Bonferroni's correction not Tukey's should be used for making pairwise comparisons in experiments with few replicates.

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Abstract:

Statistical tests can be used to help determine whether experimental manipulations produce effects. In tests of means, when more than two groups are compared the number of false positive results increases unless a correction is used. Tukey's test is thought to offer good control of the false positive rate and high statistical power when all pairwise comparisons are made. However, the number of replicates in laboratory experiments is often quite low, and small sample sizes can undermine assumptions underlying statistical methods. I used simulations to investigate how well Tukey's test controls the false positive rate when there are 3- 6 experimental groups and 2- 6 experimental replicates, conditions that span the range of typical values, and found that it generates too many false positives. I investigated 11 other approaches to controlling the false positive rate and found that none is as effective as the simple Bonferroni correction or offers much more power. I conclude that researchers should not make all pairwise comparisons using Tukey's test but instead use Bonferroni's correction on a limited number of pre-selected comparisons.

Introduction:

Statistical testing of experimental data is a key analysis step at multiple points during the process that leads to discovery of new therapeutic targets, lead compounds, chemical probes, and drugs. Statistical testing of sample means generates p values that indicate the probability of observing data with means at least as different as those obtained if the means of the underlying populations are identical (1–3). Each statistical comparison carries a risk of producing a false positive- a p value below the threshold selected for statistical significance- even if there is no true difference. When more than two means are compared, the overall false positive rate (FPR) increases above the intended significance threshold (called α and by convention usually set at 5%) unless a correction is applied. Several approaches for controlling the FPR in the context of multiple comparisons have been developed (4,5). Many can be applied directly to the results of a series of t-tests, but some advocate performing analysis of variance (ANOVA) first and only proceeding to pairwise comparisons if the ANOVA yields a significant p value as an additional safeguard against false positives (see e.g., (5)). This approach is not universally accepted, though, because it may unnecessarily decrease statistical power (6). Power, the ability to detect true differences, is discussed further below. However, a key benefit of performing ANOVA first is that models that include treatment together with experimental replicate as a blocking factor (two-factor ANOVA) can be used and comparisons between conditions made using the model's results for treatment, decreasing the effects of between-replicates variability and creating an extremely effective analysis strategy (7,8).

Approaches for controlling false positives differ in the kind of error rate they seek to manage. Some limit the probability of obtaining one or more false positives among all significant findings- a measure called the false discovery rate (FDR)- which is a relatively lenient standard. Others limit the probability of obtaining any false positives at all, known as the family-wise error rate (FWER), a stricter criterion (9). The simplest strategy for controlling the FWER is Bonferroni's correction, which involves adjusting α by dividing the level chosen for significance by the total number of comparisons. For example, conducting 10 comparisons at an α of 0.05 would require using a significance threshold of $p < 0.005$ per test. However, Bonferroni's correction reduces statistical power in a manner that increases linearly with the number of comparisons made. Power is higher when differences in means are larger, experimental variability (noise) is smaller, sample size (number of replicates) is larger, or the significance threshold is less stringent (10). To control the FWER while maximizing power, Dunnett's test is commonly recommended when multiple experimental groups are compared to a single control group. Tukey's test (Tukey's HSD) is frequently employed when making all possible pairwise comparisons. Other sophisticated methods, such as "step-up" or "step-down" modifications of Bonferroni's procedure, have been developed in an attempt to enhance power while still controlling error rates (see (4) for an overview).

Much of the work that underlies the modern discovery process involves well-controlled laboratory experiments in which researchers wish to control the FWER. Experiments typically involve relatively small sample sizes; performing 3 replicates is common, 2 replicates are not unheard of, and more than 6 replicates are rare. Concerns that small

sample sizes can compromise statistical tests have prompted evaluation of the performance of tests for 2 sample means (t tests), showing that these largely work as expected (11,12), but to my knowledge the effects of very small sample sizes when multiple means are compared has not been investigated. I examined how well procedures designed to control the FWER perform in experiments with 2- 6 replicates and found that Dunnett's test effectively maintained control of the FWER in comparisons of multiple treatment groups to a single control, but when multiple pairwise comparisons were made, Tukey's test often exceeded the intended FWER threshold, resulting in a higher-than-desired number of false positives. Evaluation of alternative procedures revealed that none provided superior control of the FWER or significantly greater power than the Bonferroni method.

Methods:

All simulation were performed using R software (13) running in the R studio IDE. Packages used include multcomp (14) and agricolae (15). To investigate the FWER, two-factor ANOVA was performed on simulated data from 3- 6 treatment groups in 2- 6 replicates, with the factors being “treatment” and “replicate”, mimicking the most effective way to analyze more than 2 group means in replicated experimental data (7,8). The mean of each of the groups was set to 100, the standard deviation (SD) of each group was set to 5, and shared variation with an SD of 20 was then added. Tests comparing individual group means were only performed if the p value of the ANOVA for “treatment” was < 0.05. Besides mimicking the most effective analysis strategy, an advantage of performing ANOVA first is that it reduced the overall computational burden by limiting the number of iterations in which further comparisons needed to be calculated. It is likely that the total FWER would have been higher for all tests if the ANOVA had not been run first. The total number of p values < 0.05 when comparing groups to a single control was summed to generate the overall FWER rate for Dunnett’s test, and for all pairwise comparisons for other tests. 100,000 simulations were performed to estimate the FWER, except in a preliminary screen conducted to eliminate computationally-intensive methods that failed to control the FWER well that used only 1000 iterations. Estimates of the error with which the FWER is measured after 100,000 iterations ranged from 1% CV for the omnibus ANOVA test to ~ 3% for the BY procedure which has a very low mean FWER (See Supplemental Table 1). To estimate power, the mean of one group was varied from 2.5 to 50 and the mean of 2-5 others was set to 100. Independent variation was set to 5% and shared variation to 20%. 10,000 simulations were performed, and for each, the number

