Life-long indeterminate growth in brittle stars is driven by a subterminal growth zone at arm tips

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Contents

Abundance of dividing (BrdU-incorporated) cells (per m3):

Importing modules and libraries:

```
library(ggplot2) # For the plots
library(ggsignif) # To perform Wilcoxon/Mann-Whitney tests on vectors of data
library(ggpubr) # For the Kruskal-Wallis test
library(multcompView) # For compact display of ANOVA results
library(FSA) # This will help with the Dunn's test
```

```
## Registered S3 methods overwritten by 'FSA':
##   method       from
##   confint.boot car
##   hist.boot    car
```

```
## ## FSA v0.9.1. See citation('FSA') if used in publication.
## ## Run fishR() for related website and fishR('IFAR') for related book.
```

```
library(PMCMRplus) # This will help with the Dwass-Steel-Critchlow-Fligner test
```

Data:

```
Cells <- c(9.45E-05, 7.23E-05, 9.84E-05, 5.53E-05, 1.18E-04,
           2.39E-04, 1.61E-04, 2.39E-04, 1.16E-04, 1.12E-04,
           1.91E-04, 8.72E-05, 1.92E-04, 1.11E-04, 1.25E-04,
           1.53E-04, 6.31E-05, 1.33E-04, 5.94E-05, 9.51E-05)
Segments <- c('Terminal', 'Terminal', 'Terminal', 'Terminal', 'Terminal',
              'Segment 2', 'Segment 2', 'Segment 2', 'Segment 2', 'Segment 2',
              'Segment 3', 'Segment 3', 'Segment 3', 'Segment 3', 'Segment 3',
              'Segment 4', 'Segment 4', 'Segment 4', 'Segment 4', 'Segment 4')
Animals <- c('Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5',
             'Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5',
             'Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5',
             'Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5')
BrdU_per_vol <- data.frame(Cells, Segments, Animals)
BrdU_per_vol$Segments <- factor(BrdU_per_vol$Segments,
                                levels = c("Terminal", "Segment 2", "Segment 3", "Segment 4")
                                )
```

One-way ANOVA:

Here we perform a one-way ANOVA followed by a Tukey's test.

```
# Compute the analysis of variance
res.aov <- aov(Cells ~ Segments, data = BrdU_per_vol)
# Summary of the analysis
summary(res.aov)
```

```
##             Df    Sum Sq   Mean Sq F value Pr(>F)
## Segments     3 2.292e-08 7.641e-09   3.567 0.0379 *
## Residuals   16 3.427e-08 2.142e-09
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
# Tukey's test
tukey <- TukeyHSD(res.aov)
print(tukey)
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = Cells ~ Segments, data = BrdU_per_vol)
##
## $Segments
##                            diff           lwr          upr     p adj
## Segment 2-Terminal   8.570e-05  1.955306e-06 1.694447e-04 0.0439664
## Segment 3-Terminal   5.354e-05 -3.020469e-05 1.372847e-04 0.2963751
## Segment 4-Terminal   1.302e-05 -7.072469e-05 9.676469e-05 0.9696834
## Segment 3-Segment 2 -3.216e-05 -1.159047e-04 5.158469e-05 0.6953538
## Segment 4-Segment 2 -7.268e-05 -1.564247e-04 1.106469e-05 0.1010972
## Segment 4-Segment 3 -4.052e-05 -1.242647e-04 4.322469e-05 0.5263107
```

```
# Compact letter display
cld <- multcompLetters4(res.aov, tukey)
print(cld)
```

```
## $Segments
## Segment 2 Segment 3 Segment 4  Terminal
```

```
##       "a"      "ab"      "ab"       "b"
```

Kruskal-Wallis test:

The `kruskal.test` performs a Kruskal-Wallis rank sum test. We follow this test with differect add hoc analyses.

The function `dunnTest` performs the Dunn's test of multiple comparisons following a Kruskal-Wallis test.

