

Analysis of Sex Differences in Morphine Deposition in the Plasma, Retina & Brain

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Reading in & tidying the data

```
sex_diff <- read.csv("../data/morphine_retina_cx_plasma_sex_diff.csv",
                     fileEncoding = 'UTF-8-BOM')
sex_diff$conc_wt <- (sex_diff$raw_conc/sex_diff$tissue_weight)
sex_diff$log_conc_wt <- log(sex_diff$conc_wt)
sex_diff$log_conc_raw <- log(sex_diff$raw_conc)

sex_diff <- sex_diff %>% mutate(tissue = factor(as.factor(tissue),
                                              c("plasma", "retina", "brain")),
                              group = factor(as.factor(group),
                                              c("male", "lowE", "highE")),
                              animal = as.factor(animal))

str(sex_diff)

## 'data.frame':   111 obs. of  11 variables:
## $ animal      : Factor w/ 37 levels "11F","12F","13F",...: 10 20 27 28 30 32 34 36 37 9 ...
## $ raw_conc    : num  56.6 22.1 36.8 49.5 87.4 ...
## $ tissue_weight: num  20.1 10.3 13.7 15.1 31.4 14.7 14.3 20.8 15.7 20.5 ...
## $ body_wt     : num  27.2 30.1 28.7 25.8 26.1 27.3 26.2 23.7 27.2 22 ...
## $ dose        : num  20.6 18.6 19.5 21.7 20 19.1 19.9 22 19.2 19.1 ...
## $ stage       : chr   "male" "male" "male" "male" ...
## $ group       : Factor w/ 3 levels "male","lowE",...: 1 1 1 1 1 1 1 1 3 ...
## $ tissue      : Factor w/ 3 levels "plasma","retina",...: 3 3 3 3 3 3 3 3 3 ...
## $ conc_wt     : num   2.82 2.14 2.68 3.28 2.78 ...
## $ log_conc_wt : num   1.035 0.763 0.987 1.188 1.024 ...
## $ log_conc_raw: num   4.04 3.09 3.6 3.9 4.47 ...

#test for outliers
test_out <- rosnerTest(sex_diff$conc_wt,
                      k = 4
)

## Warning in rosnerTest(sex_diff$conc_wt, k = 4): 37 observations with NA/NaN/Inf
## in 'x' removed.
```

```
test_out
```

```
## $distribution
## [1] "Normal"
##
## $statistic
##      R.1      R.2      R.3      R.4
## 2.398976 2.438932 2.194539 2.214792
##
## $sample.size
## [1] 74
##
## $parameters
## k
## 4
##
## $alpha
## [1] 0.05
##
## $crit.value
## lambda.1 lambda.2 lambda.3 lambda.4
## 3.277970 3.273006 3.267957 3.262821
##
## $n.outliers
## [1] 0
##
## $alternative
## [1] "Up to 4 observations are not\n                                from the same Distribution."
##
## $method
## [1] "Rosner's Test for Outliers"
##
## $data
## [1] 2.815398 2.143670 2.683080 3.278901 2.783952 2.489524 2.914196
## [8] 3.023846 2.264051 2.126278 2.158567 1.099614 2.612317 2.321724
## [15] 2.150787 5.069100 1.959265 7.416833 1.840316 2.184907 1.887165
## [22] 2.675675 1.079808 3.696573 2.867145 1.989755 3.406589 2.709242
## [29] 2.220611 2.375481 3.545806 2.180149 2.090560 3.005871 2.745674
## [36] 2.489692 2.099267 27.561395 17.246940 24.693975 22.080050 23.070419
## [43] 13.959157 19.209173 20.999789 13.090263 16.161962 11.789479 7.405556
## [50] 17.296135 14.268936 15.960467 19.374612 15.713000 26.403452 23.226366
## [57] 25.703574 19.560540 17.330184 11.049851 31.412579 22.184283 15.172353
## [64] 22.980760 17.255130 15.395250 18.666395 34.880556 21.168358 22.425167
## [71] 35.646000 25.001300 20.076692 30.730114
##
## $data.name
## [1] "sex_diff$conc_wt"
##
## $bad.obs
## [1] 37
##
## $all.stats
##      i      Mean.i      SD.i      Value Obs.Num      R.i+1 lambda.i+1 Outlier
```

```
## 1 0 11.54799 10.045123 35.64600      71 2.398976   3.277970   FALSE
## 2 1 11.21788   9.702061 34.88056      68 2.438932   3.273006   FALSE
## 3 2 10.88924   9.352005 31.41258      61 2.194539   3.267957   FALSE
## 4 3 10.60018   9.088863 30.73011      74 2.214792   3.262821   FALSE
##
## attr("class")
## [1] "gofOutlier"
```

```
SumStat_sexdiff <- dplyr::summarise(group_by(sex_diff, tissue, group),
  n = n(),
  mean_conc_raw = mean(raw_conc),
  sd_conc_raw = sd(raw_conc),
  se_conc_raw = sd_conc_raw/sqrt(n),
  mean_conc_raw_log = mean(log(raw_conc)),
  sd_conc_raw_log = sd(log(raw_conc)),
  se_conc_raw_log = sd_conc_raw_log/sqrt(n),
  mean_conc_wt = mean(conc_wt),
  sd_conc_wt = sd(conc_wt),
  se_conc_wt = sd_conc_wt/sqrt(n),
  log_conc = mean(log(conc_wt)),
  sd_conc_log = sd(log(conc_wt)),
  se_conc_log = sd_conc_log/sqrt(n),
  mean_wt = mean(body_wt),
  sd_wt = sd(body_wt),
  mean_dose = mean(dose),
  sd_dose = sd(dose),
)
```

```
## 'summarise()' has grouped output by 'tissue'. You can override using the
## '.groups' argument.
```

```
SumStat_sexdiff
```

```
## # A tibble: 9 x 19
## # Groups:   tissue [3]
##   tissue group      n mean_conc~1 sd_co~2 se_co~3 mean_~4 sd_co~5 se_co~6 mean_~7
##   <fct> <fct> <int>      <dbl>   <dbl>   <dbl>   <dbl>   <dbl>   <dbl>   <dbl>
## 1 plasma male      9      346.    63.2    21.1     5.83    0.170    0.0567    NA
## 2 plasma lowE      8      331.    59.5    21.0     5.79    0.176    0.0621    NA
## 3 plasma highE     20      345.    84.4    18.9     5.80    0.343    0.0767    NA
## 4 retina male      9      96.6    12.7     4.22     4.56    0.133    0.0444   20.2
## 5 retina lowE      8      95.6    20.9     7.39     4.54    0.243    0.0860   20.6
## 6 retina highE     20      93.1    26.4     5.91     4.49    0.320    0.0715   20.5
## 7 brain  male      9      47.7    19.3     6.43     3.79    0.398    0.133     2.71
## 8 brain  lowE      8      46.4    23.5     8.30     3.73    0.488    0.173     2.53
## 9 brain  highE     20      40.0    23.4     5.22     3.56    0.501    0.112     2.69
## # ... with 9 more variables: sd_conc_wt <dbl>, se_conc_wt <dbl>,
## #   log_conc <dbl>, sd_conc_log <dbl>, se_conc_log <dbl>, mean_wt <dbl>,
## #   sd_wt <dbl>, mean_dose <dbl>, sd_dose <dbl>, and abbreviated variable names
## #   1: mean_conc_raw, 2: sd_conc_raw, 3: se_conc_raw, 4: mean_conc_raw_log,
## #   5: sd_conc_raw_log, 6: se_conc_raw_log, 7: mean_conc_wt
```

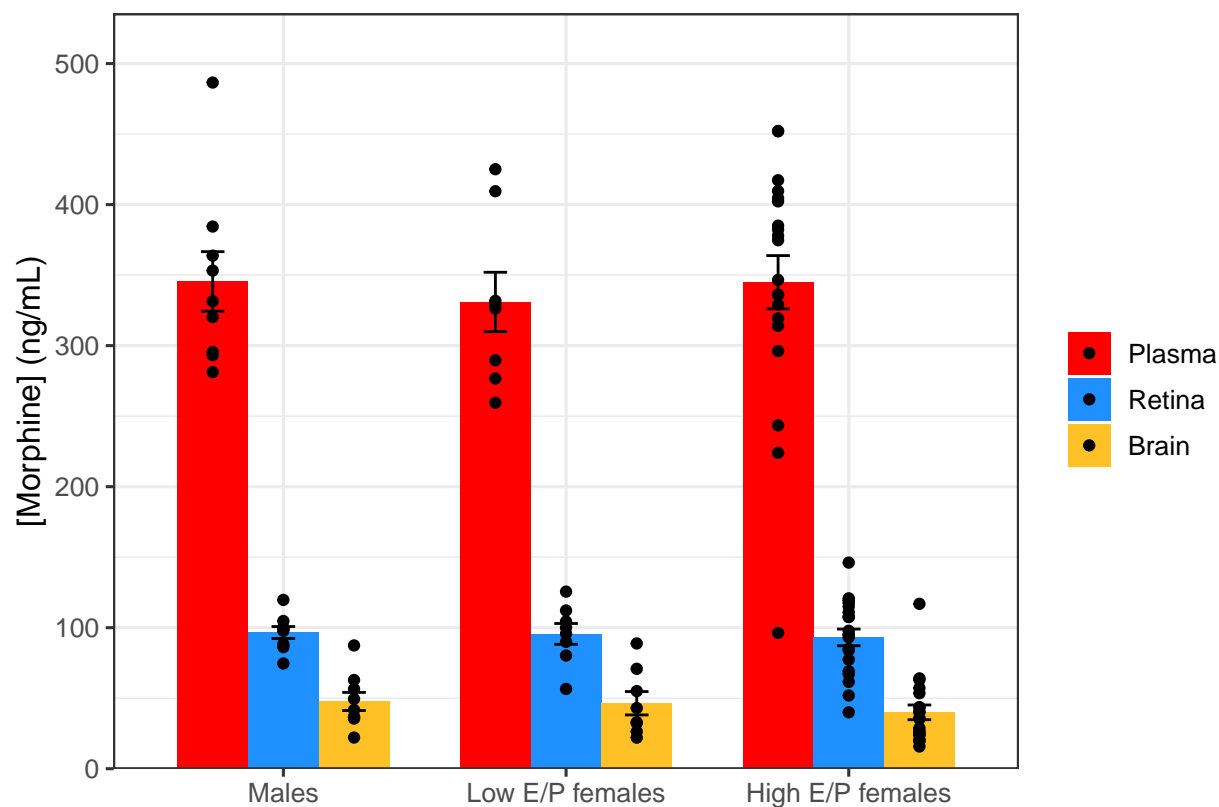
Brain, Retina & Plasma - raw concentrations

Visualizations for potential sex and tissue-dependent differences

```
plotall_tissue <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=group, y=mean_conc_raw, fill=tissue),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=group, ymin=mean_conc_raw - se_conc_raw,
    ymax=mean_conc_raw + se_conc_raw, fill=tissue),
    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=group, y=row_conc, fill=tissue),
    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="[Morphine] (ng/mL)",
    expand = expansion(mult = c(0, 0.1))) +
  theme_bw(base_size = 12) %+replace%
  theme(legend.title = element_blank()) +
  scale_fill_manual(values=c("red", "dodgerblue", "goldenrod1"),
    labels=c("plasma" = "Plasma", "retina" = "Retina",
    "brain" = "Brain")) +
  scale_x_discrete(labels=c("male" = "Males", "lowE" = "Low E/P females",
    "highE" = "High E/P females")) +
  xlab("")
```