of p values < 0.05 was measured and converted to power by dividing by the total number of p values < 0.05 expected if all real differences were found. An R script is included as supplementary information that will allow any of the calculations in this work to be made.

Results:

Dunnett's and Bonferroni's procedures control the FWER well when sample sizes are small, but Tukey's test does not.

I first examined the FWER following two-factor ANOVA, modeling "treatment" as one factor and "replicate" as the second. If the p value for the ANOVA was < 0.05, I compared all experimental conditions to a single "control" with or without Dunnett's correction (Figure 1). As mentioned previously, I chose two-factor ANOVA because it helps mitigate the impact of between-run variability on power and is applicable whether samples are independent or matched (7,8). Simulations were performed for 3- 6 experimental groups, each with 2- 6 replicates. The total number of p-values < 0.05 was used to estimate the FWER. When only two replicates were simulated, Dunnett's test resulted in a slightly elevated FWER (~6.25%) regardless of group number. However, if there were three or more replicates the FWER remained at or below 5%.

I then conducted a similar analysis making all pairwise comparisons. If the omnibus ANOVA test for treatment was significant ($p < 0.05$), I summed the number of p-values < 0.05 for pairwise comparisons calculated without correction, with Bonferroni's correction, or with Tukey's correction (Figure 2). Bonferroni's procedure consistently controlled the FWER at 5% across all conditions, but Tukey's test did not. The highest FWER values for Tukey's procedure occurred when there were only two replicates and six groups. The FWER decreased as the number of replicates increased or the number of groups decreased. I found that when there are 3 replicates, the FWER reached a maximum at 6-7 treatment groups and then stayed at a constant elevated level as additional groups

were added (See Supplemental Figure 1. I confirmed the elevated FWER with Tukey's test using three different implementations of the procedure in R: glht() from the multcomp package, TukeyHSD() from stats, and pairs() from emmeans (not shown), although I cannot be certain that these represent genuinely independent implementations of the test.

Testing the efficacy of other strategies that control the FWER.

I next tested 11 other procedures for controlling either the FWER or the false discovery rate (FDR), all of which are available in R and could be applied after two-factor ANOVA. These included the Benjamini-Hochberg (BH), Benjamini-Yekutieli (BY), Holm, Hochberg, Shaffer, Sidak, and Westfall procedures from package multcomp, and the Duncan, REGW, Scheffé, and SNK procedures from package Agricolae (see references (16,17)). Methods supported by the package multcomp make use of the full ANOVA object, while those from package agricolae use a different strategy involving an estimate of the variance associated with the factor "treatment" and thus may not be fully comparable to the multcomp methods. Initial comparisons were based on 1,000 simulations to limit computation time. Of the methods examined, the BY, Hochberg, Holm, Hommel, Scheffé, and Shaffer procedures reduced the FWER more effectively than Tukey's test. I then ran 100,000 simulations for the most promising methods (Figure 3). Among these, BY consistently kept the FWER well below 5%, Bonferroni maintained it at 5%, and Holm kept it at or below 6%.

Finally, I compared the statistical power of the BY, Holm, and Bonferroni procedures to Tukey's test. I simulated experiments in which the mean of one group differed from those

of 2- 5 other groups using 3 replicates per group. Power was calculated as the number of significant p-values < 0.05 divided by ($n_{groups} - 1$), the maximum number of true positives expected if all real differences were detected. Consistent with their respective impacts on the FWER, Tukey's test provided the highest power, and BY the lowest. The Holm procedure had higher power than Bonferroni, but the advantage decreased as the number of groups increased.

Discussion:

Using Tukey's test, the family-wise error rate (FWER) can exceed 8% when three replicates- perhaps the most common value reported in laboratory experiments- are performed. A p-value is intended to convey the probability of observing data differing as much as those collected assuming the null hypothesis, that means are the same, is true. If a p-value < 0.05 does not mean that there is a 5% chance of such data under the null, but rather some other chance, its already limited interpretive value is further diminished.

I simulated two-factor ANOVA because it is the best choice for analyzing replicated experiments. If there is variation between replicates, two-factor ANOVA minimizes its effects and results in high power when post-hoc comparisons are made using the results for the "treatment" factor. If there is no or minimal variation between replicates, two-way ANOVA gives results that differ only slightly from one-way ANOVA. However, I did confirm that the effects of the various procedures on the FWER that I observed using two-factor ANOVA held true following one-way ANOVA (Supplemental Figure 3).

