine or human genome sequences (Kukekova et al. 2009).

To increase map resolution, reduce gaps, and place markers with high PIC in regions previously mapped with markers of low informativeness, a third-generation fox meiotic linkage map was generated (Supplementary Fig. 1). In total, 196 third-generation fox pedigrees, including 916 progeny, were used for map construction. An additional 28 microsatellite markers, adapted from the recently published genetic maps of the dog genome (Sargan et al. 2007; Wong et al. 2010), increased the total number of markers on the map to 408. The total length of the fox sex-averaged map was 1,548.5 cM, highly similar to that in previous versions of the fox map (Kukekova et al. 2007, 2011a), and to the length of the fox genetic map estimated by counting MLH1 foci in pachytene cells (Basheva et al. 2010). When the new high-resolution fox map is aligned against the canine genome sequence (Supplementary Fig. 1), it allows a more detailed comparison to be drawn between the two species' genomes. This will enhance both mapping in fox pedigrees and identifying the gene content in identified quantitative trait loci (QTLs) intervals.

Measurement of fox behavioral phenotypes

To apply these new genomics tools to the behavioral genetics of the silver fox, the first issue was how to reliably measure fox behaviors and establish that they did in fact resegregate in experimental pedigrees. As noted above, over the course of the selective breeding program, the behavioral phenotypes in the selected tame and aggressive populations quickly diverged and separate scoring systems for assignment of fox behavioral phenotypes were

developed: one for the tame (Table 1) and another for the aggressive population (Table 2) (reviewed in Kukekova et al. 2006; Trut 1980a, b, 1999, 2001). Although the use of two scoring systems was successful for the purposes of selective breeding, this approach has limited resolution for measuring behavior in experimental pedigrees descended from crosses of the tame and aggressive strains. Individuals in such pedigrees have a wide range of behaviors and often exhibit fragmented or reshuffled elements of the behavioral patterns characteristic of the founder populations.

Thus, a new method had to be devised that could be used to measure fox behavior, in all fox populations, as quantitative phenotypes that differ among the different populations and resegregate in experimental pedigrees. To capture those behaviors that were under selection in the tame and aggressive fox populations, the new system was rooted in the traditional behavioral test used for selective breeding (Trut 1980b, Trut et al. 2004a, b; Vasileva and Trut 1990) (see Tables 1 and 2). Fox responses to humans were evaluated in a standard series of four sequential steps and videotaped (Kukekova et al. 2008, 2011a). Ethological survey of these videotapes identified over 300 discrete behavioral observations (traits) which could be reproducibly scored from video records in a binary fashion, e.g., presence or absence. Three examples, each simply scored yes or no, include "Wagging tail?", "Stays at the front wall of the cage?", and "Ears pinned?" (Kukekova et al. 2008). Evaluation of these traits for informativeness, redundancy, reproducibility, and consistency identified two overlapping sets of traits: (1) a minimal set of 50 traits that reliably distinguished fox populations along a tame–aggressive axis (Kukekova et al. 2008) and (2) a 98-trait set (Supplementary Table 1) that includes all traits from the minimal trait set plus further traits that capture other dimensions of fox behavior (Kukekova et al. 2011a).

In a data set comprising a total of 1,003 foxes (83 tame foxes, 80 aggressive, 93 F1, 293 backcross-to-tame, 202 backcross-to-aggressive, and 252 F2 foxes), fox behavior was evaluated using a standardized testing protocol and videotaped (Kukekova et al. 2011a). Video records were analyzed to deconstruct fox behavior by scoring for the presence or absence of each of 98 specific binary traits (Supplementary Table 1). Principal-components (PC) analysis was then undertaken, using the *prcomp* function in R (Kukekova et al. 2011a), to identify correlations among these binary behavioral variables and define an ordered set of independent (i.e., uncorrelated) underlying factors (i.e., the principal components) which accounted for decreasing amounts of the total variance in observed behavior (Kukekova et al. 2011a). Specific methodological aspects of the PC analysis are described in two reports by Kukekova et al. (2008, 2011a).

In this large data set, the first two principal components, PC1 and PC2, accounted for 33 and 9% of the total behavioral variation, respectively (Kukekova et al. 2011a). PC1 clearly distinguished tame foxes from aggressive foxes; F1 foxes yield intermediate values that extend into the ranges of both the tame and aggressive foxes, while the scores of the backcross generation resegregate (Fig. 1). Mean values of PC1 in the different populations defined a linear gradient of heritable behavior, ranging from aggressive to tame, that clearly corresponds to the relative proportions of aggressive-to-tame ancestry in each population (Fig. 2a).

