

Sequencing: Working with count data

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Contents

1	The Poisson distribution	1
1.1	The Poisson distribution in RNA-seq	2
1.2	Relative uncertainty for Poisson distributed random variables	3
2	Modeling count data: Generalized linear models	5
2.1	Why we can('t) use linear models to model count data	5
2.2	Generalized linear models	5
2.3	Statistical inference in GLMs	22
2.4	Model deviance, residuals and goodness-of-fit	26
2.5	Overdispersion	27
3	A final note and summary	33
4	References	33
5	Junk	33

In this lecture we will introduce the main principles of working with count data, and how to model these using generalized linear models. We focus on introducing the concept of generalized linear models, and how to interpret its results. We only touch briefly upon statistical inference, providing the main results rather than the theory behind it, such that they can be applied to genomics data analysis.

1 The Poisson distribution

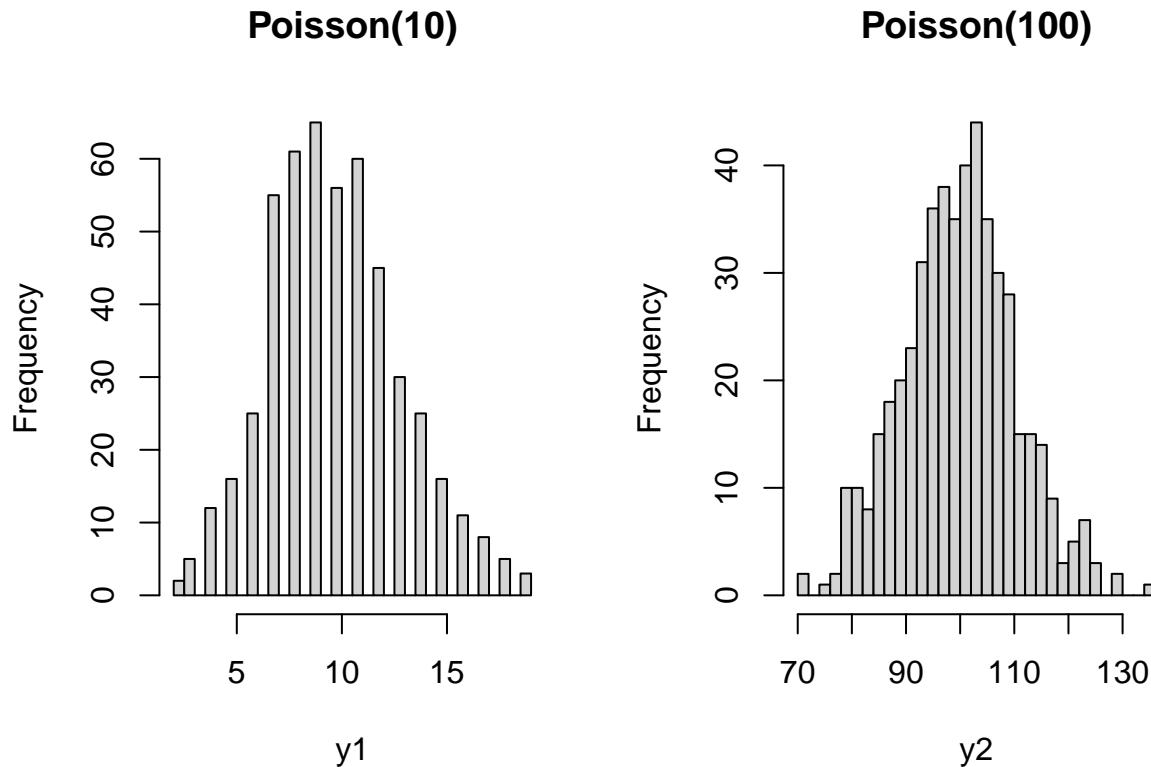
- The Poisson distribution is a typical count distribution that is generally popular and fairly easy to work with. It is defined by a single parameter: its mean μ . For a Poisson distributed random variable Y_i with observations $i \in \{1, \dots, n\}$, its variance is equal to its mean. That is, if $Y_i \sim Poi(\mu)$, then $E(Y_i) = Var(Y_i) = \mu$.
- This immediately shows an important feature of count data: the **mean-variance relationship**. Indeed, in count data, the variance will always be a function of the mean.

- This is quite intuitive. Consider the following example. You have two bird cages, where in one bird cage there are 10 birds, while in the other there are 100 birds. You let a sample of people count the number of birds in either one of the cages. It seems unlikely that a person in front of the 10-bird cage would come up with an estimate of 5, while it seems quite likely that someone in front of the 100-bird cage would come up with an estimate of 95. Even though the difference from the true value is the same, the exact value has an impact on the plausible deviation around it.

TODO: Make into discrete barplot.

```
set.seed(11)
y1 <- rpois(n=500, lambda=10)
y2 <- rpois(n=500, lambda=100)

par(mfrow = c(1,2))
hist(y1, main="Poisson(10)", breaks=40)
hist(y2, main="Poisson(100)", breaks=40)
```



1.1 The Poisson distribution in RNA-seq

- In RNA-seq, technical replicates represent different aliquots of the same sample being repeatedly. The underlying true expression of a gene can hence safely be assumed to be equal across these technical replicates.
- Marioni *et al.* (2008) have shown that, for most genes, the distribution of observed gene expression counts across technical replicates follow a Poisson distribution. A small proportion of genes ($\sim 0.5\%$) do not follow this Poisson model, however, and actually show evidence for ‘extra-Poisson variation’.

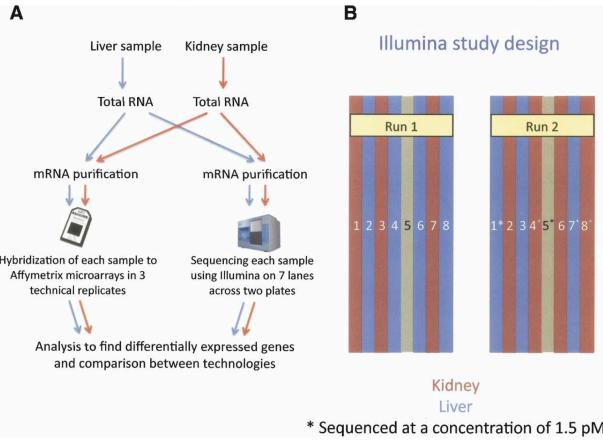


Figure 1. Graphical representation of the study design. (A) Summary of the experimental design. (B) The lanes in which each sample was sequenced across the two runs. In each run, the control sample was sequenced in lane 5. Samples were sequenced at two concentrations: 1.5 pM (indicated by an asterisk) and 3 pM (no asterisk).

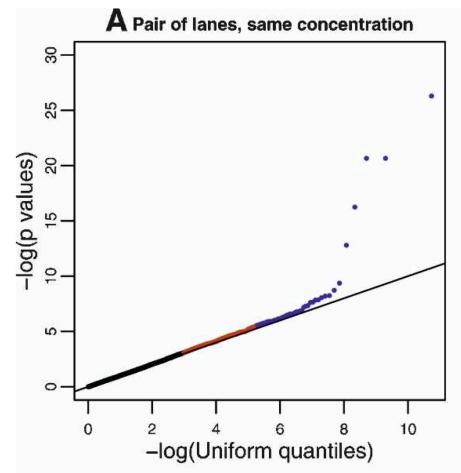


Figure 1: Figure: Technical replication in RNA-seq. Figures from Marioni et al. (2008).

1.2 Relative uncertainty for Poisson distributed random variables

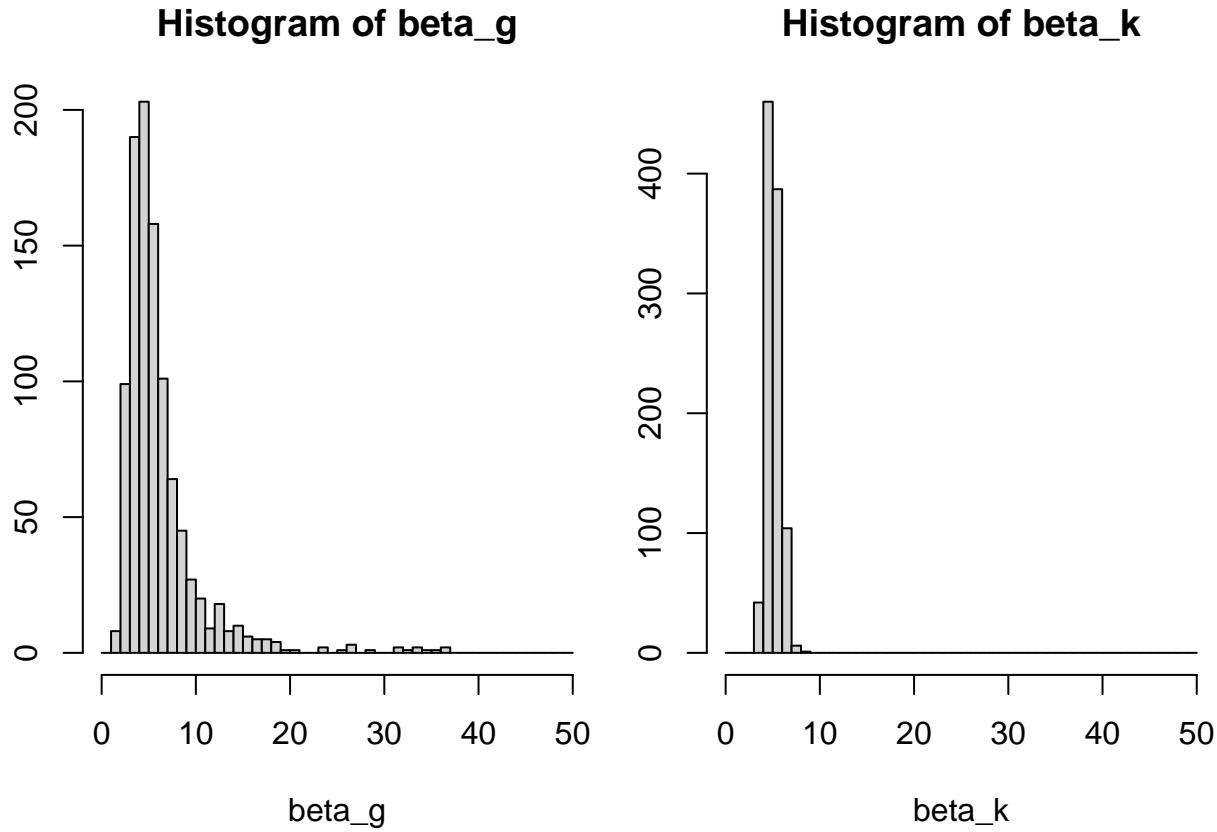
Take a minute to consider the following question:

- Suppose that we have a solid tumor sample from a cancer patient, as well as a sample of surrounding healthy tissue. For each sample, we have three technical replicates at our disposal. Let Y_{grt} denote the observed gene expression values of gene g in replicate r from tissue $t \in \{0, 1\}$, where $t = 0$ denotes healthy tissue and $t = 1$ denotes tumoral tissue.
- We then know that the random variables Y_{gr0} and Y_{gr1} follow a Poisson distribution, and we would estimate its mean as $\bar{Y}_{g0} = \frac{1}{3} \sum_{r=1}^3 Y_{gr0}$ and $\bar{Y}_{g1} = \frac{1}{3} \sum_{r=1}^3 Y_{gr1}$, respectively.
- Similar, for another gene k , we observe Y_{krt} , and estimate \bar{Y}_{k0} and \bar{Y}_{k1} correspondingly.
- Now suppose that $\beta_k = \bar{Y}_{k1}/\bar{Y}_{k0} = 5$, but also $\beta_g = \bar{Y}_{g1}/\bar{Y}_{g0} = 5$, i.e., the two genes have the same average expression ratio (also often called a fold-change) across samples. However, they are differently expressed as $\bar{Y}_{k1} = 100$, and $\bar{Y}_{g1} = 10$ (making $\bar{Y}_{k0} = 20$, and $\bar{Y}_{g0} = 2$).
- For which of the two genes is the uncertainty on the expression ratio the highest? In other words, do we trust β_k more or do we trust β_g more?

