Supplementary Information for:

# Sex separation induces differences in the olfactory sensory receptor repertoires of male and female mice

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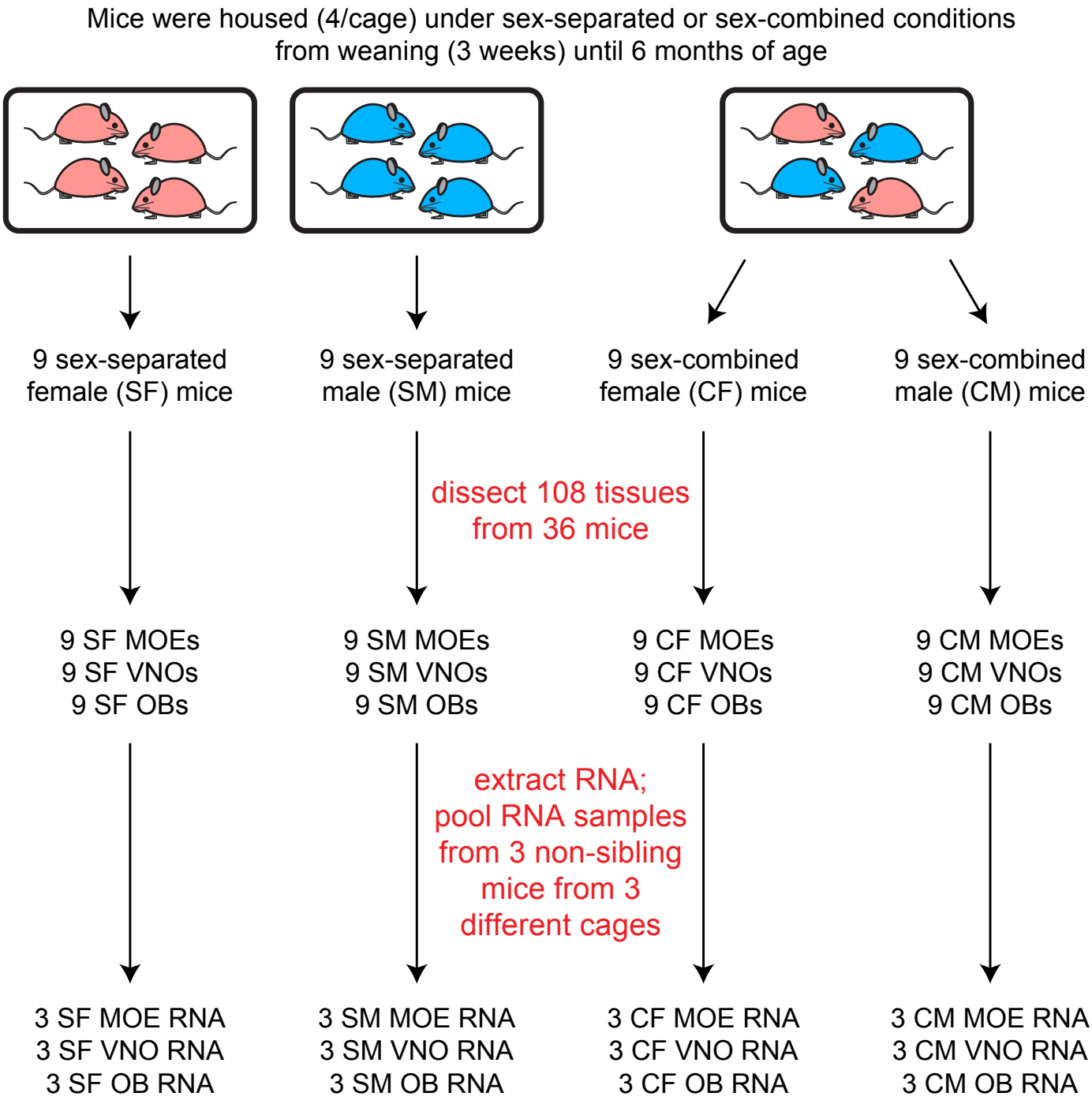
## Supplementary Figures