Warning: Ignoring unknown aesthetics: fill

plotall_tissue

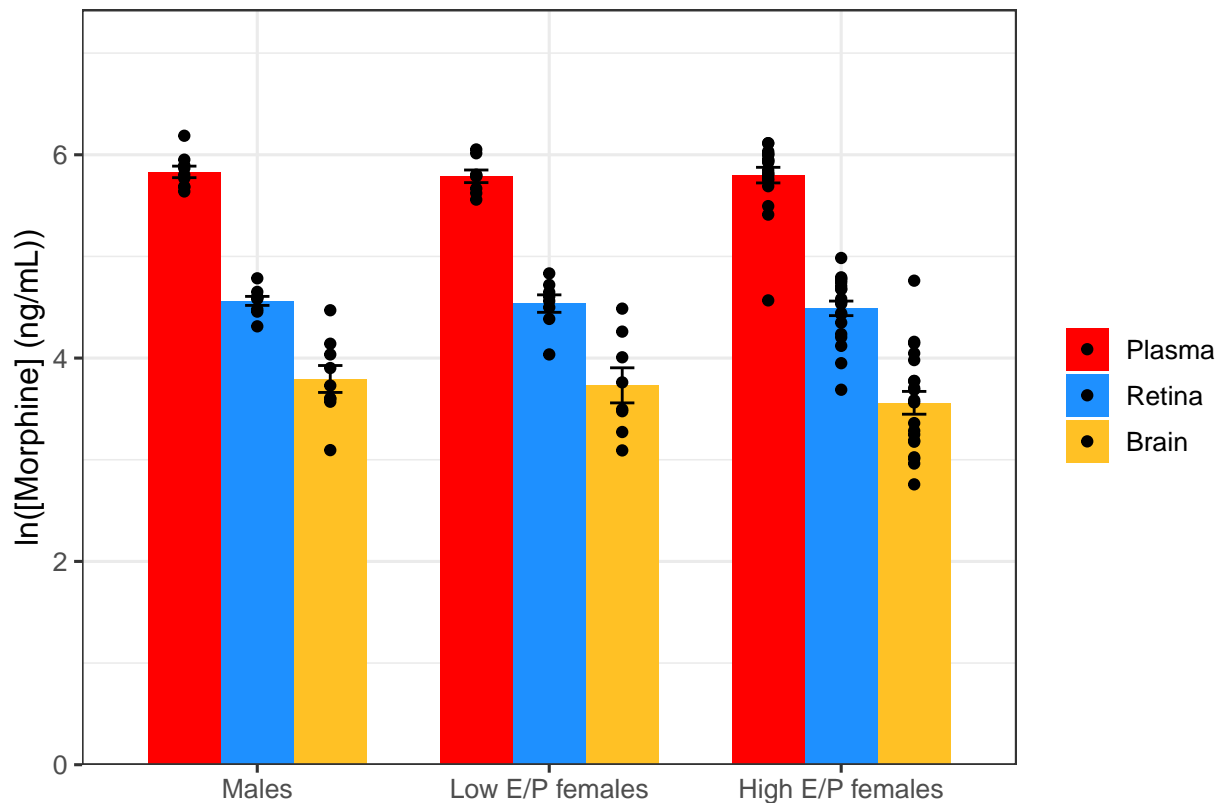


```
#ggsave("../figures/conc_alltissues.png", plot=plotall_tissue, width=6, height=4)
#ggsave("../figures/conc_alltissues.svg", plot=plotall_tissue, width=6, height=4)

plotall_tissueL <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=group, y=mean_conc_raw_log, fill=tissue),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=group, ymin=mean_conc_raw_log - se_conc_raw_log,
    ymax=mean_conc_raw_log + se_conc_raw_log, fill=tissue),
    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=group, y=log_conc_raw, fill=tissue),
    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="ln([Morphine] (ng/mL))",
    expand = expansion(mult = c(0, 0.2))) +
  theme_bw(base_size = 12) %+replace%
  theme(legend.title = element_blank()) +
  scale_fill_manual(values=c("red", "dodgerblue", "goldenrod1"),
    labels=c("plasma" = "Plasma", "retina" = "Retina",
      "brain" = "Brain")) +
  scale_x_discrete(labels=c("male" = "Males", "lowE" = "Low E/P females",
    "highE" = "High E/P females")) +
  xlab("")
```

```
## Warning: Ignoring unknown aesthetics: fill
```

```
plotall_tissueL
```



```

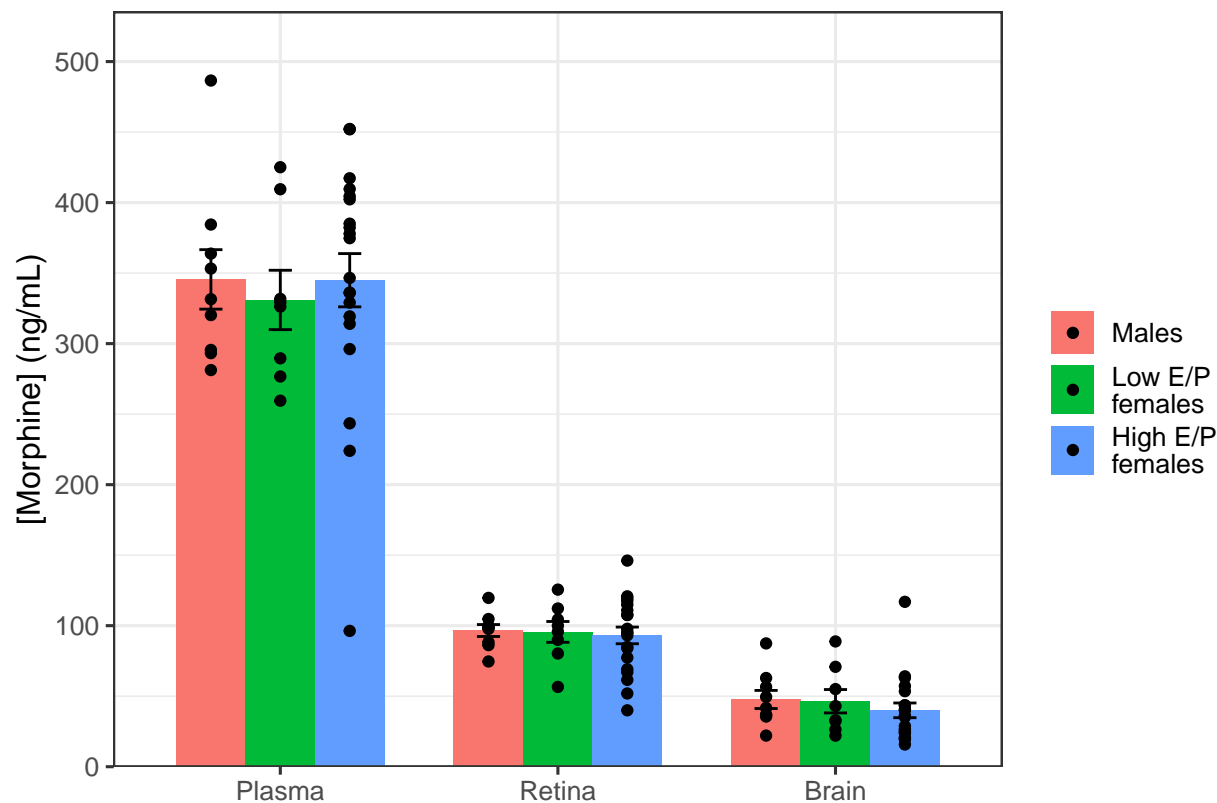
#ggsave("../figures/conc_alltissues_log.png", plot=plotall_tissueL, width=6, height=4)
#ggsave("../figures/conc_alltissues_log.svg", plot=plotall_tissueL, width=6, height=4)

plotall_stage <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=tissue, y=mean_conc_raw, fill=group),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=tissue, ymin=mean_conc_raw - se_conc_raw,
    ymax=mean_conc_raw + se_conc_raw, fill=group),
    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=tissue, y=raw_conc, fill=group),
    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="[Morphine] (ng/mL)",
    expand = expansion(mult = c(0, 0.1))) +
  theme_bw(base_size = 12) %+replace%
  theme(legend.title = element_blank(),
    legend.spacing.y = unit(0.1, 'cm')) +
  ## next line needed to make previous line work
  guides(fill = guide_legend(byrow = TRUE)) +
  scale_fill_discrete(labels=c("male" = "Males", "lowE" = "Low E/P\nfemales",
    "highE" = "High E/P\nfemales")) +
  scale_x_discrete(labels=c("plasma" = "Plasma", "retina" = "Retina",
    "brain" = "Brain")) +
  xlab("")

```

```
## Warning: Ignoring unknown aesthetics: fill
```

```
plotall_stage
```

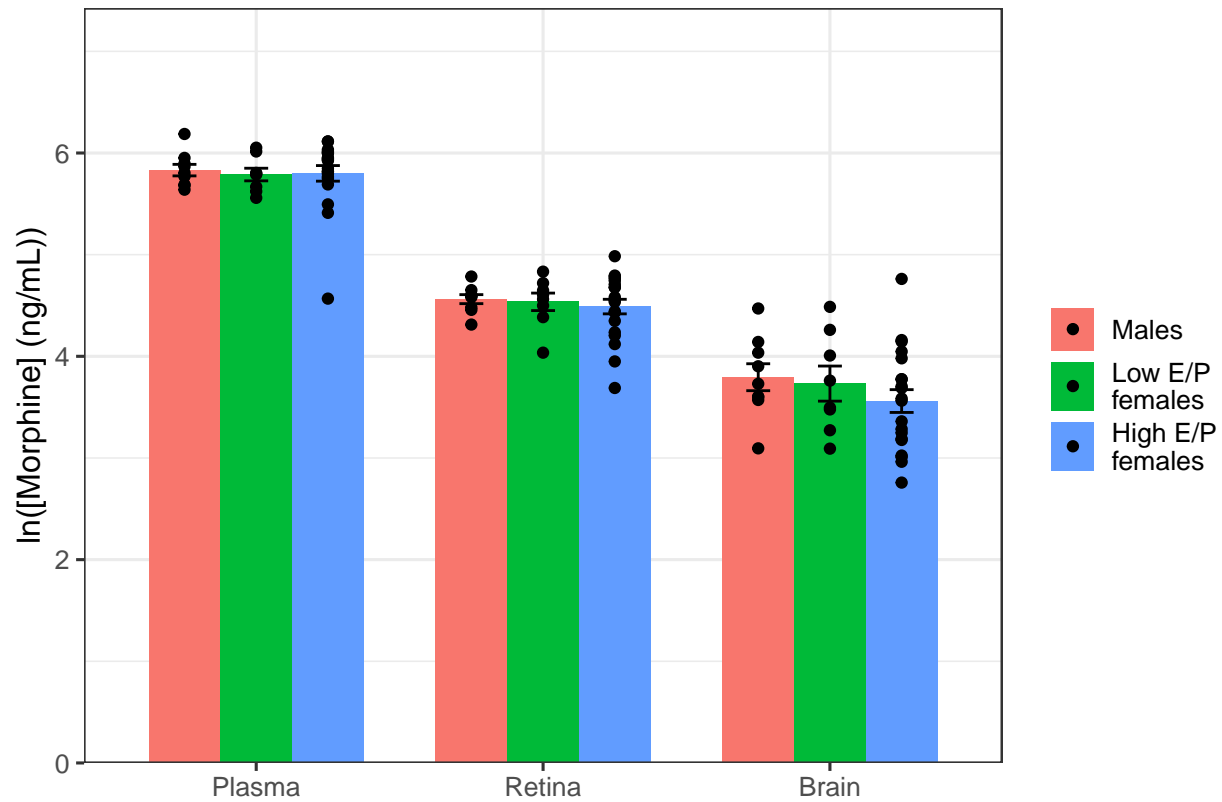


```
#ggsave("../figures/conc_all_stage.png", plot=plotall_stage, width=6, height=4)
#ggsave("../figures/conc_all_stage.svg", plot=plotall_stage, width=6, height=4)

plotall_stageL <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=tissue, y=mean_conc_raw_log, fill=group),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=tissue, ymin=mean_conc_raw_log - se_conc_raw_log,
    ymax=mean_conc_raw_log + se_conc_raw_log, fill=group),
    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=tissue, y=log_conc_raw, fill=group),
    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="ln([Morphine] (ng/mL))",
    expand = expansion(mult = c(0, 0.2))) +
  theme_bw(base_size = 12) %+replace%
    theme(legend.title = element_blank(),
      legend.spacing.y = unit(0.1, 'cm')) +
  ## next line needed to make previous line work
  guides(fill = guide_legend(byrow = TRUE)) +
  scale_fill_discrete(labels=c("male" = "Males", "lowE" = "Low E/P\nfemales",
    "highE" = "High E/P\nfemales")) +
  scale_x_discrete(labels=c("plasma" = "Plasma", "retina" = "Retina",
    "brain" = "Brain")) +
  xlab("")
```

```
## Warning: Ignoring unknown aesthetics: fill
```

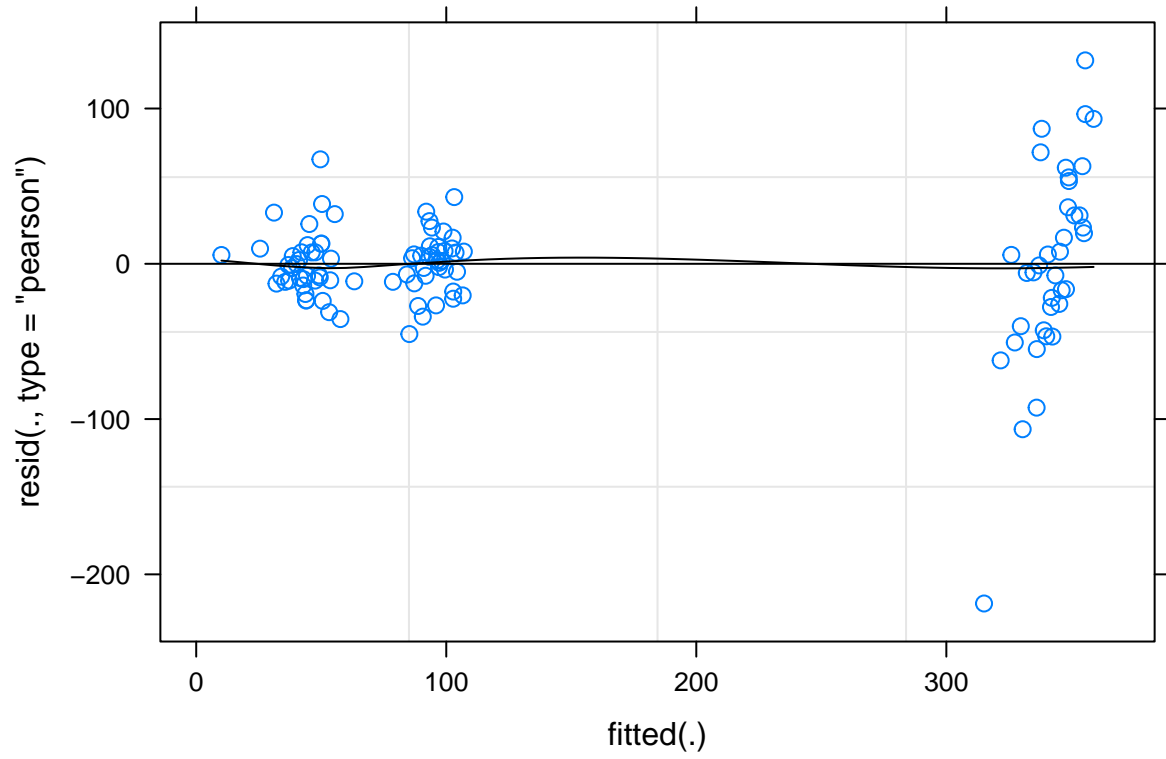
```
plotall_stageL
```



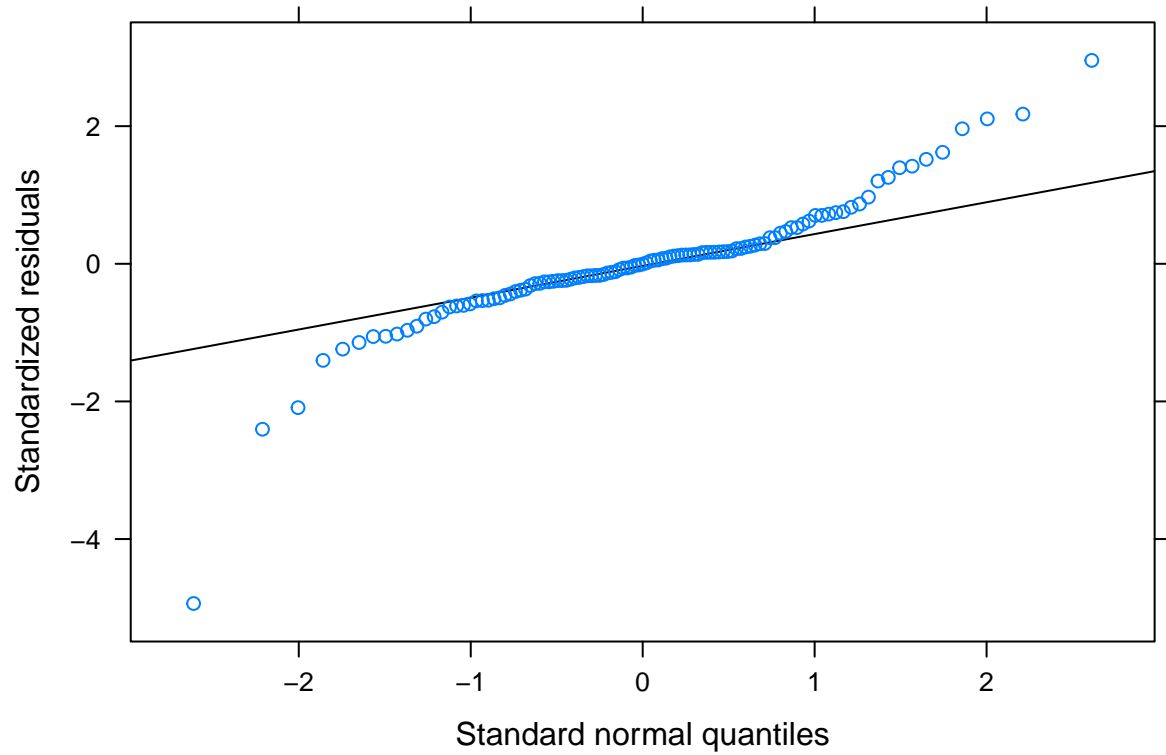
```
#ggsave("../figures/conc_all_stage_log.png", plot=plotall_stageL, width=6, height=4)
#ggsave("../figures/conc_all_stage_log.svg", plot=plotall_stageL, width=6, height=4)
```

