Experimental warming imposes sub-lethal costs in the common forest ants, *Aphaenogaster*

Curtis A. Provencher

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Contents

Rationale

Anthropogenic warming is likely to drive shifts in phenology, distribution, and performance of species in Eastern deciduous forests. Predicting these ecological cascades will depend on understanding how a primary seed disperser, the keystone ant genus Aphaenogaster, responds to warming. Temperatures surpassing a species' lethal thermal limit will clearly be detrimental, but unfavorably high temperatures may impose stress substantially before that limit is reached; characterizing such sublethal responses will be vital for predicting Aphaenogaster's future performance. Here we test for a physiological stress response in Aphaenogaster workers from a northern and southern deciduous forest under simulated climate warming.

Experimental protocol

Experimental warming sites and chambers

We collected ants from two experimental warming sites, Duke Forest (DF) in Durham, North Carolina and Harvard Forest (HF) in Petersham, Massachusetts. At both sites, twelve experimental open-top warming chambers were established in January of 2010 (Pelini et al. 2011). Each chamber is 5 m in diameter and 1.2 m tall with a 2–3 cm gap at the bottom to allow ants and other organisms to move in and out.

Nine chambers blew warmed air from 1.5–5.5°C above ambient temperatures at half-degree steps with one chamber at each temperature treatment, three chambers blew ambient, and three chamberless control plots. (see Pelini et al., 2011 for a detailed description of the chambers).

Field Collections

Samples were chosen on a relatively "hot" and "cool" day in the summer of 2013 at HF and both 2013 and 2014 at DF in order to capture as wide a temperature range as possible (See Table 1 for sampling dates). Eight artificial next boxes arrayed in pairs in each cardinal direction were placed approximately one meter apart in each chamber. Bait cards holding crumbled pecan sandwiches were placed between each set of nest boxes to sample foraging workers outside the nest box begin exposed to the warmed ground temperature. Three random foragers from each bait station were selected at random and grouped together to be immediately flash frozen in liquid nitrogen. Three replicate samples were collected from each chamber. To quantify temperatures the ants were experiencing at the time of collection, four ground temperature measurements were made for each bait collection with an infrared thermometer (ThermoScientific, USA). Samples were stored at -80° C until Hsp mRNA quantification.

Quantifying Hsp Gene Expression

We quantified hsp40, hsp70, and hsp83 gene expression fold change relative to the housekeeping gene, 18s rRNA, actin, and gapdh from Aphaenogaster samples collected at the southern (DF) and northern (HF) warming chamber sites (See Table 2 for genes tested and primers used). Total mRNA from each sample was extracted and purified using the RNeazy micro kit (QIAGEN, USA). Each sample containing three frozen ants was homogenized in a Bullet blender (Next Advance Inc., USA) for two minutes at top speed (10) in 1.4mm zirconium silicate grinding beads (Quackenbush Co., Inc., USA) and 350 uL of RLT buffer (QIAGEN, USA). RNA samples were treated with DNAse I (QIAGEN, USA) to remove DNA contamination and purified following the manufacturer's instructions. RNA concentration was verified using Qubit Fluorometric Quantitation (Invitrogen, USA) and RNA integrity was tested using a NanoDrop Bioanalyzer (ThermoScientific, USA). Samples were converted to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied BiosystemsTM). Abundance of each Hsp gene and the housekeeping gene was quantified with quantitative polymerase chain reaction (qPCR) using the ABI StepOnePlus Real-Time PCR system. Reactions took place in 10 uL volume with 1ng of cDNA, 250 nM total primer, and 5 uL of Power SYBR Green Master Mix (Life Technologies, USA). Cycling conditions began at an initial 95°C incubation for 2 min followed by 40 cycles of 95°C for 15 seconds, with 60°C annealing and extension

```
library(plyr)
library(dplyr)
```

```
##
## Attaching package: 'dplyr'
##
## The following objects are masked from 'package:plyr':
##
##     arrange, count, desc, failwith, id, mutate, rename, summarise,
##     summarize
##
## The following objects are masked from 'package:stats':
##
##     filter, lag
##
## The following objects are masked from 'package:base':
##
##     intersect, setdiff, setequal, union
```

```
library(ggplot2)
library(tidyr)
library(MASS)
```

```
##
## Attaching package: 'MASS'
##
## The following object is masked from 'package:dplyr':
##
##     select
```

```
library(formatR)
```

