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CS 152: Statistical Bioinformatics in R

Partial replication of "Coadaption of the chemosensory system with voluntary exercise behavior in mice" (Nguyen et al., 2020)

Introduction

Olfaction prompts various animal behaviors [1]. The vomeronasal organ (VNO), found in the olfactory systems of amphibians, scaled reptiles, and some mammals, detects environmental pheromones and drives physiological and behavioral changes accordingly [2]. The VNO also evolves rapidly and exhibits significant geno- and phenotypical variation across populations, even when inbred [3-19]. In "Coadaptation of the chemosensory system with voluntary exercise behavior in mice" (2020), Nguyen et al. hypothesize that if a species were selectively bred for a behavior driven by the VNO, one would expect to see coadaptive changes in the genes of and gene expression within the organ [20].

Voluntary wheel running (VWR) behavior has been widely studied in mice [21-23], and, beginning at the University of Wisconsin-Madison in 1993 (and transferred to University of California, Riverside in 2001), there has been an ongoing artificial selection experiment in which mice have been bred for high VWR. In one experiment on these mice, researchers noted VWR tended higher when the urine of other mice, which contains pheromones, was present [24]. In another, mice were shown to exhibit more VWR on wheels already used by other mice when compared to clean ones [25]. Nguyen et al. therefore thought laboratory mice might be reasonable candidates on which to test their hypothesis.

Physical exercise is an important part of a healthy lifestyle for humans. However, many people find it hard to motivate themselves to engage in regular physical activity, especially as modern conveniences make it easier to lead a sedentary lifestyle. Though humans do not have vomeronasal organs, research exploring the connection between voluntary activity and the olfactory system in general could uncover new technologies and strategies to promote and facilitate exercise among those looking to be more active.

Replicated Results

We have focused on replicating one of the three major areas of work within Nguyen et al.'s paper: analysis of differential gene expression within the VNO between High Runner (HR) and Control lines. The specific results we sought to replicate are as follow:

- Identification of 132 differentially expressed genes in the HR versus Control group, 19 being vomeronasal receptor genes
- No significant difference in expression of the olfactory marker protein (OMP) gene
- No significant differential expression by sex within HR and Control groups

Data

The data for this paper came from the 88th generation of 8 genetic lines of Hsd:ICR mice. 4 lines were selectively bred for high VWR behavior, while the other 4 were bred randomly to serve as controls. The decision to have multiple lines within each group was made to minimize the influence of genetic drift.

At the time of subject selection, mice from HR lines performed approximately 3x as many wheel rotations per day as those from controls, a difference which had plateaued, depending on the HR line, between generations 17 and 27.

For RNA sequencing analysis, VNO samples were taken from 6 mice, 3 female and 3 male, from each of the 8 lines (48 mice in total). For each line-sex triad, tissue was pooled, and total RNA samples were taken, measured for integrity, and rid of ribosomal RNA. RNA sequences were then processed.

Methods

For gene expression analysis, the authors used Cuffdiff, an algorithm within Cufflinks from the Tuxedo Suite of RNA-Seq analysis tools, to test for significance in the difference in read counts. Cuffdiff uses the negative binomial distribution to test for differential expression. Additionally, to account for false positive rate, the authors used the Benjamini-Hochberg false discovery correction.

Since Cuffdiff requires generated BAM files, which the authors have not made available and which are difficult to reverse engineer from the .txt files they did, we decided to perform our replication using DESeq2, an R package that is part of the Bioconductor project. DESeq2 also uses the negative binomial distribution for its differential expression analysis, so we thought it should make for a good comparison.

To compare gene expression across lines and sex, the authors used a two-way ANOVA test to see whether the normalized read count means were affected by these two variables. We replicate this analysis, once again, via the DESeq2 package by adding sex into our model as an additional factor.

Results

We performed quality control on the data before running differential expression analysis. We first removed 27 genes due to their names having been inadvertently converted to date format (with a few duplicates, making it hard to convert them back). We then performed a principal component analysis on the data for all 16 line-sex samples and removed sample *Cont_F4*, the female portion of one of the control lines, due to its outlier nature [Figure 1]. After these adjustments, we were left with 15 line-sex samples and 24,411 genes. Lastly, we added a binary variable to account for the batch effect contributed by samples *Runner1* and *HR_F3*, the male and female subsamples of the first HR line. Doing so resulted in what looked to be uniform distribution of p-values for null results.