Among the methods for controlling the FWER tested, none performed as well as the Bonferroni correction, and none offered a substantial gain in power. This makes Bonferroni the best option. However, it is worth noting that using Bonferroni's correction does reduce power compared to using Tukey's test (Figure 4). While power can be increased by adding replicates, this requires additional time and resources. Fortunately, power can also be enhanced simply by reducing the total number of comparisons made. Of course, this requires pre-specifying a subset of comparisons of interest before the

experiment begins, an approach that has the advantage of preventing "HARKing" (Hypothesizing After the Results are Known) and data dredging (18,19). Importantly, focusing on preselected contrasts does not mean sacrificing a valuable statistical tool, as making all pairwise comparisons is rarely a good idea. Pairwise comparisons that are expected to yield non-significant results are useless and should not be made, because when a test yields a p-value above the chosen significance threshold, the correct interpretation is simply that there is no evidence that the population means differ, not that they are the same. Moreover, two major classes of experiments that are frequently analyzed using all-pairs comparisons followed by Tukey's test are better handled in other ways. The first class includes factorial experiments where the effect of one variable (e.g., presence vs. absence of drugs, or genetic manipulations) is assessed in the context of a second variable that also has two or more levels. These studies are best analyzed using a two-factor ANOVA with an interaction term. This directly tests whether the effect of one factor depends on the level of the other, a question that pairwise comparisons cannot answer. The second class includes dose-response or time-course experiments, which may also be conducted across different conditions such as genotypes or drug types. Rather than performing all-pairs comparisons at each time point or dose, more appropriate approaches include linear regression (20), which can be performed on raw or transformed data, mixed models, analysis of theory-derived parameters (e.g., IC_{50}, K_d, V_{max}, time constants, etc.), or statistical tests performed on summary measures like area under the curve (AUC). These approaches incorporate the structure of time and/or dose into the analysis, making use of important information that Tukey's test ignores and gaining power as a result. In fact, in these contexts, using all-pairs comparisons generally

results in scientifically meaningless conclusions, because whether an observed difference between specific time points, conditions, or doses is statistically significant depends as much on sample size and variability as on the size of the effect.

When experiments of the two kinds discussed above are analyzed using methods more appropriate to their design, few scenarios remain in which all-pairs comparison is the best option. Of relevance to the readership of *SLAS Discovery*, the most appropriate context for all-pairs comparisons may be exploratory early-stage research. There, though, the goal is hypothesis generation rather than formal testing. A strong argument can be made that no correction for multiple comparisons should be applied when results are intended to guide future experiments, as those will reveal which effects are genuine, and correction for multiple comparisons might decrease power to find interesting candidates to pursue.

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Figure Legends

Figure 1: Dunnett's test controls the FWER when treatment groups are compared to a control. Family-wide error rate (FWER) with no correction (A) or Dunnett's correction (B). Two-factor ANOVA with one factor being group and the other replicate was performed with the means of 3 (dark blue squares), 4 (mustard triangles), 5 (light blue diamonds) and 6 (black circles) groups all set to 100, independent variation of 5%, and shared variation of 20%, for 2-6 simulated replicates. If the p value of the ANOVA result for "treatment" was < 0.05, comparisons between group 1 and all other groups were made. The total number of p values < 0.05 in 100,000 simulations was used to determine the FWER.

Figure 2. Tukey's test does not control the FWER well when all pairwise comparisons are made. FWER with no correction (A), Bonferroni's correction (B) or Tukey's correction (C) when all possible pairwise connections are made with the means of 3 (dark blue squares), 4 (mustard triangles), 5 (light blue diamonds) and 6 (black circles) groups all set to 100, independent variation of 5%, and shared variation of 20%, for 2-6 simulated replicates. The total number of p values < 0.05 in 100,000 simulations was used to determine the FWER.

Figure 3. FWER with a selection of other control strategies when all pairwise comparisons are made. 11 approaches to controlling the FWER were tested in an initial round using 1000 simulations (Supplemental Figure 2). 7 were selected for comparison

to Bonferroni's and Tukey's approaches using 100,000 simulations. Colors and symbols are as in Figure 1 and 2.

Figure 4. Power of Bonferroni, BY, Holm and Tukey procedures when making pairwise comparisons. Plots of power of the Bonferroni (blue squares), BY (mauve triangles), Holm (mustard diamonds) and Tukey procedures (black circles) following two-factor ANOVA. Power was calculated by dividing the total number of p values < 0.05 in each simulation by the total number of possible differences, n-1, where n is the number of groups. The x axis of each plot is the difference in mean of the first group divided by independent variation, a convenient way of normalizing effect sizes. 10,000 simulations were performed with the mean of one group set to values ranging from 2.5 to 50 and the mean of 2-5 others set to 100, with independent variation of 5% and shared variation of 20%. The total number of groups is indicated in each panel's banner.