Loading in the data and accompanying metadata

```
# Date initiated- 8/24/2015
# Date Modified- 4/18/2016
# Affiliations- University of Vermont, University of North Carolina, Harvard Forest,
  #Duke Forest, University of Tennessee
# Name and contact info: Curtis Provencher, cprovenc@uvm.edu, cprovenc3@gmail.com.
  #Andrew Nguyen, adnguyen@uvm.edu, anbe642@gmail.com
# Study name: The effects of experimental warming on forest ants
# Financial support: National Science Foundation, Division of Environmental Biology
  #(1136644)
# Methods of data collection: Experimental warming chamebrs set up at a northern (Harvard
  #Forest, MA) and southern site (Duke Forest, NC) warming groud temperature up to 5.5
  #degrees Celsius. In 2013-2014, ants were baited in each warming chamber and three ants
  #were collected per tube and flash frozen in the field. For each sample we isolated RNA
  #and quantified their stress response using heat-shock proteins (hsp70, hsp83, hsp40).
```

```
  #For housekeeping genes we quantified 18s rRNA, actin, and gapdh. Also included in this
  #worksheet is the calculations to convert RNA to cDNA.
# Experimental units for each variable: Collection Date(YearMonthDay), Site (HF- Harvard
  #Forest, DF- Duke Forest), chamber(chamber number), sample(3 ants per tube),  window(area
  #of chamber), BaitTemp(degrees Celsius), RNA conc.(ng/uL), Isolation date(Date RNA
  #isolated, YearMonthDay), CT18s, 40, 70, 83, actin, gapdh(Threshold- 0.1, basline cycle
  #9-15), RIN(RNA integrity), cDNA(ng/uL), vol cDNA and H20(uL)
warm<-read.csv("../Data//20160411_FinalExperimentalWarmingDataset.csv",skip=10)
```

Quality control of expression values

```
# Quality control

# ranges of gene expression
apply(warm[, 14:19], 2, range, na.rm = TRUE)
```

```
##      CT_18s  CT_40  CT_70  CT_83 CT_actin CT_gapdh
## [1,]  4.972 24.636 19.571 20.215   20.531   20.111
## [2,] 29.813 37.441 32.777 38.100   34.249   34.721
```

```
# filter out very lowly expressed genes
# warm.hsp70<-subset(warm,warm$CT_70<34);dim(warm.hsp70)
warm.long <- gather(warm, Genes, GXP, CT_18s:CT_83)  # converting to long format
qc.samples <- subset(warm.long, warm.long$GXP > 34)  #identifying the ones that have too low expression
n.exclude <- qc.samples$n
dim(warm[-n.exclude, ])  #excluding values that are too low in expression
```

```
## [1] 237  24
```

```
warm <- warm[-n.exclude, ]
##############################################################################
```