The fuction `dscfAllPairsTest` performs the all-pairs comparison test for different factor levels according to Dwass, Steel, Critchlow and Fligner.

```
MyTest <- kruskal.test(Cells ~ Segments,
  data = BrdU_per_vol)
MyTest
```

```
##
##  Kruskal-Wallis rank sum test
##
## data:  Cells by Segments
## Kruskal-Wallis chi-squared = 7.0739, df = 3, p-value = 0.06958
```

```
summary(MyTest)
```

```
##           Length Class  Mode
## statistic 1      -none- numeric
## parameter 1      -none- numeric
## p.value   1      -none- numeric
## method    1      -none- character
## data.name 1      -none- character
```

```
dunnTest(Cells ~ Segments,
         data = BrdU_per_vol,
         method = "bonferroni")
```

```
## Dunn (1964) Kruskal-Wallis multiple comparison
```

```
##   p-values adjusted with the Bonferroni method.
```

```
##              Comparison         Z    P.unadj      P.adj
## 1 Segment 2 - Segment 3 0.7486130 0.45409052 1.00000000
## 2 Segment 2 - Segment 4 1.8715324 0.06127132 0.36762793
## 3 Segment 3 - Segment 4 1.1229194 0.26147171 1.00000000
## 4  Segment 2 - Terminal 2.4062560 0.01611697 0.09670181
## 5  Segment 3 - Terminal 1.6576430 0.09738955 0.58433728
## 6  Segment 4 - Terminal 0.5347235 0.59284104 1.00000000
```

```
dscfAllPairsTest(Cells ~ Segments,
         data = BrdU_per_vol,
         method = "bonferroni")
```

```
##
##  Pairwise comparisons using Dwass-Steele-Critchlow-Fligner all-pairs test
```

```
## data: Cells by Segments
```

```
##           Terminal Segment 2 Segment 3
## Segment 2 0.12     -         -
## Segment 3 0.29     0.78      -
## Segment 4 0.95     0.28      0.66
```

```
##
## P value adjustment method: single-step
```

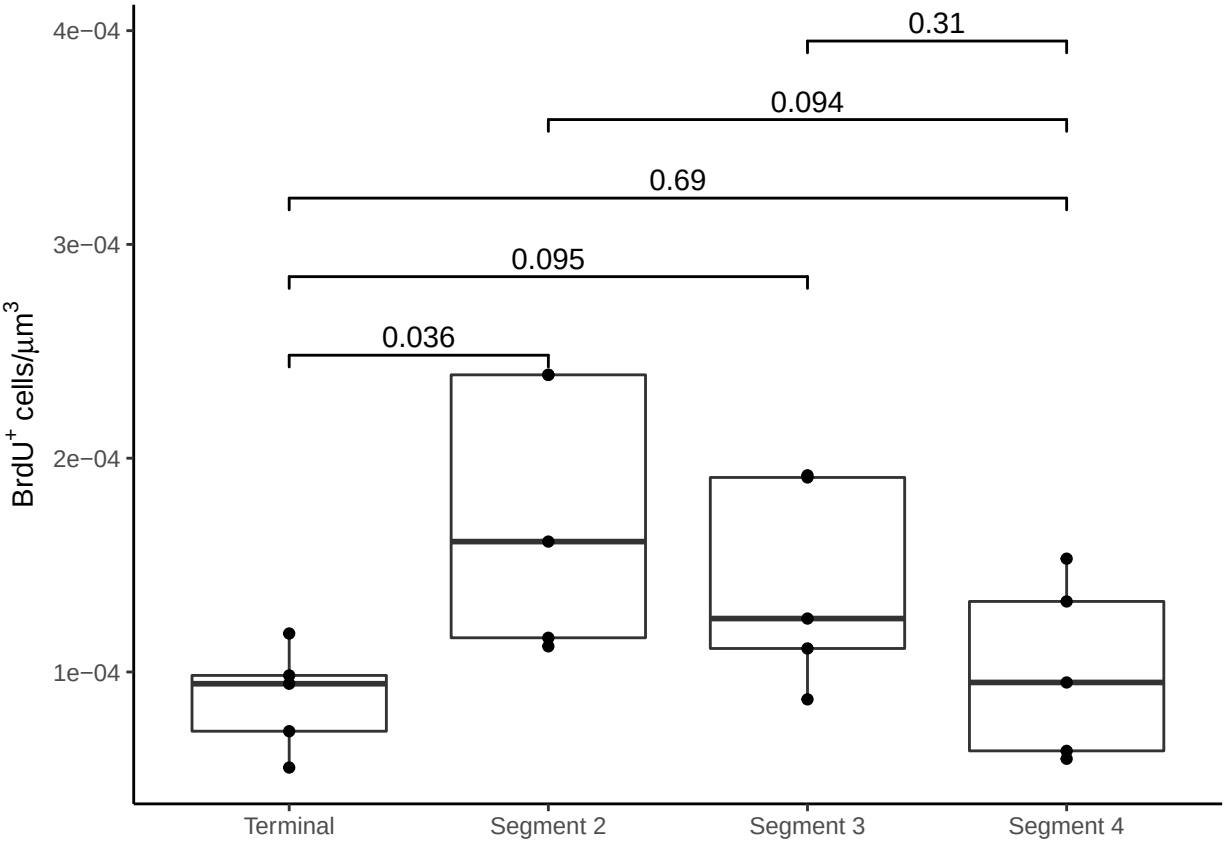
Boxplots:

Were we plot the data as a boxplot with data points on top of it. The brackets with P values correspond to the results of the Wilcoxon-Mann-Whitney test.

```
p <-ggplot(data=BrdU_per_vol, aes(x=Segments, y=Cells))
p <- p + geom_boxplot()
p <- p + geom_signif(test="wilcox.test",
  comparisons = combn(levels(BrdU_per_vol$Segments),
  2,
  simplify = F)[-4],
  step_increase = 0.2)
# p <- p + stat_compare_means(test="kruskal.test")
p <- p + geom_point()
p <- p + labs(y = expression(paste("BrdU"^'+'~'cells/', mu, 'm'^3)))
p <- p + theme_classic()
p <- p + theme(axis.title.x=element_blank())
print(p)
```

```
## Warning in wilcox.test.default(c(9.45e-05, 7.23e-05, 9.84e-05, 5.53e-05, :
## cannot compute exact p-value with ties
```

```
## Warning in wilcox.test.default(c(0.000239, 0.000161, 0.000239, 0.000116, :
## cannot compute exact p-value with ties
```



```
pdf("Figure8B.pdf")
print(p)
```

```
## Warning in wilcox.test.default(c(9.45e-05, 7.23e-05, 9.84e-05, 5.53e-05, :
## cannot compute exact p-value with ties

## Warning in wilcox.test.default(c(9.45e-05, 7.23e-05, 9.84e-05, 5.53e-05, :
## cannot compute exact p-value with ties
```

```
garbage <- dev.off()
```

Relative abundance of repeatedly proliferating cells:

The relative abundance of repeatedly proliferating cells correspond to those cells that have incorporated both thymidine analogs.

Data:

```
Percentages <- c(0.034314, 0.016949, 0.03352, 0.140625, 0.120968,
                 0.044077, 0.007212, 0.037879, 0.122807, 0.079208,
                 0.00713, 0, 0.006834, 0.14554, 0.131356, 0.004515,
                 0, 0.004396, 0.103261, 0.111111)
Segments <- c('Terminal', 'Terminal', 'Terminal', 'Terminal', 'Terminal',
              'Segment 2', 'Segment 2', 'Segment 2', 'Segment 2', 'Segment 2',
              'Segment 3', 'Segment 3', 'Segment 3', 'Segment 3', 'Segment 3',
              'Segment 4', 'Segment 4', 'Segment 4', 'Segment 4', 'Segment 4')
Animals <- c('Animal 1', 'Animal 2', 'Animal 3', 'Animal 4','Animal 5',
             'Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5',
             'Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5',
             'Animal 1', 'Animal 2', 'Animal 3', 'Animal 4', 'Animal 5')
BrdU_EdU_cells_Percentage <- data.frame(Percentages, Segments, Animals)
BrdU_EdU_cells_Percentage$Segments <- factor(BrdU_EdU_cells_Percentage$Segments,
  levels = c("Terminal", "Segment 2", "Segment 3", "Segment 4"))
```

One-way ANOVA:

Here we perform a one-way ANOVA followed by a Tukey's test.

```
# Compute the analysis of variance
res.aov <- aov(Percentages ~ Segments,
               data = BrdU_EdU_cells_Percentage)
# Summary of the analysis
summary(res.aov)
```

```
##             Df  Sum Sq  Mean Sq F value Pr(>F)
## Segments     3 0.00152 0.000508   0.146  0.931
## Residuals   16 0.05552 0.003470
```

```
# Tukey's test
tukey <- TukeyHSD(res.aov)
print(tukey)
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
```

```
##
## Fit: aov(formula = Percentages ~ Segments, data = BrdU_EdU_cells_Percentage)
##
## $Segments
##                            diff        lwr       upr     p adj
## Segment 2-Terminal  -0.0110386 -0.1176287 0.0955515 0.9905997
## Segment 3-Terminal  -0.0111032 -0.1176933 0.0954869 0.9904380
## Segment 4-Terminal  -0.0246186 -0.1312087 0.0819715 0.9101880
## Segment 3-Segment 2 -0.0000646 -0.1066547 0.1065255 1.0000000
## Segment 4-Segment 2 -0.0135800 -0.1201701 0.0930101 0.9828396
## Segment 4-Segment 3 -0.0135154 -0.1201055 0.0930747 0.9830741
```

```
# Compact letter display
cld <- multcompLetters4(res.aov, tukey)
print(cld)
```

```
## $Segments
## $Segments$Letters
##  Terminal Segment 2 Segment 3 Segment 4
##       "a"       "a"       "a"       "a"
##
## $Segments$LetterMatrix
##              a
## Terminal  TRUE
## Segment 2 TRUE
## Segment 3 TRUE
## Segment 4 TRUE
```

Kruskal-Wallis test:

The `kruskal.test` performs a Kruskal-Wallis rank sum test. We follow this test with differect add hoc analyses.

The function `dunnTest` performs the Dunn's test of multiple comparisons following a Kruskal-Wallis test.

The fuction `dscfAllPairsTest` performs the all-pairs comparison test for different factor levels according to Dwass, Steel, Critchlow and Fligner.

```
MyTest <- kruskal.test(Percentages ~ Segments,
  data = BrdU_EdU_cells_Percentage)
MyTest
```

```
##
##  Kruskal-Wallis rank sum test
##
## data:  Percentages by Segments
## Kruskal-Wallis chi-squared = 2.1473, df = 3, p-value = 0.5424
```

```
summary(MyTest)
```

```
##           Length Class  Mode
## statistic 1      -none- numeric
## parameter 1      -none- numeric
## p.value   1      -none- numeric
## method    1      -none- character
## data.name 1      -none- character
```

```r
#perform Dunn's Test with Bonferroni correction for p-values
dunnTest(Percentages ~ Segments,
         data = BrdU_EdU_cells_Percentage,
         method = "bonferroni")
```

```
## Dunn (1964) Kruskal-Wallis multiple comparison
```

```
##   p-values adjusted with the Bonferroni method.
```