Let's approximate the uncertainty in β_g and β_k using simulation:

```
N <- 1e3
beta_g <- beta_k <- vector(length=N)
for(ii in 1:N){
  ygr1 <- rpois(n=3, lambda=10)
  ygr0 <- rpois(n=3, lambda=2)
  ykr1 <- rpois(n=3, lambda=100)
  ykr0 <- rpois(n=3, lambda=20)
  beta_g[ii] <- mean(ygr1) / mean(ygr0)
  beta_k[ii] <- mean(ykr1) / mean(ykr0)
}
```

```
par(mfrow=c(1,2), mar=c(4,2,3,1))
hist(beta_g, breaks=seq(0,50,by=1), xlim=c(0,50))
hist(beta_k, breaks=seq(0,50,by=1), xlim=c(0,50))
```



We clearly see that the uncertainty on β_g is much lower than on β_k . Even though the variance on the counts of gene g is higher, since its mean is higher and it is distributed as a Poisson variable. How do we explain this?

- We may explain this by considering the relative uncertainty on the mean. Relative uncertainty can be defined as the coefficient of variation $CV = \frac{\sigma}{\mu}$ (this is, the standard deviation divided by the mean). Indeed, the CV describes the relative deviation of the distribution relative to its mean, where a low CV indicates low dispersion with respect to the mean.
- Calculating the CV shows that **the relative uncertainty for gene k than for gene g , even though the variance on the raw counts is higher for gene k than for gene g .**
- This lower relative uncertainty on the mean then propagates further to a lower uncertainty on the fold-change. This basic result will be essential for understanding the results of a differential expression analysis!

```
sqrt(100)/100 #CV for gene k
```

```
## [1] 0.1
```

```
sqrt(10)/10 #CV for gene g
```

```
## [1] 0.3162278
```

2 Modeling count data: Generalized linear models

Just like we have modeled protein abundances in the proteomics part of this course to assess differential protein abundance, we can model gene expression counts to identify genes with differences in average expression between groups of samples.

2.1 Why we can('t) use linear models to model count data

- If we're using a linear model to model a response Y_i , with $i \in \{1, \dots, n\}$ in function of a single covariate X_i , the linear model can be defined as follows:

$$\begin{cases} Y_i &= \beta_0 + \beta_1 X_i + \epsilon_i \\ Y_i | X_i &\sim N(\beta_0 + \beta_1 X_i, \sigma^2 \mathbf{I}) \end{cases}$$

- Or, equivalently, in matrix form we would write

$$\begin{cases} Y_i &= \mathbf{X}_i^T \boldsymbol{\beta} + \epsilon_i \\ Y_i | \mathbf{X}_i &\sim N(\mathbf{X}_i^T \boldsymbol{\beta}, \sigma^2 \mathbf{I}), \end{cases}$$

where \mathbf{X} now represents our $n \times p$ design matrix, with row i corresponding to observation i .

- The variance-covariance matrix of \mathbf{Y} is assumed a diagonal matrix with σ^2 on the diagonal elements and zero everywhere else. This means that the data points are uncorrelated, and that every observation has the same variance σ^2 , also referred to as homoscedasticity. The latter obviously doesn't hold for count data, due to the mean-variance relationship. This makes linear models unsuitable to model count data.

2.2 Generalized linear models

- As the name suggests, generalized linear models (GLMs) extend linear models. In GLMs, we extend two things with respect to the linear model:
 - The **conditional distribution of the response variable** $Y_i | X_i$ can be assumed to follow any distribution that belongs to the **exponential family** of distributions, which includes the Gaussian but also other commonly known distributions, such as the Binomial, Gamma and Poisson distribution.
 - The linear model assumed a linear relationship between Y_i and X_i , since we assumed that $E(Y_i | X_i) = \mathbf{X}_i^T \boldsymbol{\beta}$. In GLMs, we will allow a **link function** $g()$ that links the conditional mean to the covariates. Hence, in GLMs we have that $g(E(Y_i | X_i)) = \mathbf{X}_i^T \boldsymbol{\beta}$. Note that each family has got a canonical link function, which is the identity link function $g(\mu) = \mu$ for Gaussian, the log link function $g(\mu) = \log \mu$ for Poisson, or the logit link function $g(\mu) = \log(\frac{\mu}{1-\mu})$ for Binomial.

- Remember that for a linear model we have a closed-form solution for estimating the regression coefficients β , i.e., $\hat{\beta} = (\mathbf{X}^T \mathbf{X})^{-1} \mathbf{X}^T \mathbf{Y}$.
- Fitting a GLM is much harder, however, since for many distributions that belong to the exponential family, the variance depends on the mean, and this mean-variance relationship must be taken into account.
- The **iterative reweighted least squares (IRLS)** algorithm is usually adopted for fitting GLMs. As the name suggests, it is an iterative algorithm (so no closed-form solutions!), where each data point is reweighted in each iteration according to its variance, which is a function of its estimated mean of the previous iteration. Indeed, observations with high variance will be downweighted and vice versa.
- The objective function used in the IRLS algorithm is the log-likelihood of the data under the posited model. This function is being maximized with respect to the regression coefficients β to find a solution $\hat{\beta}$.
- In the absence of a mean-variance relationship (such as is the case with the Gaussian distribution), the IRLS algorithm reduces to the closed-form solutions of a linear model.

2.2.1 A Poisson GLM

- We can define a Poisson GLM as follows

$$\begin{cases} Y_i & \sim Poi(\mu_i) \\ \log \mu_i & = \eta_i \\ \eta_i & = \mathbf{X}_i^T \beta \end{cases}$$

where Y_i is the response variable, with mean μ_i , η_i is the linear predictor, \mathbf{X} is the $n \times p$ model matrix and β is the $p \times 1$ matrix of regression coefficients.

- It is insightful to compare this model to a linear model where Y_i is log-transformed. Indeed, in the linear model case, we would be modeling $E(\log Y_i)$, while in the GLM we are modeling $\log E(Y_i)$.
- This shows that in the GLM setting we are modeling a transformed version of the expected value, and after retransforming we can interpret the fit in terms of the mean of our response variable. In the transformed linear model, however, we are working with the expected value of a transformed version of our response variable, and we will not be able to interpret the fit in terms of the mean (because $E(\log Y_i) \neq \log E(Y_i)$). In this specific case, we would have to resort to interpreting changes in terms of a geometric mean.
- Also note that $\mathbf{X}_i^T \beta \in]-\infty, \infty[$, while Y_i must be non-negative $[0, \infty[$. The link function helps with this, since the exponential function transforms any real number to a non-negative number, i.e., $\exp(\mathbf{X}_i^T \beta) \in [0, \infty[$.

2.2.2 Generalized linear models in R

Goals: - Fit Poisson GLM on bike data - Use it to explain GLM output - Use it to interpret coefficients: show importance of link - Then look at assumptions and pinpoint overdispersion. Solution will be for next lecture.

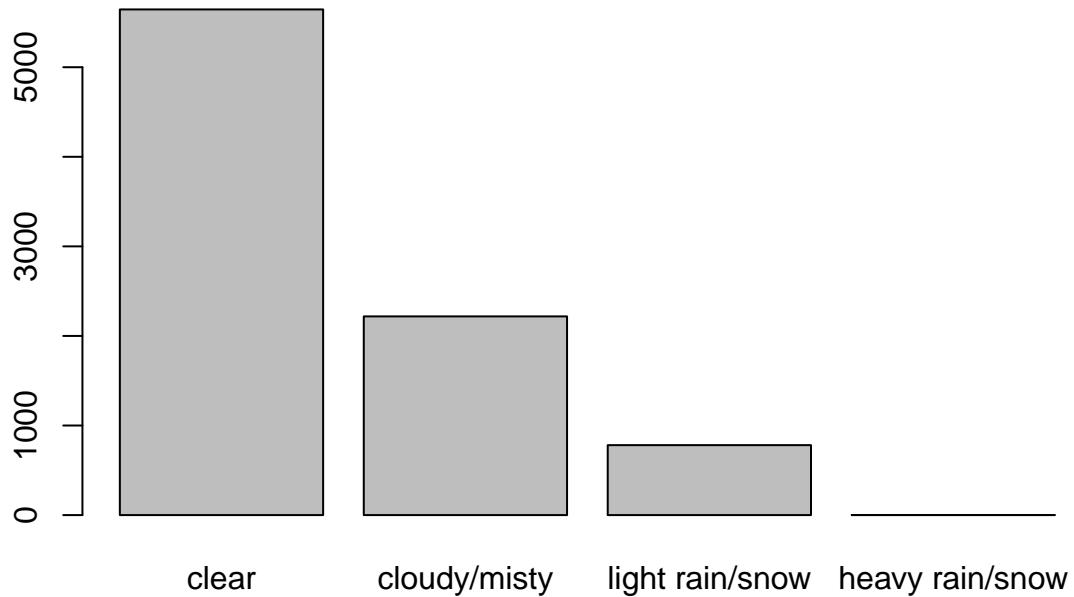
- In order to get familiar with GLMs, we will fit a Poisson GLM in R, using the **Bikeshare** dataset as part of the **ISLR2** package. This dataset records how many bikes were being used from a bike-sharing service, every hour of the day over a full year (365 days).
- Full information of the dataset is provided here. Variables of interest for us are:
 - **bikers**: Discrete count variable; the number of bikes being used that hour.
 - **hum**: Continuous variable ranging between 0 and 1; normalized humidity.
 - **hr**: Categorical variable between 0 and 23; the hour of the day. Note that one could also consider this variable to be numeric and model it as such.