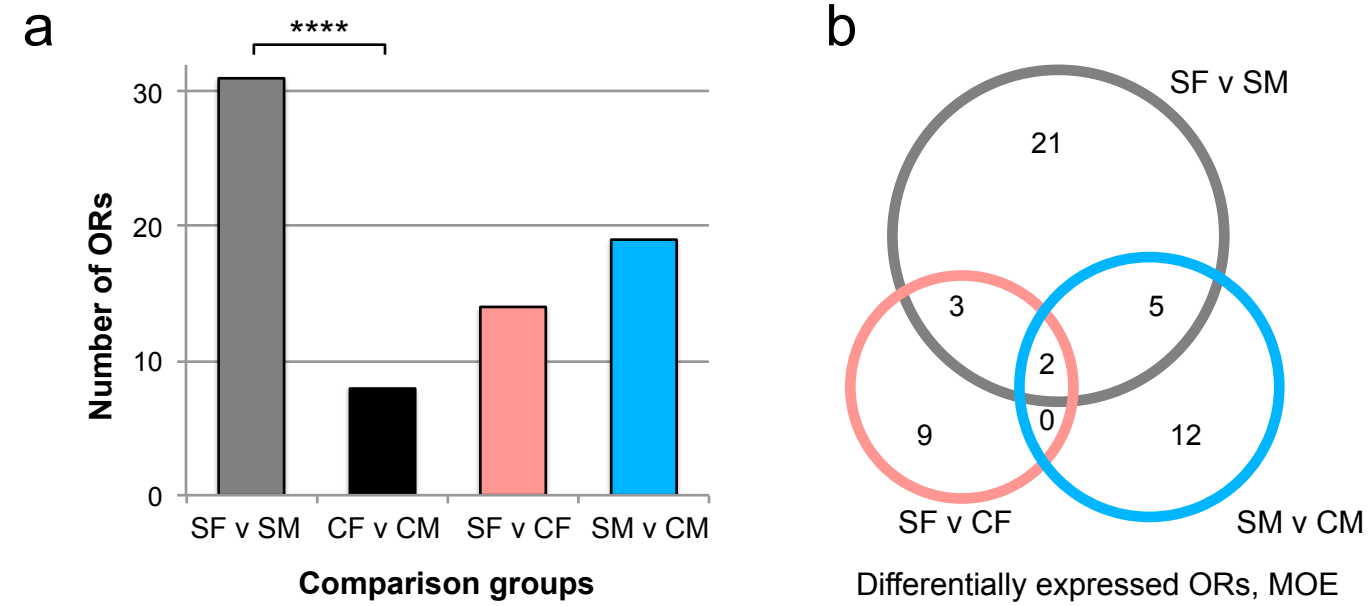
## Supplementary Table

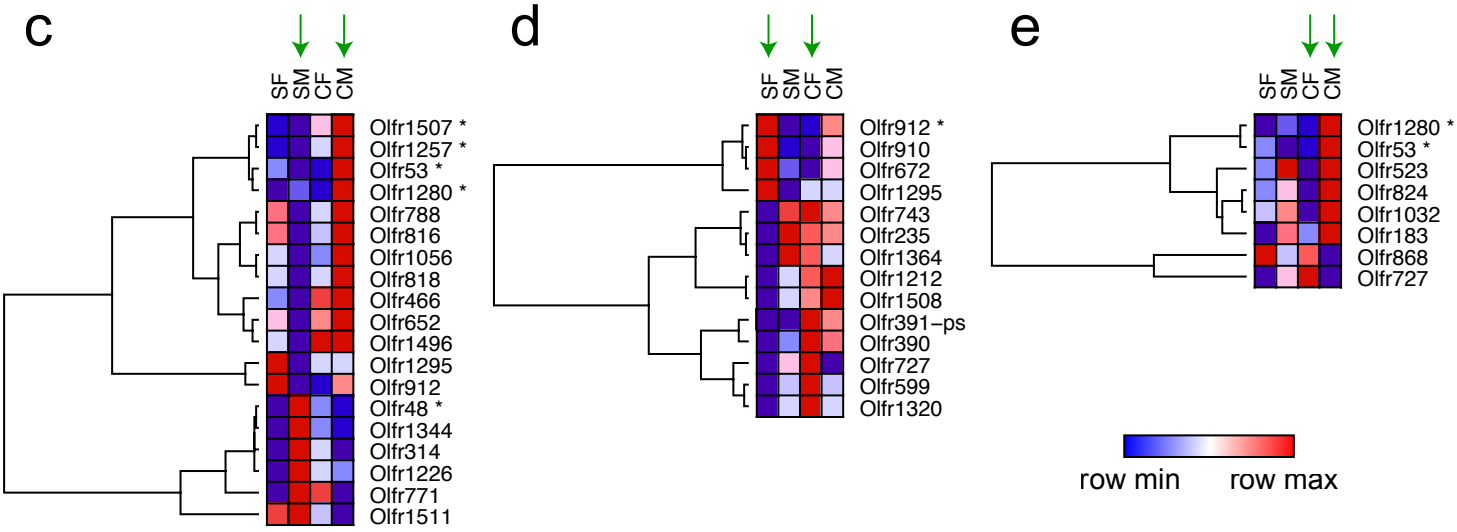
Supplementary Figure 1



**Supplementary Figure 1.** Experimental design and samples used. From weaning (P21) until 6 months of age, mice experienced either a sex-separated environment, in which they were housed either 4 females/cage (SF mice; *left*) or 4 males/cage (SM mice; *middle*), or a sex-combined environment (CF and CM mice; *right*), in which they were housed 2 females + 2 males/cage. MOE, VNO, and OB tissues were dissected from each of 9 mice per sex/condition combination, resulting in a total of 108 tissue samples. RNA was extracted from each tissue sample and pooled in groups of 3, resulting in 36 RNA samples (3 biological replicates per sex/condition/tissue combination), and used to generate RNA-seq libraries.

