# Supporting Information

# Effect of acute pesticide exposure on bee spatial working memory using an analogue of the radial-arm maze

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## Supporting Methods

### Pesticide treatment

Thiamethoxam doses were calculated to simulate a bee foraging for one hour on oilseed rape nectar contaminated with thiamethoxam at two concentrations over a range that can be found in the field: 2.4ppb, based on residues found in *B. terrestris* nectar pots (Thompson *et al.* 2013) and oilseed rape nectar in honeybee crops (Pilling *et al.* 2013) and 10ppb, based on residues in nectar of treated plants (Pohorecka *et al.* 2012; Sanchez-Bayo & Goka 2014). A foraging worker must consume 0.54 calories per minute (Heinrich 1979), so 32.4 calories are required to sustain a foraging trip of one hour. The mean sugar content of 'Samourai' oilseed rape nectar is 21.5% (Pierre *et al.* 1999), and sugar contains approximately 4cal $mg^{-1}$ (Heinrich 1979). The amount of nectar (in mg) consumed for one hour of foraging can be calculated as calories required/(sugar content x cal $mg^{-1}$ of sugar), so a bee would need

to ingest 37.7mg (≈µl) of the nectar collected during an hour on oilseed rape. This equates to 0.091ng of active ingredient per bee at a 2.4ppb concentration and 0.377ng at 10ppb. This is within the range estimated for one hour of foraging in honeybees (at 10-40% sugar concentrations; EFSA 2012).

Feeding 37.7µl of sucrose solution to a bee prior to the task is likely to lower motivation, so this volume was halved and the concentration of sugar and pesticide doubled to keep the amount of active ingredient received by each bee the same. Bees were therefore fed 18.85µl of 43% (w/w) sucrose solution containing either 0ppb (control), 4.8ppb (0.091ng per bee), 20ppb (0.377 ng per bee) and 133ppb (2.5 ng per bee) thiamethoxam. As in previous studies into acute effects of pesticides, the full dose was provided in one feed (Henry *et al.* 2012; Stanley, Smith & Raine 2015).

To obtain the required concentrations, we dissolved 100mg thiamethoxam ($C_8H_{10}ClN_5O_3S$ powder; PESTANAL® analytical standard, Sigma Aldrich, Poole, UK) in 100ml distilled water to produce a 1 ppt stock solution. Each fortnight aliquots of 1.2µl, 5µl and 33.3µl of the stock solution were diluted with 250ml 43% Brix sucrose solution to produce 4.8ppb, 20ppb and 133ppb thiamethoxam solutions respectively.

**Radial Arm Maze (RAM) design**

The RAM tests working spatial memory by requiring animals to remember which reward locations they have visited and avoid revisits (Foreman & Ermakova 1998). The original RAM was designed for rodents, for which a central chamber is appropriate to prevent animals moving from one reward location to the next in a circle; i.e. it reduces (but not eliminates) the use of stereotypical behaviour so that spatial working memory can be better identified. However, this approach is not perfectly suited to all animals; in particular, flying animals may behave unnaturally in an enclosed arm set-up. As such, previous studies on birds have used “open field” versions of the RAM to mimic a more natural setting, in which no central chamber is used (Balda & Kamil 1988; Hilton & Krebs 1990; Healy & Hurly 1995); our design refers to this approach. Bumblebee within-patch foraging typically involves flying between flowers/inflorescences (Pyke & Cartar 1992), so our RAM apparatus that requires bees to fly attempts

to represent a more ecologically relevant foraging decision than one where bees walk through maze arms.

The baffles between flowers fulfil a similar role to the rodent RAM central chamber in greatly reducing but not eliminating stereotypical behaviour (bees can fly over baffles, but cannot move directly from flower to flower), and preventing bees from seeing any other flowers when on a particular flower, meaning that each represents an independent reward location (like arms of the rodent RAM) and that bees fly out of the array to see and subsequently visit the other flowers, to some degree mimicking the return from an arm to the centre of the rodent RAM (Olton & Samuelson 1976). The apparatus was located in a cue-rich laboratory environment with constant lighting during testing; cues such as arena walls and baffles did not differ between treatments and no additional landmarks were provided in the foraging arena as cue use was not being explicitly tested.