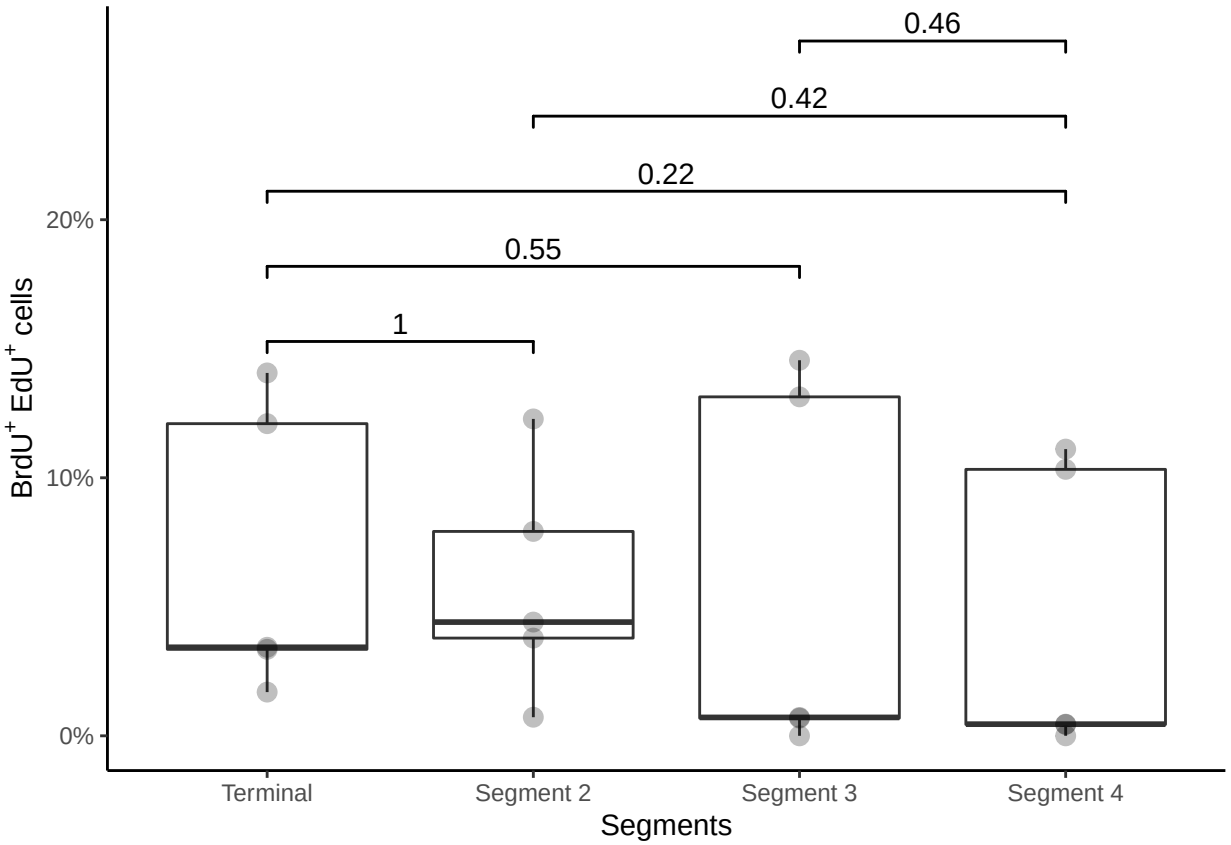
```
##              Comparison          Z   P.unadj P.adj
## 1 Segment 2 - Segment 3  0.5079874 0.6114622     1
## 2 Segment 2 - Segment 4  1.2031280 0.2289268     1
## 3 Segment 3 - Segment 4  0.6951406 0.4869672     1
## 4  Segment 2 - Terminal -0.1069447 0.9148328     1
## 5  Segment 3 - Terminal -0.6149321 0.5385996     1
## 6  Segment 4 - Terminal -1.3100727 0.1901712     1
```

Boxplots:

Were we plot the data as a boxplot with data points on top of it. The brackets with P values correspond to the results of the Wilcoxon-Mann-Whitney test.

```r
p <-ggplot(data=BrdU_EdU_cells_Percentage,
           aes(x=Segments, y=Percentages))
p <- p + geom_boxplot()
p <- p + geom_signif(test="wilcox.test",
  comparisons = combn(levels(BrdU_EdU_cells_Percentage$Segments),
  2,
  simplify = F)[-4],
  step_increase = 0.2)
# p <- p + stat_compare_means(test="kruskal.test")
p <- p + geom_point(show.legend = NA, size = 3, alpha = .25)
p <- p + scale_y_continuous(labels = scales::percent)
p <- p + labs(y = bquote('BrdU'^'+'~'EdU'^'+'~'cells'))
p <- p + theme(axis.title.x=element_blank())
p <- p + theme_classic()
print(p)
```

```
## Warning in wilcox.test.default(c(0.00713, 0, 0.006834, 0.14554, 0.131356: cannot
## compute exact p-value with ties
```




```
pdf("Figure8C.pdf")
print(p)
```

```
## Warning in wilcox.test.default(c(0.00713, 0, 0.006834, 0.14554, 0.131356: cannot
## compute exact p-value with ties
```

```
garbage <- dev.off()
```

Conclusion

As customary on the field, we performed a one-way ANOVA analysis that returns a significant result. Additional ad hoc analysis using the Tukey's test showed that the terminal is significantly different from segment 2. However, given the sample size, we may question the assumption of parametricity. In response to that, we also performed the nonparametric Kruskal-Wallis test. The Kruskal-Wallis test was insignificant, but follow-up ad hoc analysis using the nonparametric Wilcoxon-Mann-Whitney test corroborated Tukey's test results. Segment 2 is not significantly different from segments 3 and 4. However, there is a clear tendency showing that segment 2 has a higher median than segment 3 and segment 3 has a higher median than segment 4. Also, the cell count in segments 3 and 4 is not statistically significant from the terminal, but segment 4 has a larger median than the terminal. Together, these results indicate that the abundance of dividing cells is much higher in segment 2 than on the terminal. Cell proliferation in the more proximal segments (the 3rd and 4th) is gradually diminished compared to segment 2 but was also higher than in the terminal.