Supplementary Figure 2



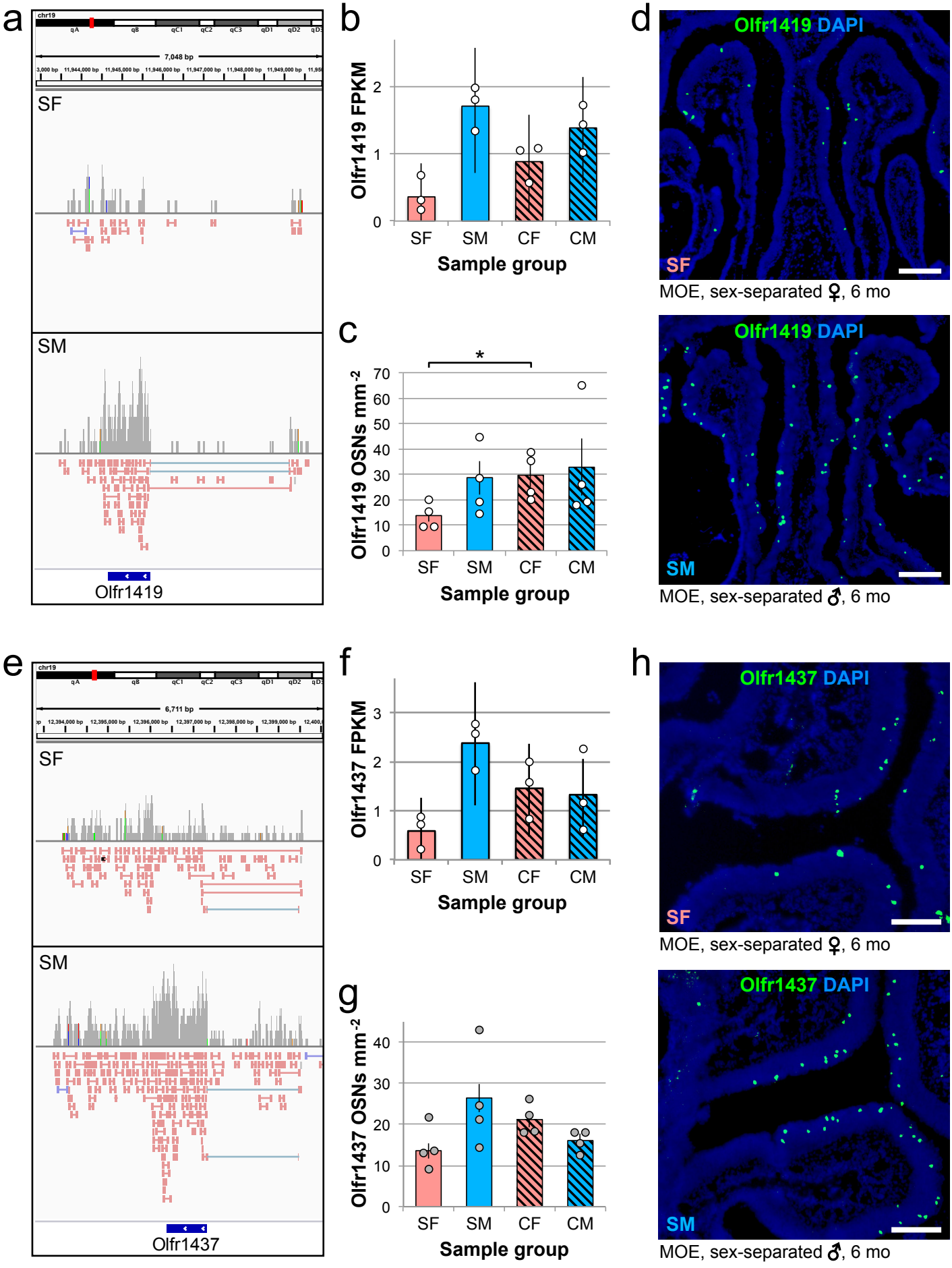




f

| OR | Frequency SF SM$^{-1}$ | P-value SF v SM 4 mice | (20 sections) | Frequency CF CM$^{-1}$ | P-value CF v CM 4 mice | (20 sections) |
|---|---|---|---|---|---|---|
| Olfr141 | 0.93 | 0.38 | (0.73) | | | |
| Olfr912 | 2.09 | 0.050 | (0.0016) | 0.84 | 0.61 | (0.030) |
| Olfr1295 | 1.08 | 0.70 | (0.61) | | | |
| Olfr976 | 0.86 | 0.12 | (0.48) | | | |
| Olfr1419 | 0.48 | 0.11 | (0.0045) | 0.90 | 0.83 | (0.64) |
| Olfr235 | 0.52 | 0.00095 | (0.0000037) | 1.27 | 0.085 | (0.080) |
| Olfr48 | 0.75 | 0.33 | (0.21) | | | |
| Olfr1437 | 0.51 | 0.140 | (0.0033) | 1.32 | 0.069 | (0.052) |