- **weathersit**: Categorical variable; the weather condition of that hour, with
 1. Clear, Few clouds, Partly cloudy.
 2. Mist + Cloudy, Mist + Broken clouds, Mist + Few clouds, Mist.
 3. Light Snow, Light Rain + Thunderstorm + Scattered clouds, Light Rain + Scattered clouds.
 4. Heavy Rain + Ice Pallets + Thunderstorm + Mist, Snow + Fog.
-

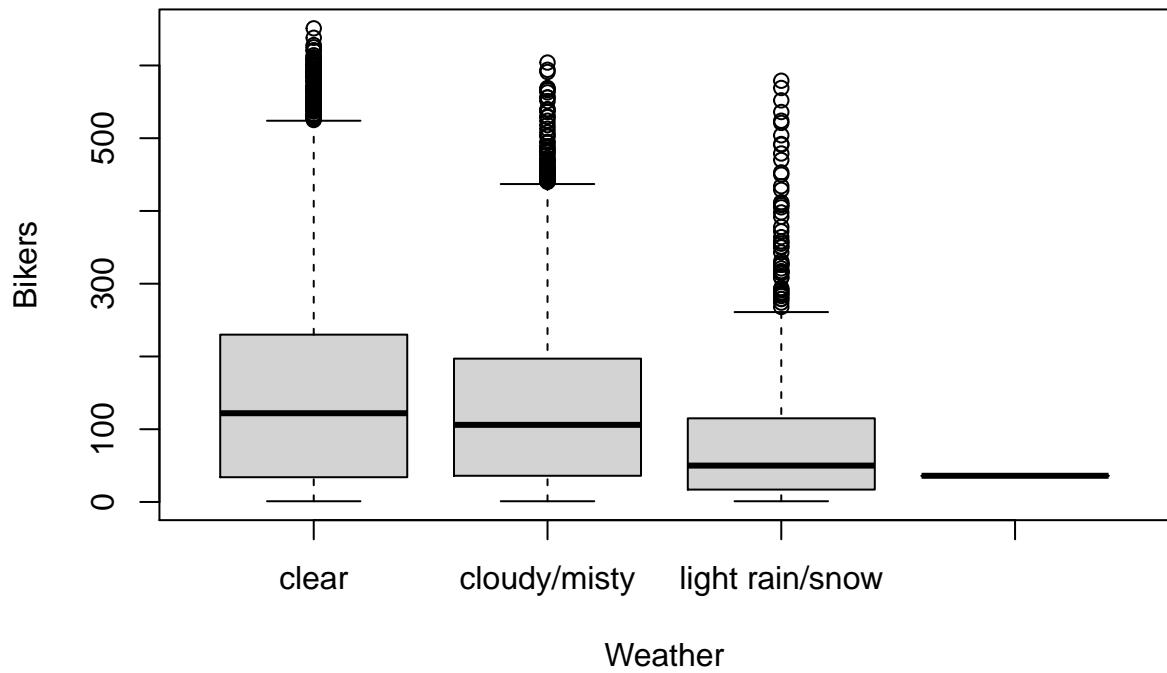
```
# if ISLR2 isn't installed, install it:
if(!"ISLR2" %in% installed.packages()[,1]){
  install.packages("ISLR2")
}
# load and preview the dataset:
data("Bikeshare", package="ISLR2")
head(Bikeshare)

##   season mnth day hr holiday weekday workingday   weathersit temp atemp hum
## 1     1 Jan  1  0       0       6       0      clear 0.24 0.2879 0.81
## 2     1 Jan  1  1       0       6       0      clear 0.22 0.2727 0.80
## 3     1 Jan  1  2       0       6       0      clear 0.22 0.2727 0.80
## 4     1 Jan  1  3       0       6       0      clear 0.24 0.2879 0.75
## 5     1 Jan  1  4       0       6       0      clear 0.24 0.2879 0.75
## 6     1 Jan  1  5       0       6      0 cloudy/misty 0.24 0.2576 0.75
##   windspeed casual registered bikers
## 1     0.0000    3      13     16
## 2     0.0000    8      32     40
## 3     0.0000    5      27     32
## 4     0.0000    3      10     13
## 5     0.0000    0       1      1
## 6     0.0896    0       1      1

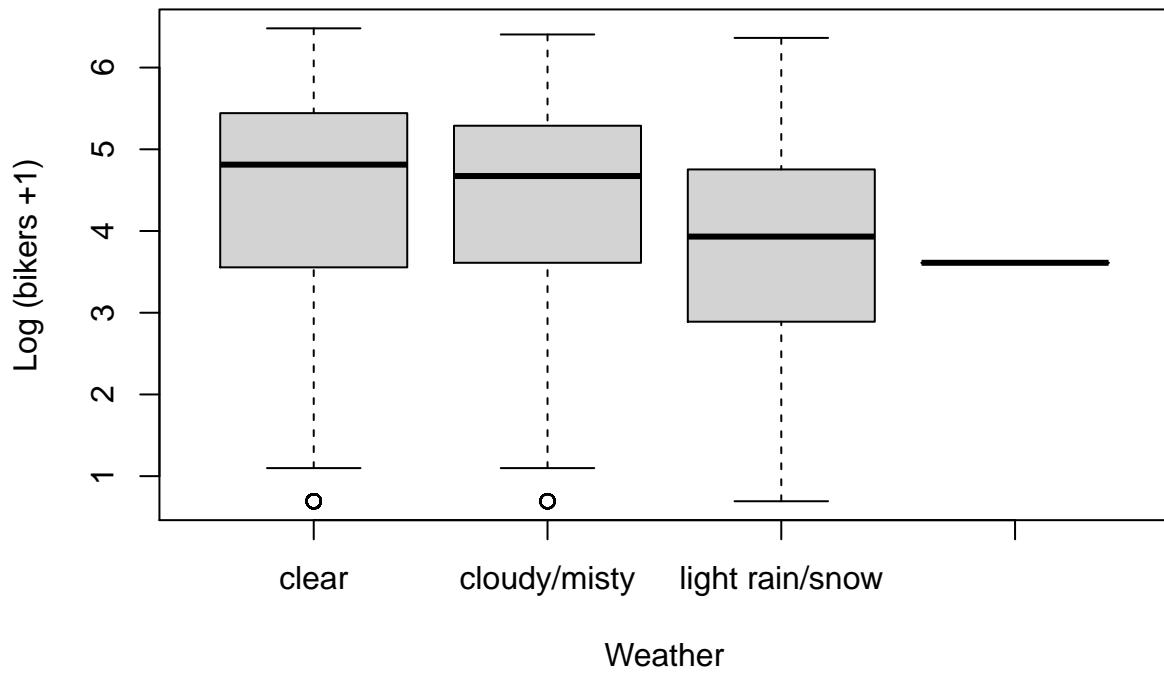
# association with weather on count and log scale
barplot(table(Bikeshare$weathersit))
```



```
boxplot(bikers ~ weathersit, data=Bikeshare,  
       xlab = "Weather", ylab = "Bikers")
```

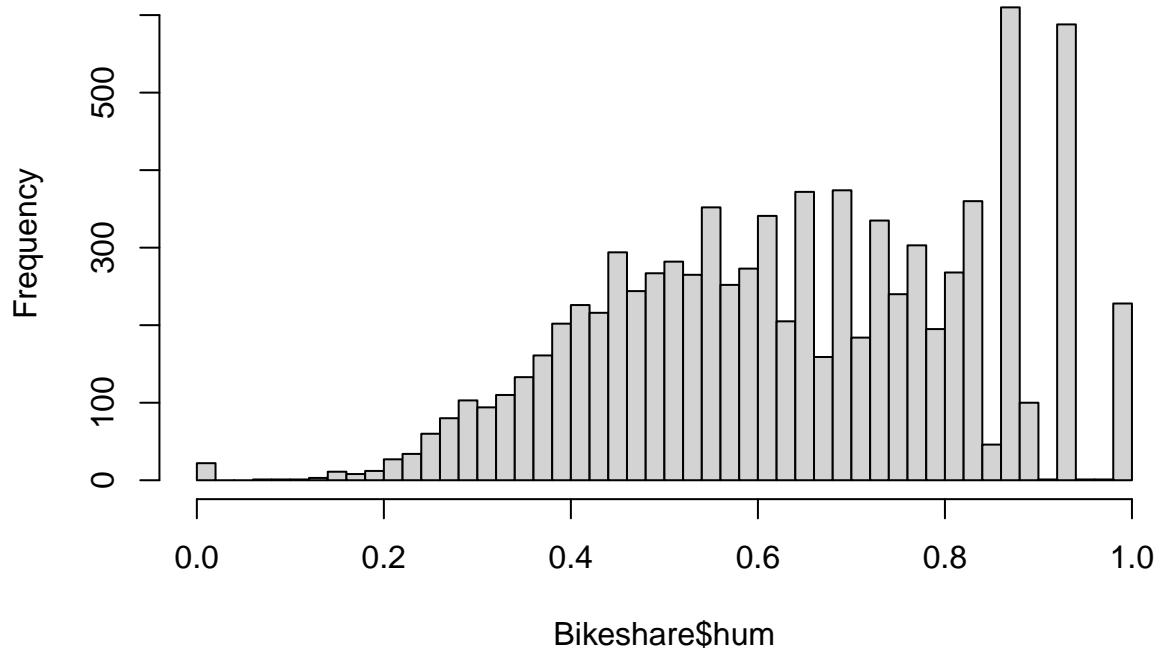


```
boxplot(log1p(bikers) ~ weathersit, data=Bikeshare,  
       xlab = "Weather", ylab = "Log (bikers +1)")
```

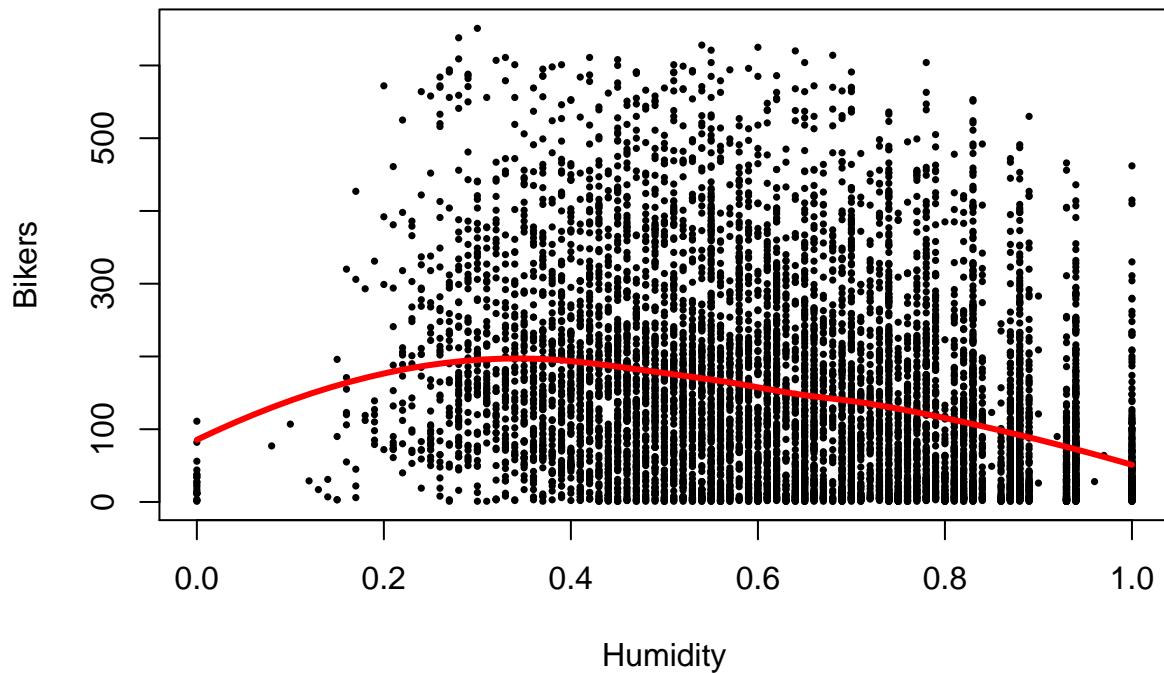


```
# association with humidity on count and log scale  
hist(Bikeshare$hum, breaks=40)
```