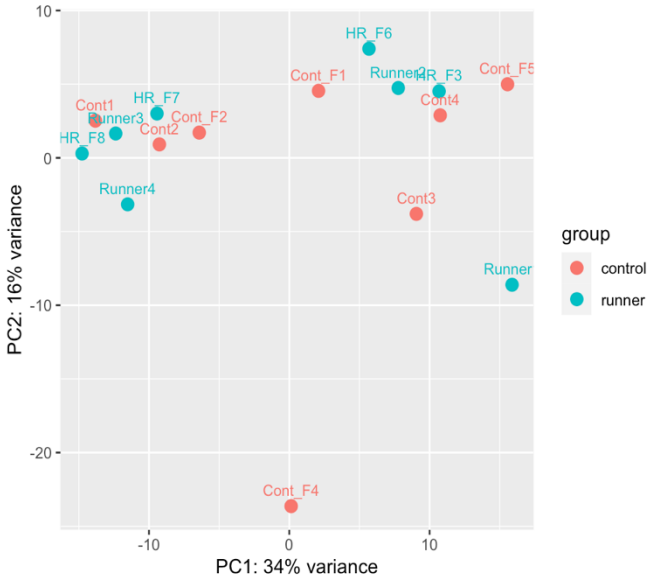

Figure 1. Results of a PCA analysis of all 16 line-sex samples.

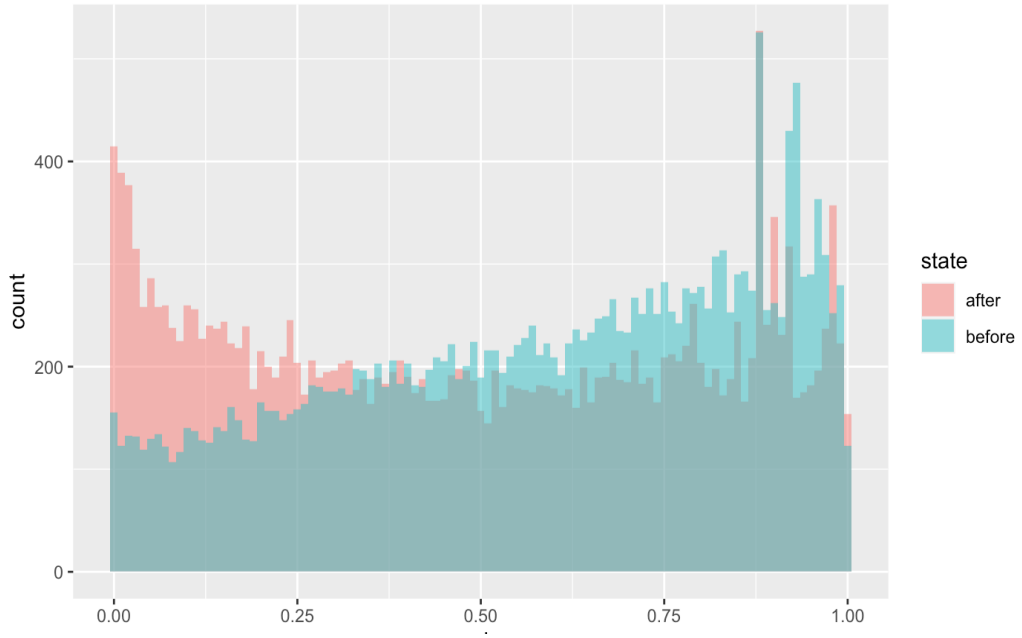

Figure 2. Comparative histograms of p-values before and after accounting for batch effects in Runner1 *and* HR_F3 *samples.*