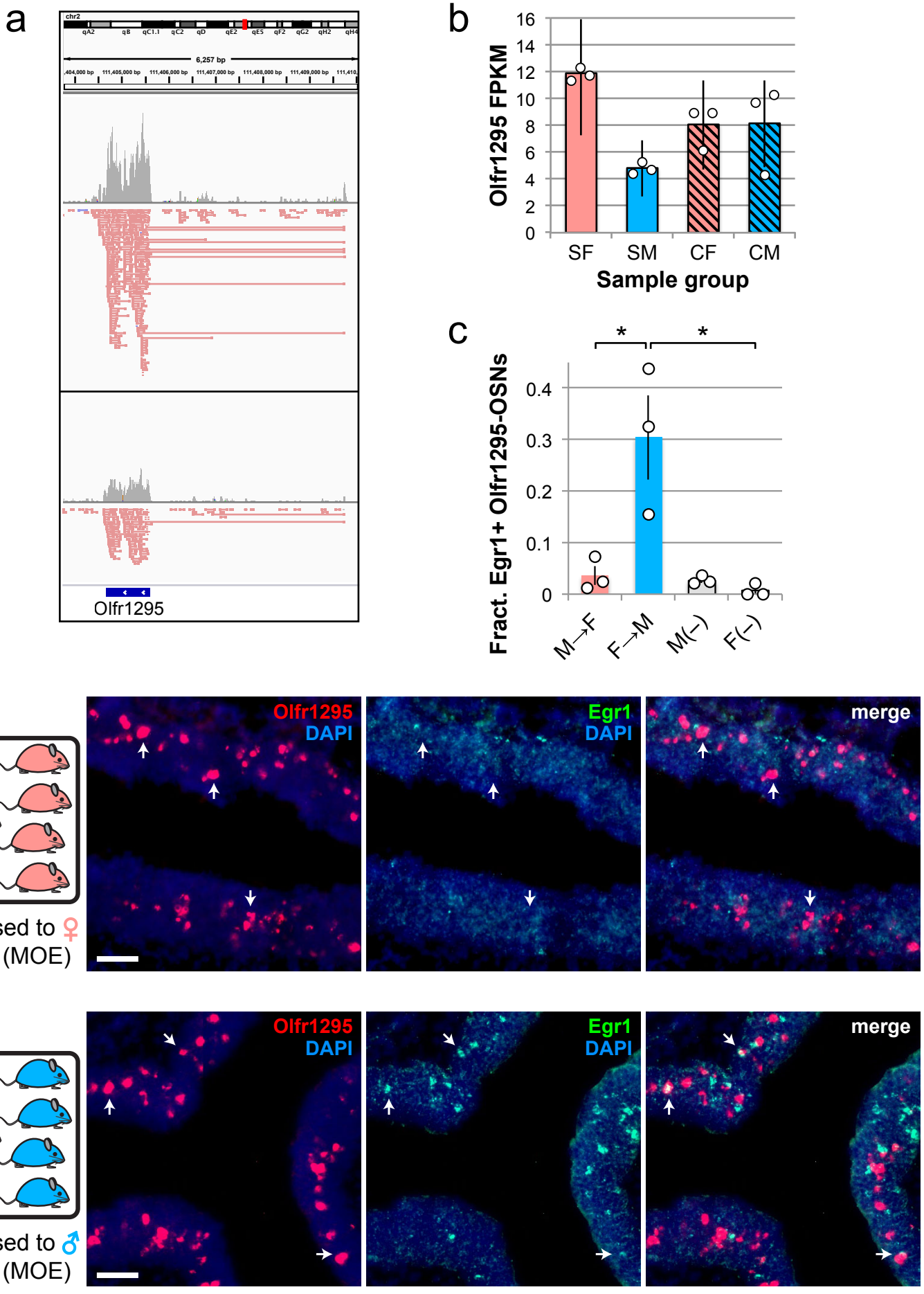
**Supplementary Figure 2.** Analysis of OR genes that are differentially expressed in the MOE between different experimental groups. (**a**) Number of OR genes that were identified *via* RNA-seq as differentially expressed (unadjusted $p < 0.01$) in the MOE between the indicated experimental groups. (**b**) Venn diagrams of OR genes identified *via* RNA-seq as differentially expressed (unadjusted $p < 0.01$) between the indicated experimental groups. (**c**–**e**) Hierarchical clustering of OR genes identified *via* RNA-seq as differentially expressed between SM and CM mice (*green arrows*; c), between SF and CF mice (*green arrows*; d), and between CF and CM mice (*green arrows*; e). ORs labeled *, FDR < 0.05; other ORs shown, unadjusted $p < 0.01$. (**f**) Quantification, by two-color RNA FISH, of the relative expression frequencies of representative OR-encoding genes that were identified *via* RNA-seq as differentially expressed (unadjusted $p < 0.01$) between SF and SM mice. OR expression frequencies for each experimental group are based on counting of specific OR-expressing cells relative to the area of all mature OSNs (detected by *Omp*). *p*-values for differential expression between the experimental groups shown were calculated using a two-tailed *t*-test based on mice: $n = 4$ (5 sections/mouse; average of 28 OSNs/section) or MOE sections (*parentheses*): $n = 20$ (average of 28 OSNs/section).