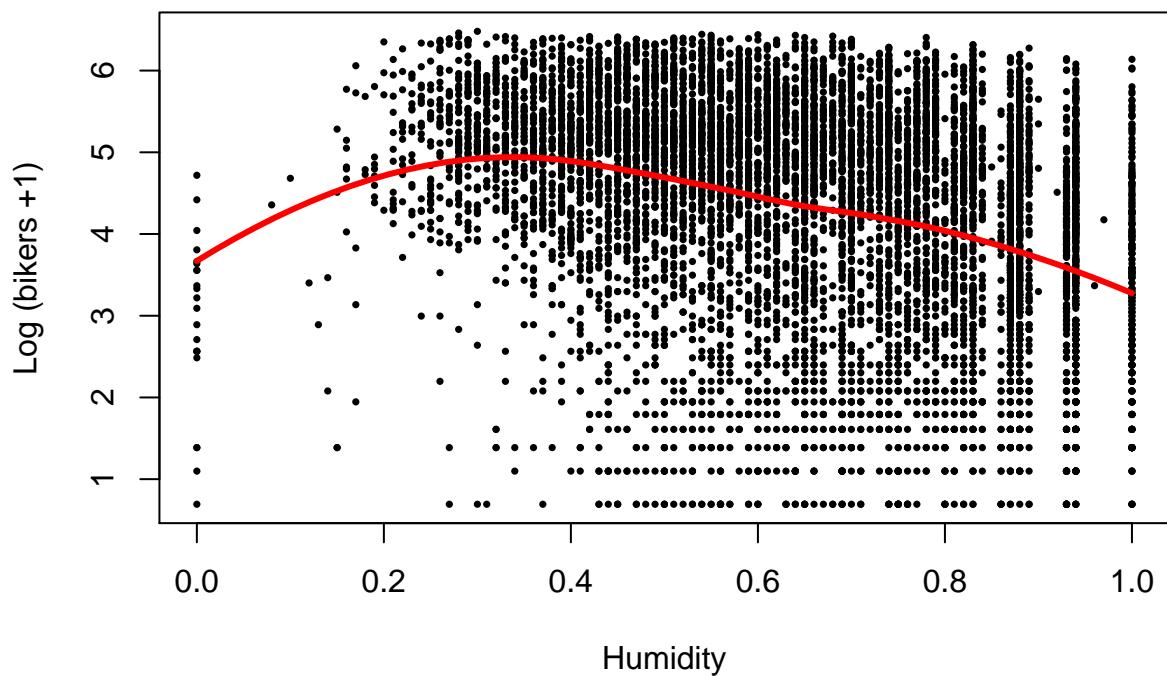
Histogram of Bikeshare\$hum



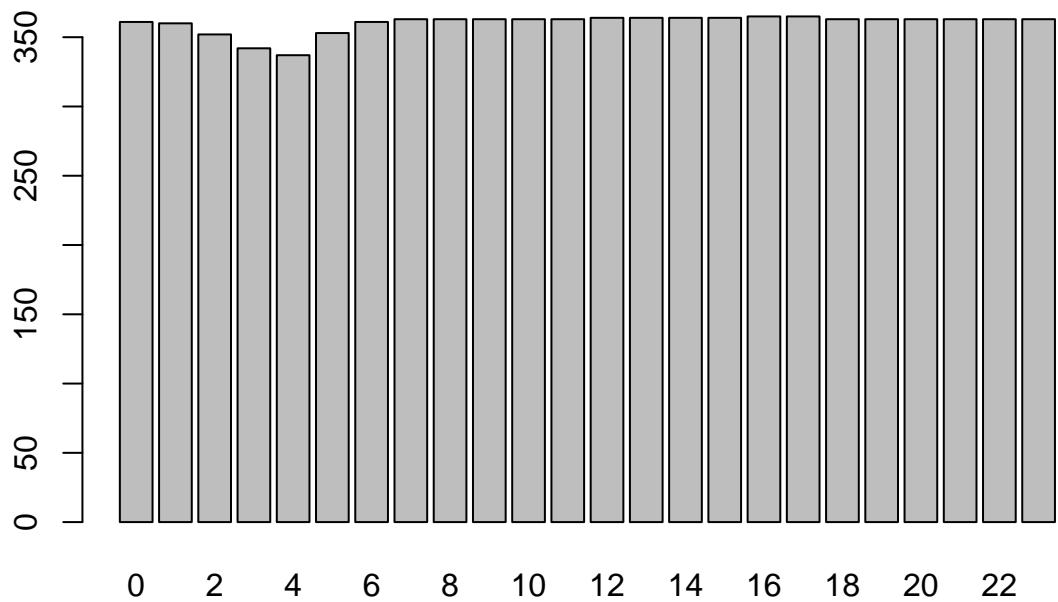
```
plot(bikers ~ hum, data=Bikeshare, pch=16, cex=1/2,
      xlab = "Humidity", ylab = "Bikers")
loHum <- loess(bikers ~ hum, data=Bikeshare)
xGrid <- seq(0, 1, length=50)
yhat <- predict(loHum, data.frame(hum = xGrid))
lines(x=xGrid, y=yhat, col="red", lwd=3)
```



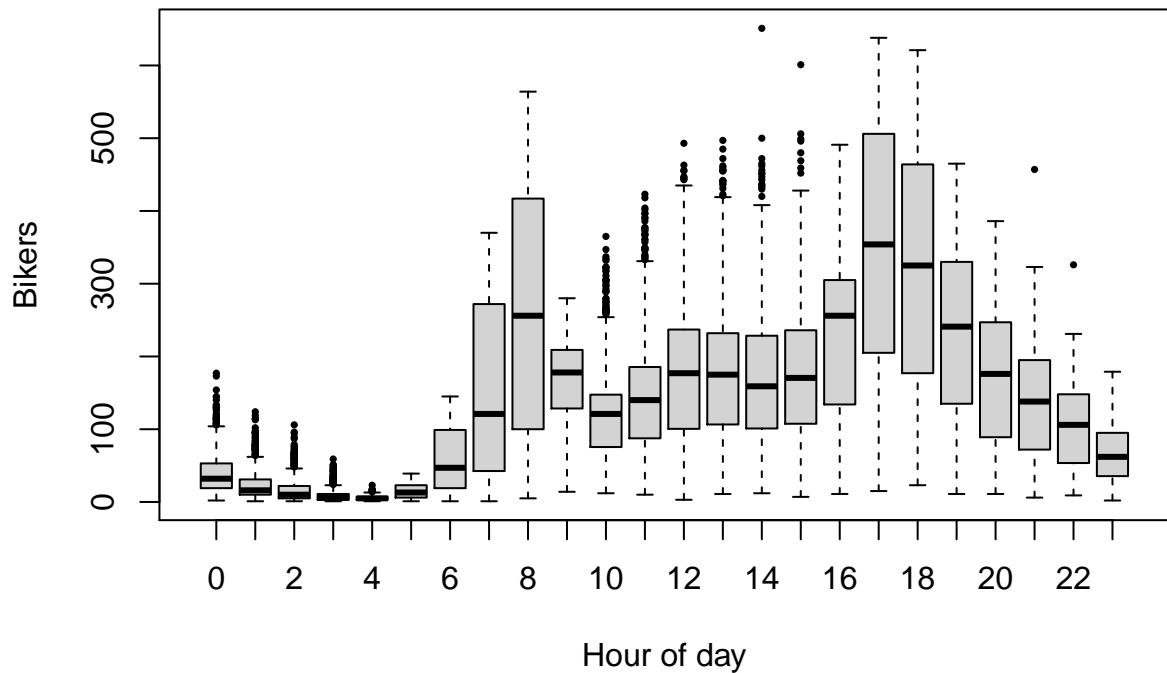
```
plot(log1p(bikers) ~ hum, data=Bikeshare, pch=16, cex=1/2,
      xlab = "Humidity", ylab = "Log (bikers +1)")
loHum <- loess(log1p(bikers) ~ hum, data=Bikeshare)
xGrid <- seq(0, 1, length=50)
yhat <- predict(loHum, data.frame(hum = xGrid))
lines(x=xGrid, y=yhat, col="red", lwd=3)
```



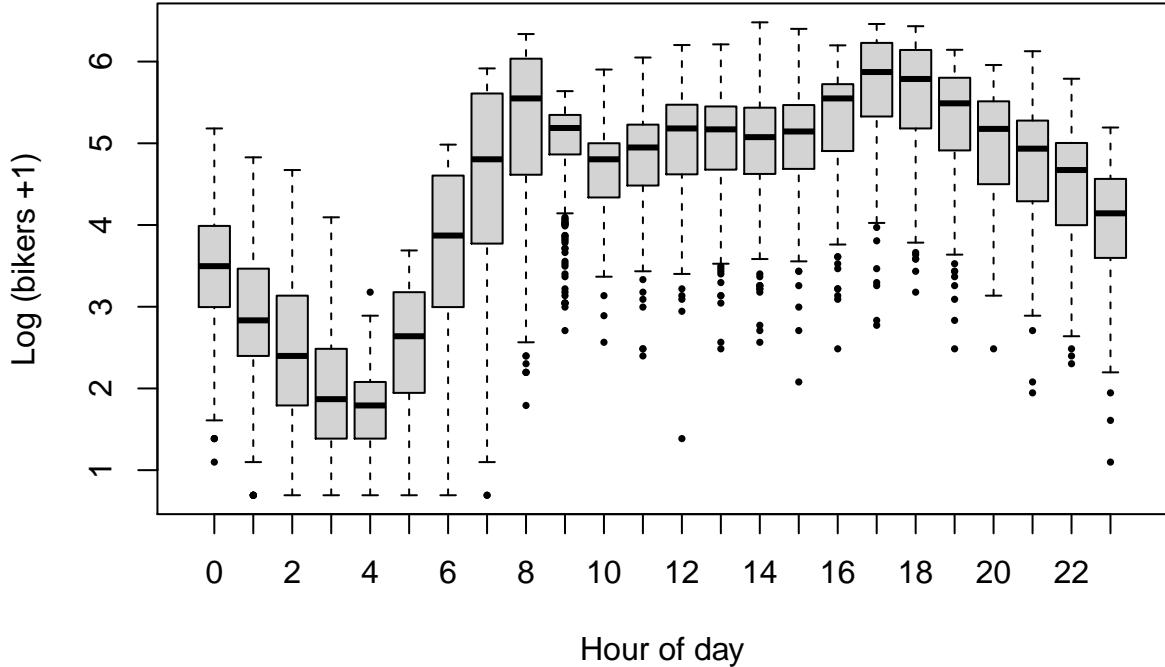
```
# association with hour on count and log scale  
barplot(table(Bikeshare$hr))
```



```
plot(bikers ~ hr, data=Bikeshare, pch=16, cex=1/2,  
      xlab = "Hour of day", ylab = "Bikers")
```



```
plot(log1p(bikers) ~ hr, data=Bikeshare, pch=16, cex=1/2,  
     xlab = "Hour of day", ylab = "Log (bikers +1)")
```



The data exploration shows that

- More bikes are being used when the weather is better.
- There seems to be a non-linear association between bicycle rentals and humidity, where in both low and high humidity conditions relatively few bikes are used, possibly reflecting very hot and very wet days respectively, while most bikes are being used at moderate humidity.
- Bicycle rental is associated to the hour of the day, however, in a non-linear way, with clear peaks in usage at typical commute hours (6h-8h and 17h-19h). Here, we will add `hr` as a categorical variable to the model, estimating one parameter for each hour. However, note that this may not be the most efficient approach statistically, as one may be able to incorporate `hr` as a numerical variable and model the non-linearity using a lower number of parameters.
- *Disclaimer:* Note that there are likely interactions between the variables, which here we will not evaluate as our goal is to introduce a Poisson GLM rather than a full analysis of the **Bikeshare** dataset. For example, it seems likely that more people commute by bike in good weather, while fewer people will commute by bike in terrible weather. This would motivate an interaction between the variables `weathersit` and `hr`.

-
- Below, we fit a Poisson GLM using the `glm` function. The number of bikers is used as a response variable, which is modeled as a function of `weathersit`, `hum` and `hr`.
 - Note that there seems to be a non-linear, though fairly simple, association between our response variable and the humidity. We will therefore add a quadratic term for humidity to the model. In order to avoid multicollinearity between the linear and quadratic humidity effects, we will first center the humidity variable and store this in a new variable called `humc`. This means that when `humc=0`, this corresponds to the average humidity in the dataset.