After these alterations, we then used a DESeq two-factor model (condition and batch) with a Benjamini-Hochberg false discovery correction and q-value threshold of 0.05, (the threshold used by the original authors) to identify 103 differentially expressed (DE) genes across the HR and Control lines. Of the 103 genes, 9 were vomeronasal receptor coding genes: *Vmn1r196, Vmn1r236*, *Vmn2r9, Vmn2r11, Vmn2r14*, *Vmn2r15*, *Vmn2r16*, *Vmn2r50,* and *Vmn2r99*. Of these, in the HR lines, 5 were significantly up-regulated compared to Controls (*Vmn1r196*, *Vmn2r9, Vmn2r11, Vmn2r14,* and *Vmn2r50*), and 4 were significantly down-regulated (*Vmn1r236*, *Vmn2r15*,

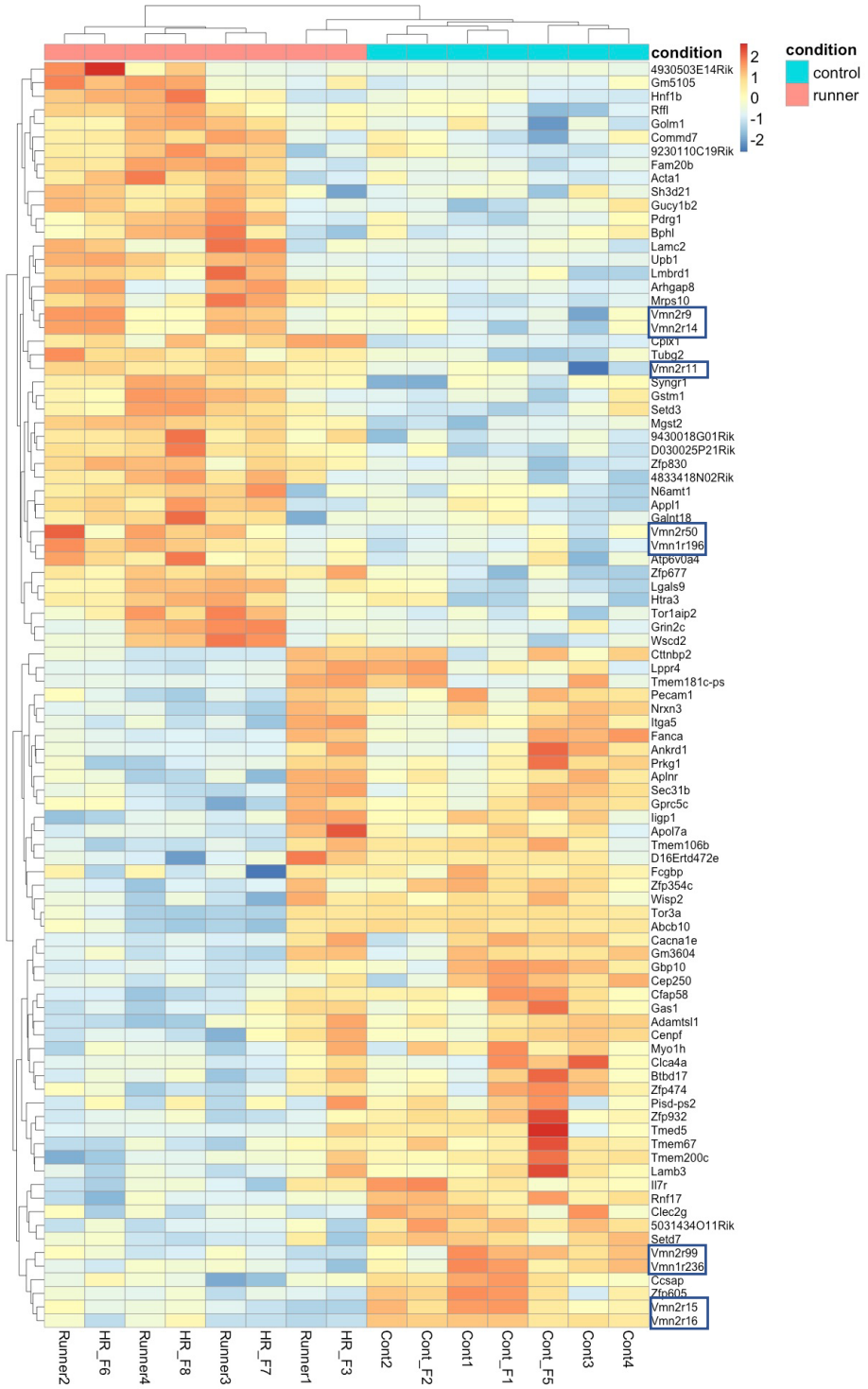

Figure 3. Heatmap of 93 differentially expressed genes between HR and Controls. Based on three-factor model including condition, sex, and batch variables. Vomeronasal receptor genes are marked with boxes.

