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SUPPLEMENTAL MATERIALS AND METHODS

In order to estimate the relative intensity of the mRNA labeling, we compared the signal in each brain region with that produced by the [^{14}C] microscales included in each film cassette. Each microscale, which is calibrated for the auto-absorptive features of intact brain grey matter (ref), contains ten values spanning 0.1 – 100 nCi/g tissue of tissue-equivalent [^{14}C] concentration. Each of the ten values represents an approximate doubling of the estimated activity (nCi/g of tissue). It was not our intention to quantify the absolute levels of signal but rather to compare the relative intensites among birds and brain regions. The signal in each labeled region was therefore estimated by comparing its optical density directly to that produced by the microscale. The digitized images of each film were opened in Photoshop CS v.8.0 and the portion containing the microscale signal was selected, copied, and pasted onto a new layer so that it could be placed anywhere on the image. For all of the films from the sparrow studies, the optical density (judged by eye) of the film itself, in areas without brain sections, was equivalent to the fifth darkest level on the microscales. That value on the microscale was therefore assigned a relative value of "0", and the remaining four darker values were assigned values 1-4 from lightest to darkest (Fig. 1). Thus, the most intense signal produced by the microscale was assigned a value of "4". The signal in each labeled brain region (signal obviously darker than in the surrounding tissue) was estimated by positioning the image of the microscale immediately adjacent to it. In every case, the signal intensity could either be matched unambiguously to one of the values 1-4 in the microscale, or the signal was darker than 4. In the latter case, the brain region was assigned a value of "5". Note that "5" refers to all labeling greater than the darkest value on the film image of the microscale and we therefore could not detect variation in the signal above this value. In no case did the signal fall clearly between two values on the microscale; all labeled regions in each bird could be unambiguously matched to a whole number between 1 and 5.

