File S1. Supplementary Materials and Methods

The C57BL/6 (B6) mouse strain used for breeding was obtained from Jackson Laboratory (Bar Harbor, Maine) and the B6 mouse strain used for behavioral testing was obtained from Velaz s.r.o. (Prague, Czech Republic, maintained in the breeding facility of the IAPG ASCR). Mice were kept in groups of littermates of the same sex with up to 5 individuals in a cage under standard laboratory conditions and water available *ad libitum*, under a 12:12 photoperiod light on 5 AM to 5 PM. Mice used as breeders were at least 45 days old and mice subjected to behavioral testing were at least 90 days old.

Producing and Maintaining the a27/bg27 *knockout line*

The targeting vector was built using a vector backbone carrying neomycin resistance for positive selection and thymidine kinase for negative selection. The *MC1NeopA* cassette was flanked by *Frt* sites for removal by Flp recombinase. The targeting vector consisted of two arms of homology adjacent to the deletion site. The left arm of homology was 4.2 kb in size and the right arm of homology was 2.1 kb. They were cloned on each side of the *MC1NeopA* cassette. The accuracy of the entire targeting vector was confirmed by DNA sequencing. The linearized targeting vector was electroporated into 129/S6 mouse embryonic stem cells (ESC). The neomycin-resistant clones were screened by PCR using primers outside the homologous arms coupled with neomycin primers in order to select for the clones that had incorporated the targeting DNA by homologous recombination. The positive ESC clones were confirmed by sequencing as well. Three targeted ESC clones were injected into B6 blastocysts for generating chimeric mice. Germline transmission of the *a27/bg27* allele was established by breeding male chimeric mice to B6 females, producing hybrid knockout mice heterozygous (+/-) for the *a27/bg27* gene module.

To create a line of B6 x *a27/bg27* knockout backcross breeders, we bred a B6 mouse with a hybrid knockout mouse heterozygous (+/-) for the *a27/bg27* gene module. Heterozygous progeny from this were mated to B6 parents for a total of five backcross generations to create a strain of mice essentially having a B6 genetic background and lacking the *a27* and *bg27* genes. The genotype of each pup was determined using a duplex PCR reaction to amplify genomic DNA extracted from ear tissues with two primer sets, *a27* and *neo*, amplifying 1019 and 493 bp fragments, respectively. The gene

products were visualized on a 1% agarose gel. Each PCR run also included a positive B6 control, a control for each possible genotype (+/+, +/-, -/-) and a negative water control. Finally, we intercrossed two heterozygous (+/-) mice from the fifth generation backcross to create an intercross breeding line to produce progeny with all three genotypes with identical B6 backgrounds. The same methods were used to determine the genotypes of progeny from the fifth generation intercross litters. Submandibular and lacrimal glands from these offspring were analyzed for *Abp* transcripts.

Gland harvesting and tissue slide preparation for histological analyses

Half of each submandibular gland was prepared for RNA extraction and the other half was placed in 10% neutral buffered formalin and allowed to fix for 24 hours for histological analysis. The lacrimal, sublingual, parotid, harderian, liver, kidney, and ovaries (female only) were also harvested and fixed for histological staining. The fixed samples were dehydrated using a series of increasing grades of alcohol and infiltrated with paraffin using xylene. The samples were then embedded into wax filled cassettes, and sectioned using a microtome. Slides were deparaffinized with xylene, subjected to decreasing grades of alcohol and allowed to rehydrate overnight in phosphate buffered saline (PBS). Slides used for histological analysis were subsequently stained with hematoxylin and eosin (H&E).

Transcript analyses

We verified the integrity of the cDNA libraries using conventional PCR to amplify a control gene (*GAPDH*) and the three salivary *Abp* transcripts (*a27, bg26* and *bg27).* The PCR products were visualized using a 2% agarose gel electrophoresis. We used primers previously verified by sequencing and optimized to amplify each *Abp* paralog (KARN *et al.* 2014). A survey using all of the *Abp* paralogs was performed with both lacrimal and salivary cDNA libraries from the three intercross genotypes as templates to determine whether any changes in gene expression had occurred in any of the genotypes. We used submandibular and lacrimal gland cDNA libraries from B6 mice as a reference. PCR amplification was performed as previously described (LAUKAITIS *et al.* 2005). Any unexpected bands were identified using Sanger sequencing by the University of Arizona Genomics Core (UAGC).