- The argument `family = "poisson"` specifies the Poisson distribution for the response variable and by default the canonical link function, which is the log link, will be used.

```
Bikeshare$humc <- Bikeshare$hum - mean(Bikeshare$hum)
m <- glm(bikers ~ weathersit + humc + I(humc^2) + I(humc^3) + hr,
          data = Bikeshare,
          family = "poisson")
summary(m)
```

```
##
## Call:
## glm(formula = bikers ~ weathersit + humc + I(humc^2) + I(humc^3) +
##       hr, family = "poisson", data = Bikeshare)
##
## Deviance Residuals:
##      Min        1Q     Median        3Q       Max
## -23.3408   -4.6201   -0.9922    3.4605   27.4153
##
## Coefficients:
##                               Estimate Std. Error z value Pr(>|z|)
## (Intercept)            3.893651  0.008083 481.708 <2e-16 ***
## weathersitcloudy/misty -0.146618  0.002277 -64.401 <2e-16 ***
## weathersitlight rain/snow -0.556153  0.004585 -121.292 <2e-16 ***
## weathersitheavy rain/snow -1.855194  0.166742 -11.126 <2e-16 ***
## humc                  0.091751  0.009233  9.938 <2e-16 ***
## I(humc^2)             -2.233919  0.029421 -75.929 <2e-16 ***
## I(humc^3)             -1.823066  0.091428 -19.940 <2e-16 ***
## hr1                  -0.476470  0.012999 -36.654 <2e-16 ***
## hr2                  -0.806959  0.014646 -55.099 <2e-16 ***
## hr3                  -1.433648  0.018842 -76.090 <2e-16 ***
## hr4                  -2.058714  0.024796 -83.027 <2e-16 ***
## hr5                  -1.061695  0.016074 -66.051 <2e-16 ***
## hr6                  0.315691  0.010607  29.761 <2e-16 ***
## hr7                  1.317856  0.009052 145.586 <2e-16 ***
## hr8                  1.830026  0.008653 211.480 <2e-16 ***
## hr9                  1.352135  0.009022 149.871 <2e-16 ***
## hr10                 1.129497  0.009271 121.831 <2e-16 ***
## hr11                 1.308554  0.009102 143.766 <2e-16 ***
## hr12                 1.522234  0.008947 170.131 <2e-16 ***
## hr13                 1.536827  0.008959 171.542 <2e-16 ***
## hr14                 1.499506  0.008999 166.633 <2e-16 ***
## hr15                 1.535043  0.008974 171.062 <2e-16 ***
## hr16                 1.745128  0.008800 198.318 <2e-16 ***
## hr17                 2.140488  0.008565 249.925 <2e-16 ***
## hr18                 2.037740  0.008588 237.279 <2e-16 ***
## hr19                 1.711545  0.008747 195.667 <2e-16 ***
## hr20                 1.393901  0.008976 155.284 <2e-16 ***
## hr21                 1.132543  0.009216 122.895 <2e-16 ***
## hr22                 0.882534  0.009537  92.539 <2e-16 ***
## hr23                 0.481354  0.010207  47.157 <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for poisson family taken to be 1)
```

```

## Null deviance: 1052921  on 8644  degrees of freedom
## Residual deviance: 375265  on 8615  degrees of freedom
## AIC: 428362
##
## Number of Fisher Scoring iterations: 5

```

2.2.2.1 Interpretation of estimated model parameters

- Remember that the Poisson GLM can be defined as

$$\begin{cases} Y_i & \sim Poi(\mu_i) \\ \log \mu_i & = \eta_i \\ \eta_i & = \mathbf{X}_i^T \boldsymbol{\beta} \end{cases}$$

Interpretation of the intercept.

- We will first interpret the intercept, in terms of the average number of bikes being used. Note that the intercept corresponds to hour 0, at good weather (`weathersit` level 1), and average humidity. We will denote the intercept as β_0 and its estimate, given the data, as $\hat{\beta}_0$. All other coefficients will thus denote a relative change with respect to that reference level.
 - The model definition shows that $\log \mu_i = \mathbf{X}_i^T \boldsymbol{\beta}$, with μ the average number of bikes being used. Since we're only working with the intercept here, we may write $\log \mu_i = \beta_0$, and thus $\mu_i = \exp \beta_0$.
 - Plugging in the estimated intercept $\hat{\beta}_0$, we have $\exp \hat{\beta}_0 = 49.09$. In other words, in clear weather with few clouds, at zero humidity and at hour 0, an average of 40.82 bikes are being used.
-

Interpretation of `weathersit`=cloudy/misty.

- We will denote this coefficient as β_1 and its estimate as $\hat{\beta}_1$.
 - Note that this coefficient defines the difference in linear predictor between `weathersit=2` and `weathersit=1`, all other variables being equal (say, at their reference level). Indeed, define η_{w2} and η_{w1} to denote the linear predictor at `weathersit=2`, and `weathersit=1`, respectively. Then, $\eta_{w2} - \eta_{w1} = (\beta_0 + \beta_1) - \beta_0 = \beta_1$.
 - This also means that $\beta_1 = \log \mu_{w2} - \log \mu_{w1} = \log \frac{\mu_{w2}}{\mu_{w1}}$, and thus $\exp \beta_1 = \frac{\mu_{w2}}{\mu_{w1}}$.
 - In our case, $\exp \hat{\beta}_1 = 0.86$. In words: All other variables being equal, the average number of bikes being used in cloudy/misty weather is 0.85 times (or, also, 85% of) the number of bikes being used in good weather.
 - This exercise has shown us that, due to the log link function, the parameters in a Poisson GLM cannot be interpreted in terms of absolute differences in averages of the response variable but instead must be interpreted in terms of multiplicative differences!
 - If you're in a meeting and you need a quick way to interpret these parameters, remember that $\exp(1) = 2.72 \approx 3$ and thus an estimated coefficient of 1 (-1) means the average of the response variable is about three times higher (lower).
-

Interpretation of the humidity effect.

- The humidity effect is a bit more involved to interpret. Due to the quadratic and cubic terms, we cannot interpret the linear term separately (nor can we interpret the quadratic or cubic term separately); we must interpret both the linear, quadratic and cubic term simultaneously.

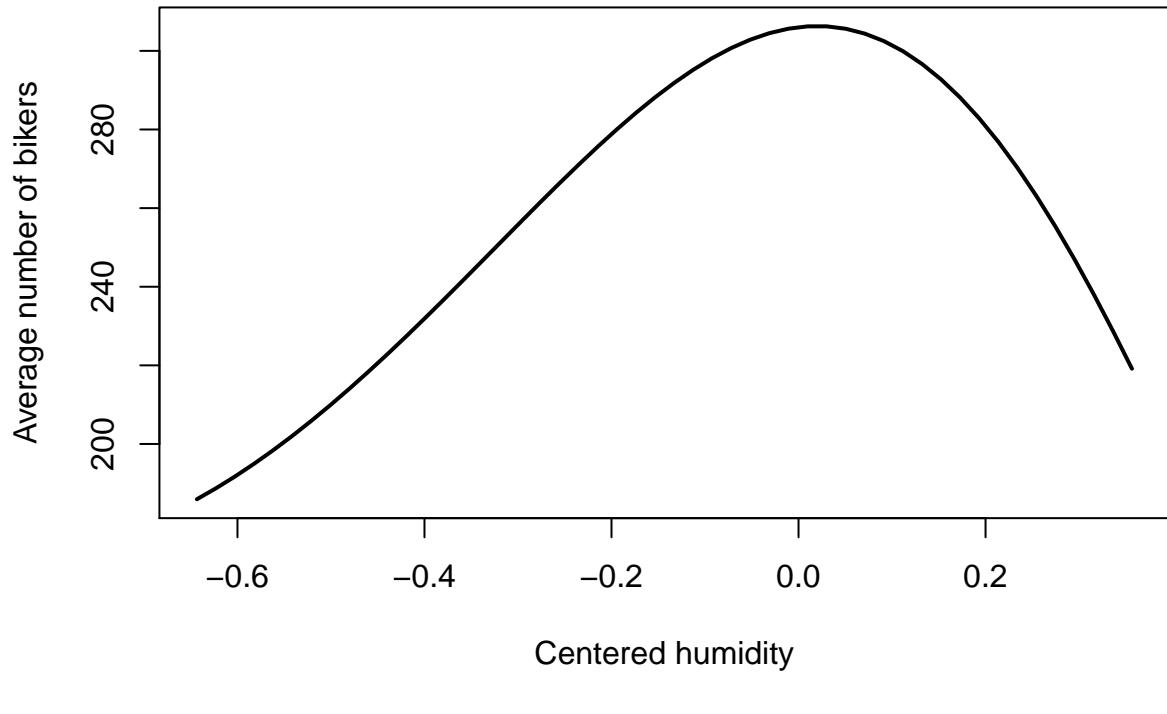
- Also due to the higher-order terms, the rate of change in average bikers will not be constant across the range of humidity. We can therefore not interpret the humidity effect using a single number as we've done previously.
- We can, however, provide some examples for specific humidity values, along with a visualization of its global effect.
- For example, let's derive the change in average bikes being used at a humidity that is 0.2 above average, versus average humidity. For average humidity $+0.2$ the linear predictor $\eta_{0.2} = \beta_0 + \beta_4 x_{hum} + \beta_5 x_{hum}^2 + \beta_6 x_{hum}^3 = \beta_0 + \beta_4 0.2 + \beta_5 0.2^2 + \beta_6 0.2^3$. For average humidity, the linear predictor $\eta_0 = \beta_0 + \beta_4 x_{hum} + \beta_5 x_{hum}^2 + \beta_6 x_{hum}^3 = \beta_0 + \beta_4 0 + \beta_5 0^2 + \beta_6 0^3 = \beta_0$. We thus have $\log \frac{\mu_{0.2}}{\mu_0} = \beta_4 0.2 + \beta_5 0.2^2 + \beta_6 0.2^3$. In our case, $\log \frac{\hat{\mu}_{0.2}}{\hat{\mu}_0} = 0.091751 * 0.2 - 2.233919 * 0.2^2 - 1.823066 * 0.2^3 = -0.0856$ and thus $\frac{\hat{\mu}_{0.2}}{\hat{\mu}_0} = 0.92$. Therefore, at humidity that is 0.2 above average, the average number of bikes being used are 0.92 times the average number of bikes used at average humidity.
- Just like with linear models, the `predict` function is extremely helpful when trying to visualize and understand a fitted GLM. In GLMs, the `type` argument becomes essential when using the `predict` function. Indeed, by default, estimates are provided on the linear predictor scale: in our case, on the log scale. If we'd like predictions on the scale of the response variable, we need to set `type="response"`. You can find more information in the help file using `?predict.glm`.
- The visualization shows that the highest number of bikes are being used at around average humidity, with a decreased usage at higher and lower humidities.

```

humidityGrid <- seq(min(Bikeshare$humc), max(Bikeshare$humc),
                      length.out = 50)
newDf <- data.frame(weatherSit = factor("clear"),
                     hr = factor(8),
                     humc = humidityGrid,
                     "I(humc^2)" = humidityGrid^2,
                     "I(humc^3)" = humidityGrid^3)
yhat <- predict(m,
                newdata = newDf,
                type = "response")

plot(x = humidityGrid,
      y = yhat,
      type = 'l', lwd=2,
      xlab = "Centered humidity",
      ylab = "Average number of bikers")

```



Setting up a contrast.