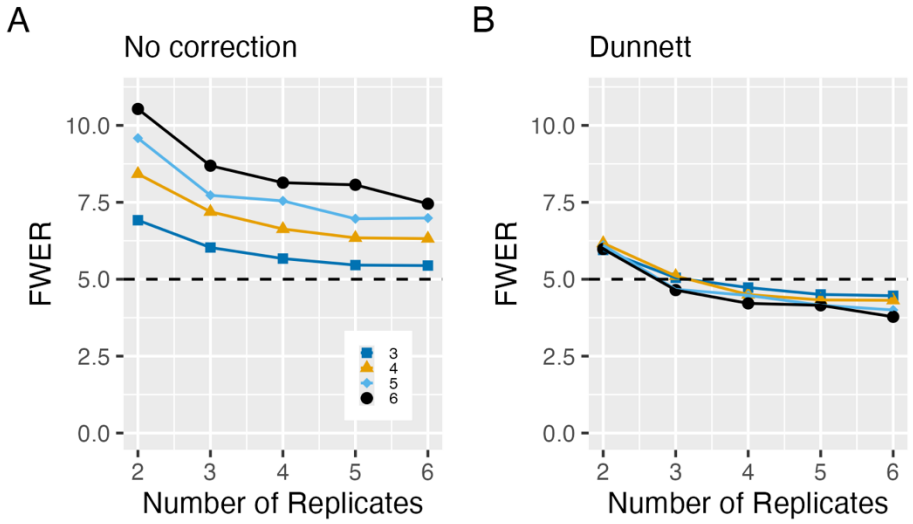

A
No correction
FWER
10.0
7.5
5.0
2.5
0.0
2
3
4
5
6
Number of Replicates
3
4
5
6
B
Dunnett
FWER
10.0
7.5
5.0
2.5
0.0
2
3
4
5
6
Number of Replicates

Figure 1

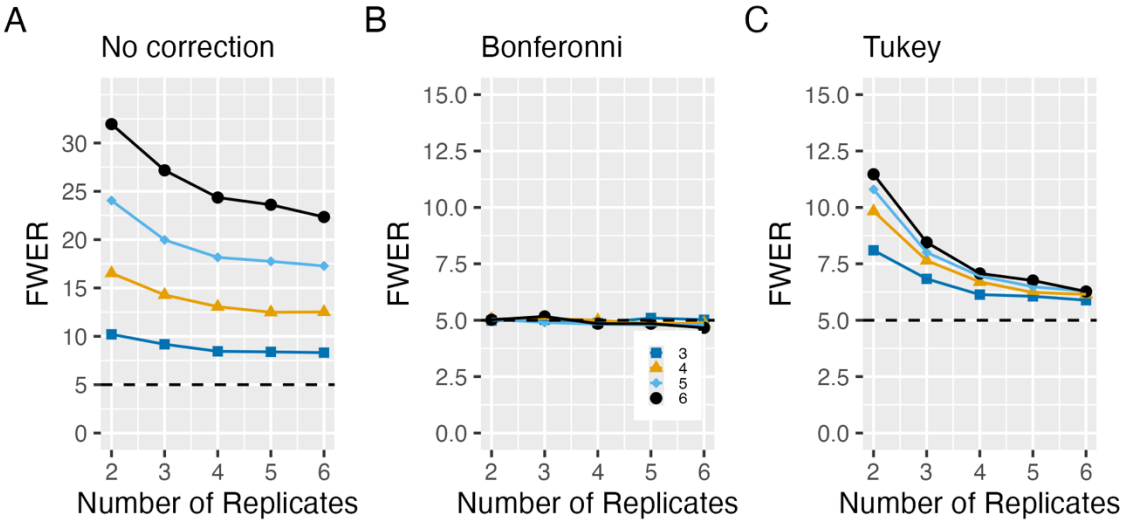
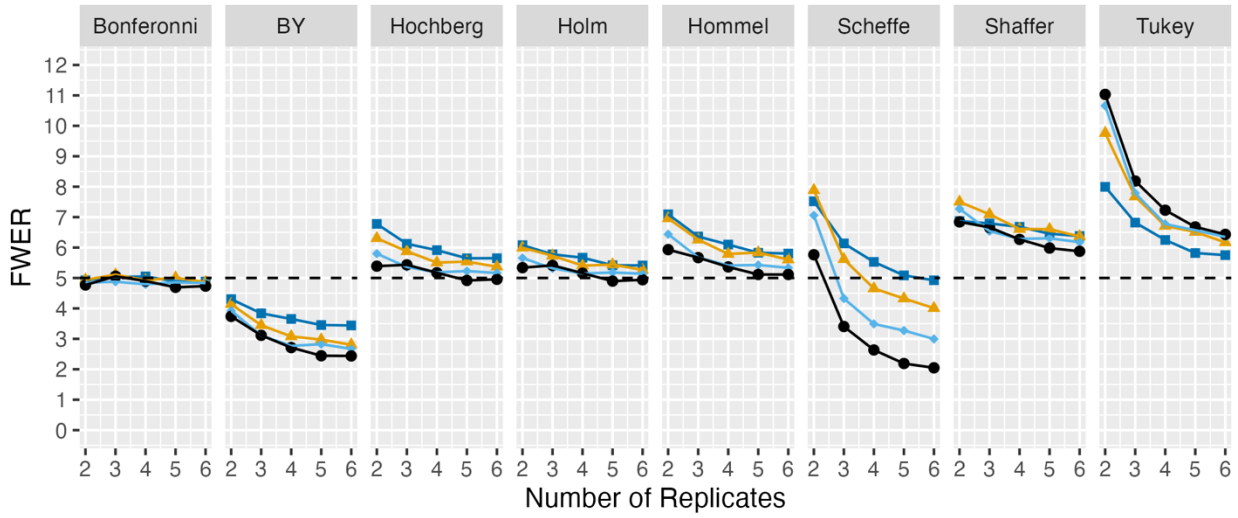