Vmn2r16, and *Vmn2r99*). Across these genes, $\log_2$ fold change ranged from -3.01 to 1.68, figures held by *Vmn2r16* and *Vmn2r9*, respectively.

A three-factor model including sex identified 93 DE genes [Figure 3], though all 9 vomeronasal receptor genes from the single-factor model remained classified as DE and with similar $\log_2$ fold changes. A model based solely on sex and batch returned 196 DE genes, 3 of which coded for vomeronasal receptors (*Vmn1r26*, *Vmn2r117*, and *Vmn2r121*). However, none of these 3 overlapped with the genes differentially expressed by condition.

Compared to Nguyen et all., we found fewer DE genes (93 vs. 132) and DE vomeronasal receptor genes, both absolutely (9 vs. 19) and in proportion to total DE genes (9.7% vs. 14.4%). Across the DE vomeronasal receptor genes, the original authors found 2 in the *Vmn1r* family, 15 in the *Vmn2r* family, and 2 in the *Fpr* family, while we found 2 in the *Vmn1r* family, 7 in the *Vmn2r* family, and

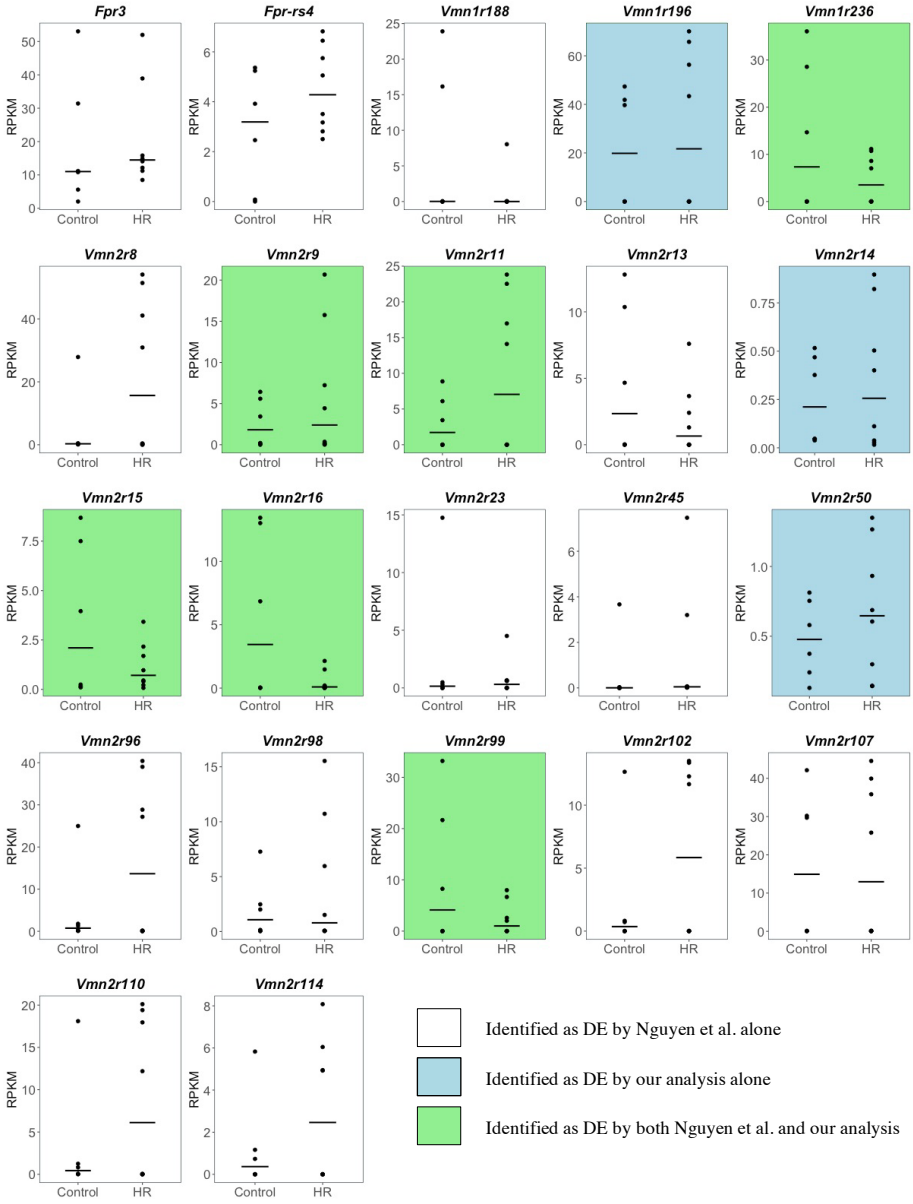

Figure 4. RPKM comparison of HR and Control lines for differentially expressed genes identified by Nguyen et al. (white), our analysis (blue), or both (green). Lines mark medians of each group.

0 in the *Fpr* family. Of the 9 DE vomeronasal receptor genes we identified, 6 were also identified by the original authors (all but *Vmn1r196, Vmn2r14,* and *Vmn2r50*) and with directionally the same up- or down-regulation when compared to Controls [Figure 4]. Also like the initial authors, we found no differential expression of the olfactory marker protein (OMP) gene, supporting their assertion that the vomeronasal receptor gene expression differences were not due to differences in number of vomeronasal sensory neurons.

Beyond the original authors' results, we also performed a functional enrichment analysis on the DE genes identified using our three-factor model [Figure 5]. As one might expect, genes coding for G protein-coupled receptor activity, a category including all the vomeronasal receptor genes we have discussed, appeared as the most significantly overrepresented class of genes. In total, 11 out of a possible 743 genes in that category were identified as differentially expressed. This included the 9 receptor genes mentioned previously as well as *Aplnr* and *Gprc5c*.

term	over_represented_pvalue	numDEInCat	numInCat	padj
G protein-coupled receptor activity	0.000178404	11	743	*1*
laminin-5 complex	0.000180986	2	4	*1*
peptidyl-lysine monomethylation	0.000702044	2	9	*1*