PC2 did not follow a gradient as PC1 did (Fig. 2b). Review of the discrete behavioral traits that contribute to these two principal components demonstrates that as expected from the statistical basis of PC analysis, PC1 and PC2 each comprise very different aspects of behavior (Fig. 3, Supplementary Table 1). PC1 comprises traits that distinguish overall aggressiveness from tameness. Most of the traits important to PC2 can be interpreted as distinguishing boldness from shyness (whether tame, aggressive, or in between).

These two principal components of behavior were then used as phenotypes to map associated QTLs in informative fox pedigrees.

GWAS and QTL mapping of fox behavior

Interval mapping was undertaken to search for QTLs cosegregating with the first two identified principal

Table 1 Established system for scoring tame behavior

Animal reaction	Scores
Passive-protection response; fox avoids experimenter or bites if stroked or handled, comes if offered food	0.5–1.0
Foxes let themselves be petted and handled, but show no emotionally friendly response to experimenter	1.5–2.0
Foxes show emotionally positive, friendly response to experimenter, wagging tail and whining	2.5–3.0
Foxes are eager to establish human contact, whimpering to attract attention and sniffing and licking experimenters like dogs. They start displaying this kind of behavior before 1 month old	3.5–4.0

This table summarizes the scoring method used for ranking foxes in the tame population and for selecting the tamest foxes for the breeding program. Tame behavior is scored from 0 (representing "neutral" behavior, an absence of both actively aggressive and actively tame responses directed toward the observer) to 4 (representing the most-tame behavior) (from Trut 1980b)

Table 2 Established system for scoring aggressive behavior

Animal reaction	Scores
Fox shows teeth, snarls, growls at first sight of human. When experimenter is near closed cage, fox attacks experimenter and other objects in field of view. Bared teeth and fixed dilated pupils	−4.0
When experimenter is near closed cage, fox shows teeth, snarls, growls, tries to attack both the experimenter and other objects in field of view. Bared teeth and fixed dilated pupils	−3.5
When experimenter is near open cage, fox shows teeth, snarls, growls, attacks experimenter and other objects in the field of view. Bared teeth and fixed dilated pupils	−3.0
When experimenter is near the open cage, fox growls but does not attack	−2.5
When experimenter, near the open cage, moves protected arm towards fox, it growls and tries to bite	−2.0
As experimenter opens cage, fox is calm, but attempts to touch the fox provoke it to show teeth and snarl	−1.5

The score is based on the critical distance between the experimenter and the caged fox when the animal first demonstrates an aggressive reaction to the experimenter's presence. Originally, the scoring system was based on a range from 0 (least aggressive) to −4 (most aggressive). However, after multiple generations of selection, no current individuals exhibit behavior that scores between −1.5 and 0 (from Trut 1980b)

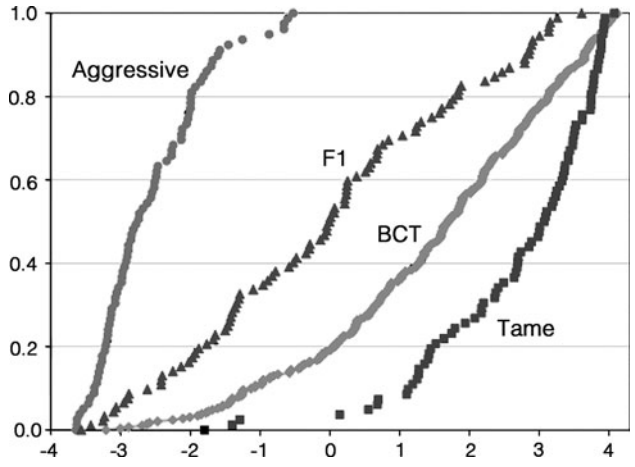

Fig. 1 Cumulative frequency distribution of the first principal component of behavior (PC1) among populations of aggressive, F1 (aggressive × tame), backcross-to-tame (BCT), and tame silver foxes. *Vertical axis* is cumulative frequency value and *horizontal axis* is first principal component of behavior (PC1). The distribution pattern of PC1 values in the different populations clearly differs by population in a manner consistent with that of a heritable trait proportionate to the relative contribution of "Aggressive" versus "Tame" ancestry

components of behavior in fox informative pedigrees from the data set of 1,003 foxes used for PC analysis described above. Fox informative pedigrees included two backcross-to-tame populations (293 progeny in the informative generation), one backcross-to aggressive population (202 progeny), and two intercross (F2) populations (250 progeny) (Kukekova et al. 2011a).