Quantitative PCR analyses

The primer sets used were the same as conventional PCR, and the primer-to-template ratio was previously optimized, as described in (KARN *et al.* 2014). Only primers that accurately amplified expressed *Abp* genes, as validated by DNA sequencing, were used in qPCR analyses. We used the relative expression analysis method with previously calculated standard curves (KARN *et al.* 2014) to search for variation in *Abp* expression between genotypes.

Previous studies have cited the variability that can arise from using reference genes despite the assumption that they are expressed at a constant level (KOZERA AND RAPACZ 2013). When we used *GAPDH* in qPCR as a reference gene to normalize samples, it increased variability and fluctuation in expression levels of the *Abp* paralogs. Other studies have also shown inconsistent expression levels of *GAPDH* when comparing both inter-tissue and inter-sample values in human tissues (BARBER *et al.* 2005). We tested the concentration of each RNA extraction prior to cDNA synthesis, and standardized the input RNA to ensure equal amounts of template for each library and, for these reasons, we decided to exclude normalization to *GAPDH* in our calculations.

Relative standard curve method

We produced a standard curve for each primer using male C57BL/6 lacrimal library as a template, because it expresses more *Abp* paralogs than do the salivary glands. Male salivary C57BL/6 cDNA template was used to produce standard curves only for three *Abp* paralog primers (*a27*, *bg26* and *bg27)*. To create a standard curve, we made a serial dilution spanning five orders of magnitude for each cDNA template. The cDNA template concentrations ranged from 10 ng to 0.001 ng including a no-template control for each primer set. The standard curves were plotted on Microsoft Excel as graphs of the threshold cycle (Ct) values vs. log ng total RNA. The Ct value of each sample was assigned as the cycle number that crossed a threshold line using the ABI Prism 7000 Sequence Detector Software. This was arbitrarily set in the linear portion of the amplification plot because the linear section correlates to the region of exponential amplification of the template. We obtained the line of best fit using the trendline function on Excel. We used the slope of each line to calculate the efficiency (E) of the primer using the following equation:

$$E = (10^{-1/slope} - 1) \times 100$$

The efficiency, which indicates the rate at which a PCR amplicon is produced, and an efficiency of 100% indicates that a PCR amplicon is doubling for every cycle. This is also a measure of the overall performance of the primers and PCR assay. The correlation coefficient (R^2 value), which measures the linear relationship between two variables, was obtained from the line of best fit using the built-in graphing function in Excel.

To analyze the qPCR results, the threshold for each reaction was set within the linear portion of the curve using the ABI Prism 7000 Sequence Detection System software. As previously stated, the linear portion indicates an exponential amplification of the template. After setting the threshold, any samples with a Ct value greater than 35 were designated as non-detectable. We calculated the relative quantity of RNA using the Ct value from each sample run and plotting the value on the standard curve specific for each primer. First, we input the equation for the standard curve in an online graphing calculator, Desmos (desmos.com). The Ct value (an x-value) generated from each sample was found on the standard curve graph to determine the log ng RNA value (y-value) that we converted into ng of total RNA using the following formula, provided by the ABI quantitative PCR guide:

$$\text{ng RNA} = 10^{-\log \text{ng RNA}}$$

After obtaining the ng of RNA per sample using the above formula, we normalized each primer sample to the reference gene, *GAPDH*, and this ratio served as a test sample. The ratio of the *Abp* primer to *GAPDH* primer using a C57BL6 submandibular or lacrimal gland cDNA library template was treated as a calibrator sample. We calculated the fold difference of each paralog amplified in the each of the various cDNA libraries by taking the ratio of the test sample to the calibrator sample. Using the graphing function of Excel, we plotted the fold differences for the libraries of each genotype and gland on a bar chart to visualize the changes of paralog expression across the submandibular and lacrimal glands.

Immunohistochemical analyses

Antigen retrieval was performed by placing previously prepared sections in a container of 0.01 M, pH 6.0 sodium citrate submerged in a 95 C^{o} hot water bath for 30 minutes. Non-specific peroxidase

activity was quenched by subjecting slides to four 5-minute baths in PBS with a 10 minute bath in 0.3% H_20_2-PBS in between. They were then subjected to alternating baths in PBS and applications of 1) a blocking solution comprised of blocking serum diluted 3:200 in PBS plus 0.5 % polysorbate-20 (PBS-T) for one hour; 2) anti-ABP antibody diluted 1:500 in blocking solution overnight at 4 Cº; 3) a biotinylated secondary goat anti-rabbit antibody (Santa Cruz BioTechnology) diluted 1:200 in blocking solution for 30 minutes; 4) avidin and biotinylated HRP diluted in PBS in a 1:1:50 ratio for 30 minutes.