Supplementary Figure 3

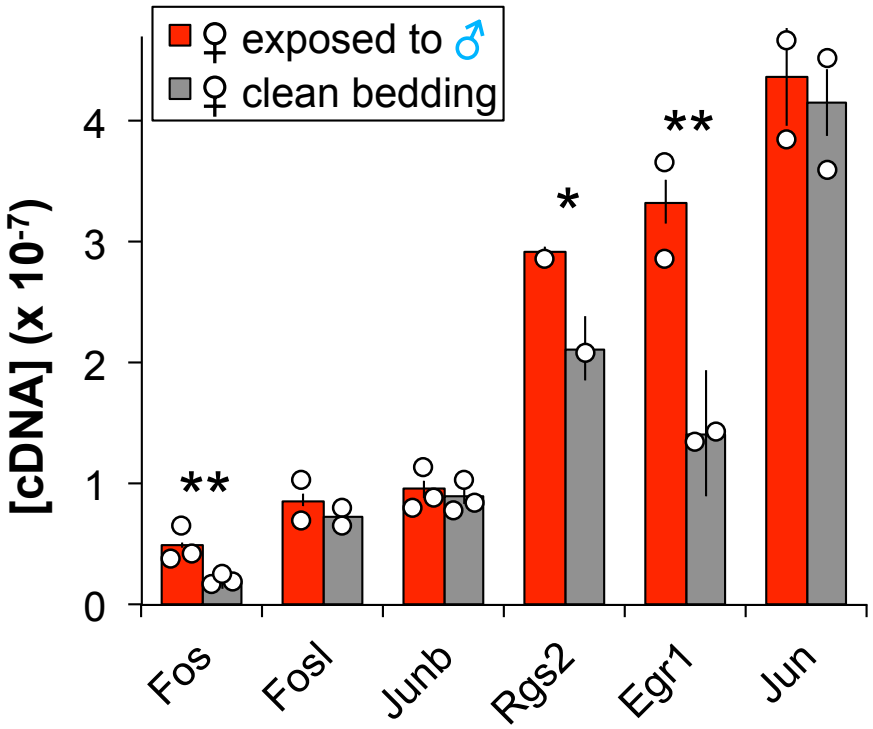


**Supplementary Figure 3.** Differences in OR expression between SF and SM mice reflect differences in the abundance of corresponding OSN subtypes, as shown for two additional ORs. (**a**, **e**) RNA-seq read alignments to *Olfr1419* (a) and *Olfr1437* (e) for SF and SM samples. For simplicity, alignments from the three biological replicates in each experimental group were combined (SF, *top*; SM; *bottom*). Strand orientation: - strand, *pink*; + strand, *blue*. (**b**, **f**) Expression levels, determined by RNA-seq FPKM values for *Olfr1419* (b) and *Olfr1437* (f) in the MOEs of the experimental mouse groups shown. Error bars: 95% c.i.. (**c**, **g**) Quantification, using two-color RNA-FISH, of the frequency of *Olfr1419*-expressing (c) and *Olfr1437*-expressing (g) OSNs relative to the area of all mature OSNs (based on *Omp* expression) for each experimental group. Error bars: s.e.m. *$p < 0.05$ (two-tailed *t*-test); n = 4 mice (5 sections/mouse; average of 21 *Olfr1419*-expressing and 17 *Olfr1437*-expressing OSNs/section). Dots represent average values for individual mice. (**d**, **h**) Representative images of *Olfr1419* (d) and *Olfr1437* (h) expression in the MOEs of the experimental mouse groups shown. Scale bars: 200 μm.

Supplementary Figure 4



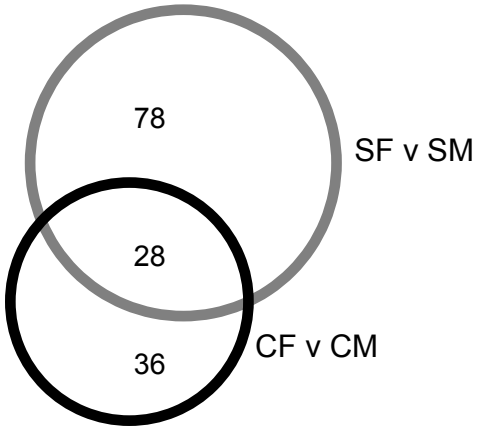
**Supplementary Figure 4.** *Olfr1295* is expressed in a female-biased manner in sex-separated mice and responds to male-specific odors. (**a**) RNA-seq read alignments to *Olfr1295* for SF and SM samples. For simplicity, alignments from the three biological replicates in each experimental group were combined (SF, *top*; SM; *bottom*). Strand orientation is indicated by color: - strand, *pink*; + strand, *blue*. (**b**) Expression levels, determined by RNA-seq FPKM values for *Olfr1295* in the MOEs of the experimental mouse groups shown. Error bars: 95% c.i. (**c**) Quantification, based on two-color RNA FISH, of the fraction of *Olfr1295*-expressing OSNs that co-express *Egr1* within the MOE of a male mouse exposed to 4 female mice for 40 min. (M→F), a female mouse exposed to 4 male mice for 40 min. (F→M), a male mouse exposed to clean bedding (M(-)), or a female mouse exposed to clean bedding (F(–)). *$p < 0.05$ (two-tailed *t*-test); $n = 3$ MOE sections (average of 73 *Olfr1295*-expressing OSNs/section). Error bars: s.e.m. Dots represent average values for individual sections. (**d**, **e**) Representative images of two-color RNA FISH analyses of *Olfr1295* co-expression with *Egr1* following exposure of a female mouse to a group of male mice (♂ exposed to ♀; d) or a male mouse to a group of female mice (♀ exposed to ♂; e). *Arrows*: locations of representative *Olfr1295*-expressing cells. Scale bars: 50 μm.




**Supplementary Figure 5.** Quantitative PCR (qPCR) analysis of the expression of immediate-early genes (IEGs) in the MOEs of female mice exposed to either groups of male mice (♀ exposed to ♂) or to clean bedding (♀ clean bedding). *$p < 0.05$, **$p < 0.01$ (two-tailed *t*-test; $n$ = 2 mice). Error bars: s.d.