Interval mapping in a combined data set that included all experimental pedigrees identified a locus for PC1 on fox chromosome 12 (VVU12), broadly in a region between 10 and 60 cM (Fig. 4a). In this data set, PC2 did not yield a significant peak on VVU12. However, in backcross-to-tame pedigrees, PC2 also mapped to VVU12 (Fig. 4b).

Although at first glance these results may be surprising, they provide insight into the complex expression of behavioral phenotypes in different populations. They suggest that although independent by definition, the phenotypes measured by PC1 ("tame" vs. "aggressive") and PC2 ("bold" vs. "shy") are not entirely unrelated, as PC2 can enhance the expression of PC1. That is, if an animal is aggressive, passive behavior will reduce the perceived expression of that trait, whereas active behavior will enhance the expression; the same effect applies if an animal is tame. In backcross populations, the distribution of behavior is skewed toward the extreme of the recurrent parent, thus reducing the range of tame vs. aggressive behaviors. Under these circumstances, PC2 acts to increase that range. We would therefore expect that whereas PC1 and PC2 are distinct principal components in a matrix composed of all populations, they could be correlated in particular backcross populations. This is in fact the case for the backcross-to-tame populations ($r = 0.75$–0.8). In contrast, in F2 populations, where the behaviors are more normally distributed, this is not the case ($r = -0.06$). It could well be that the PC2 QTL on VVU12 in the backcross-to-tame population reflects enhanced expression of PC1.

Differences in the amplitudes and peak locations of the PC1 and PC2 QTL signals on VVU12 in different segregating populations were evident when interval mapping was undertaken for individual steps of the behavioral testing protocol or for individual behavioral traits (for details, see Kukekova et al. 2011a). Particularly unexpected were the different VVU12 mapping profiles observed between the two backcross-to-tame populations. The frequencies of individual trait phenotypes remained very similar between these two populations (Kukekova et al. 2011a), but the presence of the tame allele in the backcross-to-tame offspring often resulted in quite different effects between the two populations (for details see Kukekova et al. 2011a). The two backcross-to-tame populations were produced using mostly the same F1 parents but different tame parents, with only 20% of the latter