**Behaviour on the RAM**

The vast majority of the time, bees fed on the full 10µl of sucrose solution once the proboscis made contact. On the rare occasions some solution remained, the flower was exchanged for a clean, empty one as usual once the bee had left. A revisit to the most recent flower visited was only counted if >20 seconds of flying occurred between visits. Exchanging visited flowers for clean ones was done from the back of the maze while the bee was feeding on its next flower to minimise disturbance.

For the first bout, 20µl droplets of sucrose solution were used to increase motivation; for the subsequent nine training bouts and the final testing bout 10µl drops were used to ensure all flowers could be visited before satiation. On the last flower, the experimenter increased the size of the drop to allow the bee to fill its crop and return to the colony (Burmeister, Couvillon & Bitterman 1995).

Access to the nest box was blocked while a bee was in the arena to encourage it to visit all eight flowers; however, if the bee made three attempts to return before visiting all eight flowers the entrance was unblocked and the bee allowed to return to the nest box to minimise loss of motivation. The entrance was also unblocked after a bee had been in the arena for longer than 20 minutes; if 30 minutes elapsed

the bee was guided towards the entrance with a plastic pot. In the final bout, recording of visits was stopped after 30 minutes, as a longer period in the maze may not be an accurate test of working memory.

To administer the pesticide dose after the tenth bout, the bee was intercepted on its way to the arena by manipulating the tunnel doors to direct the bee into a plastic pot (diameter: 60mm). After allowing the bee to acclimatise for approximately one minute, 18.85µl of sucrose solution containing the relevant dose of pesticide was fed to the bee using a pipette inserted through a hole in the pot.

**Statistical analysis**

We employed the AIC-IT approach as this does not only test the support for the null hypothesis but also the plausibility of the alternative hypothesis or hypotheses, making it appropriate for use in controlled experiments as well as observational studies (Lukacs *et al.* 2007; Richards, Whittingham & Stephens 2011). In addition, we view the AIC-IT approach as providing further advantages over the Fisherian approach because in cases where two or more models are almost as good (have similar AIC values), it is possible to perform model averaging to get a more accurate estimate of effect size based on weighted support (Johnson & Omland 2004). Quoting effect sizes and confidence intervals rather than p-values is based on current recommended practice (Nakagawa & Cuthill 2007; Halsey *et al.* 2015).

AIC was chosen over AICc (corrected AIC for small sample sizes) as AICc can be overly conservative and has been shown to have little advantage over AIC (Richards 2005; Raffalovich *et al.* 2008). The R packages glmmADMB (Skaug *et al.* 2014), survival (Therneau 2015), MuMIn (Barton 2015), plotrix (Lemon 2006) and lattice (Sarkar 2008) were used for model fitting and producing graphs.

## Pilot study

Before the experiment began, 14 bees from a separate colony were tested on the RAM to confirm that bees would interact with the maze and feed from the artificial flowers and to ascertain the number of training bouts needed before asymptotic performance was reached. Ten training bouts were used because the data for both correct choices in first the eight choices and total revisits showed performance reached an asymptote by bout ten (Fig. S2).

Six bees were also tested on a partially baited version of the maze to ensure bees were not able to detect the presence of a reward behind the flowers (e.g. by olfaction). Four flowers were randomly selected to be baited and the first four unique (non-repeated) flower visits recorded. Mean number of visits to baited flowers in the first four unique choices was 1.83, which was not significantly different from the chance expectation of 2 (one-sample t-test, t=-0.30715, df=5, p=0.7711).

## Supporting Results and Discussion

Repeating the analysis excluding bees that did not visit all eight flowers (n=13) did not affect the outcome so the final analysis includes all 61 bees.