Statistical analysis

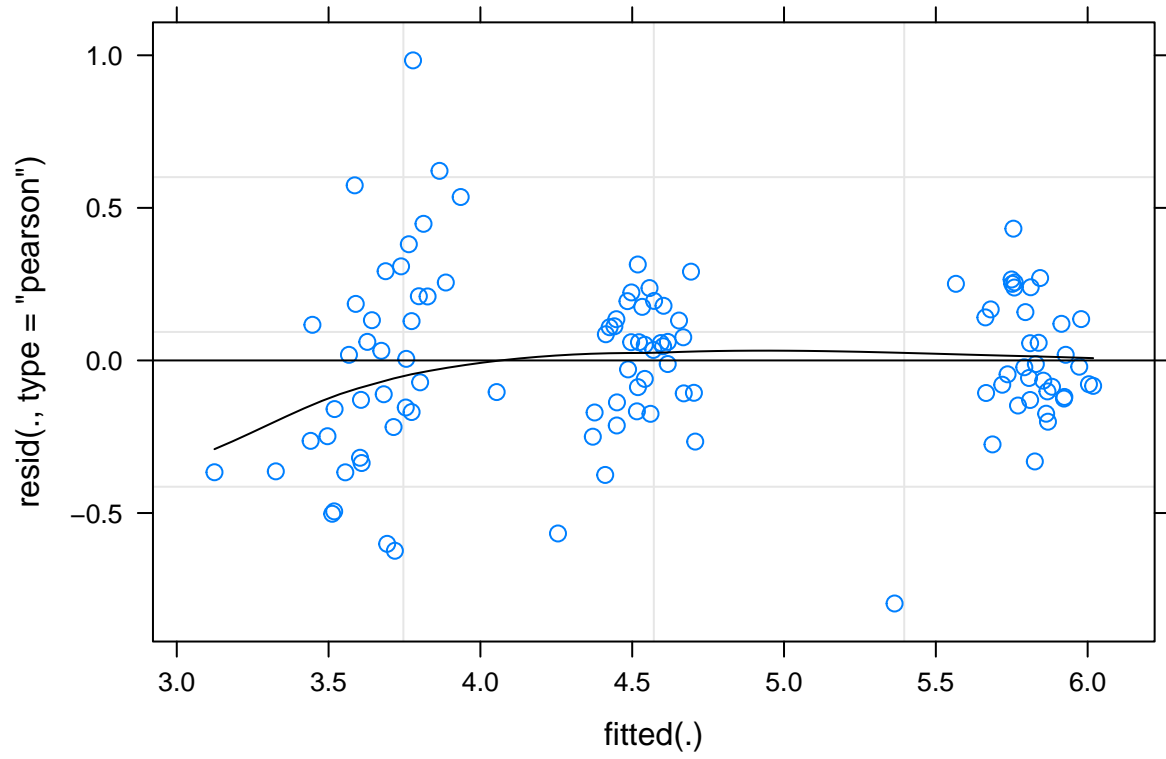
```
lmer_diff_all <- lmer(raw_conc ~ group*tissue+(1|animal), data =sex_diff)
plot(lmer_diff_all, type=c("p","smooth"), col.line=1)
```

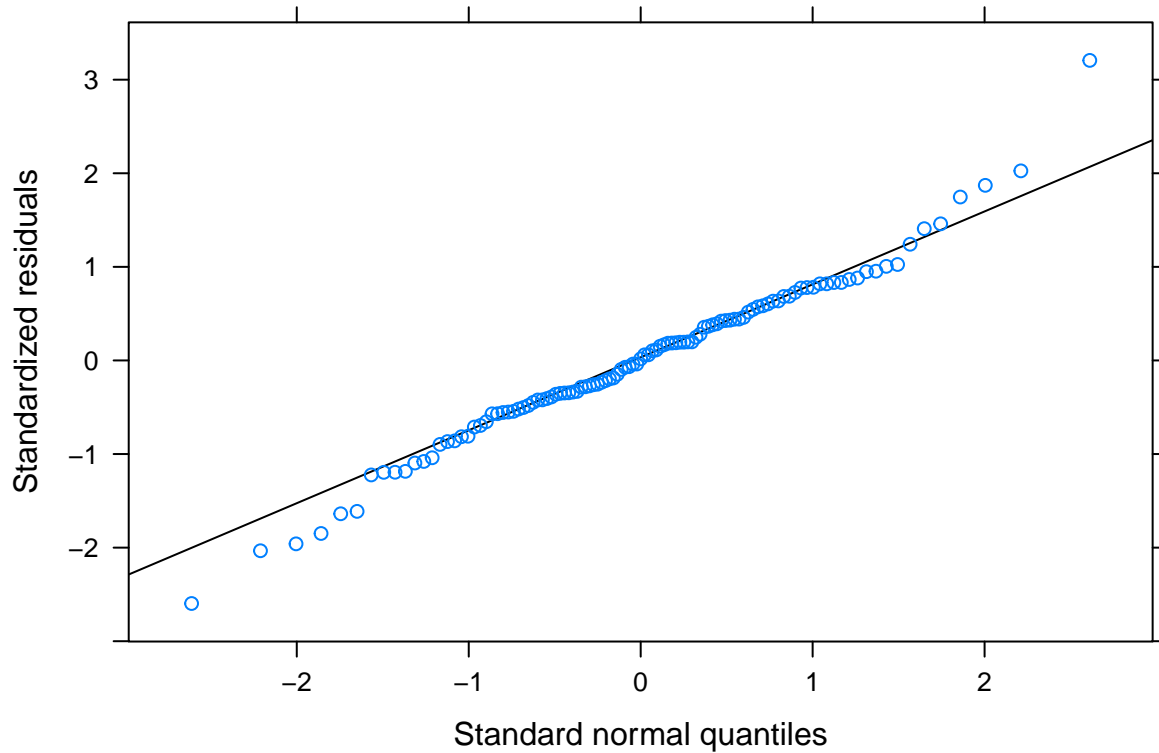
```
lattice::qqmath(lmer_diff_all)
```



```
lmer_diff_all_log <- lmer(log(raw_conc) ~ group*tissue+(1|animal), data =sex_diff)
plot(lmer_diff_all_log, type=c("p","smooth"), col.line=1)
```



```
lattice::qqmath(lmer_diff_all_log)
```



```
Anova(lmer_diff_all_log, type="3")
```

```
## Analysis of Deviance Table (Type III Wald chisquare tests)
##
## Response: log(raw_conc)
##           Chisq Df Pr(>Chisq)
## (Intercept) 2420.9212 1    <2e-16 ***
## group        0.0722 2     0.9645
## tissue       202.5510 2    <2e-16 ***
## group:tissue  2.0070 4     0.7345
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
emmeans::emmeans(lmer_diff_all_log, pairwise ~ tissue | group)$contrasts
```

```
## group = male:
## contrast      estimate    SE df t.ratio p.value
## plasma - retina  1.269 0.145 68   8.778 <.0001
## plasma - brain   2.037 0.145 68  14.091 <.0001
## retina - brain    0.768 0.145 68   5.313 <.0001
##
## group = lowE:
## contrast      estimate    SE df t.ratio p.value
## plasma - retina  1.252 0.153 68   8.166 <.0001
```

```
## plasma - brain      2.057 0.153 68  13.411 <.0001
## retina - brain      0.804 0.153 68   5.245 <.0001
##
## group = highE:
## contrast      estimate      SE df t.ratio p.value
## plasma - retina  1.311 0.097 68  13.513 <.0001
## plasma - brain   2.240 0.097 68  23.095 <.0001
## retina - brain   0.929 0.097 68   9.583 <.0001
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## P value adjustment: tukey method for comparing a family of 3 estimates
```

```
emmeans::emmeans(lmer_diff_all_log, pairwise ~ group | tissue)$contrasts
```

```
## tissue = plasma:
## contrast      estimate      SE df t.ratio p.value
## male - lowE    0.0433 0.173 90.2  0.251  0.9660
## male - highE   0.0318 0.143 90.2  0.222  0.9731
## lowE - highE  -0.0116 0.149 90.2 -0.078  0.9967
##
## tissue = retina:
## contrast      estimate      SE df t.ratio p.value
## male - lowE    0.0265 0.173 90.2  0.153  0.9872
## male - highE   0.0732 0.143 90.2  0.513  0.8653
## lowE - highE   0.0467 0.149 90.2  0.314  0.9471
##
## tissue = brain:
## contrast      estimate      SE df t.ratio p.value
## male - lowE    0.0627 0.173 90.2  0.363  0.9301
## male - highE   0.2345 0.143 90.2  1.643  0.2331
## lowE - highE   0.1718 0.149 90.2  1.155  0.4831
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## P value adjustment: tukey method for comparing a family of 3 estimates
```

```
#shapiro.test(resid(lmer_diff))
```

Brain & Retina normalized by tissue weight

```
sex_diff <- sex_diff %>% filter(tissue != "plasma")
SumStat_sexdiff <- SumStat_sexdiff %>% filter(tissue != "plasma")

plot_tissue <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=group, y=mean_conc_wt, fill=tissue),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=group, ymin=mean_conc_wt - se_conc_wt,
    ymax=mean_conc_wt + se_conc_wt, fill=tissue),
    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=group, y=conc_wt, fill=tissue),
```

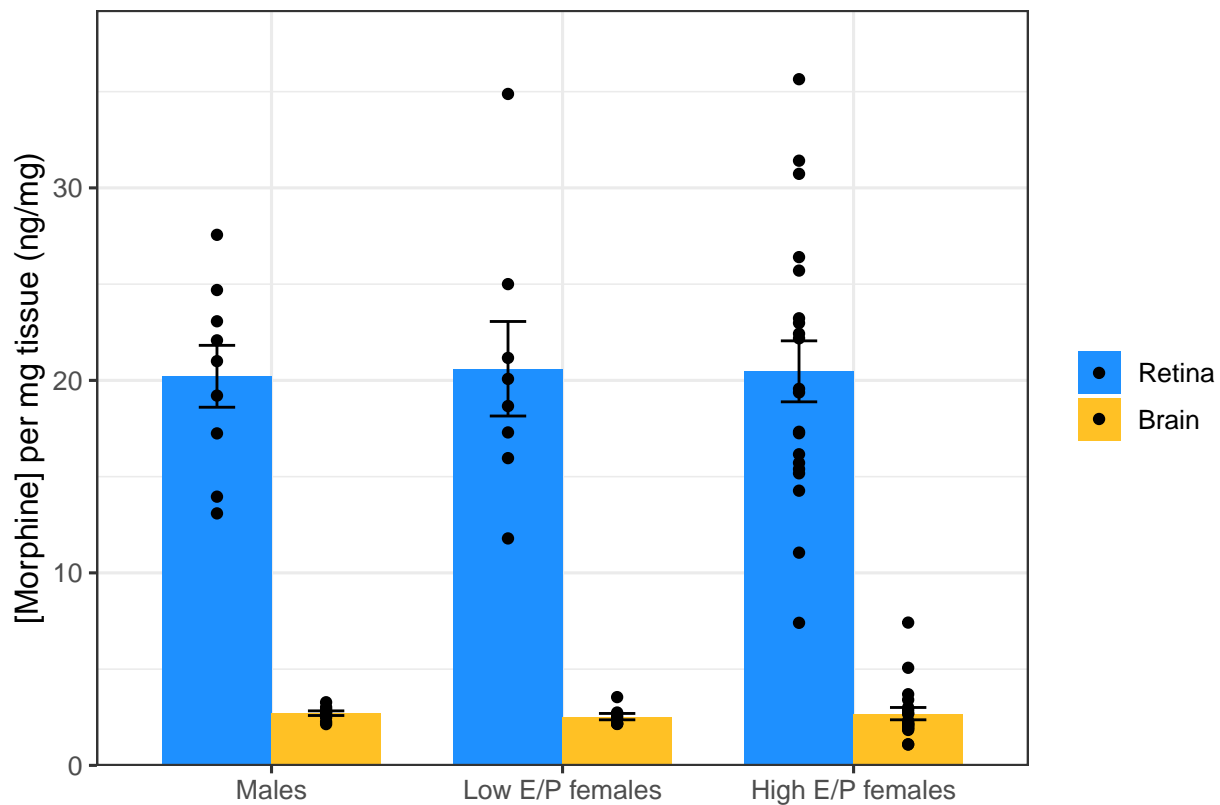
```

    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="[Morphine] per mg tissue (ng/mg)",
    expand = expansion(mult = c(0, 0.1))) +
  theme_bw(12) %+replace%
  theme(legend.title = element_blank()) +
  scale_fill_manual(values=c("dodgerblue", "goldenrod1"),
    labels=c("retina" = "Retina", "brain" = "Brain")) +
  scale_x_discrete(labels=c("male" = "Males", "lowE" = "Low E/P females",
    "highE" = "High E/P females")) +
  xlab("")

```

```
## Warning: Ignoring unknown aesthetics: fill
```

```
plot_tissue
```



```

ggsave("../figures/conc_tissue.png", plot=plot_tissue, width=6, height=4)
ggsave("../figures/conc_tissue.svg", plot=plot_tissue, width=6, height=4)

plot_tissueL <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=group, y=log_conc, fill=tissue),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=group, ymin=log_conc - se_conc_log,
    ymax=log_conc + se_conc_log, fill=tissue),
    position=position_dodge(width=0.75), width=.25) +