A
No correction
FWER
Number of Replicates
B
Bonferonni
FWER
Number of Replicates
3
4
5
6
C
Tukey
FWER
Number of Replicates

Figure 2


Bonferonni
BY
Hochberg
Holm
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Scheffe
Shaffer
Tukey
FWER
0
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2 3 4 5 6
Number of Replicates

Figure 3

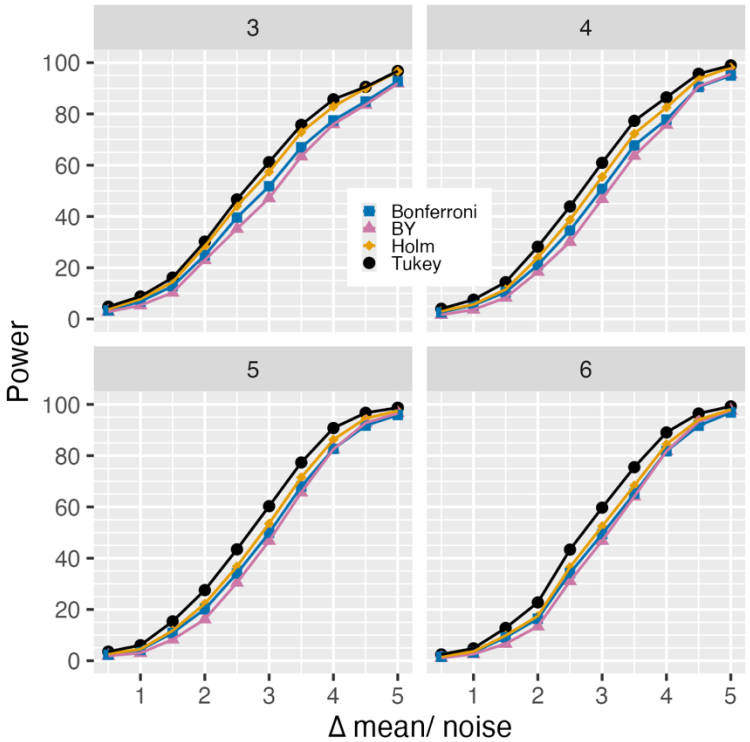
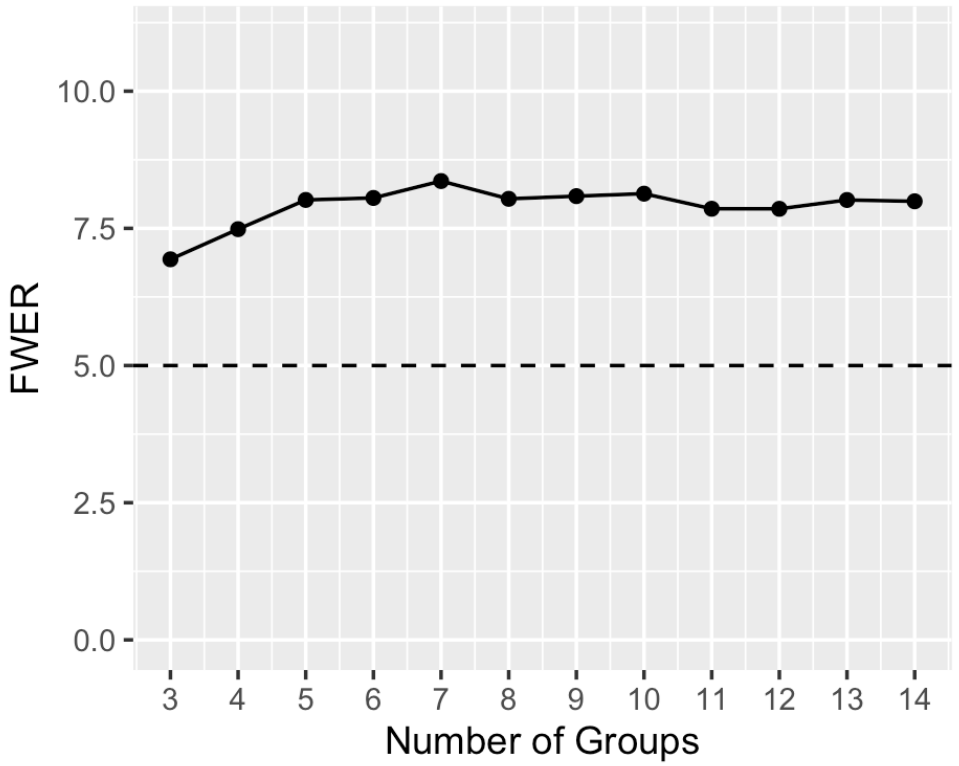