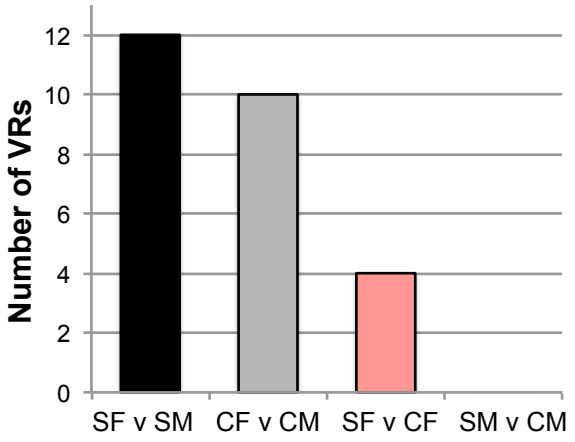
Supplementary Figure 6

a

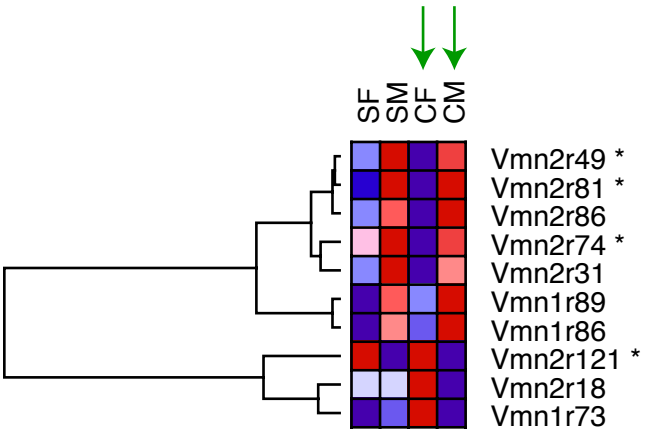



Differentially expressed genes, VNO

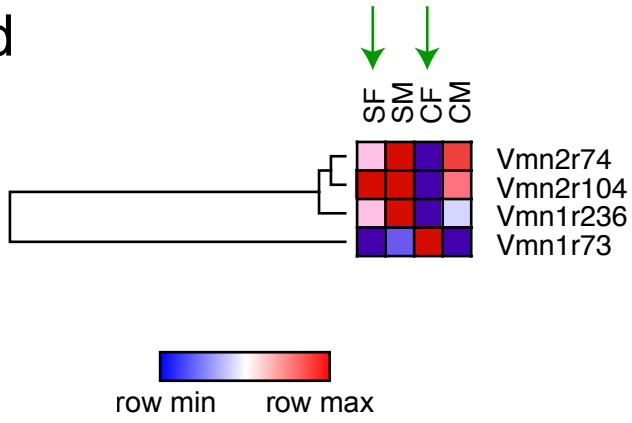
b



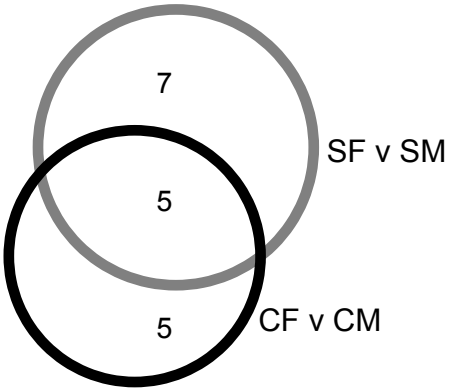

c




d




e



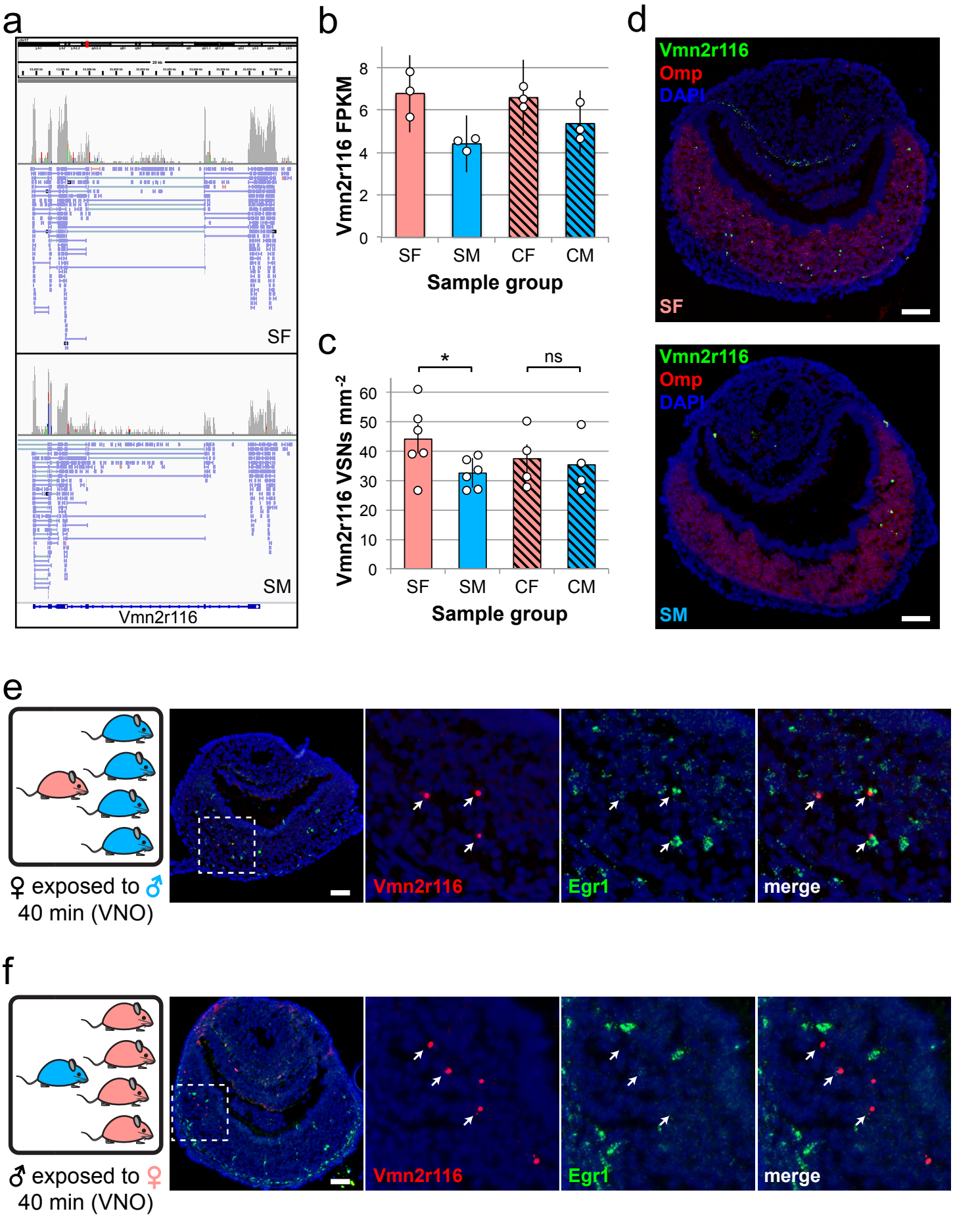

Differentially expressed VRs, VNO

f

| VR | Count ratio SF SM$^{-1}$ | P-value SF v SM 6 mice | (30 sections) | Count ratio CF CM$^{-1}$ | P-value CF v CM 6 mice | (30 sections) |
|---|---|---|---|---|---|---|
| Vmn2r116 | 1.40 | 0.050 | (0.025) | 1.06 | 0.78 | (0.70) |
| Vmn1r89 | 0.66 | 0.0013 | (0.0000091) | 0.85 | 0.16 | (0.073) |
| Vmn1r185 | 1.03 | 0.83 | (0.74) | | | |
| Vmn1r233 | 0.87 | 0.33 | (0.20) | | | |
| Vmn2r81 | 0.91 | 0.75 | (0.54) | | | |
| Vmn1r69 | 0.90 | 0.37 | (0.42) | | | |

**Supplementary Figure 6.** Analysis of genes, including VRs, that are differentially expressed in the VNO between different experimental groups. (**a**) Venn diagram of overlap of genes identified *via* RNA-seq as significantly differentially expressed (FDR < 0.05) between male and female sex-separated (SF vs. SM) and sex combined (CF vs. CM) mice. (**b**) Number of VR-encoding genes that were identified *via* RNA-seq as differentially expressed (unadjusted $p < 0.01$) in the VNO between the indicated experimental groups. (**c**, **d**) Hierarchical clustering of VR-encoding genes identified *via* RNA-seq as differentially expressed between SF and SM mice (*green arrows*; c) and between SF and CF mice (*green arrows*; d). VRs labeled *, FDR < 0.05; other ORs shown, unadjusted $p < 0.01$. (**e**) Venn diagram of VR-encoding genes identified *via* RNA-seq as differentially expressed (unadjusted $p < 0.01$) between male and female sex-separated (SF vs. SM) and sex combined (CF vs. CM) mice. (**f**) Quantification, using two-color RNA FISH, of the relative expression frequencies of representative VRs that were identified *via* RNA-seq