*Total revisits*

Analysing the full dataset but omitting the positive control still showed an effect on total revisits at field-realistic doses. Removing the high dose reduced support for the model containing a size * treatment interaction but increased support for the other models including treatment (Table S2). Model averaged effect size estimates (MAE) showed a significant difference between LD.091 and the control (MAE = 0.417, 95% CIs = [0.027– 0.807], Table S3), indicating that this field-realistic dose increased total revists.

One observation included a bee in the high treatment group with thorax width 6.07mm and 28 total revisits. To examine the effect of this observation on the treatment * size interaction found for total revisits, we repeated the analysis without this data point. Removing the observation reduced support for the model containing the treatment * size interaction (ΔAIC to best model: 2.84) although the treatment * size interaction at the high treatment level was still significant in that model (estimate = 2.291, 95% CIs = [0.311 – 4.271]) . Model averaging of the new best model set (treatment, size and basic) showed a significant positive effect of treatment on total revisits in the high dose (model averaged estimate = 0.434, 95% CIs = [0.001 – 0.867]), despite the highest total revisits value having been omitted.

*Correct choices before first revisit*

Analysing the data without the positive control did not change the outcome, with the model containing treatment only having the lowest AIC (ΔAIC to next best model = 2) with the hazard ratio (HR) for LD.091 positive and significantly different to the control (HR = 2.276, 95% CIs = [1.077– 4.810], Table S3).

*Monte Carlo simulation and behaviour on the RAM*

Holding the bee in the tunnel for 45 minutes following pesticide exposure did not visibly decrease motivation (bees flew directly to the array on entering the arena) and had no effect on maze performance (no difference between final training bout and testing bout in control bees for total revisits ($t = 0.458$, $df = 27.0$, $p = 0.651$), correct choices in the first eight choices ($t = -1.0132$, $df = 27.908$, $p = 0.3197$) and correct choices before first revisit ($t = -1.395$, $df = 29.5$, $p = 0.174$)).

The Monte Carlo simulations may overestimate total revisits as choices are made continuously until all eight flowers have been visited, whereas bees have physical limitations which are likely to restrict the total number of choices that can be made. To assess this, a conservative upper limit for number of revisits (18; two standard deviations above the observed mean) was applied to both the observed and C+S simulation data and the means recalculated. The simulated means remained outside the 95% CIs of the observed data (C = 9.94, C+S = 9.95, observed = 5.14[3.84-6.45]). Only bouts where all eight flowers were visited were included in the total revisits analysis (n=30). Every arrival at a flower ("visit", "revisit", "land" and "approach") was included in the transition probability matrix (Fig. S1a).

Stereotypical behaviour improved simulated RAM performance in terms of correct choices before the first revisit and correct choices in the first eight choices (see Fig. 5 in main text), due in part to the moderate contiguity ("nearest neighbour") preference and a low probability of revisiting the flower last visited. However, stereotypical behaviour increased total revisits; the most likely explanation for this is the unequal total frequencies with which each flower was visited (Fig. S1a). The contiguity preference and tendency to travel upwards is consistent with known bumblebee foraging behaviour (Pyke 1978).

147 Interestingly, bumblebee RAM performance (mean correct choices in the first eight choices (C) = 5.9)
148 was not as high as in rats, for whom the maze was designed (C = 7.6; Bond, Cook & Lamb 1981), or
149 other vertebrates including blue tits (C = 6.8; Hilton & Krebs 1990), *Betta splendens* (C = 6.6; Roitblat,
150 Tham & Golub 1982) and pigeons (C = 6.3; Bond, Cook & Lamb 1981). This comparatively low but
151 better-than-chance performance is consistent with two previous studies using an analogue of the RAM
152 in honeybees (Brown & Demas 1994; Brown *et al.* 1997).