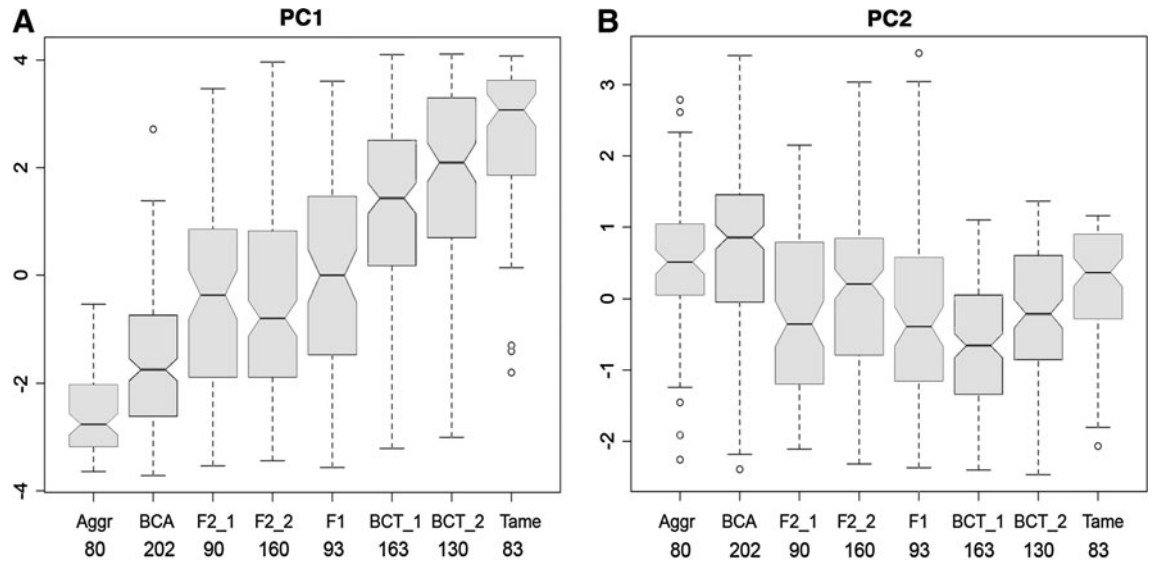

Fig. 2 Population distributions for the first two principal components of silver fox behavior. **a** Distributions for principal component 1 (PC1). **b** Distributions for PC2. Aggr, "aggressive" founder population; BCA, backcross-to-aggressive; F2_1 and F2_2, two F2 populations (F1 × F1); F1, F1 population ("tame" × "aggressive"); BCT_1 and BCT_2, two backcross-to-tame populations; Tame, "tame" founder population. *Horizontal bars within each box* indicate the population median. Confidence intervals for the medians are shown as notches such that two distributions with nonoverlapping notches are significantly different (at $P \leq 0.05$). The bottom and top edges of the boxes indicate the 25 and 75 percentiles. The whiskers indicate the range of data up to 1.5 times the interquartile range. Outliers are shown as *individual circles*. In each of the populations, the PC1 distribution pattern conforms to that expected for a heritable trait, reflecting the proportional contributions from "Tame" and "Aggressive" ancestry. This is clearly not the case for PC2

A (Sorted by C.PC1)

	Trait	Trait description	C.PC1	C.PC2
*	C37	Aggressive sounds	**-86**	8
	C34	Follows the hand (aggr.)	**-84**	14
	C31	Attack alert	**-83**	12
	C32	Pinned ears (aggr.)	**-56**	8
	C36	Triangle ears directed back (aggr.)	**-42**	4
	C33	Trying to bite	**-33**	12
	C30	Attack	**-33**	20
	C55	Leans on back or side walls in zones 5-6	**-5**	-14
	C3	Fox is in zones 3-4-5-6 at the beginning of step C	**-4**	-24
	C4	Spends more than 30 seconds in zones 3-4-5-6	**-3**	-33
**	C7	Observer can first touch fox in zones 5-6	**-2**	-41
	C38	Fox remains only in zones 3-5-6	**-1**	-39
	C50	Tail is up for at least for 3 seconds	**-1**	1
	C35	Narrow ears directed back	**3**	-26
***	C39	Moved forward at least one zone during the step	**3**	29
	C2	Fox is in zones 1-2-3-4 at the beginning of step C	**6**	24
	C6	Observer can first touch fox in zones 3-4	**18**	4
	C204	Tame sounds (combined)	**18**	10
	C19	Comes into zone 2 at the end of step C	**21**	19
	C25	Wagging tail	**25**	13