as differentially expressed (unadjusted $p < 0.01$) between SF and SM mice. VR expression frequencies for each experimental group are based on counting of specific VR-expressing cells relative to the area of all mature VSNs (detected by *Omp*). *p*-values for differential expression between the experimental mouse groups shown were calculated using two-tailed *t*-tests based on mice: $n = 6$ (5 sections/mouse; average of 9 VSNs/section), or VNO sections (*parentheses*): $n = 30$ (average of 9 VSNs/section).

Supplementary Figure 7



**Supplementary Figure 7.** *Vmn2r116* is expressed in a female-biased manner in both sex-separated and -combined mice and responds to male-specific odors. (**a**) RNA-seq read alignments to *Vmn2r116* for SF and SM samples. For simplicity, alignments from the three biological replicates in each experimental group were combined (SF, *top*; SM; *bottom*). Strand orientation is indicated by color: - strand, *pink*; + strand, *blue*. (**b**) Expression levels, determined by RNA-seq FPKM values for *Vmn2r116* in the VNOs of the experimental mouse groups shown. Error bars: 95% c.i. (**c**) Quantification, using two-color RNA-FISH, of the frequency of *Vmn2r116*-expressing VSNs relative to all mature VSNs (based on *Omp* expression). $^{**}p < 0.01$ (two-tailed *t*-test); $n = 4 – 6$ mice (5 sections/mouse; average of 6 *Vmn2r116*-expressing VSNs/section). Error bars: s.e.m. Dots represent average values for individual mice. (**d**) Representative images of *Vmn2r116* expression in the VNOs of the experimental mouse groups shown (SF, *top*; SM; *bottom*). (**e**, **f**) Representative images of two-color RNA FISH analyses of *Vmn2r116* co-expression with *Egr1* following exposure of a female mouse to a group of male mice (♀ exposed to ♂; e) or a male mouse to a group of female mice (♂ exposed to ♀; f). *Arrows*: locations of representative *Vmn2r116*-expressing cells. Scale bars: 100 μm.