3
4
5
6
Power
100
80
60
40
20
0
1
2
3
4
5
Δ mean/ noise
Bonferroni
BY
Holm
Tukey

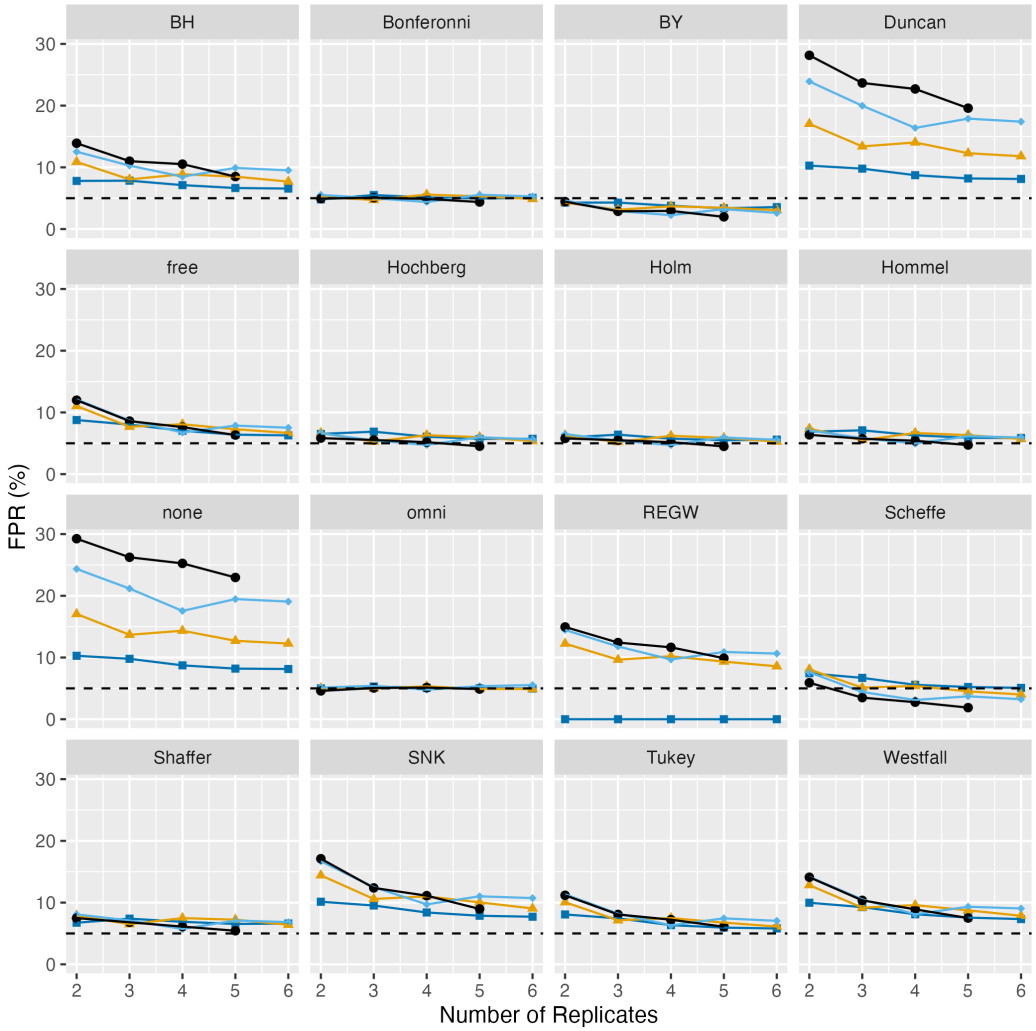
Figure 4

Supplemental information

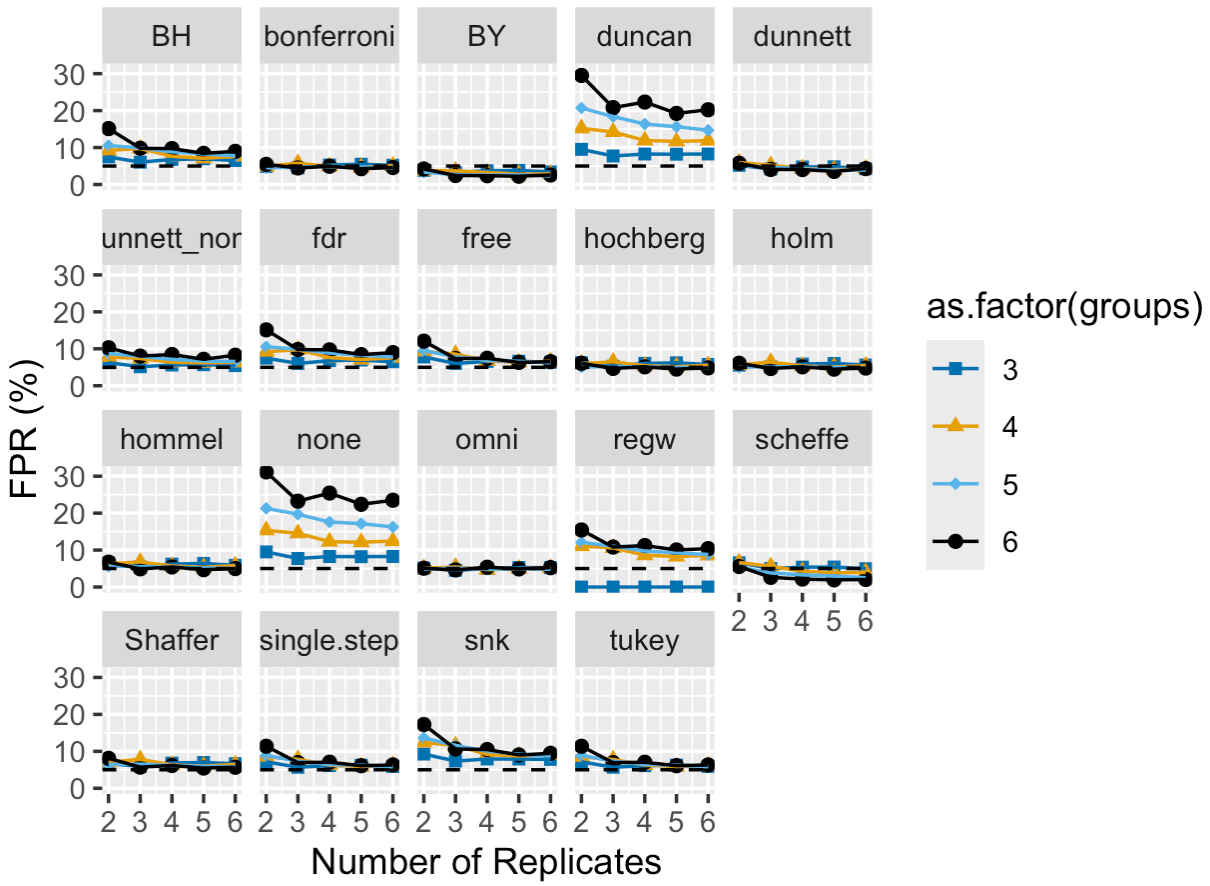
Supplementary Figure 1: FWER of Tukey's test with 3 replicates and from 3-15 sample groups.