The signal intensities for the zebra finches were estimated as described above for the sparrows, except that for the finches, the background on the films was equivalent to the sixth darkest value on the microscales. Thus, that value was deemed "0" and there were five levels of detectable signal (1-5 from lightest to darkest) represented on the microscales rather than four (Fig. 1). Because there was no signal that clearly exceeded the top level (deemed "5") on the microscale for the finches, it was not necessary to assign higher values. As for the sparrows, each labeled region in each bird could be unambiguously matched to an intensity corresponding to a whole number between 1 and 5. All values for both the sparrows and the finches were assigned by the same person (DLM) using the same computer, monitor, and ambient lighting conditions.

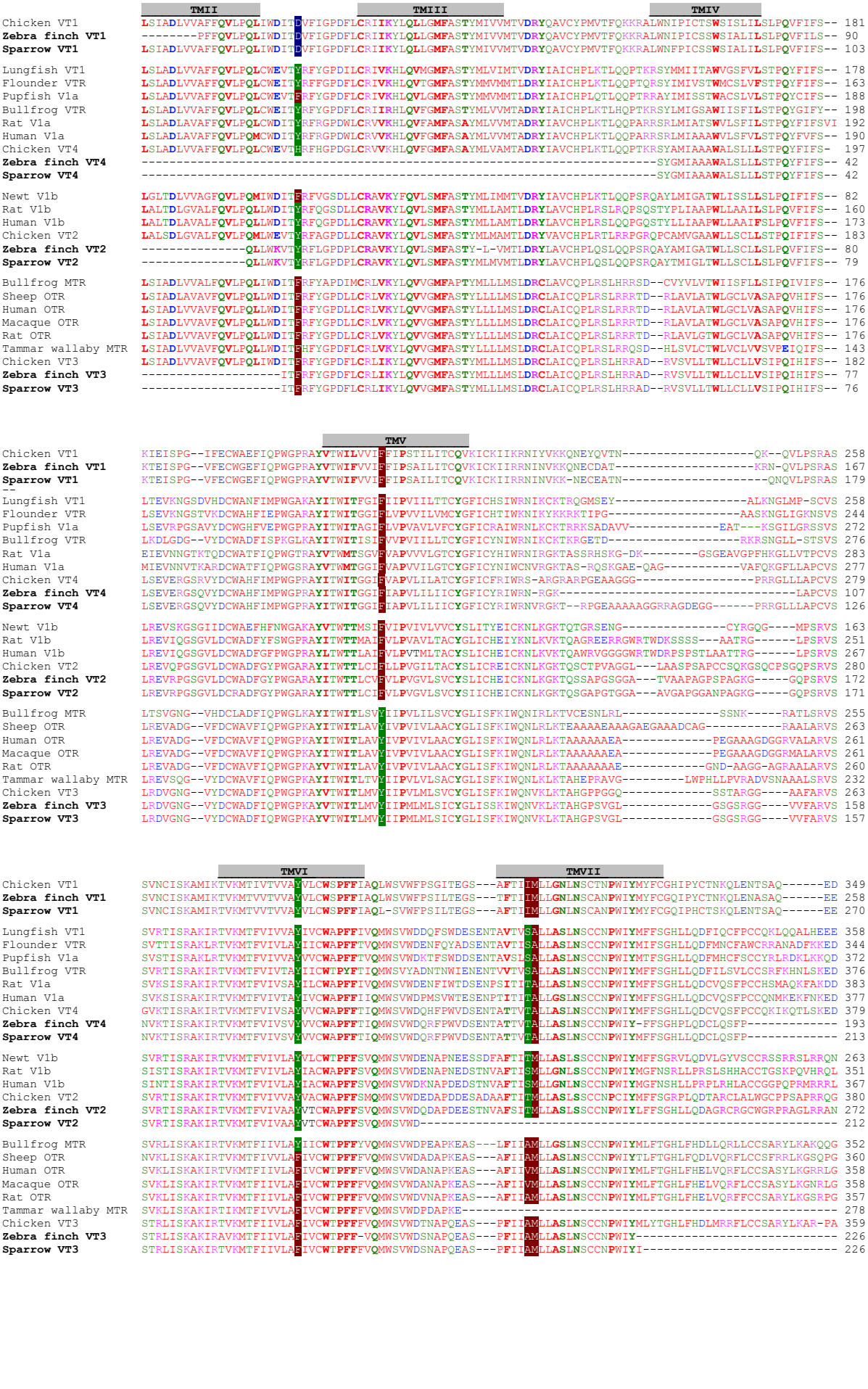
In order to show that individual variation in signal intensity was not explained by overall lighter or darker values throughout the brains of some individuals, we performed the following analysis separately for each species and mRNA. First, the median signal intensity was calculated for each brain region. Then, the values for each individual were subtracted from each median. This calculation resulted in a series of "difference scores" for each individual, one score per brain region, representing how much ligher or darker the region was in that individual compared to the others. The difference scores were then

averaged across regions for each individual to estimate the degree to which the values from each individual tended to be higher or lower than the group medians. For the sparrows, all average difference scores were zero for all individuals and all mRNAs, showing that there were no birds for which the labeling was uniformly lighter or darker than the others. All sparrows were therefore included in the subsequent analyses for all three mRNAs. For the finches, the calculation of difference scores revealed one male in the VT1 study and another in the VT3 study with lower than average signal intensities overall. Those males were excluded from subsequent analysis only for the mRNA for which their values were low.

We next inspected the ranges of signal intensities in each brain region for each mRNA in order to identify regions with notable individual variation. Ranges of one unit or less (e.g. 2-3 or 3-4) were not considered notable and those cases were not investigated further. For each mRNA, the values for every region in which the range spanned two or more units (e.g. 2-4) were (1) plotted in a histogram to show the varation and (2) subjected to a Mann-Whitney U test to determine whether the variation was explained by sex differences. The histograms, MWU and P values are shown in Supplemental Figs. 2-5.