153 The success of applying our modified RAM to bumblebees is likely due to their ecological requirements
154 as nectarivores, as the apparatus has been shown to be most relevant in species for whom the test mimics
155 natural foraging ecology (Bond, Cook & Lamb 1981; Sulikowski & Burke 2010). Furthermore, bees
156 perform a repeated foraging bout behaviour that can be exploited for this task. As such there is
157 considerable potential for the application of this established lab technique in the study of the quantitative
158 effects of pesticides and other stressors such as parasites on bumblebee cognition and learning
159 behaviours.

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| **a** | Correct choices in first eight choices | | |
|---|---|---|---|
| Model | AIC | ΔAIC | $w_i$ |
| **Basic** | **542** | **0** | **0.525** |
| **Size** | **543** | **0.92** | **0.331** |
| Treatment | 545.6 | 3.52 | 0.09 |
| Treatment + size | 546.7 | 4.66 | 0.051 |
| Treatment * size | 552.1 | 10.09 | 0.003 |
| **b** | Time per visit | | |
| Model | AIC | ΔAIC | $w_i$ |
| **Size** | **15** | **0** | **0.913** |
| Basic | 20.2 | 5.26 | 0.066 |
| Treatment + size | 22.7 | 7.71 | 0.019 |
| Treatment * size | 28.3 | 13.34 | 0.001 |
| Treatment | 32 | 17.06 | 0 |

**Table S1.** Tables of candidate models to investigate the effect of pesticide treatment and bee size on a) correct choices in first eight choices using binomial GLMMs and b) duration divided by total visits using linear mixed models. The basic model included the constant and the residual variance, with all other models containing the basic model plus the indicated covariates. Models are presented in order of ΔAIC from the best model alongside their respective Akaike weights ($w_i$). The best set of models (models <2 ΔAIC from the model with the lowest AIC) is highlighted in bold.

| **a** | Total revisits | | |
|---|---|---|---|
| Model | AIC | ΔAIC | $w_i$ |
| **Treatment** | **285.3** | **0** | **0.303** |
| **Treatment + Size** | **285.7** | **0.3** | **0.256** |
| **Size** | **286.1** | **0.7** | **0.209** |
| **Basic** | **286.3** | **1.0** | **0.186** |
| Treatment * Size | 289.1 | 3.8 | 0.046 |
| **b** | Choices before first revisit | | |
| Model | AIC | ΔAIC | $w_i$ |
| **Treatment** | **273** | **0** | **0.474** |
| Treatment + Size | 275 | 2 | 0.174 |
| Size | 275.5 | 2.5 | 0.136 |
| Basic | 275.6 | 2.6 | 0.129 |
| Treatment * Size | 276.4 | 3.4 | 0.087 |
| **c** | Time per visit | | |
| Model | AIC | ΔAIC | $w_i$ |
| **Size** | **13** | **0** | **0.75** |
| Basic | 15.6 | 2.55 | 0.209 |
| Treatment + Size | 19.3 | 6.32 | 0.032 |
| Treatment * Size | 22.3 | 9.33 | 0.007 |
| Treatment | 24.6 | 11.55 | 0.002 |

**Table S2.** Analysis including field-realistic doses only of the effect of pesticide on RAM performance measures. Tables show candidate models a) using negative binomial GLMMs to investigate the effect of pesticide treatment and bee size on total revisits, b) using Cox proportional hazards models to investigate the effect of pesticide treatment and bee size on choices before first revisit and c) using linear mixed models to investigate the effect of pesticide treatment and bee size on log-transformed duration divided by total visits. In all cases, the basic model included the constant and the residual variance, with all other models containing the basic model plus the indicated covariates. Models are presented in order of ΔAIC from the best model alongside their respective Akaike weights (wi). The best sets of models which were averaged to obtain model averaged estimates (models <2 ΔAIC from the model with the lowest AIC) are highlighted in bold.