	C18	Fox holds observer's hand with its mouth	**26**	14
	C17	Fox rolls onto its side, inviting observer to touch its belly	**27**	20
	C29	Comes to and sniffs observer's hand at the end of step C	**32**	14
	C24	Loud breathing	**36**	20
	C13	Fox allows observer to touch the rear part of its back	**49**	-18
	C14	Fox allows observer to touch its back	**73**	-15
	C8	Lies down during a contact for at least 5 seconds	**88**	0
	C16	Fox allows observer to touch its head	**105**	-12
	C12	Tame ears	**107**	7
****	C15	Fox allows observer to touch its nose	**115**	-10

B (Sorted by C.PC2)

Trait	Trait description	C.PC1	C.PC2	
C7	Observer can first touch fox in zones 5-6	-2	**-41**	**
C38	Fox remains only in zones 3-5-6	-1	**-39**	
C4	Spends more than 30 seconds in zones 3-4-5-6	-3	**-33**	
C35	Narrow ears directed back	3	**-26**	
C3	Fox is in zones 3-4-5-6 at the beginning of step C	-4	**-24**	
C13	Fox allows observer to touch the rear part of its back	49	**-18**	
C14	Fox allows observer to touch its back	73	**-15**	
C55	Leans on back or side walls in zones 5-6	-5	**-14**	
C16	Fox allows observer to touch its head	105	**-12**	
C15	Fox allows observer to touch its nose	115	**-10**	****
C8	Lies down during a contact for at least 5 seconds	88	**0**	
C50	Tail is up for at least for 3 seconds	-1	**1**	
C6	Observer can first touch fox in zones 3-4	18	**4**	
C36	Triangle ears directed back (aggr.)	-42	**4**	
C12	Tame ears	107	**7**	
C37	Aggressive sounds	-86	**8**	*
C32	Pinned ears (aggr.)	-56	**8**	
C204	Tame sounds (combined)	18	**10**	
C33	Trying to bite	-33	**12**	
C31	Attack alert	-83	**12**	
C25	Wagging tail	25	**13**	
C18	Fox holds observer's hand with its mouth	26	**14**	
C29	Comes to and sniffs observer's hand at the end of step C	32	**14**	
C34	Follows the hand (aggr.)	-84	**14**	
C19	Comes into zone 2 at the end of step C	21	**19**	
C30	Attack	-33	**20**	
C17	Fox rolls onto its side, inviting observer to touch its belly	27	**20**	
C24	Loud breathing	36	**20**	
C2	Fox is in zones 1-2-3-4 at the beginning of step C	6	**24**	
C39	Moved forward at least one zone during the step	3	**29**	***