Supplemental Table 1 (see next page). Partial amino acid sequences of zebra finch and sparrow receptors VT1, VT2 (V1b), VT3 (OT-like), and VT4 (V1a) aligned with published sequences of nonapeptides receptors in other species. The locations of transmembrane (TM) domains are approximate. Nonpolar neutral residues are shown in red, polar neutral in green, polar positive in purple and polar negative in blue. Residues involved in ligand binding, i.e. the "binding pocket" [1] are indicated in bold. Residues thought to be important for ligand specificity [2, 3, 4] are highlighted with white lettering. Sequence between the N-terminus and TMII is not shown because our cloned fragments did not contain that region. Our zebra finch VT2 receptor sequence ended at residue 212, so the rest of that sequence has been filled in from the published sequence (XM_002195382).

1. **Chini B, Mouillac B, Ala Y, Balestre MN, Trumpp-Kallmeyer S, Hoflack J, Elands J, Hibert M, Manning M, Jard S, Barberis C** 1995 Tyr115 is the key residue for determining agonist selectivity in the V1a vasopressin receptor. EMBO J 14:2176-2182
2. **Chini B, Mouillac B, Balestre MN, Trumpp-Kallmeyer S, Hoflack J, Hibert M, Andriolo M, Pupier S, Jard S, Barberis C** 1996 Two aromatic residues regulate the response of the human oxytocin receptor to the partial agonist arginine vasopressin. FEBS Lett 397:201-206
3. **Cotte N, Balestre MN, Aumelas A, Mahé E, Phalipou S, Morin D, Hibert M, Manning M, Durroux T, Barberis C, Mouillac B** 