Supplementary Figure 2: Screening other strategies for controlling the FWER with 10000 iterations prior to select a subset for more detailed analysis.



Supplementary Figure 3: FWER following one-way ANOVA assessed with 10,000 iterations.



Supplemental Table: Errors associated with estimates of the FWER.

Procedure	Mean	CI lower	CI upper	%_CV
Tukey	7559.66667	7412.49303	7706.8403	1.85511811
Hommel	6141.33333	5985.83733	6296.82934	2.41268572

Holm	5600.16667	5452.35573	5747.97761	2.51506848
Hochberg	5763.33333	5607.24353	5919.42314	2.58074443
BY	3364.5	3248.41942	3480.58058	3.28763223
BH	8809.16667	8650.49781	8967.83553	1.71633109
Bonferonni	4972.83333	4844.2213	5101.44537	2.46446075
Shaffer	6926.66667	6765.86153	7087.47181	2.21217676
Westfall	9758.16667	9555.37028	9960.96306	1.98032366
SNK	11217	11022.7552	11411.2448	1.65012544
REGW	10052.3333	9862.24271	10242.424	1.80193034
Scheffe	5496.83333	5357.74701	5635.91965	2.41110454
Duncan	13879.3333	13653.7143	14104.9524	1.54899985
Anova omnibus	5010.16667	4926.70013	5093.6332	1.58746592
free	8200.83333	8016.97243	8384.69424	2.1363657
No correction	14180.6667	13950.5116	14410.8217	1.5465646

6 repeats of 100,000 simulations of the FWER were performed with 4 groups and 3 replicates and the mean, 95% CI and percent CV of the responses were calculated.