Fig. 3 Comparison of the contributions of discrete behavioral traits to the first two principal components of silver fox behavior in step C (C.PC1 and C.PC2) of the standardized behavior testing protocol (step C: "Observer attempts to touch the fox"). For each of the 30 traits recorded in step C, its contribution (i.e., loading, from principal component analysis) to C.PC1 and C.PC2 is listed twice; the same data are presented in **a** and **b** but are sorted in order of contribution to C.PC1 and C.PC2, respectively. The values for each trait loading are calculated as the number of standard errors from zero (negative or positive) as established by bootstrap trials. Note in particular the difference in ranking for the traits marked with asterisks. C.PC1 and C.PC2 represent very different behavioral patterns. C.PC1 comprises traits that contribute to overall aggressiveness (*) versus tameness (****), whereas C.PC2 represents traits that are marked by either passivity (**) or activity (***) and may be interpretable as distinguishing boldness from shyness (whether tame, aggressive, or in between)

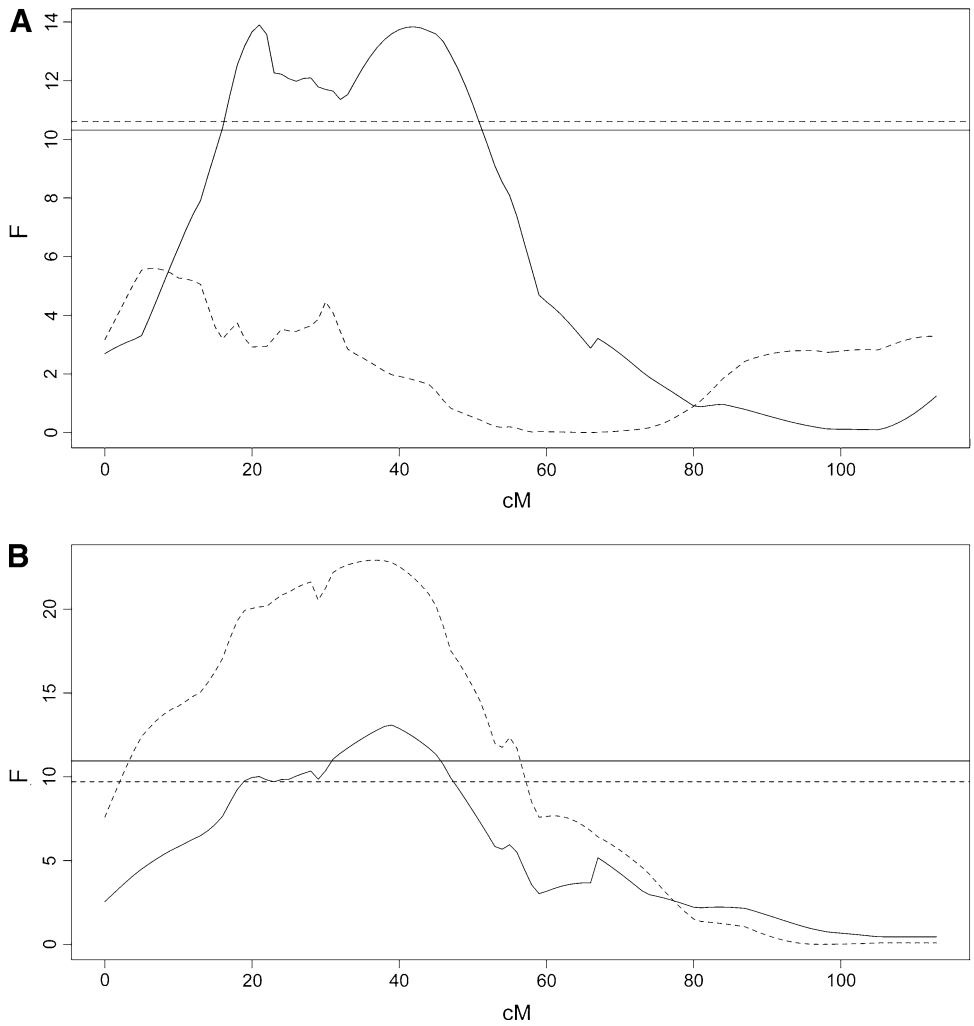

Fig. 4 Interval mapping of the first two principal components of silver fox behavior (PC1 and PC2) to fox chromosome 12 (VVU12). Interval mapping using GridQTL software was undertaken on **a** a combined data set, including all experimental silver fox populations, and **b** backcross-to-tame populations (BCT_1 and BCT_2) only (i.e., excluding backcross-to-aggressive and F2). *Solid lines* = PC1; *dashed lines* = PC2. The F stat (*y*-axis) is graphed as a function of cM distance across VVU12 (data and map distances are from Kukekova et al. 2011a). *Horizontal lines* (*Solid* = PC1, *dashed* = PC2) are the thresholds for genome-wide significance at $P < 0.01$. Interval mapping across all populations (**a**) yields support for PC1-associated loci on VVU12, located broadly between 10 and 60 cM, that exceeds the threshold for genome-wide significance; however, support for PC2 does not achieve significance. Interval mapping restricted to backcross-to-tame populations (**b**) yields support for PC2-associated loci on VVU12, located broadly between 10 and 60 cM, that exceeds the threshold for significance; however, support for PC1 does not achieve significance

being common to both populations. This finding suggests that the loci on VVU12 may be expressed differently in different genomic contexts, depending perhaps on alleles elsewhere in the genome. This would be consistent with the results from rats (Albert et al. 2009) that demonstrated the existence of a five-locus epistatic network influencing tameness.

Intriguingly, a part of this identified QTL interval on VVU12 is homologous to canine chromosome 5 (Fig. 5) and includes a region corresponding to a genomic location recently implicated in the domestication of dogs from wolves (VonHoldt et al. 2010). On the fox meiotic map, this conserved syntenic region lies between markers CM5.41 and CM5.60, or between 27.2 and 28.4 cM, respectively. Among individual behavioral traits that map precisely to this interval are B3 (touches observer's hand) and C17 (allows observer to touch belly), both traits of trustfulness that will be immediately familiar to persons familiar with dogs and other domesticated species.

A fox SNP map and transcriptome sequence

Although genetic mapping with canine-derived microsatellite markers allowed identification of several behavioral loci in the fox genome, this type of genetic analysis has