2000 Conserved aromatic residues in the transmembrane region VI of the V1a vasopressin receptor differentiate agonist vs. antagonist ligand binding. Eur J Biochem 267:4253-4263
4. **Tahtaoui C, Balestre MN, Klotz P, Rognan D, Barberis C, Mouillac B, Hibert M. Brownstein MJ, Saito N, MacLeod V, Baeyens DA, Mayeux PR** 2003 Identification of the binding sites of the SR49059 nonapeptide antagonist into the V1a vasopressin receptor using sulfydryl-reactive ligands and cysteine mutants as chemical sensors. J Biol Chem 278:40010-40009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TMIII
TMIV
TMV
TMVI
TMVII
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Zebra finch VT1
Sparrow VT1
Lungfish VT1
Flounder VTR
Pupfish V1a
Bullfrog VTR
Rat V1a
Human V1a
Chicken VT4
Zebra finch VT4
Sparrow VT4
Newt V1b
Rat V1b
Human V1b
Chicken VT2
Zebra finch VT2
Sparrow VT2
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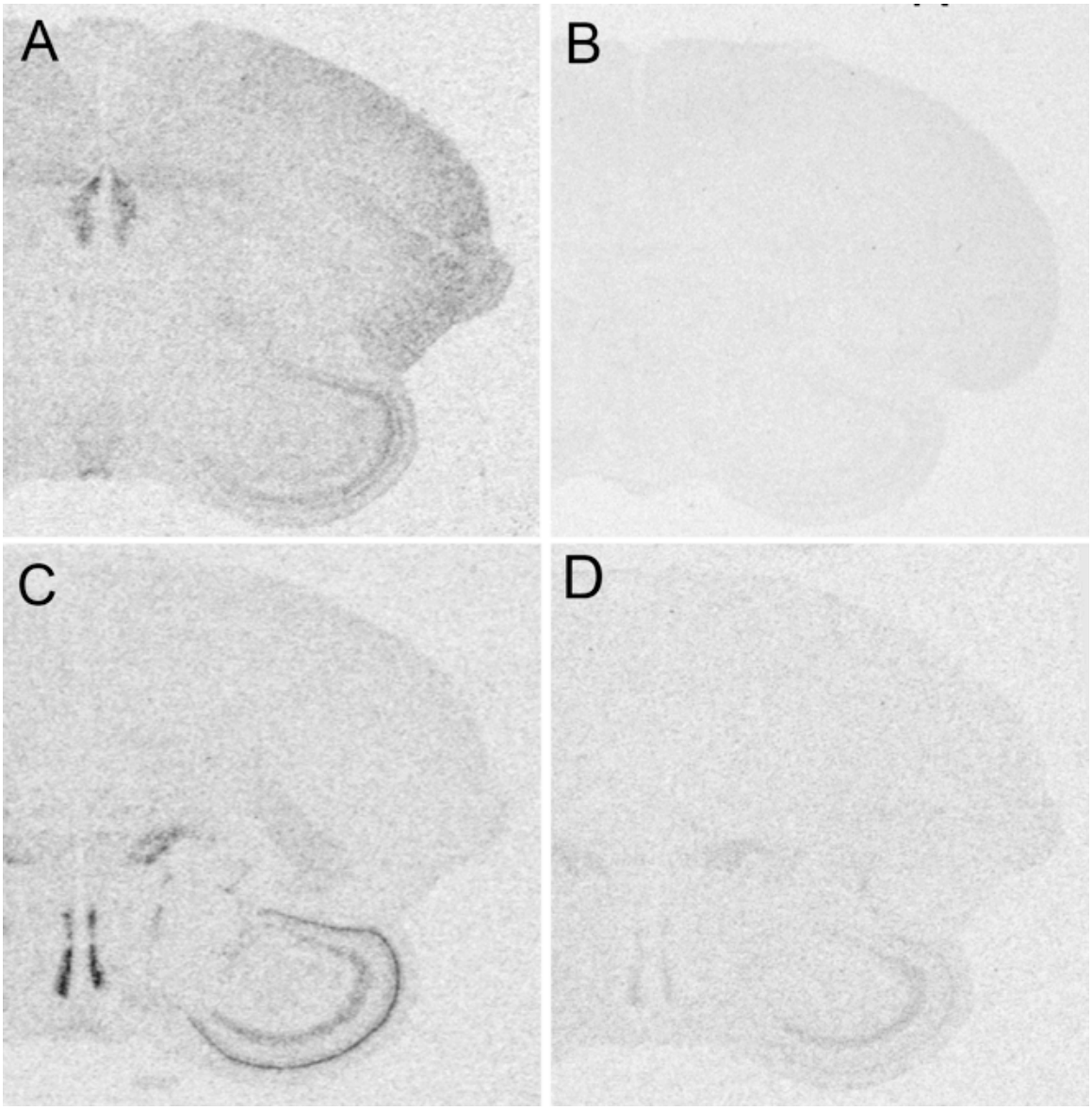
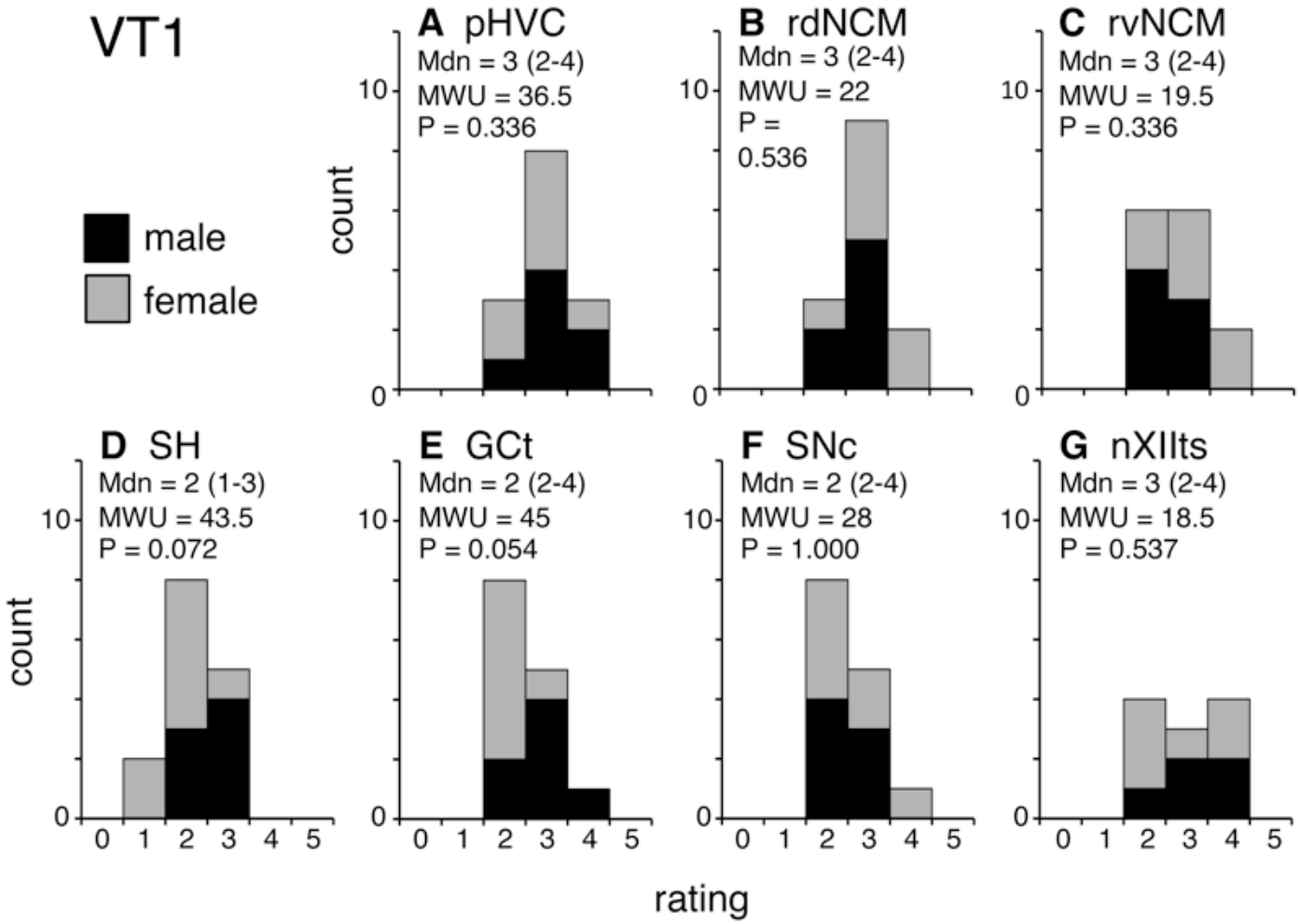
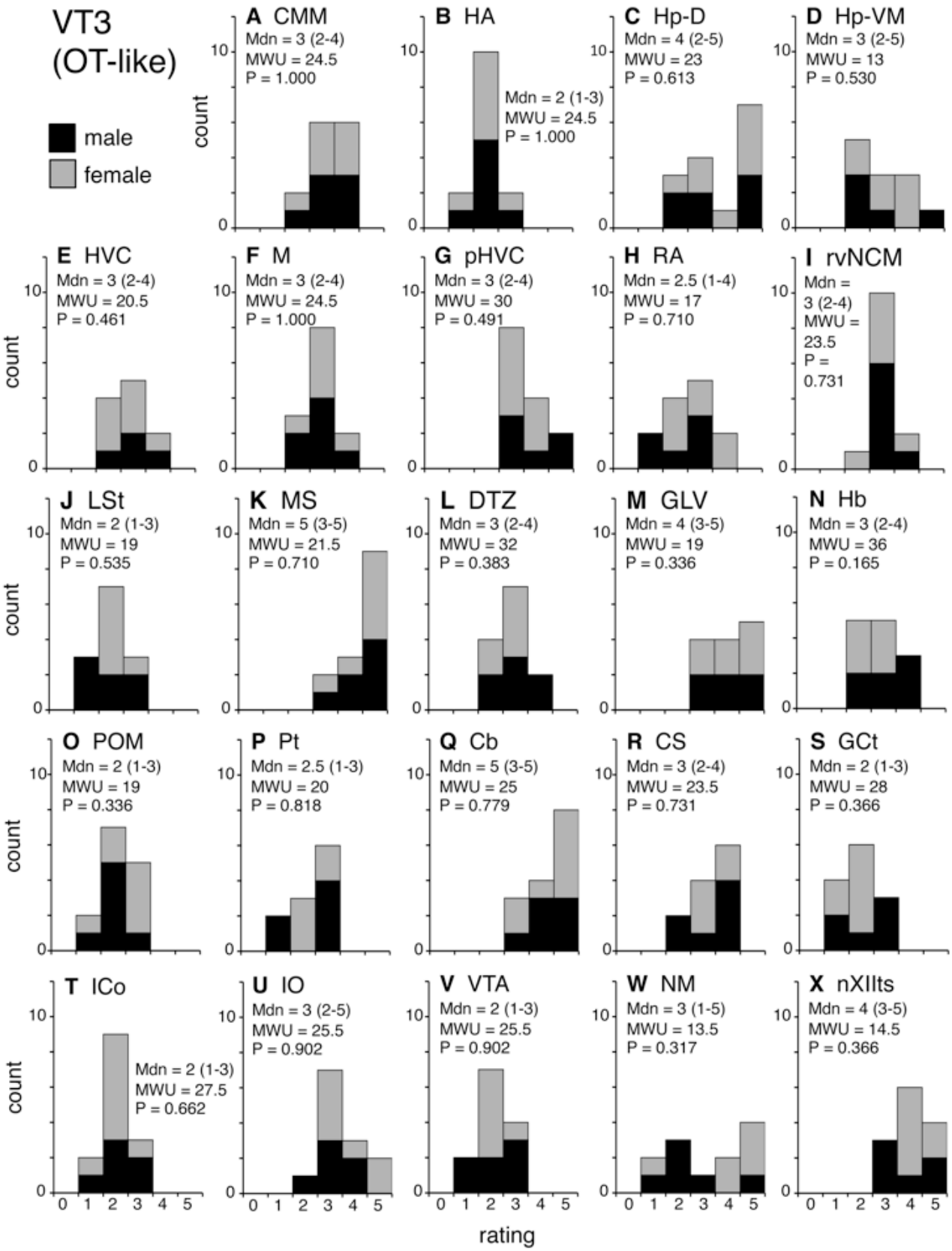
Macaque OTR
Rat OTR
Tammar wallaby MTR
Chicken VT3
Zebra finch VT3
Sparrow VT3



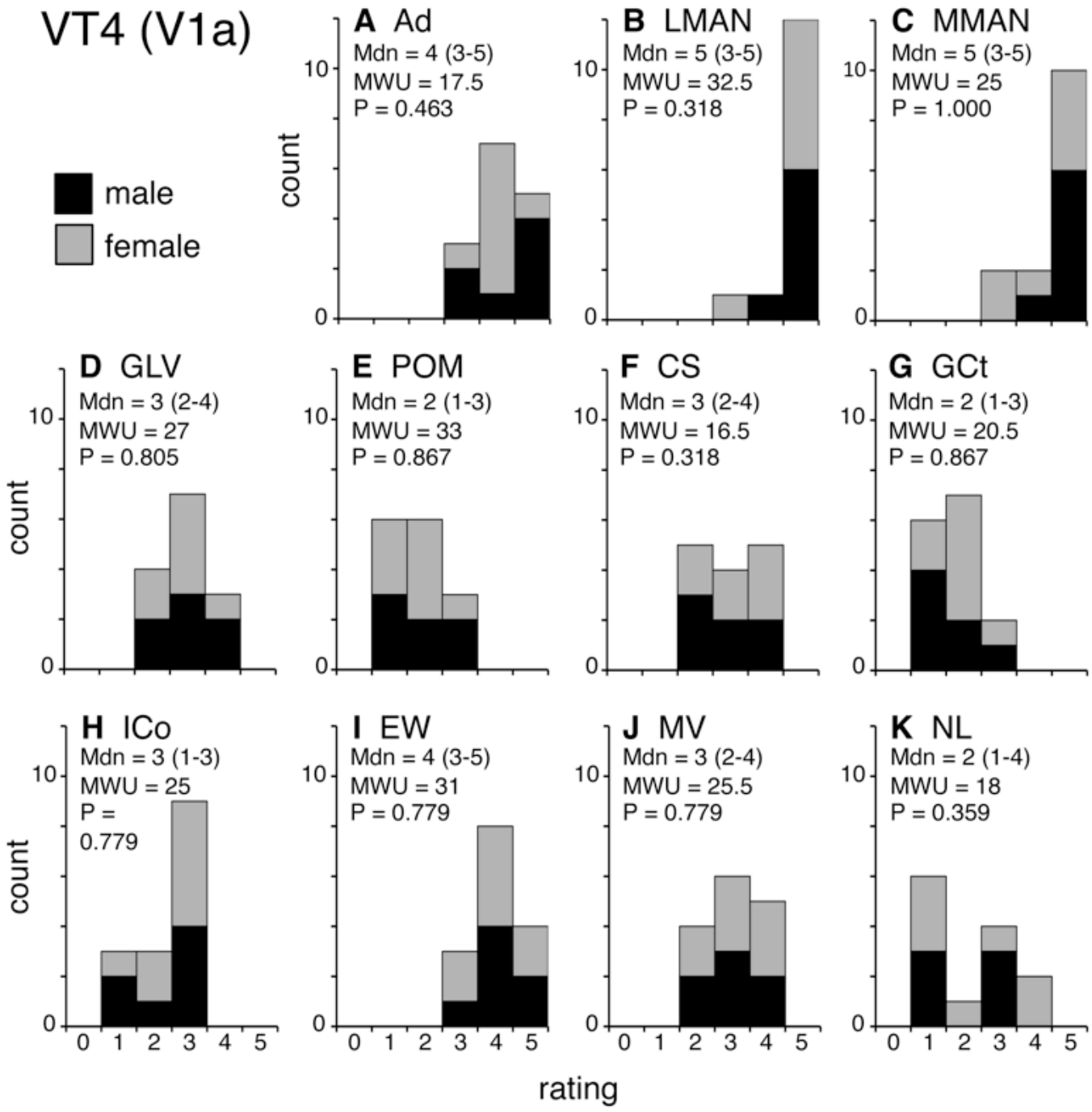
Supplemental Fig. 1. Comparison of *in situ* hybridization signal seen with antisense riboprobes (A, C) and sense (control) riboprobes (B, D). For most mRNAs in both species, no signal was observed with the sense riboprobe. For example, compare the signal obtained with the antisense VT3 riboprobe (A) to that obtained with the sense VT3 riboprobe (B) in white-throated sparrow. In some cases, for example VT2 receptor mRNA, identical signal was obtained in the cerebellum with both sense and antisense probes (not shown) and was thus considered nonspecific. Only one sense riboprobe produced a discernable signal that overlapped with antisense signal outside the cerebellum: in sparrow, weak labeling was obtained using the VT4 (V1a) sense riboprobe (D; compare to antisense in C). The same distribution of VT4 receptor mRNA was observed using a second antisense riboprobe containing a different region of the VT4 receptor sequence (shown in Fig. 10F). Note that the distribution of labeling in D is quite different from the VT3 receptor distribution in an adjacent section from the same brain (A).