Visualizing the properties of the dataset

```
# Visualizing the properties of the dataset
str(warm)
```

```
## 'data.frame':    237 obs. of  24 variables:
##  $ n                      : int  1 3 4 5 6 7 8 9 11 12 ...
##  $ Collection.Date        : int  20130702 20130702 20130702 20130702 20130702 20130702 20130702 201
##  $ Site                   : Factor w/ 2 levels "DF","HF": 1 1 1 1 1 1 1 1 1 1 ...
##  $ Vial.Name              : Factor w/ 170 levels "","DF 1.1","DF 1.2",..: 54 52 79 80 78 83 82 84 8
##  $ Cham                   : int  1 1 2 2 2 3 3 3 4 4 ...
##  $ Sample                 : int  1 3 1 2 3 1 2 3 2 3 ...
##  $ Window                 : Factor w/ 6 levels "","A","B","C",..: 4 3 3 4 2 3 2 4 5 2 ...
##  $ BaitTemp1              : num  24.2 25.2 23 23.8 22.4 23 23 22.8 24 23.8 ...
##  $ BaitTemp2              : num  24.2 25.2 23.2 23.8 23.6 23 22.8 22.8 24.2 23.8 ...
```

```
##  $ BaitTemp3                : num  24.4 25.2 23.2 23.6 23.6 23.6 23 22.8 23.8 24.2 ...
##  $ BaitTemp4                : num  24.4 25 23.4 23.6 23.6 23.2 23.8 22.4 23.8 23.8 ...
##  $ RNA.conc.                : Factor w/ 139 levels "","<2","10","10.4",..: 118 14 83 136 122 19 121 9
##  $ Isolation.Date           : int  20150811 20150731 20150814 20150813 20150813 20150730 20150814 201
##  $ CT_18s                   : num  NA 18.5 13.1 NA 15.3 ...
##  $ CT_40                    : num  28.5 32.1 32.1 NA 31.6 ...
##  $ CT_70                    : num  25.8 26.1 28.4 NA 26.9 ...
##  $ CT_83                    : num  27.4 31.3 31.1 NA 32.7 ...
##  $ CT_actin                 : num  24.4 27.8 33.6 32.7 29.3 ...
##  $ CT_gapdh                 : num  23.5 28.8 30.8 NA 29.2 ...
##  $ RIN_Value                : num  2.1 3.7 2.8 2.4 7 2.6 2.1 2.4 2.9 1 ...
##  $ CDNA                     : num  2.5 2.5 2.5 2.5 2.5 2.5 2.5 2.5 2.5 2.5 ...
##  $ dilution.factor          : Factor w/ 13 levels "#DIV/0!","10",..: 2 2 2 2 2 2 2 2 2 2 ...
##  $ vol.cDNA.for.dilution    : Factor w/ 13 levels "#DIV/0!","10.86956522",..: 4 4 4 4 4 4 4 4 4 4 ..
##  $ vol.of.water.for.dilution: Factor w/ 13 levels "#DIV/0!","37.5",..: 13 13 13 13 13 13 13 13 13 13
```

```
# Calculating # of samples per site per chamber
knitr::kable(ddply(warm, .(Site, as.factor(Cham)), summarize, num = length(n)))
```

Site	as.factor(Cham)	num
DF	1	9
DF	2	12
DF	3	12
DF	4	11
DF	5	11
DF	6	12
DF	7	8
DF	8	9
DF	9	11
DF	10	9
DF	11	12
DF	12	9
DF	13	11
DF	14	9
DF	15	8
HF	1	7
HF	2	8
HF	3	7
HF	4	5
HF	5	8
HF	6	8
HF	7	5
HF	8	7
HF	9	7
HF	10	7
HF	11	7
HF	12	8

```
# Number of samples per site
knitr::kable(ddply(warm, .(Site), summarize, num = length(n)))
```

Site	num
DF	153
HF	84

```
# Calculating the bait temperatures
warm$baittemp.ave <- apply(warm[, 8:11], 1, mean, na.rm = TRUE)
## knitr::kable(ddply(warm,.(Site),summarize,range(na.exclude(baittemp.ave))))

# Range of temperatures for duke forest
range(subset(warm, warm$Site == "DF")$baittemp.ave)
```

```
## [1] 22.30667 32.90000
```

```
# Range of temperatures for Harvard forest
range(subset(warm, warm$Site == "HF")$baittemp.ave)
```

```
## [1] 23.60 32.95
```

```
# Looking at the dimensions...rows,columns
dim(warm)
```

```
## [1] 237  25
```

```
# Number of samples for hsp70
length(na.exclude(warm$CT_70))
```

```
## [1] 145
```

```
# Number of samples for hsp83
length(na.exclude(warm$CT_83))
```

```
## [1] 144
```

```
# Number of samples for hsp40
length(na.exclude(warm$CT_40))
```

```
## [1] 139
```

```
# the overall theme for ggplot
T <- theme_bw() + theme(text = element_text(size = 22), axis.text = element_text(size = 22),
    axis.title.y = element_text(margin = margin(20, 20, 20, 20)), axis.title.x = element_text(margin = m
        0, 0, 0)), plot.margin = unit(c(1, 1, 1, 0), "lines"), panel.grid.major = element_blank(),
    panel.grid.minor.x = element_blank(), panel.grid = element_blank(), legend.key = element_blank(),
    legend.position = c(0.1, 0.85))
```

Checking internal control

```r
# standard deviation in CT value for each gene
apply(warm[, 14:19], 2, sd, na.rm = TRUE)
```

```
##   CT_18s    CT_40    CT_70    CT_83 CT_actin CT_gapdh
## 5.079533 2.399703 2.860830 4.186841 3.150696 3.279284
```