| a | Total revisits | | | |
|---|---|---|---|---|
| Parameters | Estimate | Std. Error | 95% CIs | |
| | | | Lower | Upper |
| (Intercept) | 3.167 | 1.513 | 0.202 | 6.132 |
| Treatment (LD.377) | 0.344 | 0.205 | -0.058 | 0.745 |
| **Treatment (LD.091)** | **0.417** | **0.199** | **0.027** | **0.807** |
| Size | -0.358 | 0.259 | -0.865 | 0.150 |

| b | Correct choices before first revisit | | | | |
|---|---|---|---|---|---|
| Parameters | Regression coefficient (b) | Std. Error (SE(b)) | Hazard Ratio ($e^b$) | 95% CIs on Hazard Ratio | |
| | | | | Lower | Upper |
| Treatment (LD.377) | 0.295 | 0.366 | 1.343 | 0.656 | 2.751 |
| **Treatment (LD.091)** | **0.823** | **0.382** | **2.276** | **1.077** | **4.810** |

| c | Time per visit | | | |
|---|---|---|---|---|
| Parameters | Estimate | Std. Error | 95% CIs | |
| | | | Lower | Upper |
| (Intercept) | 1.563 | 0.643 | 0.302 | 2.824 |
| **Size** | **-0.323** | **0.118** | **-0.555** | **-0.091** |

**Table S3.** Analysis including field-realistic doses only of the effect of pesticide on RAM performance measures. Tables show model averaged coefficients and 95% confidence intervals (CIs) for the optimal model set to predict a) total revisits (treatment, treatment + size, size only and basic models with colony as a random effect), b) correct choices before first revisit (treatment only model) and c) time per visit (duration divided by total visits; size only model with colony as a random effect). Parameters highlighted in bold are considered important to the model based on 95% CIs.

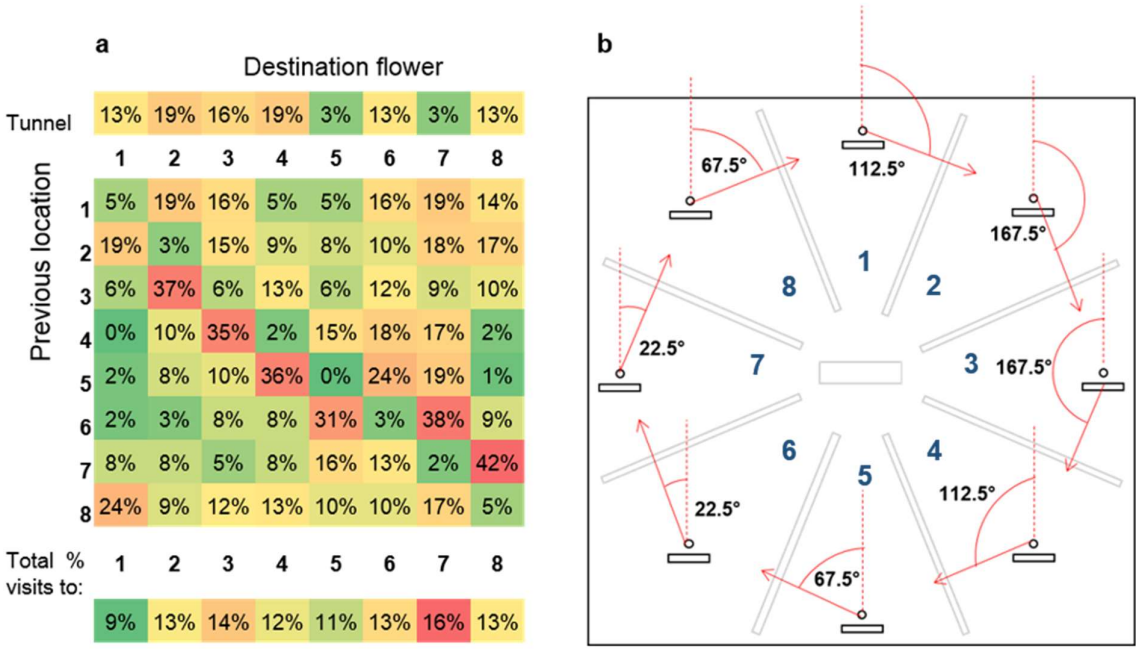


**Figure S1**. a) Global transition probability matrix showing probabilities of moving from the tunnel (arena entrance) or a flower to each other possible flower. This was used to construct a Monte Carlo simulation of performance on the radial arm maze (RAM) using stereotypical behaviour only. b) Schematic of the RAM with the angle from vertical of each transition between neighbouring flowers. Angle from vertical was negatively correlated with transition probability (Spearman's rho = -0.87).