Supplementary Table 1. *In situ* hybridization probe design

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Olfr141 | OSN counting | 3' of CDS | + 1182 | ACGTTGGCTTTG CATGTGTTC | AAAGTCCCAGTC TCTGACTCC | 992 | 1 | 0 | 1095 |
| Olfr912 | OSN counting, Egr1 assay | CDS/3' of CDS | + 483 | TGCATCCTGAGA CTGACTTTCT | GCTGTGTCAAAT GCAAGATTTTAAA | 500 | 46 | 3 | 44, 145,146, 147, 229, 874, 875, 878, 883, 884, 885, 887, 888, 889, 890, 901, 902, 906, 907, 908, 910, 914, 915, 916, 917, 918, 920, 921, 922, 923, 933, 935, 936, 937, 943, 952, 954, 955, 957, 967, 968, 969, 970, 971, 983 |
| Olfr976 | OSN counting | CDS/3' of CDS | + 949 | AAGCAATGCAAC CTTTGGGG | TCAGGTTCTTCCA GGAGGCA | 705 | 0 | 0 | |
| Olfr1419 | OSN counting, Egr1 assay | CDS | + 33 | TTCCACTTCCGC CCCTTTTC | ACTTCTTCTGCGA CATGCCT | 753 | 0 | 0 | |
| Olfr1437 | OSN counting, Egr1 assay | 3' of CDS | + 1190 | TTCAGCCCTTGG TCTTGTCC | CCTGACGGTGAG AAGTGTCC | 753 | 1 | 0 | 1436 |
| Olfr235 | OSN counting, Egr1 assay | 5' of CDS | - 7217 | TCAACAGCATGT TCAGGGGA | GCCTTTTTCTCAC CTGGGCT | 673 | 2 | 0 | 1431, 1433, |
| Olfr48 | OSN counting, Egr1 assay | CDS/3' of CDS | + 691 | CCCTCTCCACCT GTGTCTCT | TGGAGGCACTGT CTGTTTCA | 904 | 3 | 0 | 1258, 1254, 1240 |
| Olfr374 | Egr1 assay | CDS | + 37 | TGCTGCTAGGCT TGTCACAG | ACTGCTGTGAAG ATGCGTGT | 637 | 31 | 6 | 8, 18, 39, 54, 57, 191, 351, 353, 365, 371, 412, 527, 531, 707, 708, 741, 742, 845, 853, 860, 870, 871, 1351, 1352, 1353, 1354, 1356, 1375, 1377, 1378, 1402 |
| Olfr12 | Egr1 assay | 5' of CDS | - 1992 | ACTGGAGGCTAC ACATCCCT | GGTGTGGTTAGAT GCAGCCT | 502 | 0 | 0 | |
| Olfr771 | Egr1 assay | CDS | + 245 | AGCAACCGGAG ACAGGTCAA | GGTTCAACATGG GAGCAATCG | 605 | 26 | 0 | 777, 800, 770, 780, 781, 772, 773, 799, 769, 791, 794, 768, 821, 792, 814, 815, 798, 774, 805, 776, 805, 810, 816, 775, 802, 820 |
| Olfr183 | Egr1 assay | CDS | + 252 | TGCAGGGTGCA CATACATGA | CTCTTGGACAAG GGCAAGGT | 537 | 13 | 4 | 196, 186, 187, 197, 193, 191, 190, 192, 194, 203, 205, 198, 209 |
| Olfr222 | Egr1 assay | 5' of CDS/CDS | - 342 | AGCCAGTGGAA AATGGCGTA | ACTGAACGTGAAT GACGCCT | 674 | 8 | 0 | 317, 1360, 1359, 1367, 138, 136, 124, 15 |
| Olfr325 | Egr1 assay | CDS/3' of CDS | + 853 | TCACCCCTCTGT TGAACCCA | TAGGGAAAACCA CGGACCAT | 522 | 0 | 0 | |
| Olfr358 | Egr1 assay | CDS | + 248 | ACACACACTCTC TGTGGTTCG | GATGGCTTGAGG TACATGCG | 537 | 2 | 1 | 360, 361 |
| | | | | | | | | | |
| Vmn2r116 | VSN counting, Egr1 assay | Intron | + 3212 | AGACACAGAGA GAGATGGGTTA | TATGTTTGTGCTT GATAATGTCTTAG A | 582 | 6 | 11 | 2r112, 2r111, 2r115, 2r117, 2r114, 2r113 |
| Vmn2r81 | VSN counting, Egr1 assay | 3' of CDS | + 46247 | ACAAATTTCACA CACTATGACTGC | TGCTCCAAAAATC AGTGCCC | 577 | 2 | 0 | 2r82, 2r80 |
| Vmn1r233 | VSN counting, Egr1 assay | 5' of CDS | - 1709 | TCCATATGACCT TTCTGGGGC | GTTTCACAAGGG CCCCTGAT | 402 | 0 | 0 | |
| Vmn1r185 | VSN counting, Egr1 assay | 5' of CDS/CDS | - 103 | TGAAGCTCCATG GTTAATGGC | CACAATGGCTGTT CCATTTCCC | 518 | 7 | 4 | 1r184, 1r71, 1r69, 1r67, 1r68, 1r225, 1r228 |
| Vmn1r69 | VSN counting, Egr1 assay | 5' of CDS | - 1354 | TATGGGCCCCTT TCTGGTTC | AGTGATCTGCATG GATGGGC | 531 | 2 | 0 | 1r68, 1r67 |
| Vmn1r89 | VSN counting, Egr1 assay | 5' of CDS | - 6123 | GCAGCCTTTCCA AGACACTTTGT | ACTACTGTTTTCT GCAGGATAGTGG | 828 | 2 | 0 | 1r87, 1r88 |