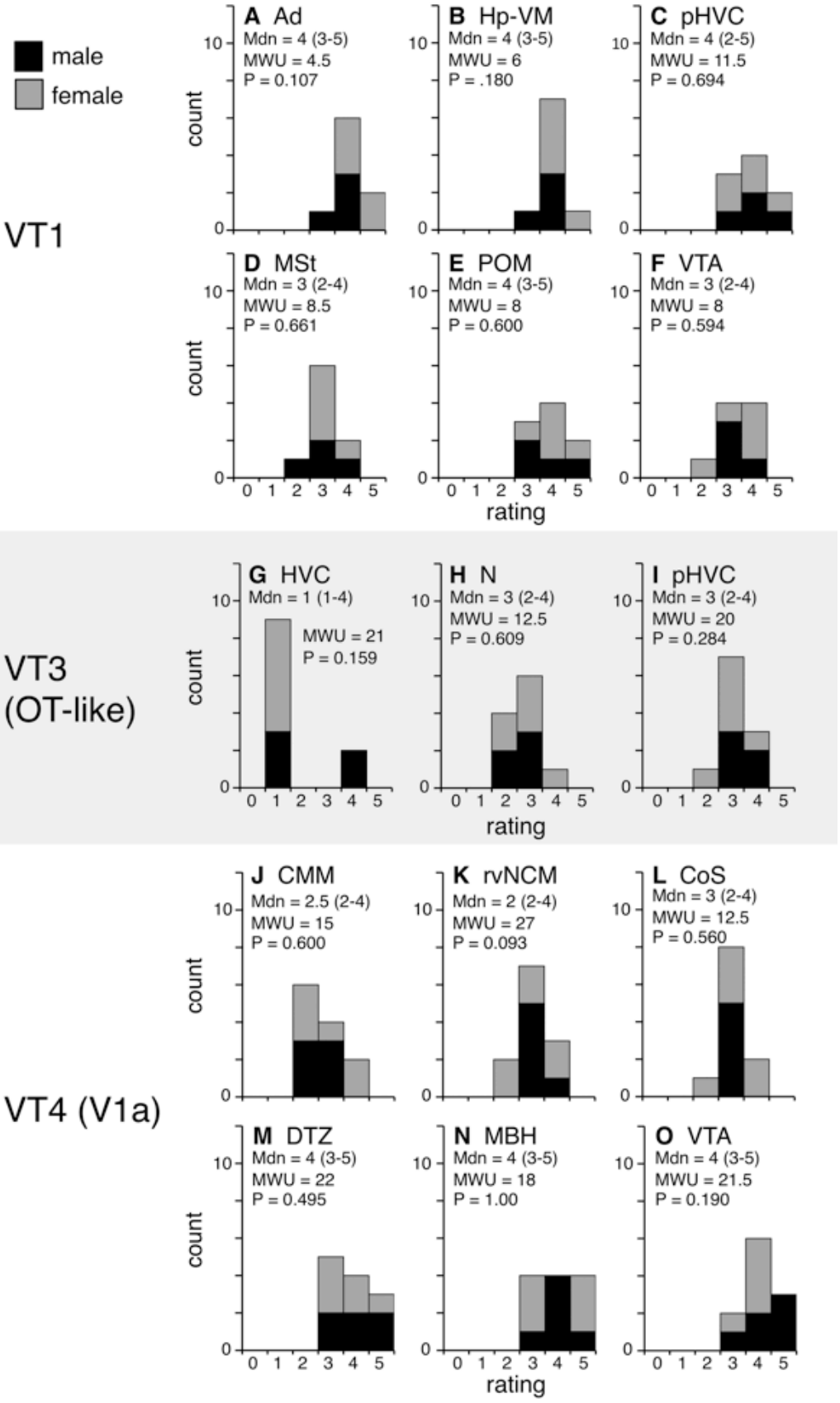
Supplemental Fig. 2. Histograms showing individual variation in intensity of VT1 mRNA signal in white-throated sparrow. Medians (Mdn) and ranges are given for each region, as well as the results (MWU and P values) of a comparison between males and females. There were no sex differences in any of the regions of interest. Regions are listed in the order they appear in Table 2.



Supplemental Fig. 3. Histograms showing individual variation in intensity of VT3 mRNA signal in white-throated sparrow. Medians (Mdn) and ranges are given for each region, as well as the results (MWU and P values) of a comparison between males and females. There were no sex differences in any of the regions of interest. Regions are listed in the order they appear in Table 2.



Supplemental Fig. 4. Histograms showing individual variation in intensity of VT4 mRNA signal in white-throated sparrow. Medians (Mdn) and ranges are given for each region, as well as the results (MWU and P values) of a comparison between males and females. There were no sex differences in any of the regions of interest. Regions are listed in the order they appear in Table 2.



Supplemental Fig. 5. Histograms showing individual variation in intensity of VT receptor mRNA signal in zebra finch. Medians (Mdn) and ranges are given for each region, as well as the results (MWU and P values) of a comparison between males and females. There were no sex differences in any of the regions of interest. Within each mRNA, regions are listed in the order they appear in Table 2.