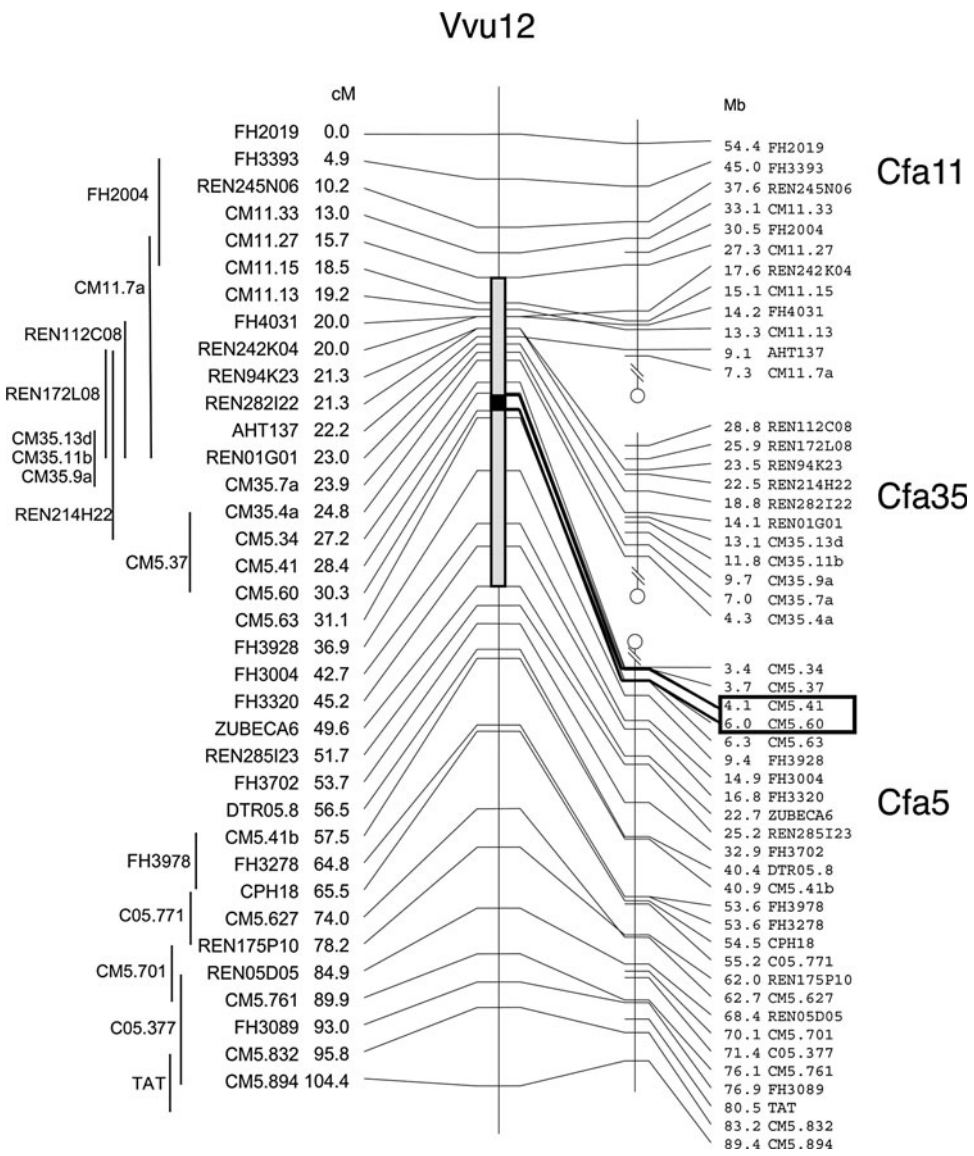

Fig. 5 **a** Locus for the first principal component of behavior (PC1) on fox chromosome 12 (VVU12). The meiotic linkage map of VVU12 (*left side*) is aligned to the genome sequence of the homologous canine chromosomes (CFA11, CFA35, and CFA5) on the right side (data and map distances are from Kukekova et al. 2011a). In the middle, the *gray interval* indicates the broad support interval for PC1 in the silver fox, and the *black interval* indicates a region homologous to a locus on CFA5 identified with domestication of the wolf (VonHoldt et al. 2010)

inherent limitations in its mapping power. Single nucleotide polymorphism (SNP) markers would be much more powerful for fine mapping these behavioral loci and for identification of haplotypes cosegregating with QTLs that were under selection in the founder strains. Unfortunately, the majority of dog SNPs are not informative in foxes, and fox-specific SNPs need to be identified to move this study forward.

To this end, deep transcriptome sequencing of prefrontal brain samples from one tame and one aggressive fox was undertaken (Kukekova et al. 2011b). Because prefrontal cortex is a brain area relatively easy to identify, samples from different individuals can be readily collected in a relatively consistent manner. Furthermore, the role of this brain region in aspects of behavior is well established (Clark et al. 2010) and thus particularly relevant to the study.

cDNA from mRNA from prefrontal cortex of a tame and an aggressive fox was sequenced using the Roche 454 FLX Titanium platform (>2.5 million reads yielding 0.9 Gb of tame fox sequence and >3.3 million reads yielding 1.2 Gb of aggressive fox sequence). Over 80% of the fox reads were assembled into contigs. Mapping fox reads against the fox transcriptome assembly and the dog genome identified over 30,000 high-confidence fox-specific SNPs. Fox transcripts for approximately 14,000 genes were identified using SwissProt and the dog RefSeq databases. Comparison of gene expression profiles between a tame and an aggressive individual offers insights into functional differences in the prefrontal brain cortex. An at least twofold expression difference between the two samples ($P < 0.05$) was observed for 335 genes, fewer than 3% of the total number of genes identified in the fox transcriptome. Transcriptome sequencing significantly expanded genomic

resources available for the fox, a species without a sequenced genome. In a very cost-efficient manner this yielded a large number of fox-specific SNP markers for genetic studies and provided significant insights into the gene expression profile of the fox prefrontal cortex, expression differences between the two fox samples, and a catalog of potentially important gene-specific sequence variants (Kukekova et al. 2011b). All the sequence reads have been deposited in the GenBank Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) under accession number SRA029285.1 in two files: one for sequences from the tame fox and one for sequences from the aggressive fox.