```

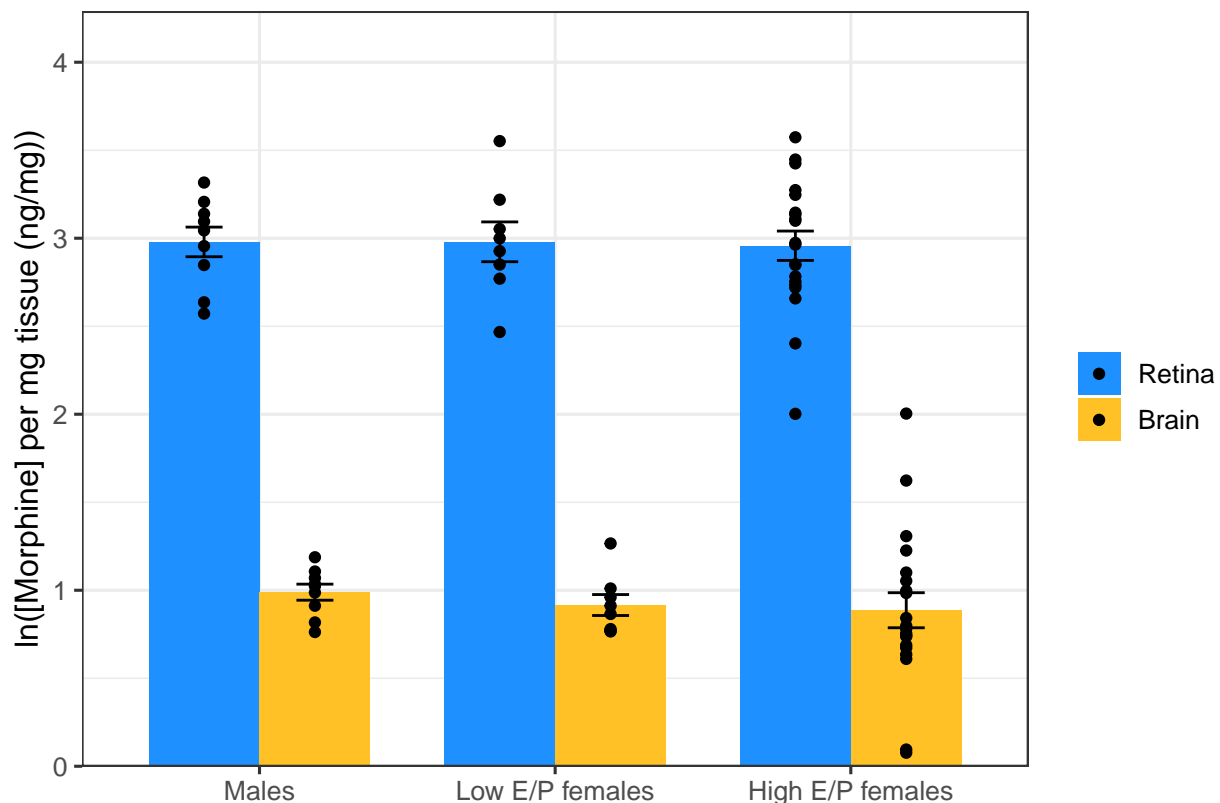
```

geom_point(data=sex_diff, aes(x=group, y=log_conc_wt, fill=tissue),
           position=position_dodge(width=0.75)) +
scale_y_continuous(name="ln([Morphine] per mg tissue (ng/mg))",
                  expand = expansion(mult = c(0, 0.2))) +
theme_bw(12) %+replace%
  theme(legend.title = element_blank()) +
scale_fill_manual(values=c("dodgerblue", "goldenrod1"),
                 labels=c("retina" = "Retina", "brain" = "Brain")) +
scale_x_discrete(labels=c("male" = "Males", "lowE" = "Low E/P females",
                          "highE" = "High E/P females")) +
xlab("")

```

Warning: Ignoring unknown aesthetics: fill

plot_tissueL



```

ggsave("../figures/conc_tissue_log.png", plot=plot_tissueL, width=6, height=4)
ggsave("../figures/conc_tissue_log.svg", plot=plot_tissueL, width=6, height=4)

plot_stage <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=tissue, y=mean_conc_wt, fill=group),
           stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=tissue, ymin=mean_conc_wt - se_conc_wt,
                                           ymax=mean_conc_wt + se_conc_wt, fill=group),

```

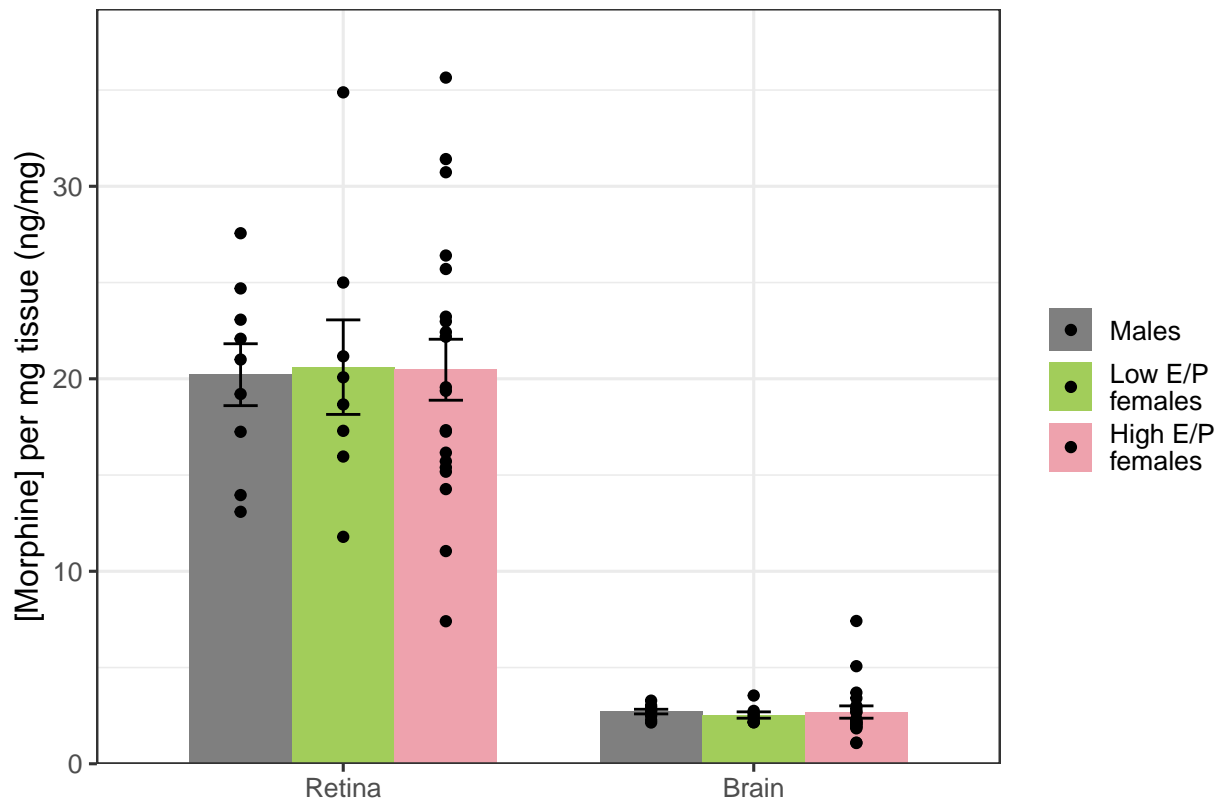
```

    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=tissue, y=conc_wt, fill=group),
    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="[Morphine] per mg tissue (ng/mg)",
    expand = expansion(mult = c(0, 0.1))) +
  theme_bw(base_size = 12) %+replace%
    theme(legend.title = element_blank(),
      legend.spacing.y = unit(0.1, 'cm')) +
  ## next line needed to make previous line work
  guides(fill = guide_legend(byrow = TRUE)) +
  scale_fill_manual(values=c("gray50", "darkolivegreen3", "lightpink2"),
    labels=c("male" = "Males", "lowE" = "Low E/P\nfemales",
      "highE" = "High E/P\nfemales")) +
  scale_x_discrete(labels=c("retina" = "Retina", "brain" = "Brain")) +
  xlab("")

```

```
## Warning: Ignoring unknown aesthetics: fill
```

```
plot_stage
```



```

ggsave("../figures/conc_stage.png", plot=plot_stage, width=6, height=4)
ggsave("../figures/conc_stage.svg", plot=plot_stage, width=6, height=4)

```



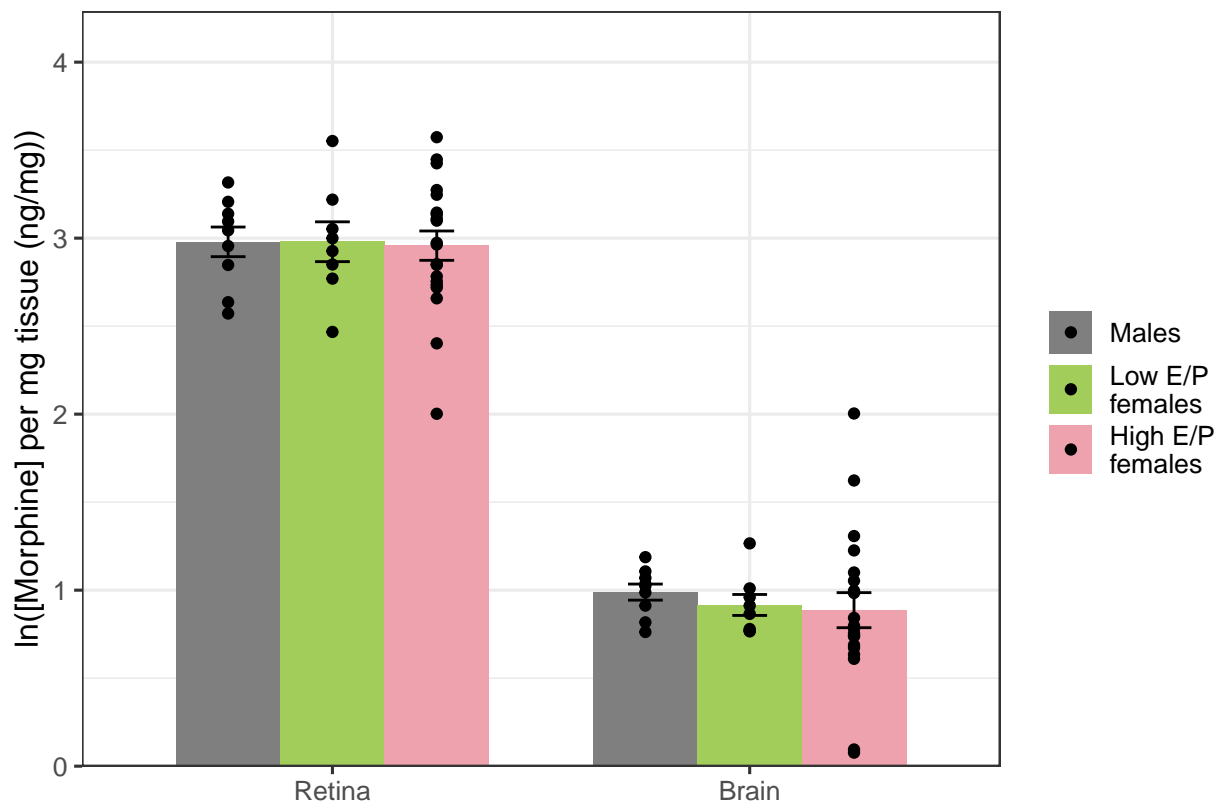
```

plot_stageL <- ggplot() +
  geom_bar(data=SumStat_sexdiff, aes(x=tissue, y=log_conc, fill=group),
    stat="identity", position=position_dodge(width=0.75), width=0.75) +
  geom_errorbar(data=SumStat_sexdiff, aes(x=tissue, ymin=log_conc - se_conc_log,
    ymax=log_conc + se_conc_log, fill=group),
    position=position_dodge(width=0.75), width=.25) +
  geom_point(data=sex_diff, aes(x=tissue, y=log_conc_wt, fill=group),
    position=position_dodge(width=0.75)) +
  scale_y_continuous(name="ln([Morphine] per mg tissue (ng/mg))",
    expand = expansion(mult = c(0, 0.2))) +
  theme_bw(base_size = 12) %+replace%
  theme(legend.title = element_blank(),
    legend.spacing.y = unit(0.1, 'cm')) +
  ## next line needed to make previous line work
  guides(fill = guide_legend(byrow = TRUE)) +
  scale_fill_manual(values=c("gray50", "darkolivegreen3", "lightpink2"),
    labels=c("male" = "Males", "lowE" = "Low E/P\nfemales",
      "highE" = "High E/P\nfemales")) +
  scale_x_discrete(labels=c("retina" = "Retina", "brain" = "Brain")) +
  xlab("")