R Code.

```
# ============================== ==============================
# Use this to perform calculations of the FWER (delta_vals <-c(0)), or power
# (e.g. delta_vals<- seq(0,25,5)). First, load the packages.
# Then, go to section 2 and choose the correction methods (the full list is
# commented out, so either un-comment it or create a subset), the parameters for
# the simulation, and the number of iterations to be performed. Then highlight
# the entire text and run. Bear in mind that these simulations can take hours to
# in some cases days, depending on the number of conditions and the number of
# iterations.
#
# 1. LOAD REQUIRED PACKAGES
packages <- c("multcomp", "agricolae", "dplyr", "stats")
lapply(packages, require, character.only = TRUE)

# 2. DEFINE SIMULATION PARAMETERS
samp_vals   <- seq(2,6,1)
group_vals  <- seq(3, 6,1)
sd_vals     <- c(5)
sds_vals    <- c(20)
delta_vals  <- c(0)

# methods_to_use <- c("none", "bonferroni", "holm", "hochberg", "hommel", "BH", "BY",
#                     "fdr", "single-step", "Westfall", "Shaffer", "free", "tukey",
#                     "scheffe", "duncan", "snk", "regw", "dunnett")

methods_to_use <- c("none", "bonferroni","dunnett","tukey","Westfall")

scenarios <- expand.grid(samp = samp_vals, groups = group_vals,
                         sd_val = sd_vals, sds_val = sds_vals, delta = delta_vals)
```

```
n_scenarios <- nrow(scenarios)

numb <- 100000
results_mat_overall <- matrix(NA, nrow = n_scenarios,
                              ncol = length(methods_to_use) + 7)
colnames(results_mat_overall) <- c(methods_to_use, "Dunnett_none", "omni", "samp",
                                   "groups", "sd_val", "sds_val", "delta")

# 3. HELPER FUNCTIONS
standardize_contrast <- function(x) {
  parts <- unlist(strsplit(x, "\\s*-\\s*"))
  paste(sort(parts), collapse = " - ")
}

get_pairwise_pvals <- function(res_aov2, vframe, methods_vec) {
  expt_levels <- levels(vframe$expt)
  all_pairs <- combn(expt_levels, 2, simplify = FALSE)
  contr_names <- sapply(all_pairs, function(x) paste(sort(x), collapse = " - "))

  pvals_list <- vector("list", length(methods_vec) + 1)
  names(pvals_list) <- c(methods_vec, "Dunnett_none")
  for (m in names(pvals_list)) {
    pvals_list[[m]] <- setNames(rep(NA, length(contr_names)), contr_names)
  }

  p_omni <- summary(res_aov2)[[1]][["Pr(>F)"]][1]
  if (p_omni >= 0.05) return(pvals_list)

  post_test_tukey <- glht(res_aov2, linfct = mcp(expt = "Tukey"))
```

```
get_glht_pvals <- function(x) {
  comp_names_std <- sapply(names(x$test$coefficients), standardize_contrast)
  setNames(x$test$pvalues, comp_names_std)[contr_names]
}

valid_multcomp_methods <- c("none", "bonferroni", "holm", "hochberg",
                            "hommel", "BH", "BY", "fdr",
                            "single-step", "Westfall", "Shaffer", "free", "dunnett")

agricolae_methods <- c("scheffe", "duncan", "snk", "regw")

for (meth in methods_vec) {
  if (meth %in% valid_multcomp_methods && meth != "dunnett") {
    res <- summary(post_test_tukey, test = adjusted(meth))
    pvals_list[[meth]] <- get_glht_pvals(res)

  } else if (meth == "tukey") {
    res <- summary(post_test_tukey, test = adjusted("single-step"))
    pvals_list[[meth]] <- get_glht_pvals(res)

  } else if (meth == "dunnett") {
    post_test_dunnett <- glht(res_aov2, linfct = mcp(expt = "Dunnett"))
    res <- summary(post_test_dunnett)
    comp_names_std <- sapply(names(res$test$coefficients), standardize_contrast)
    pvals_list[[meth]] <- setNames(res$test$pvalues, comp_names_std)[contr_names]
    pvals_list[["Dunnett_none"]] <- setNames(summary(post_test_dunnett, test =
adjusted("none"))$test$pvalues, comp_names_std)[contr_names]

  } else if (meth %in% agricolae_methods) {
    if (meth == "scheffe") {
      pvals_list[[meth]] <- scheffe.test(res_aov2, "expt", group = FALSE)$comparison$pvalue
```

```
      } else if (meth == "duncan") {
        pvals_list[[meth]] <- duncan.test(res_aov2, "expt", group = FALSE)$comparison$pvalue
      } else if (meth == "snk") {
        pvals_list[[meth]] <- SNK.test(res_aov2, "expt", group = FALSE)$comparison$pvalue
      } else if (meth == "regw") {
        if (nlevels(vframe$expt) > 3) {
          pvals_list[[meth]] <- REGW.test(res_aov2, "expt", group = FALSE)$comparison$pvalue
        }
      }
    }
  }
  return(pvals_list)
}

# 4. MAIN SIMULATION LOOP
set.seed(123)
for (i in 1:n_scenarios) {
  samp_val   <- scenarios$samp[i]
  groups_val <- scenarios$groups[i]
  sd_val     <- scenarios$sd_val[i]
  sds_val    <- scenarios$sds_val[i]
  delta      <- scenarios$delta[i]

  iter_with_sig <- setNames(rep(0, length(methods_to_use) + 1), c(methods_to_use, "Dunnett_none"))
  omni_count <- 0

  for (j in 1:numb) {
    base <- c(rnorm(samp_val, 100 + delta, sd_val),
              rnorm(samp_val * (groups_val - 1), 100, sd_val))
    noise <- rep(rnorm(samp_val, 0, sds_val), groups_val)
```

```
    vframe <- data.frame(
      expt = factor(rep(1:groups_val, each = samp_val)),
      pair = factor(rep(1:samp_val, groups_val)),
      value = base + noise
    )

    res_aov2 <- aov(value ~ expt + pair, data = vframe)
    p_omni <- summary(res_aov2)[[1]][["Pr(>F)"]][1]
    if (p_omni < 0.05) omni_count <- omni_count + 1

    posthoc_pvals <- get_pairwise_pvals(res_aov2, vframe, methods_to_use)
    for (mn in c(methods_to_use, "Dunnett_none")) {
      current_pvals <- posthoc_pvals[[mn]]
      if (!is.null(current_pvals)) {
        iter_with_sig[mn] <- iter_with_sig[mn] + sum(current_pvals < 0.05, na.rm = TRUE)
      }
    }
  }

  results_mat_overall[i, c(methods_to_use, "Dunnett_none")] <- iter_with_sig
  results_mat_overall[i, "omni"]        <- omni_count
  results_mat_overall[i, "samp"]        <- samp_val
  results_mat_overall[i, "groups"]      <- groups_val
  results_mat_overall[i, "sd_val"]      <- sd_val
  results_mat_overall[i, "sds_val"]     <- sds_val
  results_mat_overall[i, "delta"]       <- delta
}

# 5. DISPLAY RESULTS
```

```
results_df <- as.data.frame(results_mat_overall)

View(results_df)
```