Salivary protein analyses

Nonreducing SDS electrophoresis was performed according to a modification of (DLOUHY *et al.* 1986) using the Mini-PROTEAN II (Bio-Rad) system with a 10% running gel layer and 5% stacking gel layer. Saliva samples were mixed 1:1 with a 2x loading dye (Amresco) and boiled at 90 Cº for 5 minutes. The samples were then loaded in individual wells in the gel with PageRuler pre-stained protein standards (Fermentas). Electroblotting (western blotting) was performed with Mini Trans-Blot electrophoretic transfer kit onto PVDF membrane (Millipore). The resulting membrane was rehydrated with PBS-T for 30 minutes before undergoing consecutive baths in 1) 1:1000 rabbit anti-ABP antibody in PBS-T for 30 minutes; 2) PBS-T for three 10-minute intervals; 3) 1:2000 goat anti-rabbit HRP-conjugate secondary antibody in PBS-T for 30 minutes; 4) PBS-T for three 10-minute intervals prior to exposure with a DAB chromagen and hydrogen peroxide included in the ImmunoCruz ABC staining system.

Sexual preference tests

The experimental apparatus consisted of a habituating box (35 x 25 x 13 cm) connected to the stem of a Y-maze (diameter: 5 cm; stem length: 35 cm; side arm's length: 23 cm) and an electric pump ensuring one-way air circulation in the apparatus [**Fig. S2**; (BÍMOVÁ *et al.* 2005; BÍMOVÁ *et al.* 2009)]. Each tested individual was allowed to choose between saliva from animals of the opposite sex.of the +/+ and -/- genotype. Salivary signals were collected using the pilocarpine stimulated salivation method (KARN *et al.* 2014) and stored at -20°C until they were shipped to BVB on dry ice.

Prior to each experiment, the tested animal was placed in a habituation box for a 5-10 minute habituation period. We spotted 10 µl of defrosted saliva in the middle of a 1.5 x 20 cm sterile strip of

filter paper and positioned it at the bottom of the side arms of the Y-maze immediately before the animal was released from the habituation chamber. To eliminate a sidedness bias, the right/left position of +/+ and -/- signal cues (salivas) in the arms were randomized between experiments. After the habituation period, the door leading to the Y-maze was opened and the animal was free to explore the Y-maze. Its behavior was recorded on video for five minutes after it crossed the stem of the Y-maze and displayed the first choice of arm. Observer software [Noldus Technologies, (NOLDUS *et al.* 2000)] was used to analyze each individual sexual preference in the Y-maze. Tests lasting longer than 20 minutes were excluded from analysis.

We assessed three parameters: 1) a first choice of the Y-maze arm the animal entered after it first traversed the Y-maze stem, 2) the first choice of the signal target the animal sniffed for the first time in the experiment (LAUKAITIS *et al.* 1997; TALLEY *et al.* 2001), and 3) the coefficient of preference of the signal (R_{Sn}) calculated as a difference of total time the animal sniffed each signal target (SMADJA AND GANEM 2002; BÍMOVÁ *et al.* 2005; VOŠLAJEROVÁ BÍMOVÁ *et al.* 2011).

Coefficient of preference of the signal (R_{Sn}) was calculated as follows:

R_{Sn} = (Time spent sniffing +/+ signal - Time spent sniffing -/- signal)/ (Time spent sniffing both signals)

The first choices of signal were analyzed using a χ^2 test. The normality of the distributions of R_{Sn} values were verified by the Shapiro-Wilk and Kolmogorov-Smirnov tests. Subsequently a parametric one-way t-test (H_0: $\mu=0$) was used to test the difference of the variable R_{Sn} from zero separately for both sexes. All tests were two-tailed, the cutoff level of significance was 0.05, and tests were performed using the Statistica software package (STATSOFT 2001).

Proteomic analyses

Data-dependent scanning was performed by the Xcalibur v 2.1.0 software (ANDON *et al.* 2002) using a survey mass scan at 60,000 resolution in the Orbitrap analyzer scanning m/z 400–1600, followed by collision-induced dissociation tandem mass spectrometry (MS/MS) of the most intense ions in the linear ion trap analyzer. Precursor ions were selected as described in (KARN *et al.* 2014). Dynamic exclusion was used to minimize masking of low-abundance peptides by the more abundant ones. MS/MS

spectra were searched against the Uniprot *Mus musculus* database downloaded July 20, 2016 (http://www.uniprot.org/taxonomy/10090), appended with ABP sequences provided by RCK, using Thermo Proteome Discoverer 1.3.

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