```

Warning: Ignoring unknown aesthetics: fill

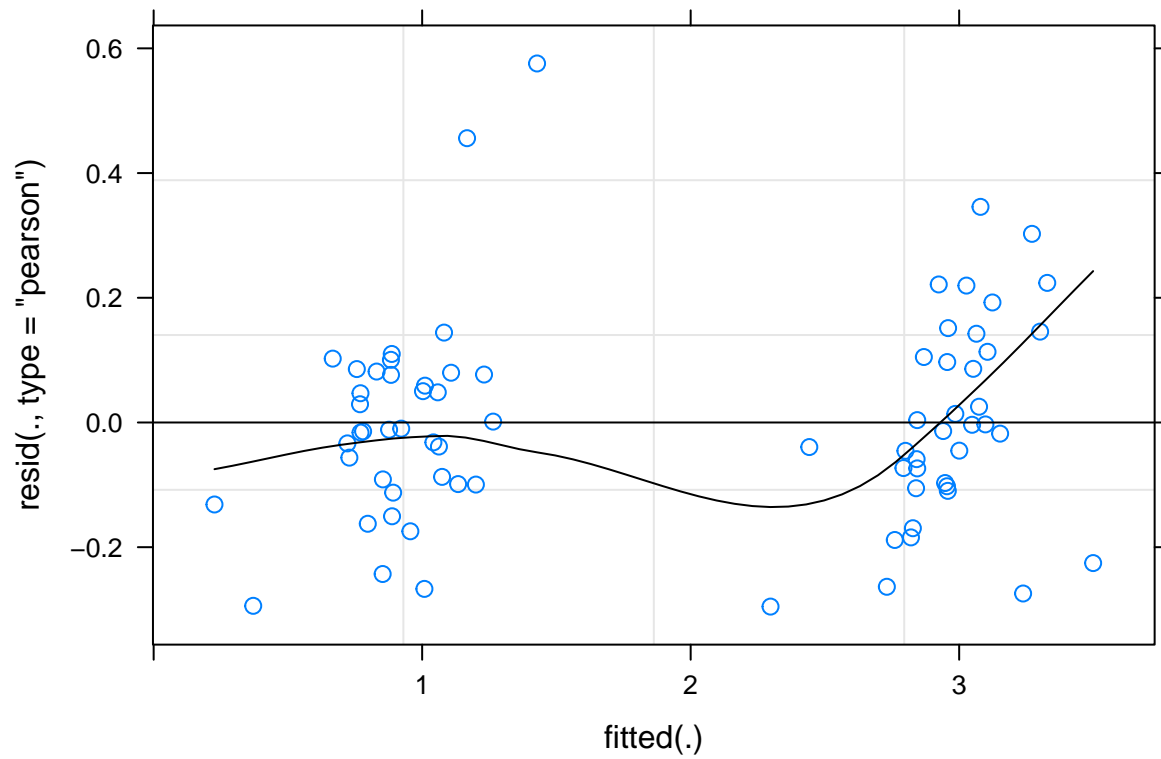
plot_stageL



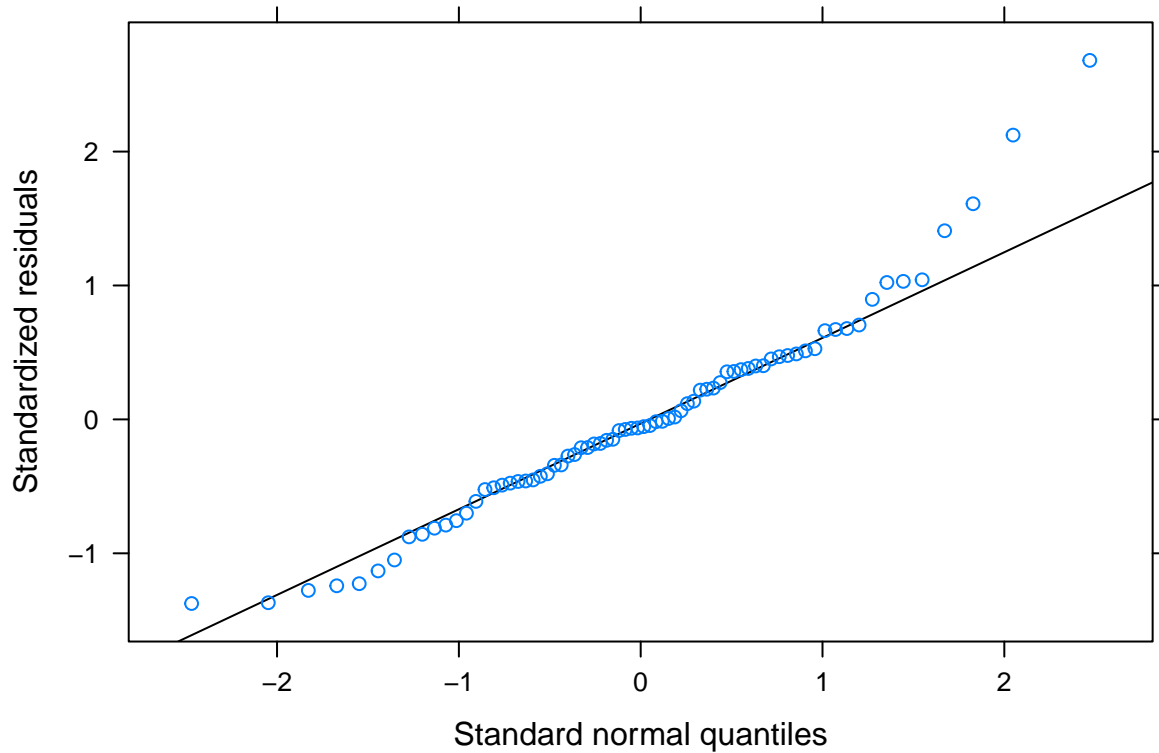
```
ggsave("../figures/conc_stage_log.png", plot=plot_stageL, width=6, height=4)
ggsave("../figures/conc_stage_log.svg", plot=plot_stageL, width=6, height=4)
```

Statistical analysis for differences between groups and tissues

```
lmer_diff <- lmer(log(conc_wt) ~ group*tissue+(1|animal), data =sex_diff)
plot(lmer_diff, type=c("p","smooth"), col.line=1)
```



```
lattice::qqmath(lmer_diff)
```



```
Anova(lmer_diff, type="3")
```

```
## Analysis of Deviance Table (Type III Wald chisquare tests)
##
## Response: log(conc_wt)
##           Chisq Df Pr(>Chisq)
## (Intercept)  680.1794  1    <2e-16 ***
## group         0.0379  2     0.9812
## tissue       386.3966  1    <2e-16 ***
## group:tissue   0.4625  2     0.7935
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
emmeans::emmeans(lmer_diff, pairwise ~ tissue | group)$contrasts
```

```
## group = male:
## contrast      estimate      SE df t.ratio p.value
## retina - brain    1.99 0.1012 34  19.657 <.0001
##
## group = lowE:
## contrast      estimate      SE df t.ratio p.value
## retina - brain    2.06 0.1074 34  19.218 <.0001
##
## group = highE:
```

```
## contrast      estimate      SE df t.ratio p.value
## retina - brain      2.07 0.0679 34  30.495  <.0001
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
```

```
emmeans::emmeans(lmer_diff, pairwise ~ group | tissue)$contrasts
```

```
## tissue = retina:
## contrast      estimate      SE  df t.ratio p.value
## male - lowE   -0.0006 0.167 49.7  -0.004  1.0000
## male - highE    0.0217 0.138 49.7   0.158  0.9863
## lowE - highE    0.0223 0.143 49.7   0.156  0.9867
##
## tissue = brain:
## contrast      estimate      SE  df t.ratio p.value
## male - lowE     0.0730 0.167 49.7   0.439  0.8996
## male - highE     0.1027 0.138 49.7   0.746  0.7371
## lowE - highE     0.0296 0.143 49.7   0.207  0.9767
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## P value adjustment: tukey method for comparing a family of 3 estimates
```

```
#shapiro.test(resid(lmer_diff))
```

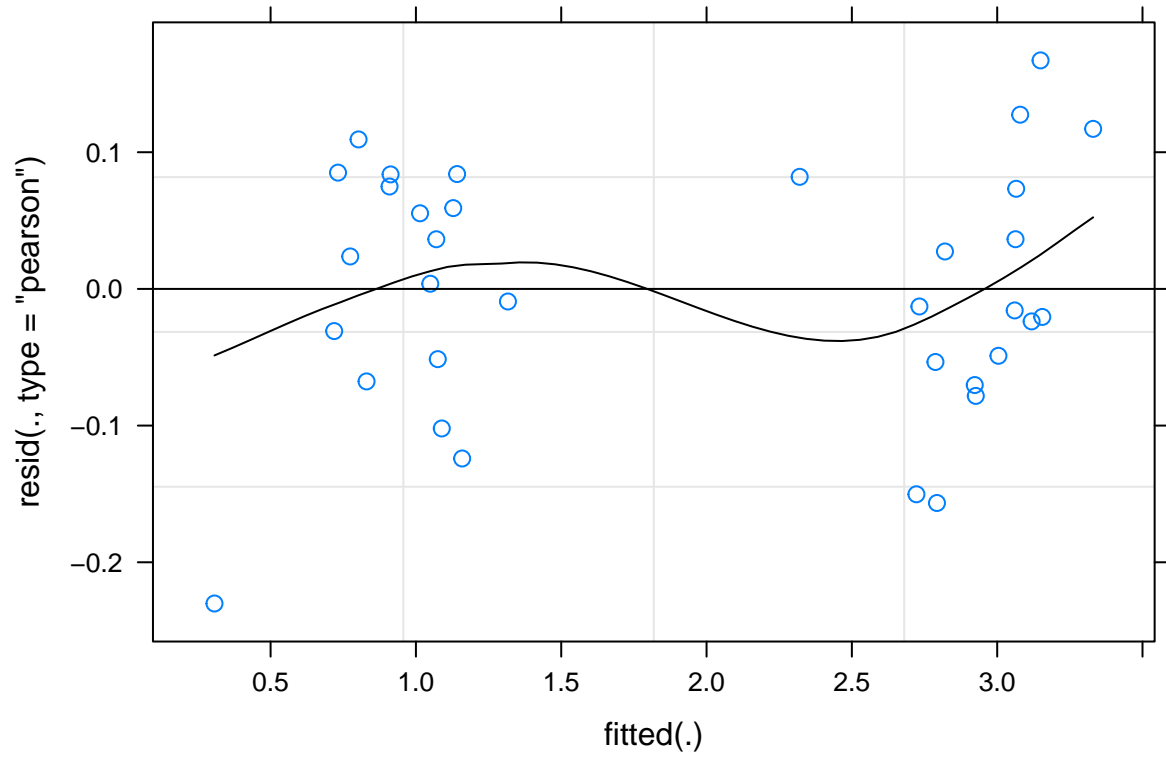
Sex differences analysis exclusively for animals that were littermates

```
litter <- subset(sex_diff, animal %in% c("1M", "2M", "3M", "4M", "5M", "6M", "7M", "8M", "9M", "18F", "19F", "20F"))
litter
```

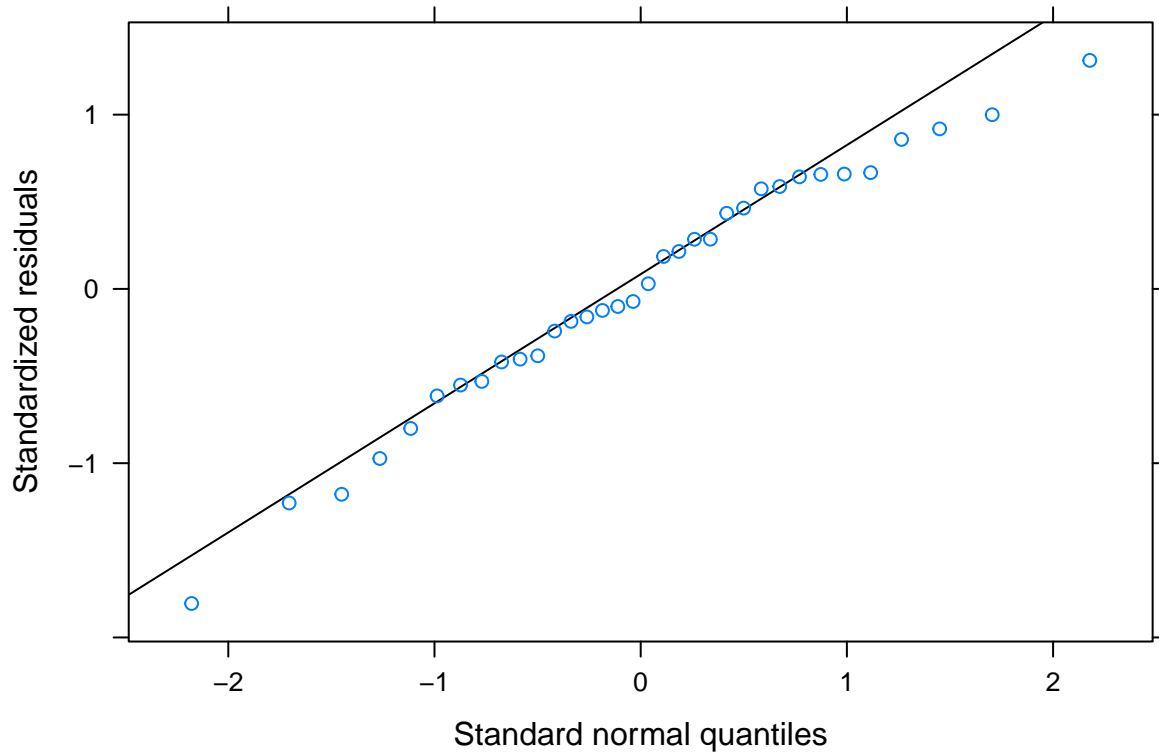
##	animal	raw_conc	tissue_weight	body_wt	dose	stage	group	tissue	conc_wt
## 1	1M	56.5895	20.1	27.2	20.60	male	male	brain	2.815398
## 2	2M	22.0798	10.3	30.1	18.60	male	male	brain	2.143670
## 3	3M	36.7582	13.7	28.7	19.50	male	male	brain	2.683080
## 4	4M	49.5114	15.1	25.8	21.70	male	male	brain	3.278901
## 5	5M	87.4161	31.4	26.1	20.00	male	male	brain	2.783952
## 6	6M	36.5960	14.7	27.3	19.10	male	male	brain	2.489524
## 7	7M	41.6730	14.3	26.2	19.90	male	male	brain	2.914196
## 8	8M	62.8960	20.8	23.7	22.00	male	male	brain	3.023846
## 9	9M	35.5456	15.7	27.2	19.20	male	male	brain	2.264051
## 22	18F	116.9270	43.7	21.3	20.40	estrus	highE	brain	2.675675
## 23	19F	15.7652	14.6	22.9	19.00	estrus	highE	brain	1.079808
## 24	20F	40.6623	11.0	20.9	20.80	proestrus	highE	brain	3.696573
## 25	21F	43.5806	15.2	20.4	21.30	proestrus	highE	brain	2.867145
## 26	22F	64.0701	32.2	23.2	18.70	estrus	highE	brain	1.989755
## 27	24F	57.2307	16.8	21.2	20.50	proestrus	highE	brain	3.406589
## 28	25F	25.7378	9.5	20.3	21.40	estrus	highE	brain	2.709242
## 29	26F	23.9826	10.8	21.7	20.00	estrus	highE	brain	2.220611
## 38	1M	104.7333	3.8	27.2	20.55	male	male	retina	27.561395