- Suppose we're interested in whether there are more bikers at (A) maximum humidity (centered humidity value of 0.357), hour 17, in the `light rain/snow` weather category, versus (B) average humidity, hour 8, in the `clear` weather category. This requires us to set up a contrast in terms of a linear combination of the model parameters.
- **Manually:**

$$\log \mu_A = \beta_0 + \beta_2 x_{rainSnow} + \beta_4 x_{hum} + \beta_5 x_{hum}^2 + \beta_6 x_{hum}^3 + \beta_{23} x_{hr17} = 3.894 - 0.556 + 0.092 * 0.357 - 2.234 * 0.357^2 - 1.823 * 0.357^3$$

Thus, at maximum humidity, hour 17, in the `light rain/snow` weather category the average number of bikers is 56% times the average number of bikers in the average humidity, hour 8, in the `clear` weather category.

- **Manually in R:** We can also use matrix multiplication to derive the estimates. We know from our manual calculations above, that the contrast of interest is $(\beta_0 + \beta_2 x_{rainSnow} + \beta_4 x_{hum} + \beta_5 x_{hum}^2 + \beta_6 x_{hum}^3 + \beta_{23} x_{hr17}) - (\beta_0 + \beta_{14} x_{hr8})$. We can store this in a contrast matrix, and then multiply it with the coefficients of our model:

```
L <- matrix(0,
            nrow = length(coef(m)),
            ncol = 1)
rownames(L) <- names(coef(m))
```

```

L["weathersitlight rain/snow",1] <- 1
L["humc",1] <- 0.357
L["I(humc^2)", 1] <- 0.357^2
L["I(humc^3)", 1] <- 0.357^3
L["hr17", 1] <- 1
L["hr8",1] <- -1
L

## [,1]
## (Intercept) 0.00000000
## weathersitcloudy/misty 0.00000000
## weathersitlight rain/snow 1.00000000
## weathersitheavy rain/snow 0.00000000
## humc 0.35700000
## I(humc^2) 0.12744900
## I(humc^3) 0.04549929
## hr1 0.00000000
## hr2 0.00000000
## hr3 0.00000000
## hr4 0.00000000
## hr5 0.00000000
## hr6 0.00000000
## hr7 0.00000000
## hr8 -1.00000000
## hr9 0.00000000
## hr10 0.00000000
## hr11 0.00000000
## hr12 0.00000000
## hr13 0.00000000
## hr14 0.00000000
## hr15 0.00000000
## hr16 0.00000000
## hr17 1.00000000
## hr18 0.00000000
## hr19 0.00000000
## hr20 0.00000000
## hr21 0.00000000
## hr22 0.00000000
## hr23 0.00000000

beta <- matrix(coef(m), ncol=1)
exp(t(L) %*% beta) # equals our manual calculation.

```

```

## [,1]
## [1,] 0.5595652

```

- Using `predict` in R:

```

# set up data frames with relevant predictor variables' values.
dfa <- data.frame(weather = factor("light rain/snow"),
                   hr = factor(17),
                   humc = 0.357,

```

```

    "I(humc^2)" = 0.357^2,
    "I(humc^3)" = 0.357^3)
dfB <- data.frame(weatherSit = factor("clear"),
                   hr = factor(8),
                   humc = 0,
                   "I(humc^2)" = 0,
                   "I(humc^3)" = 0)

# calculate estimated average number of bikers
yhatA <- predict(m,
                  newdata = dfA,
                  type = "response")
yhatB <- predict(m,
                  newdata = dfB,
                  type = "response")

yhatA / yhatB # also equal to above.

##           1
## 0.5595652

```

Exercise: try to derive the change in average number of bikers between (a) humidity of 0.1 above average, clear weather (`weatherSit=1`), at hour 10 and (b) humidity of 0.1 below average, cloudy weather (`weatherSit=2`), at hour 20, using all three methods.

2.3 Statistical inference in GLMs

2.3.1 Wald test and likelihood ratio test

- In our interpretation above we have focussed on deriving changes in the average number of bikers between groups of interest. However, we have not yet tested whether these changes are statistically significant.
- In genomics applications, statistical inference in GLMs is often adopted to test for differential expression between conditions for each gene (e.g., *is gene A differently expressed in healthy versus tumoral tissue?*), which amounts to testing the null hypothesis of whether a (linear combination of) coefficient(s) equals zero.
- In this course, we will mainly work with two types of statistical tests for GLMs:
 - **Wald test:** The Wald test may be viewed as being analogous to the *t*-test we are using in linear models. The Wald test relies on the following asymptotic result

$$\hat{\beta} | \beta \sim N(\beta, \text{Var}(\hat{\beta}))$$

. The Wald test statistic for testing a single parameter $\hat{\beta}$

$$W = \frac{\hat{\beta}}{\hat{SE}(\hat{\beta})} \sim N(0, 1) | H_0$$

or, equivalently, letting \mathbf{C} denote the $1 \times p$ contrast matrix denoting the contrast for the single parameter β we would like to test, and $\hat{\Sigma}_{\hat{\beta}}$ the variance-covariance matrix of the parameters,

$$W = \mathbf{C}\hat{\beta}(\mathbf{C}\hat{\Sigma}_{\hat{\beta}}\mathbf{C}^T)^{-1}\hat{\beta}^T\mathbf{C}^T \sim \chi^2_1 | H_0.$$

The null and alternative hypothesis can therefore in general be written as

$$H_0 : \mathbf{C}\hat{\beta} = 0$$

$$H_1 : \mathbf{C}\hat{\beta} \neq 0$$

If $c \geq 1$ contrasts are tested, then the test statistic $W \sim \chi_c^2 | H_0$, provided that the c contrasts are linearly independent (i.e., the contrast matrix is full rank).

- **Likelihood ratio test:** The likelihood ratio test (LRT) measures the discrepancy in log-likelihood between our current model (sometimes also referred to as full model) and a reduced model (sometimes also referred to as null or alternative model). The reduced model must be nested in (and therefore of lower dimension as compared to) the full model. While adding more covariates will always explain more variability in our response variable, the LRT tests whether this is actually significant. For example, in the example of gene differential expression between healthy versus tumoral tissue, the full model could be a GLM where the mean is modeled according to an intercept and a tissue indicator variable (healthy / tumoral), while the alternative model could be a GLM with just an intercept. Indeed, if the gene is similarly expressed between healthy and tumoral tissue, the log-likelihood of the alternative model will decrease only a little as compared to the full model. As the name suggests, the likelihood ratio test assesses whether the ratio of the log-likelihoods provides sufficient evidence for a worse fit of the alternative versus full model

$$L = 2 \log \left\{ \ell(\hat{\beta}_{full}) - \ell(\hat{\beta}_{alternative}) \right\}.$$

Asymptotically, under the null hypothesis it can be shown that

$$L \sim \chi_c^2 | H_0,$$

with c the number of parameters dropped in the alternative model versus the full model. If we again let \mathbf{C} denote the $c \times p$ contrast matrix denoting the contrast for the parameters being dropped, the null and alternative hypothesis are as in the Wald test setting:

$$H_0 : \mathbf{C}\hat{\beta} = 0$$

$$H_1 : \mathbf{C}\hat{\beta} \neq 0$$

Finally, note that while, in this explanation, I have focussed on reducing a more complex model, but of course the LRT can also be adopted to check whether adding a covariate significantly improves the fit.

- Statistical inference theory in GLMs always works asymptotically in terms of the sample size. Thus we need many data points in order for the theory to hold in practice and, in addition, all other assumptions such as the distributional assumption, must hold in order for the p -values to be correct.
- In bulk RNA-seq, we are often working with a limited number of samples and so we typically do not expect asymptotic theory to hold yet. In single-cell RNA-seq, we often perform several preprocessing steps before calculating p -values for each gene and so we may be ‘using the data multiple times’. Rather than attaching strong probabilistic interpretations to the p -values, we therefore advice to view the p -values simply as useful numerical summaries for ranking the genes for further inspection in genomics applications.

TODO: add schematic comparing Wald with LRT using log-likelihood function

2.3.2 Wald test and likelihood ratio test in R

Let’s use a Wald test and a likelihood ratio test to test whether the average number of bikers differs between a working day or a weekend day, using a simple GLM with only that variable as a covariate. This amounts to testing