Conclusions

The strength of the silver fox as a model system for investigating the genetics of domestication lies primarily in the richness of the resource, and the increasing availability of tools to mine this resource. Mapping the fox genome both depended upon and further enabled exploitation of the assembled canine genome sequence. Behavioral analysis has provided discrete quantitative principal components of fox behaviors to be mapped. QTL mapping and association testing has already identified regions of the fox genome on which to focus. Transcriptome sequencing has provided both a first-generation SNP map of the fox genome and a list of genes with either sequence differences, expression differences, or both between one tame and one aggressive fox's prefrontal cortex. Progress has been facilitated by exploiting dog genomic research, a synergism that continues to be advantageous.

In attempting to understand behavior, an important guiding principle, indeed a mantra worth adopting, is that "behavior is complex." Furthermore, even as certain aspects of behavior yield to various analyses, there still remains unresolved complexity. When Belyaev began the farm fox experiment, the goal was straightforward. Farm foxes naturally responded to humans in a manner that was considered undesirable and was broadly categorized as a combination of fearful, aggressive, or avoidance responses. In contrast, the desired behavior was simply the antithesis of these reactions, i.e., a fox that was neither fearful, aggressive, nor shy but responded to humans in a manner like that of a friendly domesticated dog. The rapid success of this experiment was truly astounding. His "tame" and "aggressive" strains were developed in the blink of an eye on an evolutionary time scale, and breed true to this day. That certainly attests to the genetic determination of the selected behaviors, but also inherently conflates aspects of behavior that are generally regarded as separate or intrinsically different. That is, fearfulness, aggressiveness, and shyness are in fact very different behaviors and do not necessarily arise from a common mechanism. Similarly, the various aspects of behavior exhibited by "tame" foxes, whether regarded as sociability, confidence, or friendliness, are also intrinsically different and do not necessarily arise from a common mechanism. In the studies reviewed herein, principal component 1 (PC1) clearly captures the essence of the behavioral differences between "tame" and "aggressive" foxes and accurately measures the phenotype under selection. It remains to be seen how much biological complexity underlies this phenotype. PC2 is not as easy to categorize: it is clearly very different from PC1. To some extent it corresponds to behavioral factors that may be interpreted as representing a "boldness–shyness axis," but in some respects this interpretation does not capture the essence of PC2. Unraveling these complexities will doubtless be challenging but rewarding.

Although domestication has for many years been of great interest to scientists, notably including Darwin, until recently the basic question of what genetic mechanisms are involved has been largely unaddressable. With the advances in modern molecular genetic technologies, however, it is now feasible to pursue research on diverse organisms to find the links between genes, brain function, and a wide range of social behaviors (Robinson et al. 2005, 2008).

The concept that humans are a self-domesticated species, i.e., that processes akin to the domestication of animals may, to some degree, have been involved in shaping human nature, has been long considered (Brüne 2007; Eickstedt 1934; Hare and Tomasello 2005a, b, c, 2006; Kaminski 2009; Morioka 2003; Omoto 2004; Wrangham 2003) but difficult to test. More recently, however, comparison of social cognitive behaviors among chimpanzees, bonobos, humans, dogs, wolves, and tame and aggressive silver foxes has drawn some intriguing parallels (Hare and Tomasello 2005a, b, c, 2006).

Moreover, some conditions in humans are characterized by unusual social cognitive behaviors that may have parallels in canids. For example, Williams-Beuren syndrome is a complex human phenotype that includes hypersociable traits such as exceptional gregariousness (Doyle et al. 2004; Järvinen-Pasley et al. 2008). VonHoldt et al. (2010) identified a polymorphic difference between dogs and wolves near the canine homolog of WBSCR17, one of the genes located on human chromosome 7 in the interval responsible for Williams–Beuren syndrome (Merla et al. 2002), that demonstrated a selection signal in dogs, perhaps implicating this gene in canine domestication and temperament. At the other end of the spectrum, persons with autism spectrum disorders often exhibit impaired social reciprocity, a reduced ability to communicate with and to recognize the intentions of other people, traits sometimes referred to as impairments of the "Theory of Mind"

mechanism (Baron-Cohen et al. 1985; Leslie 2000). If studies of domestication in canids and other mammals can yield insight into the genetic mechanisms of such conditions, then that would be a very worthwhile outcome.

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