## 39	2M	86.2347	5.0	30.1	18.57	male	male	retina	17.246940
## 40	3M	98.7759	4.0	28.7	19.48	male	male	retina	24.693975
## 41	4M	88.3202	4.0	25.8	21.67	male	male	retina	22.080050
## 42	5M	99.2028	4.3	26.1	20.00	male	male	retina	23.070419
## 43	6M	97.7141	7.0	27.3	19.12	male	male	retina	13.959157
## 44	7M	99.8877	5.2	26.2	19.92	male	male	retina	19.209173
## 45	8M	119.6988	5.7	23.7	22.03	male	male	retina	20.999789
## 46	9M	74.6145	5.7	27.2	19.19	male	male	retina	13.090263
## 59	18F	84.9179	4.9	21.3	20.38	estrus	highE	retina	17.330184
## 60	19F	51.9343	4.7	22.9	18.95	estrus	highE	retina	11.049851
## 61	20F	119.3678	3.8	20.9	20.77	proestrus	highE	retina	31.412579
## 62	21F	117.5767	5.3	20.4	21.27	proestrus	highE	retina	22.184283
## 63	22F	77.3790	5.1	23.2	18.71	estrus	highE	retina	15.172353
## 64	24F	114.9038	5.0	21.2	20.47	proestrus	highE	retina	22.980760
## 65	25F	93.1777	5.4	20.3	21.38	estrus	highE	retina	17.255130
## 66	26F	61.5810	4.0	21.7	20.00	estrus	highE	retina	15.395250
##	log_conc_wt	log_conc_raw							
## 1	1.03510364	4.035823							
## 2	0.76251927	3.094663							
## 3	0.98696550	3.604361							
## 4	1.18750820	3.902203							
## 5	1.02387158	4.470679							
## 6	0.91209145	3.599939							
## 7	1.06959390	3.729853							
## 8	1.10652958	4.141483							
## 9	0.81715567	3.570816							
## 22	0.98420171	4.761550							
## 23	0.07678345	2.757805							
## 24	1.30740610	3.705301							
## 25	1.05331667	3.774612							
## 26	0.68801134	4.159978							
## 27	1.22571158	4.047090							
## 28	0.99666893	3.247961							
## 29	0.79778243	3.177329							
## 38	3.31641605	4.651417							
## 39	2.84763474	4.457073							
## 40	3.20655929	4.592854							
## 41	3.09467449	4.480969							
## 42	3.13855122	4.597166							
## 43	2.63613572	4.582046							
## 44	2.95538793	4.604047							
## 45	3.04451241	4.784979							
## 46	2.57186868	4.312335							
## 59	2.85244970	4.441685							
## 60	2.40241695	3.949979							
## 61	3.44720842	4.782209							
## 62	3.09938407	4.767091							
## 63	2.71947489	4.348715							
## 64	3.13465734	4.744095							
## 65	2.84810947	4.534508							
## 66	2.73405902	4.120353							

```
lmer_litt <- lmer(log(conc_wt) ~ group*tissue+(1|animal), data =litter)
plot(lmer_litt, type=c("p","smooth"), col.line=1)
```



```
lattice::qqmath(lmer_litt)
```



```
anova(lmer_litt)
```

```
## Analysis of Variance Table
##               npar Sum Sq Mean Sq    F value
## group           1  0.007   0.007    0.4345
## tissue           1 34.037  34.037 2093.6731
## group:tissue     1  0.001   0.001    0.0716
```

```
emmeans::emmeans(lmer_litt, pairwise ~ tissue | group)
```

```
## $emmeans
## group = male:
##   tissue emmean      SE    df lower.CL upper.CL
##   retina  2.979 0.0945 18.3    2.781    3.18
##   brain   0.989 0.0945 18.3    0.791    1.19
##
## group = highE:
##   tissue emmean      SE    df lower.CL upper.CL
##   retina  2.905 0.1002 18.3    2.694    3.12
##   brain   0.891 0.1002 18.3    0.681    1.10
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## Confidence level used: 0.95
##
```

```
## $contrasts
## group = male:
## contrast      estimate      SE df t.ratio p.value
## retina - brain    1.99 0.0601 15  33.109 <.0001
##
## group = highE:
## contrast      estimate      SE df t.ratio p.value
## retina - brain    2.01 0.0638 15  31.583 <.0001
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
```

```
emmeans::emmeans(lmer_litt, pairwise ~ group | tissue)
```

```
## $emmeans
## tissue = retina:
## group emmean      SE    df lower.CL upper.CL
## male  2.979 0.0945 18.3    2.781    3.18
## highE 2.905 0.1002 18.3    2.694    3.12
##
## tissue = brain:
## group emmean      SE    df lower.CL upper.CL
## male  0.989 0.0945 18.3    0.791    1.19
## highE 0.891 0.1002 18.3    0.681    1.10
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## Confidence level used: 0.95
##
## $contrasts
## tissue = retina:
## contrast      estimate      SE    df t.ratio p.value
## male - highE   0.0744 0.138 18.3    0.540 0.5958
##
## tissue = brain:
## contrast      estimate      SE    df t.ratio p.value
## male - highE   0.0978 0.138 18.3    0.710 0.4866
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
```

```
shapiro.test(resid(lmer_litt))
```

```
##
## Shapiro-Wilk normality test
##
## data:  resid(lmer_litt)
## W = 0.97782, p-value = 0.7028
```

Visualizations


```
SumStat_sex1itt <- dplyr::summarise(group_by(litter, tissue, group),
  n = n(),
  mean_conc_wt = mean(conc_wt),
  sd_conc_wt = sd(conc_wt),
  se_conc_wt = sd_conc_wt/sqrt(n),
  log_conc = mean(log(conc_wt)),
  sd_conc_log = sd(log(conc_wt)),
  se_conc_log = sd_conc_log/sqrt(n),
  mean_wt = mean(body_wt),
  sd_wt = sd(body_wt),
  mean_dose = mean(dose),
  sd_dose = sd(dose),
)
```

'summarise()' has grouped output by 'tissue'. You can override using the
'.groups' argument.

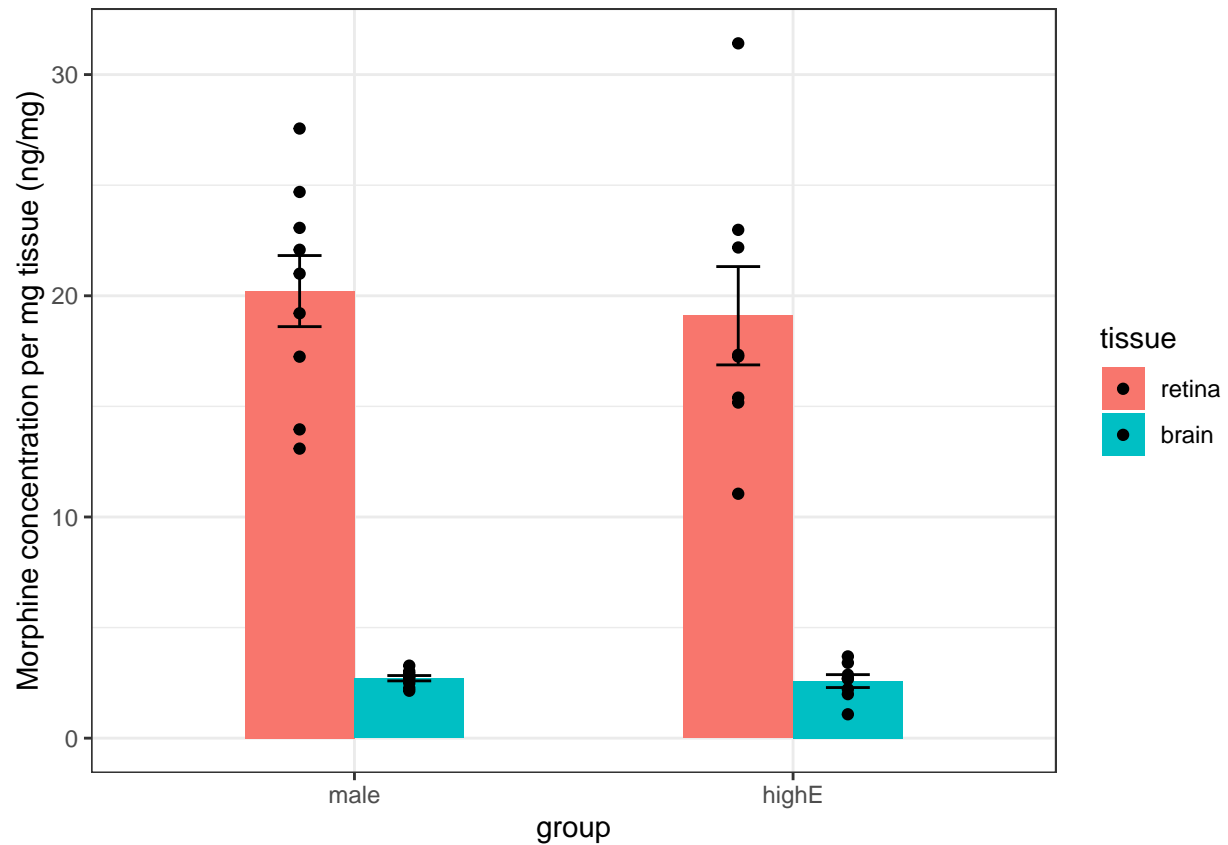
```
SumStat_sex1itt
```

```
## # A tibble: 4 x 13
## # Groups:   tissue [2]
##   tissue group      n mean_conc~1 sd_co~2 se_co~3 log_c~4 sd_co~5 se_co~6 mean_wt
##   <fct> <fct> <int>      <dbl>  <dbl>  <dbl>  <dbl>  <dbl>  <dbl>  <dbl>
## 1 retina male      9      20.2   4.82   1.61   2.98   0.252  0.0842   26.9
## 2 retina highE     8      19.1   6.29   2.22   2.90   0.318  0.112   21.5
## 3 brain  male      9       2.71  0.362  0.121  0.989  0.137  0.0456   26.9
## 4 brain  highE     8       2.58  0.825  0.292  0.891  0.386  0.137   21.5
## # ... with 3 more variables: sd_wt <dbl>, mean_dose <dbl>, sd_dose <dbl>, and
## #   abbreviated variable names 1: mean_conc_wt, 2: sd_conc_wt, 3: se_conc_wt,
## #   4: log_conc, 5: sd_conc_log, 6: se_conc_log
```

```
plot_tissue_litt <- ggplot() +
  geom_bar(data=SumStat_sex1itt, aes(x=group, y=mean_conc_wt, fill=tissue), stat="identity", position=position_dodge(width=0.5)) +
  geom_errorbar(data=SumStat_sex1itt, aes(x=group, ymin=mean_conc_wt - se_conc_wt, ymax=mean_conc_wt + se_conc_wt), position=position_dodge(width=0.5)) +
  geom_point(data=litter, aes(x=group, y=conc_wt, fill=tissue), position=position_dodge(width=0.5)) +
  scale_y_continuous(name="Morphine concentration per mg tissue (ng/mg)") + theme_bw()
```

Warning: Ignoring unknown aesthetics: fill

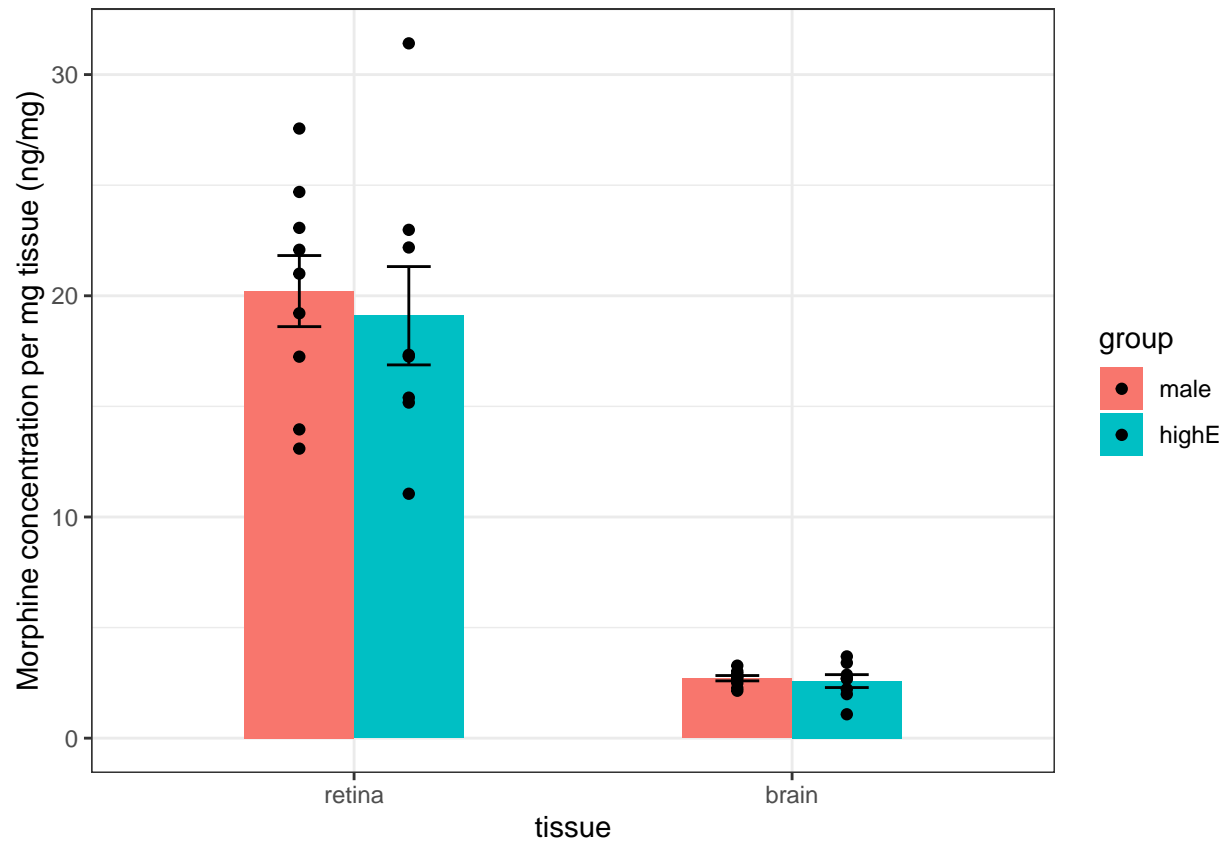
```
plot_tissue_litt
```



```
plot_stage_litt <- ggplot() +
  geom_bar(data=SumStat_sex1itt, aes(x=tissue, y=mean_conc_wt, fill=group), stat="identity", position=position_dodge(width=0.5)) +
  geom_errorbar(data=SumStat_sex1itt, aes(x=tissue, ymin=mean_conc_wt - se_conc_wt, ymax=mean_conc_wt + se_conc_wt, fill=group), position=position_dodge(width=0.5)) +
  geom_point(data=litter, aes(x=tissue, y=conc_wt, fill=group), position=position_dodge(width=0.5)) +
  scale_y_continuous(name="Morphine concentration per mg tissue (ng/mg)") + theme_bw()
```

```
## Warning: Ignoring unknown aesthetics: fill
```