$$H_0 : \beta_{workingday} = 0$$

$$H_1 : \beta_{workingday} \neq 0$$

TODO: z-test versus Wald test

```
mSimple <- glm(bikers ~ workingday,
                 family = "poisson",
                 data = Bikeshare)
summSimple <- summary(mSimple)
summSimple$coefficients[["workingday"],]

##      Estimate Std. Error     z value   Pr(>|z|)
## 2.352087e-02 1.937241e-03 1.214143e+01 6.370326e-34

# Wald test manually
W <- summSimple$coefficients[["workingday", "Estimate"] / summSimple$coefficients[["workingday", "Std. Err"]]
pval <- 2*(1 - pnorm(W))
W

## [1] 12.14143

pval

## [1] 0

# Wald test through a contrast
C <- matrix(0, nrow=1, ncol=length(coef(mSimple)))
colnames(C) <- names(coef(mSimple))
C[, "workingday"] <- 1
beta <- matrix(coef(mSimple), ncol=1)
Sigma <- vcov(mSimple)

W2 <- C %*% beta %*% solve(C %*% Sigma %*% t(C)) %*% t(beta) %*% t(C)
W2

## [1,] [,1]
## [1,] 147.4143

# note this being equal to
W^2

## [1] 147.4143

pval <- 1-pchisq(W, df=1)
pval

## [1] 0.0004931396
```

```
# finally, we can also read the Wald test result from the summary of the model
summSimple
```

```
##  
## Call:  
## glm(formula = bikers ~ workingday, family = "poisson", data = Bikeshare)  
##  
## Deviance Residuals:  
##      Min       1Q   Median       3Q      Max  
## -16.666  -11.361   -3.026    5.214   30.729  
##  
## Coefficients:  
##             Estimate Std. Error z value Pr(>|z|)  
## (Intercept) 4.952243  0.001608 3080.12 <2e-16 ***  
## workingday  0.023521  0.001937   12.14 <2e-16 ***  
## ---  
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1  
##  
## (Dispersion parameter for poisson family taken to be 1)  
##  
## Null deviance: 1052921  on 8644  degrees of freedom  
## Residual deviance: 1052773  on 8643  degrees of freedom  
## AIC: 1105815  
##  
## Number of Fisher Scoring iterations: 5
```

```
mFull <- glm(bikers ~ workingday,  
              family = "poisson",  
              data = Bikeshare)  
  
mReduced <- glm(bikers ~ 1,  
                  family = "poisson",  
                  data = Bikeshare)  
  
# manual LRT  
llFull <- logLik(mFull)  
llReduced <- logLik(mReduced)  
  
lrt <- as.numeric(2 * (llFull - llReduced))  
lrt
```

```
## [1] 147.8481
```

```
pval <- 1 - pchisq(lrt, df=1)  
pval
```

```
## [1] 0
```

```
# using anova function  
anova(mReduced, mFull, test = "Chisq") # note test statistic is equal
```

```

## Analysis of Deviance Table
##
## Model 1: bikers ~ 1
## Model 2: bikers ~ workingday
##   Resid. Df Resid. Dev Df Deviance Pr(>Chi)
## 1      8644    1052921
## 2      8643    1052773  1    147.85 < 2.2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

2.4 Model deviance, residuals and goodness-of-fit

- In linear models, we often use residuals $e_i = y_i - \hat{\mu}_i$ to check model assumptions (linearity, homoscedasticity). However, in a GLM setting, we know that the variance of our residuals will depend on the mean, i.e., $Var(\epsilon_i) = f(\mu_i)$. Using ordinary residuals such as e_i therefore is no longer appropriate.
- We have seen that the objective function that is used to fit a GLM is the log-likelihood of the data under the posited model. For example, the log likelihood of a Poisson GLM with response variable \mathbf{Y} , with elements $Y_i, i \in \{1, \dots, n\}$ and model matrix \mathbf{X} is

$$\ell(\mathbf{Y}; \beta) = \log \prod_{i=1}^n \left(\frac{\exp(\mathbf{X}_i^T \beta)^{Y_i} \exp(-\exp(\mathbf{X}_i^T \beta))}{Y_i!} \right) = \sum_{i=1}^n \log \left(\frac{\exp(\mathbf{X}_i^T \beta)^{Y_i} \exp(-\exp(\mathbf{X}_i^T \beta))}{Y_i!} \right) = \sum_{i=1}^n Y_i(\mathbf{X}_i^T \beta) + \exp(\mathbf{X}_i^T \beta)$$

The estimates $\hat{\beta}$ are then found by maximizing $\ell(\beta | \mathbf{Y}, \mathbf{X})$ with respect to β . This is analogous to maximizing a Gaussian likelihood in the linear model setting.

- A goodness-of-fit measure used in the GLM setting is then the **residual deviance** D (sometimes referred to simply as ‘deviance’), that is twice the difference in log-likelihood between a ‘saturated model’ and the current model. Here, a saturated model, is a model where we fit one parameter per data point and therefore fit the data perfectly, in other words $\hat{\mu}_i = y_i$. This is,

$$D = 2 * \{\ell(\mathbf{Y}; \beta | \hat{\mu}_i = y_i) - \ell(\mathbf{Y}; \beta | \hat{\mu}_i = \exp(\mathbf{X}_i^T \beta))\}.$$

- From the equation above it becomes clear that the residual deviance is actually a ratio in log-likelihoods and therefore a likelihood ratio test statistic! This will become important in assessing goodness of fit.
- A low residual deviance can thus be interpreted as a model that is fitting the data well, since your current model will be close in log-likelihood to the saturated model. The deviance is a very useful statistic that is also important in statistical inference and model selection, e.g., for testing if a smaller model fits significantly worse than a larger model.
- Finally, a **deviance residual** D_i can then be defined as the square root of the contribution of the i th datum to the residual deviance

$$D_i = \sqrt{2 * \{\ell(\mathbf{Y}; \beta | \hat{\mu}_i = y_i) - \ell(\mathbf{Y}; \beta | \hat{\mu}_i = \exp(\mathbf{X}_i^T \beta))\}}$$

- Another type of residuals commonly used in a GLM setting are **Pearson residuals**. A Pearson residual is defined as

$$e_i = \frac{y_i - \hat{\mu}_i}{\sqrt{Var(\hat{\mu}_i)}},$$

and we can see that it has the form of a regular residual such as used in liner models (numerator), but normalized according to the variance of the estimated mean (denominator), to correct for the mean-variance relationship.

- **Goodness-of-fit** (GOF) analyses serve to assess how well the model actually fit the observed data. One may view the fitting of a GLM as replacing a set of observed data points \mathbf{y} by a set of fitted values $\hat{\mathbf{y}}$ derived from a model. In general $\hat{\mathbf{y}} \neq \mathbf{y}$ and the question arises as to how well $\hat{\mathbf{y}}$ approximates \mathbf{y} . This naturally raises the question of how much of a discrepancy we believe to be tolerable. Two important discrepancy measures are often used in a GLM setting.
- Note that the **residual deviance** was a likelihood ratio test statistic between a saturated and our current model. This saturated model actually provides us with a baseline as to how well a model can fit the observed data (even if we know that the saturated model is uninformative for summarizing the data). This motivates a statistical test with
 - H_0 : The current model does not fit significantly worse than the saturated model.
 - H_1 : The current model fits significantly worse than a saturated model.

- The residual deviance immediately tests this hypothesis using a likelihood ratio test and is therefore a useful goodness-of-fit measure.
- Another measure of discrepancy is the generalized Pearson χ^2 statistic

$$X^2 = \sum_{i=1}^n \frac{(y_i - \hat{\mu}_i)^2}{Var(\hat{\mu}_i)} = \sum_{i=1}^n e_i^2,$$

with e_i the Pearson residual of observation i .

- Asymptotic theory shows that both the residual deviance $D \sim \chi^2_{n-p}|H_0$ and $X^2 \sim \chi^2_{n-p}|H_0$, with n the number of observations in our dataset (and, hence, the number of parameters fitted in our saturated model), and p the number of parameters fitted in our current model.

Exercise: - See if you can come up with the residual deviance that is reported in the summary of our model above. - Also check if you can recover the correct deviance and Pearson residuals by calculating them yourself. You can get the deviance residuals in R by `resid(m, type="deviance")` and `resid(m, type="pearson")`. - Does your model fit significantly worse than a saturated model?

2.5 Overdispersion

```
library(MASS)
m <- glm.nb(bikers ~ weathersit + hum + hr,
             data = Bikeshare)

summary(m)

##
## Call:
## glm.nb(formula = bikers ~ weathersit + hum + hr, data = Bikeshare,
##        init.theta = 2.390699586, link = log)
##
## Deviance Residuals:
##    Min      1Q  Median      3Q     Max 
## -3.9691 -0.9332 -0.1646  0.5012  5.2091 
##
## Coefficients:
##              Estimate Std. Error z value Pr(>|z|)    
## (Intercept) 3.64207   0.04649  78.334 < 2e-16 ***
##
```

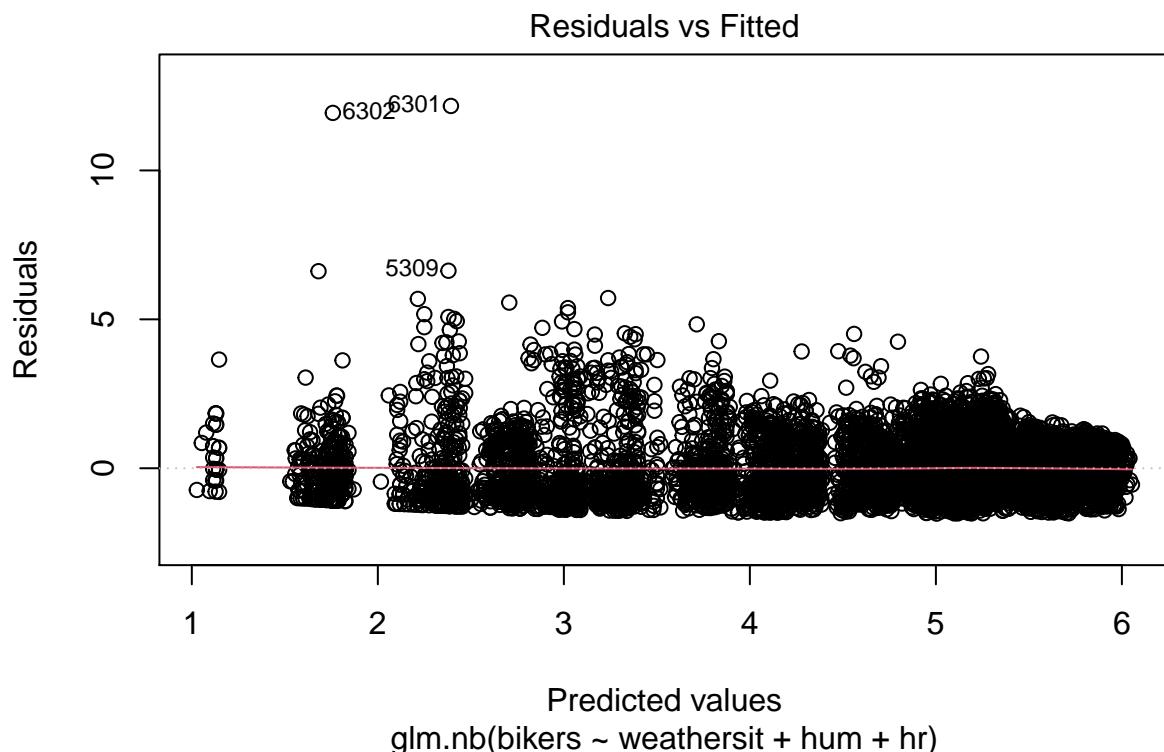
```

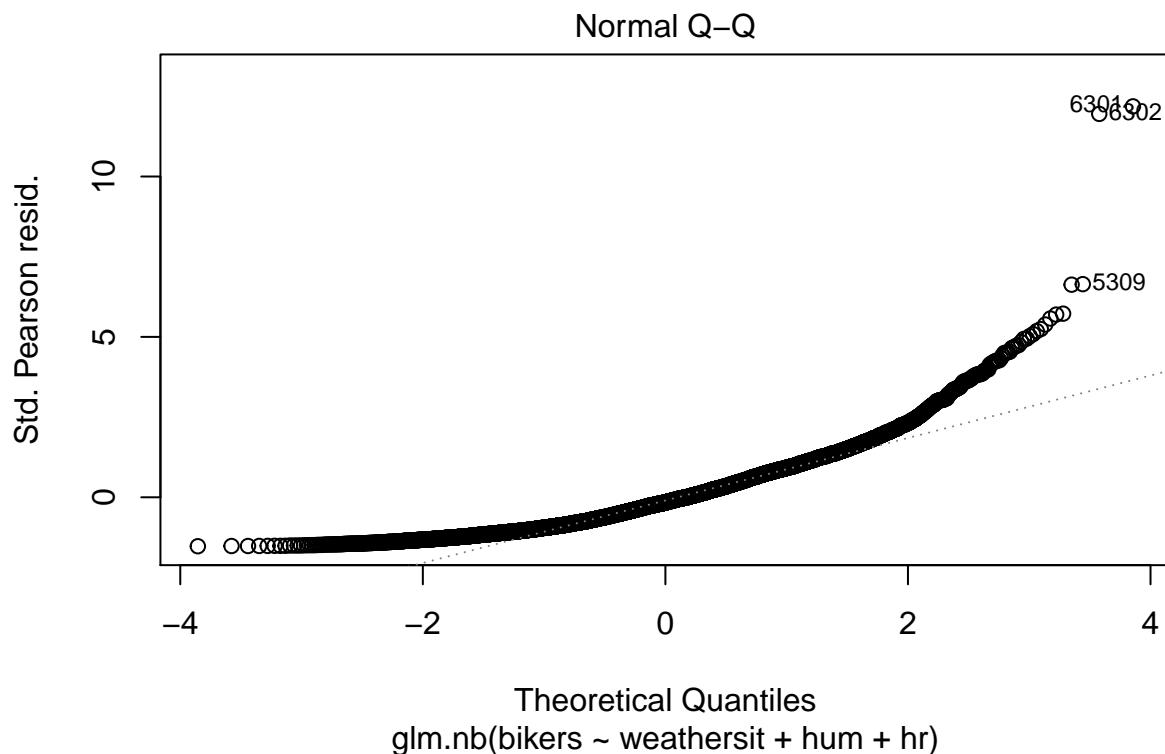
## weathersitcloudy/misty    -0.16140    0.01757   -9.184 < 2e-16 ***
## weathersitlight rain/snow -0.69553    0.02826   -24.613 < 2e-16 ***
## weathersitheavy rain/snow -2.06808    0.66910   -3.091   0.002 **
## hum                      0.26576    0.04562    5.825  5.71e-09 ***
## hr1                      -0.47649    0.04995   -9.539 < 2e-16 ***
## hr2                      -0.80343    0.05068   -15.853 < 2e-16 ***
## hr3                      -1.43821    0.05243   -27.431 < 2e-16 ***
## hr4                      -2.06572    0.05504   -37.529 < 2e-16 ***
## hr5                      -1.07629    0.05112   -21.055 < 2e-16 ***
## hr6                      0.30646    0.04936    6.208  5.35e-10 ***
## hr7                      1.32138    0.04897   26.984 < 2e-16 ***
## hr8                      1.85945    0.04890   38.023 < 2e-16 ***
## hr9                      1.39286    0.04904   28.402 < 2e-16 ***
## hr10                     1.15904    0.04924   23.537 < 2e-16 ***
## hr11                     1.33625    0.04937   27.066 < 2e-16 ***
## hr12                     1.54463    0.04954   31.182 < 2e-16 ***
## hr13                     1.55491    0.04971   31.282 < 2e-16 ***
## hr14                     1.52081    0.04979   30.543 < 2e-16 ***
## hr15                     1.54852    0.04980   31.094 < 2e-16 ***
## hr16                     1.76237    0.04968   35.477 < 2e-16 ***
## hr17                     2.17811    0.04947   44.026 < 2e-16 ***
## hr18                     2.07910    0.04936   42.122 < 2e-16 ***
## hr19                     1.75246    0.04917   35.643 < 2e-16 ***
## hr20                     1.42493    0.04910   29.023 < 2e-16 ***
## hr21                     1.15080    0.04903   23.470 < 2e-16 ***
## hr22                     0.89518    0.04907   18.243 < 2e-16 ***
## hr23                     0.49554    0.04919   10.073 < 2e-16 ***
##
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for Negative Binomial(2.3907) family taken to be 1)
##
## Null deviance: 26318.1 on 8644 degrees of freedom
## Residual deviance: 9315.3 on 8617 degrees of freedom
## AIC: 93328
##
## Number of Fisher Scoring iterations: 1
##
##
##          Theta:  2.3907
##          Std. Err.: 0.0369
##          ##
## 2 x log-likelihood: -93269.5260