histone-lysine N-methyltransferase activity	0.000821177	3	38	*1*
glutathione binding	0.001187668	2	17	*1*

Figure 5. Top five overrepresented gene categories according to functional enrichment analysis.

After correcting this analysis with a BH false discovery rate adjustment (with a q-value threshold of 0.05), however, no functional class of genes remained over- or underrepresented to a statistically significant level. In fact, q-values for every category equaled 1. This was a strange result, as it stands to reason that, even if vomeronasal receptors were not part of the mechanism responsible for higher VWR activity in mice, as Nguyen et al. hypothesize, one would expect the DE population of this class of genes to be overrepresented in the VNO, an organ whose most notable functions rely on them. The lack of significance, then, likely indicates that we have too little data for this sort of analysis, and not, instead, that the differential expression of vomeronasal receptor genes holds no signal.

Conclusions and Further Work

We largely replicate the differential gene expression results of Nguyen et al., though to a more modest extent. It would not be surprising if, like the batch effect we discovered, there were additional idiosyncrasies to the data that the original authors were able to account for but went unmentioned in the paper. It is also plausible that some portion of the discrepancy arises from the genes and sample we removed from the analysis, as well as potential non-obvious differences between DESeq and Cuffdiff. That said, directionally, we came to the same conclusions, and, with more data, it appears we would be able to underscore the significance of differential expression of vomeronasal receptor genes within the HR mouse lines.

In terms of future work, continued research into a potential mechanism connecting the differentially expressed vomeronasal receptors and VWR in mice would go a long way toward confirming or refuting the link the original authors propose. Nguyen et al. mention a handful of papers showing that the chemosensory system (or VNO specifically) in mice prompts activity in

specific regions of their brains [26] and others that connect these regions to VWR behavior [27-31], but they do not list any that have been able to draw a more direct relationship. With further, more directed research, it is possible we could gain more insight into this potential connection and ultimately shed light on novel ways to promote physical exercise in humans.