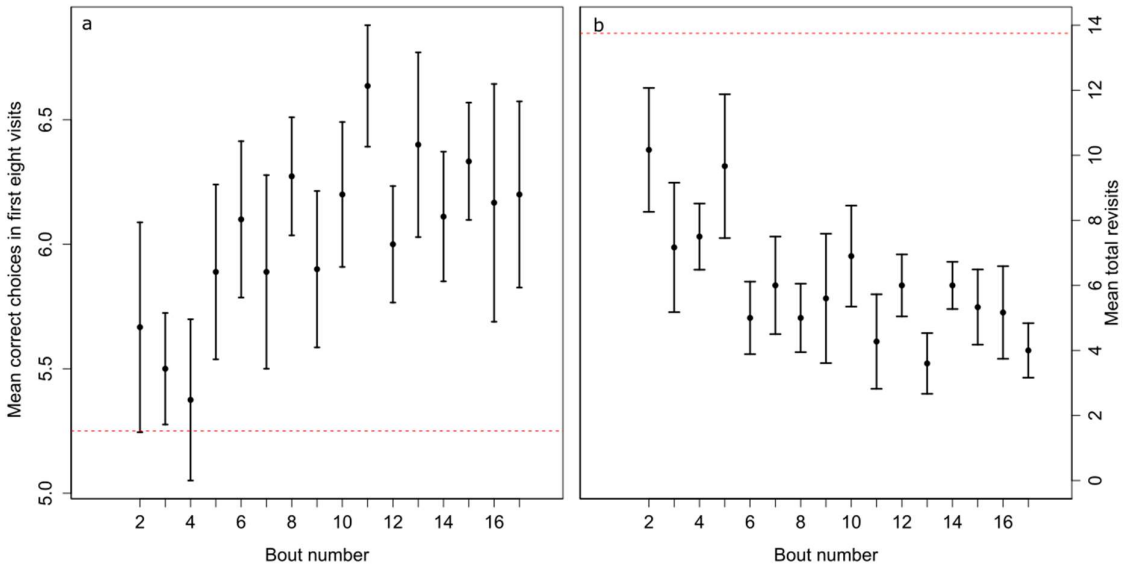


**Figure S2**. Data from a pilot study using 14 bees showing a) mean correct choices in first eight choices (±SE) and b) mean total revisits (±SE) by bout number. Expected chance performance for both measures is indicated by a red dashed line.

**Figure S3.** *(Below)* Outputs of two Monte Carlo simulations run with 1,000,000 iterations simulating a) pure chance (C) and b) chance plus stereotypical behaviour (C+S), showing frequency tables and means for three measures of RAM performance: correct choices in first eight choices, correct choices before first revisit and total revisits. The frequency tables for total revisits are truncated at 75 revisits, although the actual data from which the means are calculated include values greater than 75. Mean total revisits with revisits capped at 18 is also shown to account for physical limitations on maximum number of revisits in the observed data. The simulations were coded using C++.