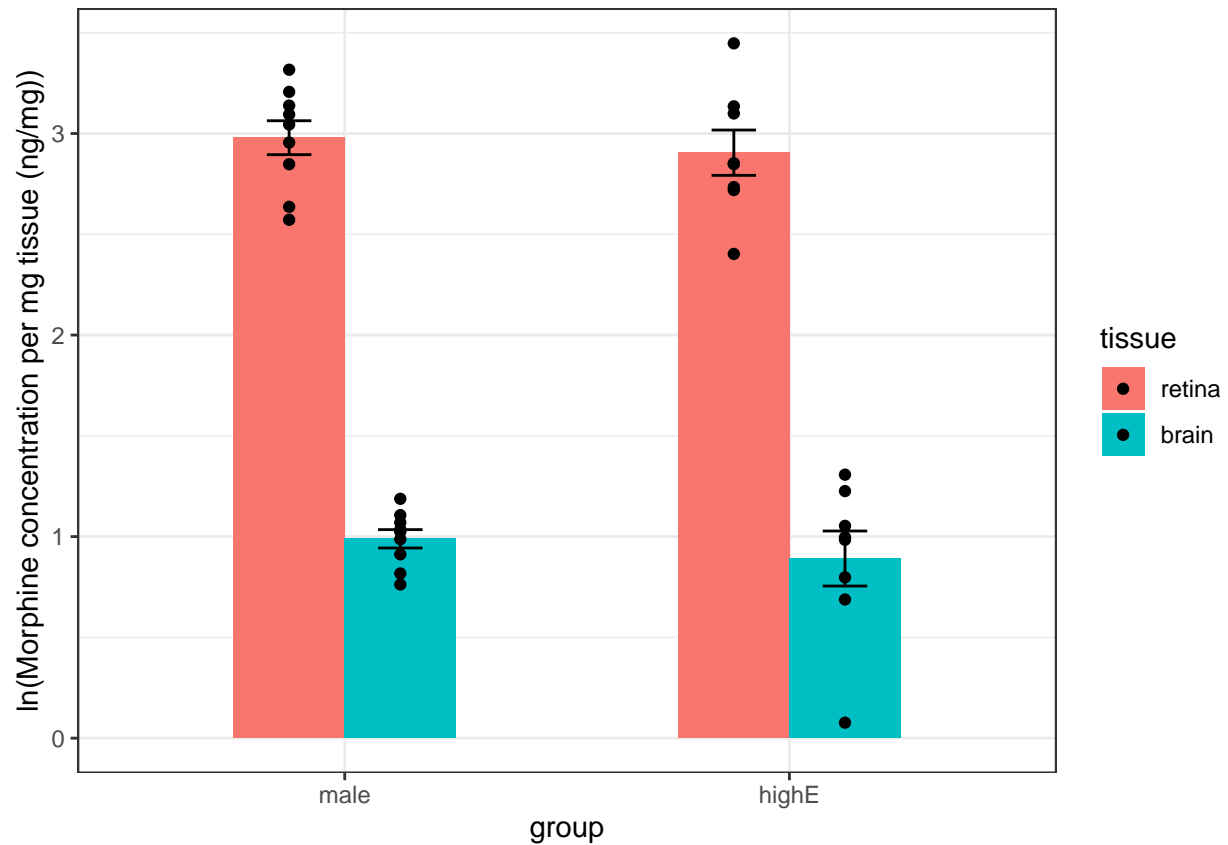
```
plot_stage_litt
```



```
plot_tissueL_litt <- ggplot() +
  geom_bar(data=SumStat_sexLitt, aes(x=group, y=log_conc, fill=tissue), stat="identity", position=position_dodge()) +
  geom_errorbar(data=SumStat_sexLitt, aes(x=group, ymin=log_conc - se_conc_log, ymax=log_conc + se_conc_log), width=0.5) +
  scale_y_continuous(name="ln(Morphine concentration per mg tissue (ng/mg))") + theme_bw()
```

```
## Warning: Ignoring unknown aesthetics: fill
```

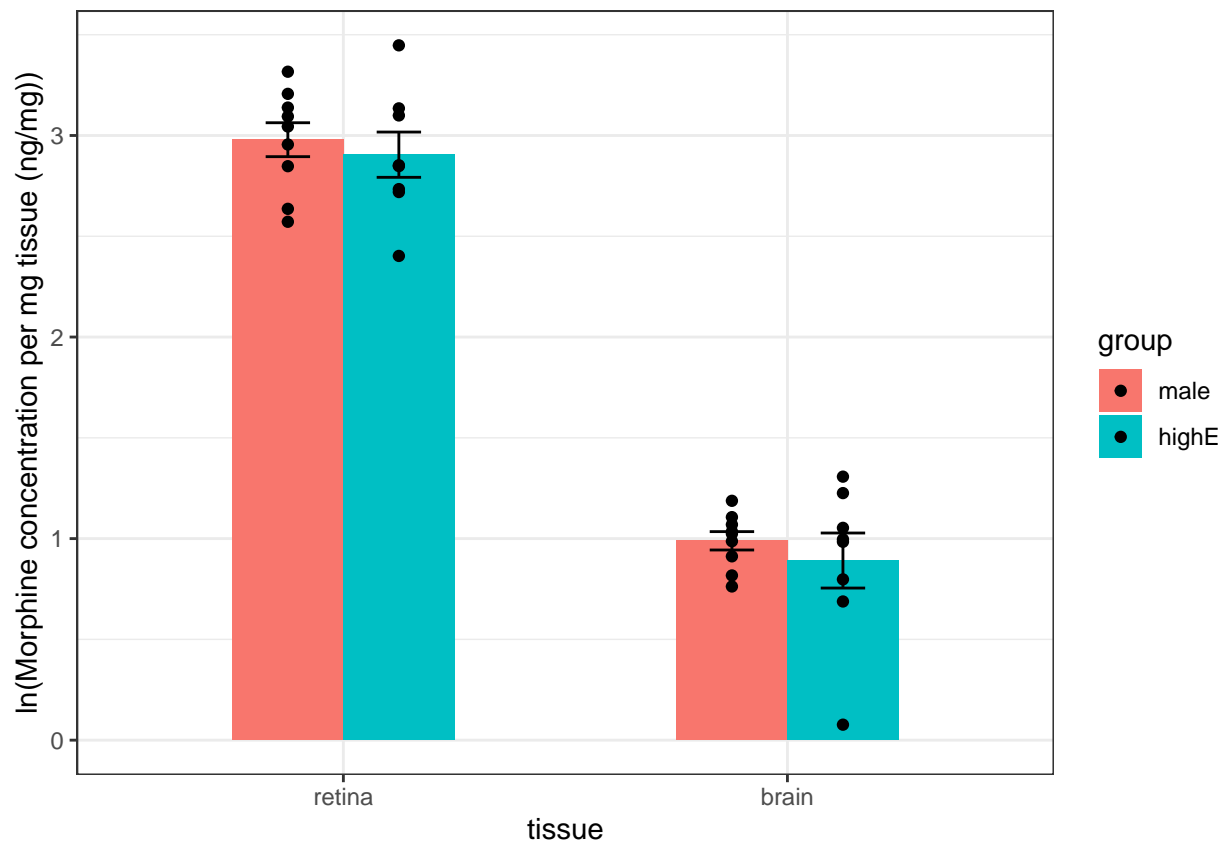
```
plot_tissueL_litt
```



```
plot_stageL_litt <- ggplot() +
  geom_bar(data=SumStat_sexLitt, aes(x=tissue, y=log_conc, fill=group), stat="identity", position=position_dodge()) +
  geom_errorbar(data=SumStat_sexLitt, aes(x=tissue, ymin=log_conc - se_conc_log, ymax=log_conc + se_conc_log), width=0.5) +
  scale_y_continuous(name="ln(Morphine concentration per mg tissue (ng/mg))") + theme_bw()
```

```
## Warning: Ignoring unknown aesthetics: fill
```

```
plot_stageL_litt
```



Comparisons between estrus stages

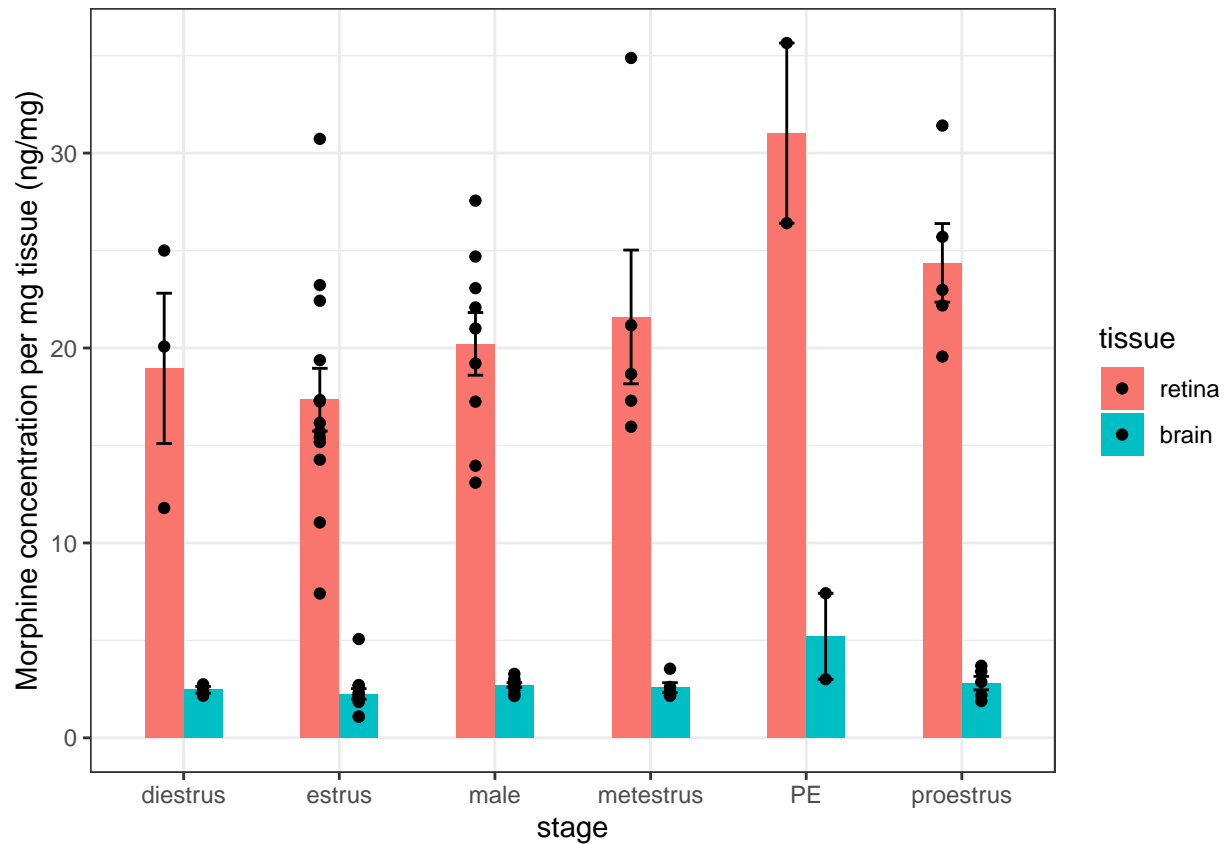
```
SumStat_sexstage <- dplyr::summarise(group_by(sex_diff, tissue, stage),
  n = n(),
  mean_conc_wt = mean(conc_wt),
  sd_conc_wt = sd(conc_wt),
  se_conc_wt = sd_conc_wt/sqrt(n),
  log_conc = mean(log(conc_wt)),
  sd_conc_log = sd(log(conc_wt)),
  se_conc_log = sd_conc_log/sqrt(n),
  mean_wt = mean(body_wt),
  sd_wt = sd(body_wt),
  mean_dose = mean(dose),
  sd_dose = sd(dose),
)
```

'summarise()' has grouped output by 'tissue'. You can override using the
'.groups' argument.

```
plot_stage_tissue <- ggplot() +
  geom_bar(data=SumStat_sexstage, aes(x=stage, y=mean_conc_wt, fill=tissue), stat="identity", position=position_dodge(width=0.5)) +
  geom_errorbar(data=SumStat_sexstage, aes(x=stage, ymin=mean_conc_wt - se_conc_wt, ymax=mean_conc_wt + se_conc_wt, fill=tissue), position=position_dodge(width=0.5)) +
  geom_point(data=sex_diff, aes(x=stage, y=conc_wt, fill=tissue), position=position_dodge(width=0.5)) +
  scale_y_continuous(name="Morphine concentration per mg tissue (ng/mg)") + theme_bw()
```

```
## Warning: Ignoring unknown aesthetics: fill
```