References

1. Nielsen BL. Olfaction in animal behaviour and welfare: CABI; 2017 2017/6/29. 233 p.
2. Liberles SD. Mammalian pheromones. Annu Rev Physiol. 2014; 76(1):151–75. https://doi.org/10.1146/ annurev-physiol-021113-170334 PMID: 23988175
3. Wynn EH, Sa´nchez-Andrade G, Carss KJ, Logan DW. Genomic variation in the vomeronasal receptor gene repertoires of inbred mice. BMC Genomics. 2012; 13:415. https://doi.org/10.1186/1471-2164-13- 415 PMID: 22908939
4. Zhang J, Webb DM. Evolutionary deterioration of the vomeronasal pheromone transduction pathway in catarrhine primates. Proc Natl Acad Sci U S A. 2003; 100(14):8337–41. https://doi.org/10.1073/pnas. 1331721100 PMID: 12826614
5. Grus WE, Zhang J. Rapid turnover and species-specificity of vomeronasal pheromone receptor genes in mice and rats. Gene. 2004; 340(2):303–12. https://doi.org/10.1016/j.gene.2004.07.037 PMID: 15475172
6. Shi P, Bielawski JP, Yang H, Zhang Y-P. Adaptive diversification of vomeronasal receptor 1 genes in rodents. J Mol Evol. 2005; 60(5):566–76.
7. Shi P, Zhang J. Comparative genomic analysis identifies an evolutionary shift of vomeronasal receptor gene repertoires in the vertebrate transition from water to land. Genome Res. 2007; 17(2):166–74. https://doi.org/10.1101/gr.6040007 PMID: 17210926
8. Grus WE, Zhang J. Origin of the genetic components of the vomeronasal system in the common ancestor of all extant vertebrates. Mol Biol Evol. 2009; 26(2):407–19. https://doi.org/10.1093/molbev/msn262 PMID: 19008528
9. Grus WE, Zhang J. Origin and evolution of the vertebrate vomeronasal system viewed through systemspecific genes. Bioessays. 2006; 28(7):709–18. https://doi.org/10.1002/bies.20432 PMID: 16850401
10. Grus WE, Shi P, Zhang J. Largest vertebrate vomeronasal type 1 receptor gene repertoire in the semiaquatic platypus. Mol Biol Evol. 2007; 24(10):2153–7. https://doi.org/10.1093/molbev/msm157 PMID: 17666439
11. Young JM, Trask BJ. V2R gene families degenerated in primates, dog and cow, but expanded in opossum. Trends Genet. 2007; 23(5):212–5. https://doi.org/10.1016/j.tig.2007.03.004 PMID: 17382427
12. Young JM, Massa HF, Hsu L, Trask BJ. Extreme variability among mammalian V1R gene families. Genome Res. 2010; 20(1):10–8. https://doi.org/10.1101/gr.098913.109 PMID: 19952141
13. Young JM. Divergent V1R repertoires in five species: Amplification in rodents, decimation in primates, and a surprisingly small repertoire in dogs. Genome Res 2005; 15(2):231–40. https://doi.org/10.1101/ gr.3339905 PMID: 15653832
14. Lane RP, Cutforth T, Axel R, Hood L, Trask BJ. Sequence analysis of mouse vomeronasal receptor gene clusters reveals common promoter motifs and a history of recent expansion. Proc Natl Acad Sci. 2002; 99(1):291–6. https://doi.org/10.1073/pnas.012608399 PMID: 11752409