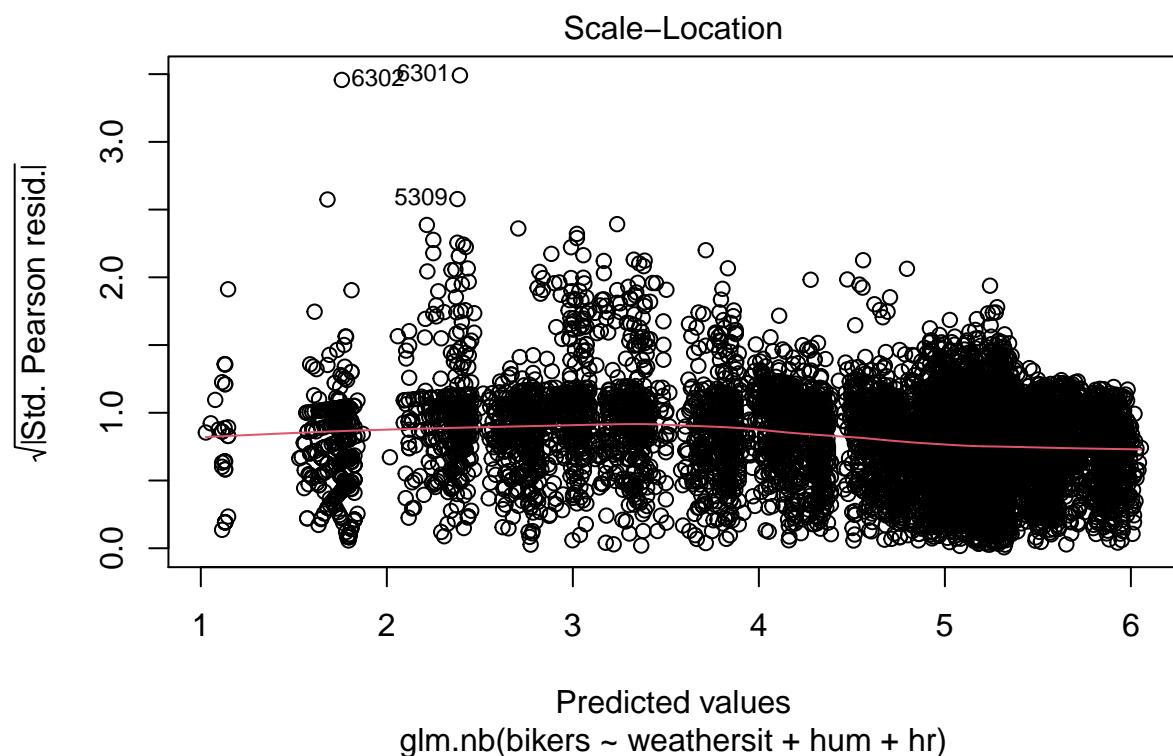
plot(m)

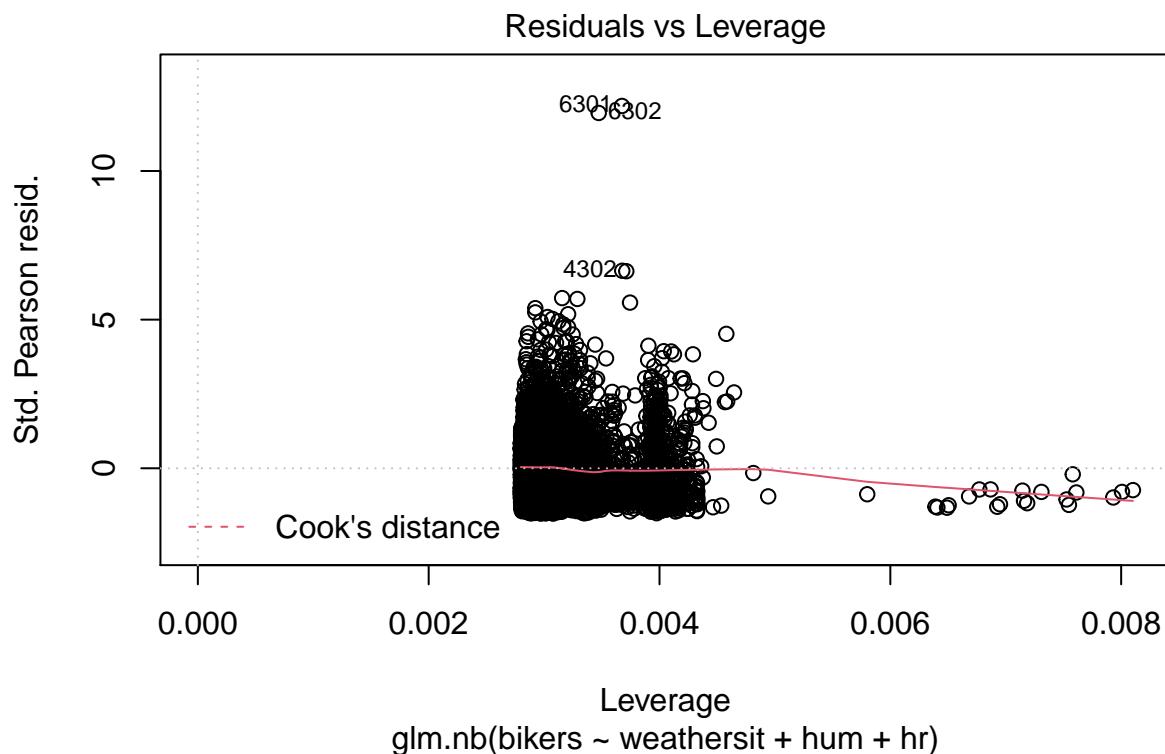
## Warning: not plotting observations with leverage one:
##      586

```

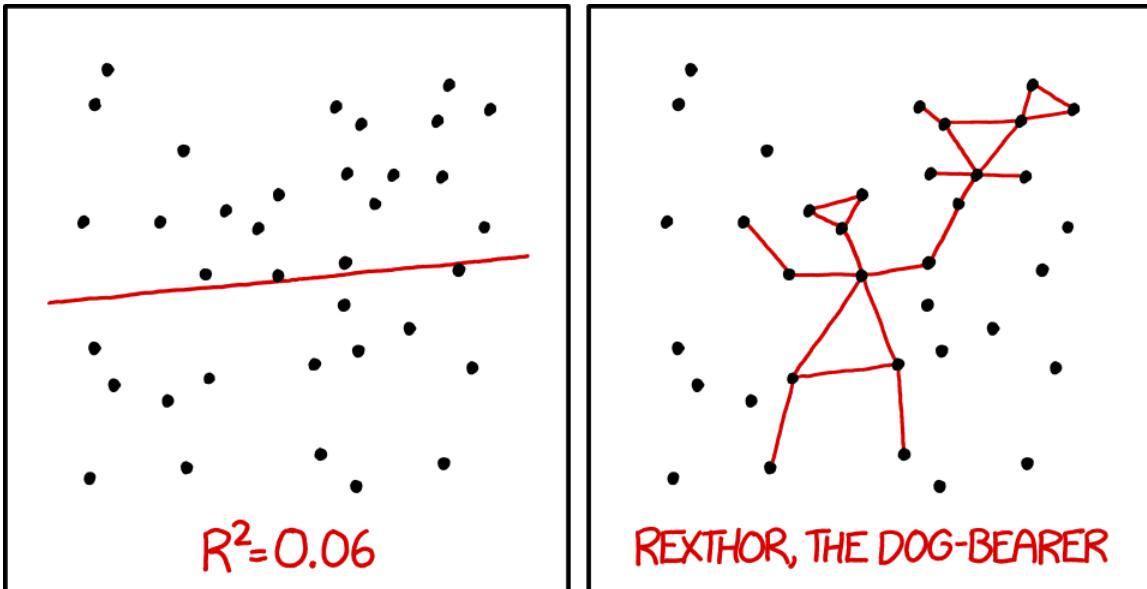








3 A final note and summary



I DON'T TRUST LINEAR REGRESSIONS WHEN IT'S HARDER
TO GUESS THE DIRECTION OF THE CORRELATION FROM THE
SCATTER PLOT THAN TO FIND NEW CONSTELLATIONS ON IT.

4 References

- Marioni *et al.* (2008) describe the distribution of gene expression counts across technical replicates, and in addition discuss lane effects in RNA-seq, as well as a comparison between RNA-seq and array-based platforms.
- The count data chapter of Modern Statistics for Modern Biology by Wolfgang Huber and Susan Holmes handles similar topics also in the context of RNA-seq: <https://www.huber.embl.de/msmb/Chap-CountData.html>

5 Junk

```
library(dplyr)
# downloaded from https://epistat.wiv-isp.be/covid/
data <- read.csv("~/Downloads/COVID19BE_CASES_AGESEX.csv")
# remove rows with NA
data <- data[!apply(data, 1, function(x) any(is.na(x))),]
# convert to R Date format
data$DATE <- as.Date(data$DATE)
# convert date to day of the week
data$day <- weekdays(data$DATE)

dataGrouped <- group_by(data, DATE) %>%
  summarize(nCases = sum(CASES),
```

```
day = unique(day)

plot(x=dataGrouped$DATE, y=dataGrouped$nCases,
      pch=16, cex=1/2)

# less reporting on weekends
plot(x=dataGrouped$DATE, y=dataGrouped$nCases,
      pch=16, cex=1/2, col=factor(dataGrouped$day))
```