287 Fig. S3 cont.

a) Chance

| Mean total revisits | |
|---|---|
| Total revisits | Frequency |
| 0 | 2440 |
| 1 | 8565 |
| 2 | 17274 |
| 3 | 27222 |
| 4 | 37588 |
| 5 | 46498 |
| 6 | 52243 |
| 7 | 56392 |
| 8 | 58477 |
| 9 | 58648 |
| 10 | 58021 |
| 11 | 55353 |
| 12 | 52128 |
| 13 | 48313 |
| 14 | 44875 |
| 15 | 41354 |
| 16 | 37258 |
| 17 | 33944 |
| 18 | 30152 |
| 19 | 27091 |
| 20 | 24379 |
| 21 | 21657 |
| 22 | 19033 |
| 23 | 17094 |
| 24 | 15037 |
| 25 | 13217 |
| 26 | 11703 |
| 27 | 10420 |
| 28 | 9009 |
| 29 | 7976 |
| 30 | 6876 |
| 31 | 6327 |
| 32 | 5438 |
| 33 | 4666 |
| 34 | 4123 |
| 35 | 3666 |
| 36 | 3159 |
| 37 | 2745 |
| 38 | 2497 |
| 39 | 2162 |
| 40 | 1887 |
| 41 | 1684 |
| 42 | 1418 |
| 43 | 1254 |
| 44 | 1023 |
| 45 | 922 |
| 46 | 846 |
| 47 | 777 |
| 48 | 710 |
| 49 | 586 |
| 50 | 503 |
| 51 | 453 |
| 52 | 381 |
| 53 | 303 |
| 54 | 267 |
| 55 | 240 |
| 56 | 217 |
| 57 | 198 |
| 58 | 160 |
| 59 | 147 |
| 60 | 120 |
| 61 | 114 |
| 62 | 95 |
| 63 | 75 |
| 64 | 88 |
| 65 | 57 |
| 66 | 56 |
| 67 | 54 |
| 68 | 42 |
| 69 | 38 |
| 70 | 37 |
| 71 | 28 |
| 72 | 15 |
| 73 | 28 |
| 74 | 15 |
| 75 | 13 |

b) Chance + stereotypical behaviour

| Mean total revisits | |
|---|---|
| Total revisits | Frequency |
| 0 | 2791 |
| 1 | 9202 |
| 2 | 17527 |
| 3 | 26408 |
| 4 | 34862 |
| 5 | 41101 |
| 6 | 46002 |
| 7 | 48663 |
| 8 | 50387 |
| 9 | 50541 |
| 10 | 49129 |
| 11 | 47999 |
| 12 | 45453 |
| 13 | 42794 |
| 14 | 40797 |
| 15 | 37913 |
| 16 | 34899 |
| 17 | 32403 |
| 18 | 29809 |
| 19 | 27403 |
| 20 | 24663 |
| 21 | 22993 |
| 22 | 20865 |
| 23 | 19027 |
| 24 | 17081 |
| 25 | 15801 |
| 26 | 14321 |
| 27 | 12811 |
| 28 | 11901 |
| 29 | 10642 |
| 30 | 9948 |
| 31 | 8940 |
| 32 | 8235 |
| 33 | 7399 |
| 34 | 6695 |
| 35 | 6144 |
| 36 | 5649 |
| 37 | 5181 |
| 38 | 4696 |
| 39 | 4208 |
| 40 | 3875 |
| 41 | 3595 |
| 42 | 3268 |
| 43 | 2938 |
| 44 | 2740 |
| 45 | 2474 |
| 46 | 2350 |
| 47 | 2053 |
| 48 | 1900 |
| 49 | 1767 |
| 50 | 1593 |
| 51 | 1467 |
| 52 | 1395 |
| 53 | 1298 |
| 54 | 1098 |
| 55 | 1102 |
| 56 | 936 |
| 57 | 882 |
| 58 | 783 |
| 59 | 763 |
| 60 | 692 |
| 61 | 597 |
| 62 | 598 |
| 63 | 550 |
| 64 | 472 |
| 65 | 437 |
| 66 | 409 |
| 67 | 376 |
| 68 | 351 |
| 69 | 325 |
| 70 | 302 |
| 71 | 262 |
| 72 | 247 |
| 73 | 224 |
| 74 | 214 |
| 75 | 205 |

288 Fig. S3 cont.

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