15. Lane RP, Young J, Newman T, Trask BJ. Species specificity in rodent pheromone receptor repertoires. Genome Res. 2004; 14(4):603–8. https://doi.org/10.1101/gr.2117004 PMID: 15060001
16. Yang H, Shi P, Zhang Y-P, Zhang J. Composition and evolution of the V2r vomeronasal receptor gene repertoire in mice and rats. Genomics. 2005; 86(3):306–15. https://doi.org/10.1016/j.ygeno.2005.05. 012 PMID: 16024217
17. Couger MB, Are´valo L, Campbell P. A high quality genome for mus spicilegus, a close relative of house mice with unique social and ecological adaptations. G3. 2018; 8(7):2145–52. https://doi.org/10.1534/ g3.118.200318 PMID: 29794166
18. Grus WE, Shi P, Zhang YP, Zhang J. Dramatic variation of the vomeronasal pheromone receptor gene repertoire among five orders of placental and marsupial mammals. Proc Natl Acad Sci. 2005; 102 (16):5767–72. https://doi.org/10.1073/pnas.0501589102 PMID: 15790682
19. Duyck K, DuTell V, Ma L, Paulson A, Yu CR. Pronounced strain-specific chemosensory receptor gene expression in the mouse vomeronasal organ. BMC Genomics. 2017; 18(1):965. https://doi.org/10. 1186/s12864-017-4364-4 PMID: 29233099
20. Nguyen, Quynh Anh Thi et al. "Coadaptation of the chemosensory system with voluntary exercise behavior in mice." *PloS one* vol. 15,11 e0241758. 25 Nov. 2020, doi:10.1371/journal.pone.0241758
21. Sherwin CM. Voluntary wheel running: a review and novel interpretation. Anim Behav. 1998; 56(1):11– 27. https://doi.org/10.1006/anbe.1998.0836 PMID: 9710457
22. Garland T Jr., Schutz H, Chappell MA, Keeney BK, Meek TH, Copes LE, et al. The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. The Journal of experimental biology. 2011; 214(Pt 2):206–29. https:// doi.org/10.1242/jeb.048397 PMID: 21177942
23. Novak CM, Burghardt PR, Levine JA. The use of a running wheel to measure activity in rodents: relationship to energy balance, general activity, and reward. Neuroscience and biobehavioral reviews. 2012; 36(3):1001–14. https://doi.org/10.1016/j.neubiorev.2011.12.012 PMID: 22230703
24. Drickamer LC, Evans TR. Chemosignals and activity of wild stock house mice, with a note on the use of running wheels to assess activity in rodents. Behav Processes. 1996; 36(1):51–66. https://doi.org/10. 1016/0376-6357(95)00015-1 PMID: 24896417
25. Dewan I, Garland T, Hiramatsu L, Careau V. I smell a mouse: indirect genetic effects on voluntary wheel-running distance, duration and speed. Behavior Genetics. 2019; 49(1):49–59. https://doi.org/10. 1007/s10519-018-9930-2 PMID: 30324246
26. Ben-Shaul Y, Katz LC, Mooney R, Dulac C. In vivo vomeronasal stimulation reveals sensory encoding of conspecific and allospecific cues by the mouse accessory olfactory bulb. Proc Natl Acad Sci U S A. 2010; 107(11):5172–7. https://doi.org/10.1073/pnas.0915147107 PMID: 20194746
27. Rhodes JS, Garland T Jr., Gammie SC. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav Neurosci. 2003; 117(6):1243–56. https://doi.org/10.1037/0735-7044. 117.6.1243 PMID: 14674844
28. King JM. Effects of lesions of the amygdala, preoptic area, and hypothalamus on estradiol-induced activity in the female rat. Journal of comparative and physiological psychology. 1979; 93(2):360–7. https://doi.org/10.1037/h0077559 PMID: 457955

29. Fahrbach S, Meisel R, Pfaff D. Preoptic implants of estradiol increase wheel running but not the open field activity of female rats. 1985; 35(6):985–92. https://doi.org/10.1016/0031-9384(85)90270-7 PMID: 4095192
30. Ogawa S, Chan J, Gustafsson J-Å, Korach KS, Pfaff DW. Estrogen Increases Locomotor Activity in Mice through Estrogen Receptor α: Specificity for the Type of Activity. Endocrinology. 2003; 144 (1):230–9. https://doi.org/10.1210/en.2002-220519 PMID: 12488349
31. Spiteri T, Ogawa S, Musatov S, Pfaff DW, Ågmo A. The role of the estrogen receptor α in the medial preoptic area in sexual incentive motivation, proceptivity and receptivity, anxiety, and wheel running in female rats. 2012; 230(1):11–20.