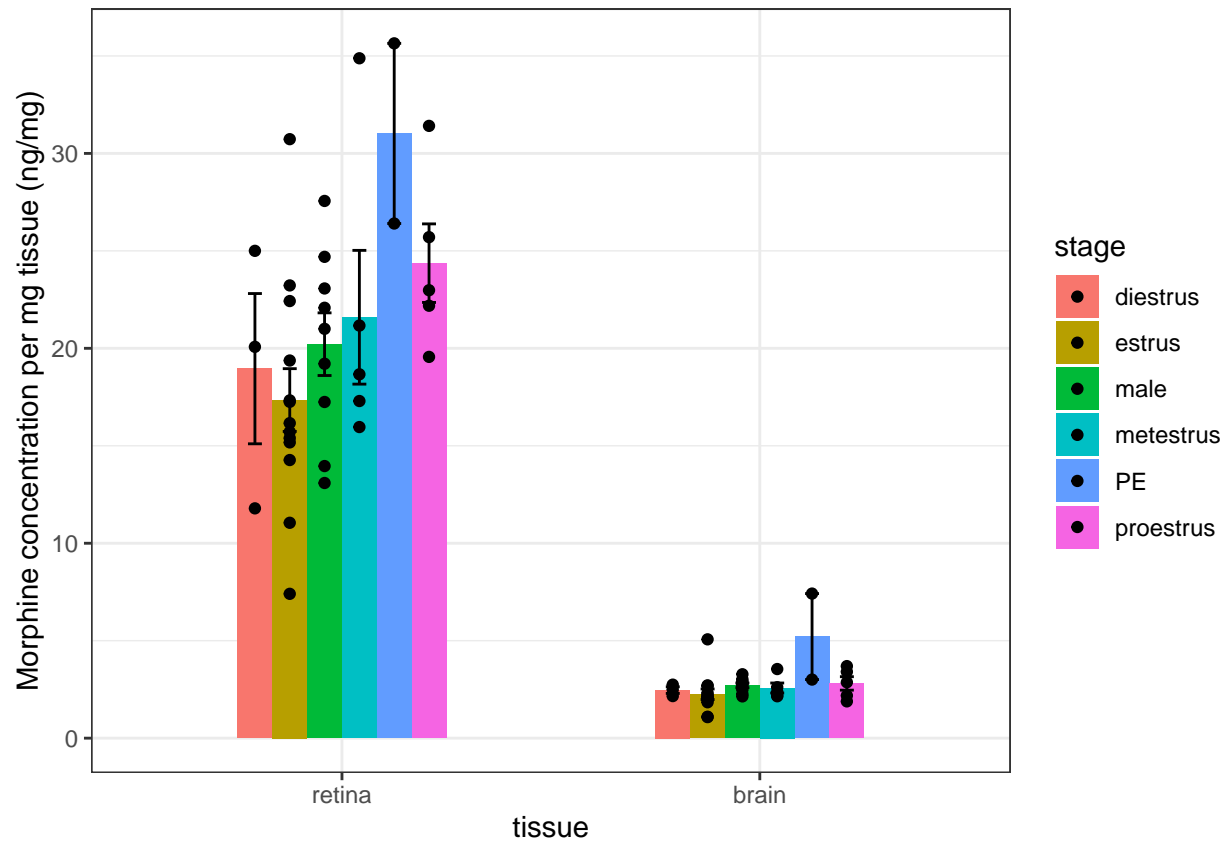
```
plot_stage_tissue
```



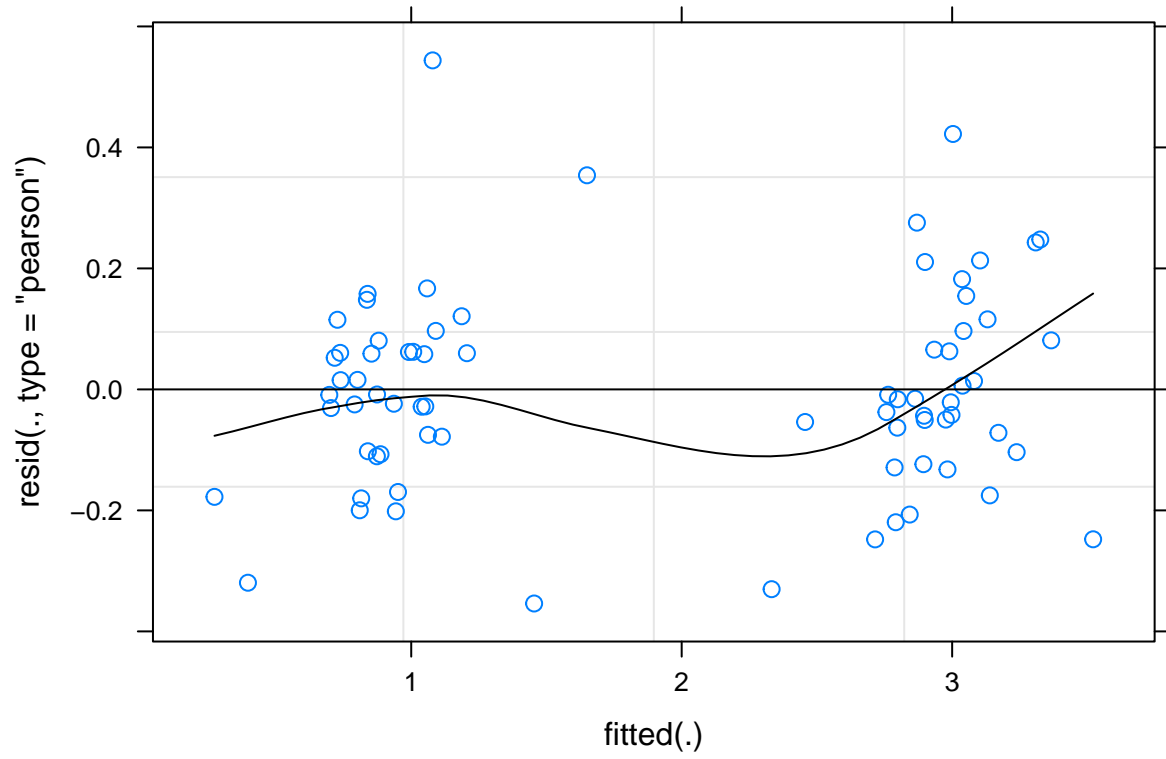
```
plot_stages <- ggplot() +
  geom_bar(data=SumStat_sexstage, aes(x=tissue, y=mean_conc_wt, fill=stage), stat="identity", position=position_dodge(width=0.5)) +
  geom_errorbar(data=SumStat_sexstage, aes(x=tissue, ymin=mean_conc_wt - se_conc_wt, ymax=mean_conc_wt + se_conc_wt, fill=stage), position=position_dodge(width=0.5)) +
  geom_point(data=sex_diff, aes(x=tissue, y=conc_wt, fill=stage), position=position_dodge(width=0.5)) +
  scale_y_continuous(name="Morphine concentration per mg tissue (ng/mg)") + theme_bw()
```

```
## Warning: Ignoring unknown aesthetics: fill
```

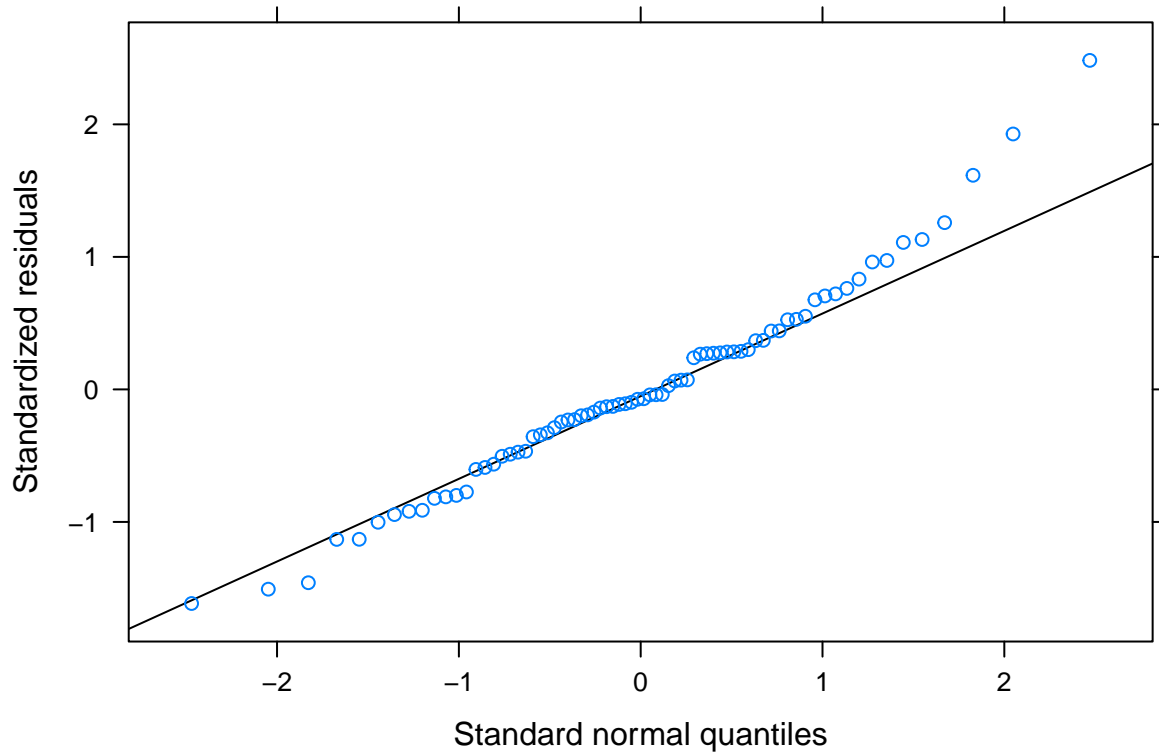
```
plot_stages
```



```
lmer_stage <- lmer(log(conc_wt) ~ stage*tissue+(1|animal), data =sex_diff)
plot(lmer_stage, type=c("p","smooth"), col.line=1)
```



```
lattice::qqmath(lmer_stage)
```

```
anova(lmer_stage)
```

```
## Analysis of Variance Table
##           npar Sum Sq Mean Sq  F value
## stage           5  0.789   0.158    3.2863
## tissue           1 77.725  77.725 1619.6736
## stage:tissue     5  0.102   0.020    0.4244
```

```
emmeans::emmeans(lmer_stage, pairwise ~ tissue | stage)
```

```
## $emmeans
## stage = diestrus:
##   tissue emmean      SE    df lower.CL upper.CL
##   retina  2.895 0.1753 50.4    2.543    3.247
##   brain   0.897 0.1753 50.4    0.545    1.249
##
## stage = estrus:
##   tissue emmean      SE    df lower.CL upper.CL
##   retina  2.800 0.0842 50.4    2.631    2.969
##   brain   0.740 0.0842 50.4    0.571    0.909
##
## stage = male:
##   tissue emmean      SE    df lower.CL upper.CL
##   retina  2.979 0.1012 50.4    2.776    3.182
```

```

## brain 0.989 0.1012 50.4 0.786 1.192
##
## stage = metestrus:
## tissue emmean SE df lower.CL upper.CL
## retina 3.030 0.1358 50.4 2.758 3.303
## brain 0.927 0.1358 50.4 0.655 1.200
##
## stage = PE:
## tissue emmean SE df lower.CL upper.CL
## retina 3.424 0.2147 50.4 2.993 3.855
## brain 1.552 0.2147 50.4 1.121 1.983
##
## stage = proestrus:
## tissue emmean SE df lower.CL upper.CL
## retina 3.180 0.1358 50.4 2.908 3.453
## brain 1.001 0.1358 50.4 0.728 1.273
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## Confidence level used: 0.95
##
## $contrasts
## stage = diestrus:
## contrast estimate SE df t.ratio p.value
## retina - brain 2.00 0.1789 31 11.171 <.0001
##
## stage = estrus:
## contrast estimate SE df t.ratio p.value
## retina - brain 2.06 0.0859 31 23.974 <.0001
##
## stage = male:
## contrast estimate SE df t.ratio p.value
## retina - brain 1.99 0.1033 31 19.271 <.0001
##
## stage = metestrus:
## contrast estimate SE df t.ratio p.value
## retina - brain 2.10 0.1385 31 15.179 <.0001
##
## stage = PE:
## contrast estimate SE df t.ratio p.value
## retina - brain 1.87 0.2191 31 8.543 <.0001
##
## stage = proestrus:
## contrast estimate SE df t.ratio p.value
## retina - brain 2.18 0.1385 31 15.732 <.0001
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.

emmeans::emmeans(lmer_stage, pairwise ~ stage | tissue)

## $emmeans
## tissue = retina:
## stage emmean SE df lower.CL upper.CL

```

```

## diestrus 2.895 0.1753 50.4 2.543 3.247
## estrus 2.800 0.0842 50.4 2.631 2.969
## male 2.979 0.1012 50.4 2.776 3.182
## metestrus 3.030 0.1358 50.4 2.758 3.303
## PE 3.424 0.2147 50.4 2.993 3.855
## proestrus 3.180 0.1358 50.4 2.908 3.453
##
## tissue = brain:
## stage emmean SE df lower.CL upper.CL
## diestrus 0.897 0.1753 50.4 0.545 1.249
## estrus 0.740 0.0842 50.4 0.571 0.909
## male 0.989 0.1012 50.4 0.786 1.192
## metestrus 0.927 0.1358 50.4 0.655 1.200
## PE 1.552 0.2147 50.4 1.121 1.983
## proestrus 1.001 0.1358 50.4 0.728 1.273
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## Confidence level used: 0.95
##
## $contrasts
## tissue = retina:
## contrast estimate SE df t.ratio p.value
## diestrus - estrus 0.0953 0.194 50.4 0.490 0.9963
## diestrus - male -0.0839 0.202 50.4 -0.414 0.9983
## diestrus - metestrus -0.1351 0.222 50.4 -0.609 0.9899
## diestrus - PE -0.5283 0.277 50.4 -1.907 0.4102
## diestrus - proestrus -0.2850 0.222 50.4 -1.286 0.7913
## estrus - male -0.1792 0.132 50.4 -1.361 0.7494
## estrus - metestrus -0.2305 0.160 50.4 -1.443 0.7012
## estrus - PE -0.6237 0.231 50.4 -2.705 0.0920
## estrus - proestrus -0.3804 0.160 50.4 -2.381 0.1825
## male - metestrus -0.0513 0.169 50.4 -0.303 0.9996
## male - PE -0.4445 0.237 50.4 -1.873 0.4302
## male - proestrus -0.2012 0.169 50.4 -1.188 0.8404
## metestrus - PE -0.3932 0.254 50.4 -1.548 0.6354
## metestrus - proestrus -0.1499 0.192 50.4 -0.781 0.9695
## PE - proestrus 0.2433 0.254 50.4 0.958 0.9289
##
## tissue = brain:
## contrast estimate SE df t.ratio p.value
## diestrus - estrus 0.1572 0.194 50.4 0.809 0.9646
## diestrus - male -0.0918 0.202 50.4 -0.454 0.9974
## diestrus - metestrus -0.0301 0.222 50.4 -0.136 1.0000
## diestrus - PE -0.6549 0.277 50.4 -2.363 0.1889
## diestrus - proestrus -0.1034 0.222 50.4 -0.466 0.9971
## estrus - male -0.2491 0.132 50.4 -1.892 0.4188
## estrus - metestrus -0.1873 0.160 50.4 -1.172 0.8477
## estrus - PE -0.8122 0.231 50.4 -3.522 0.0112
## estrus - proestrus -0.2606 0.160 50.4 -1.632 0.5821
## male - metestrus 0.0618 0.169 50.4 0.365 0.9991
## male - PE -0.5631 0.237 50.4 -2.373 0.1854
## male - proestrus -0.0116 0.169 50.4 -0.068 1.0000
## metestrus - PE -0.6249 0.254 50.4 -2.460 0.1557

```

```
## metestrus - proestrus -0.0733 0.192 50.4 -0.382 0.9989
## PE - proestrus      0.5515 0.254 50.4  2.172 0.2690
##
## Degrees-of-freedom method: kenward-roger
## Results are given on the log (not the response) scale.
## P value adjustment: tukey method for comparing a family of 6 estimates
```

```
shapiro.test(resid(lmer_stage))
```

```
##
## Shapiro-Wilk normality test
##
## data:  resid(lmer_stage)
## W = 0.97701, p-value = 0.196
```