Evaluating 18s rRNA

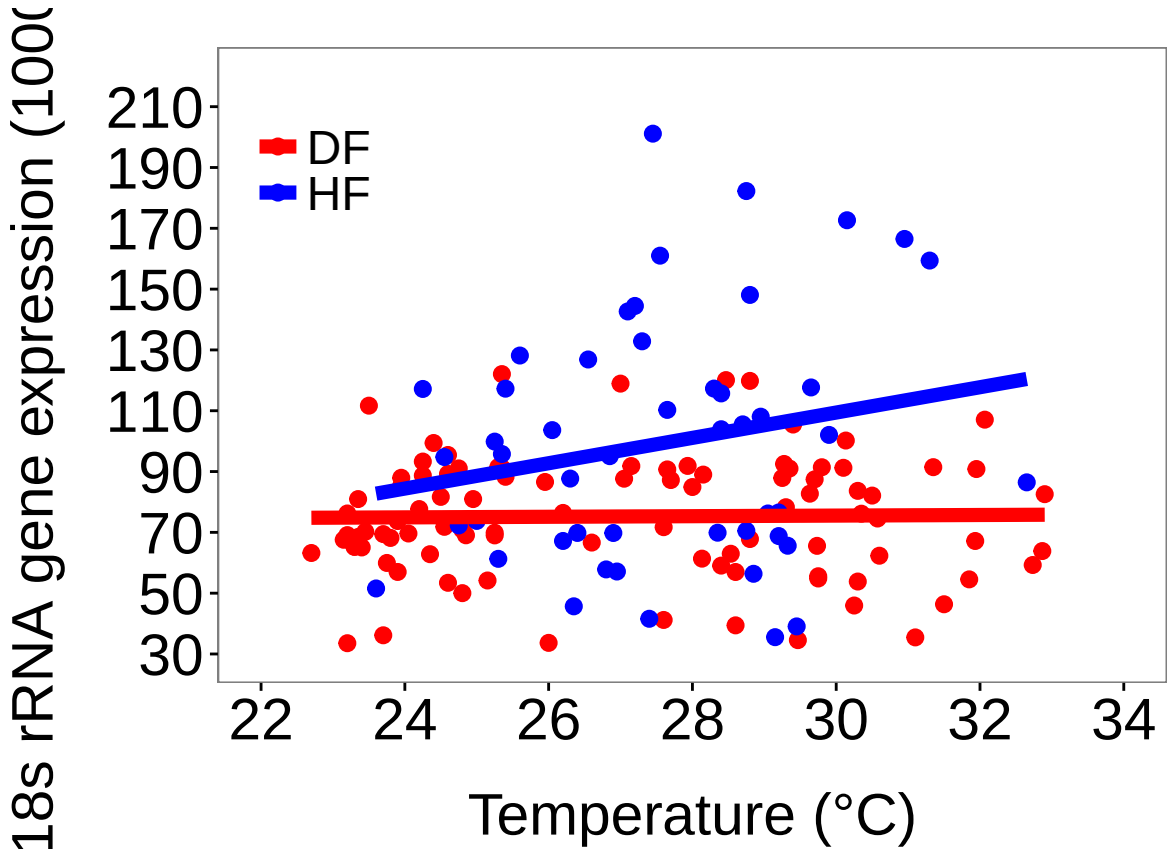
```r
hkg.mod1 <- lm((1000/warm$CT_18s) ~ baittemp.ave * Site + RIN_Value, data = warm)
summary(hkg.mod1)
```

```
##
## Call:
## lm(formula = (1000/warm$CT_18s) ~ baittemp.ave * Site + RIN_Value,
##     data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -71.241 -16.995  -0.165  14.626 103.578
##
## Coefficients:
##                      Estimate Std. Error t value Pr(>|t|)
## (Intercept)          73.06161   26.90000   2.716  0.00744 **
## baittemp.ave         -0.01859    1.02861  -0.018  0.98560
## SiteHF              -79.88765   65.42935  -1.221  0.22415
## RIN_Value             0.60839    1.23882   0.491  0.62412
## baittemp.ave:SiteHF   3.76111    2.37819   1.581  0.11602
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 28.27 on 140 degrees of freedom
##   (92 observations deleted due to missingness)
## Multiple R-squared:  0.168,  Adjusted R-squared:  0.1442
## F-statistic: 7.068 on 4 and 140 DF,  p-value: 3.266e-05
```

```r
# 18s expression by site
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_18s), colour = factor(Site))) +
    geom_point(size = 2.5) + T + geom_smooth(method = "lm", se = FALSE, size = 2.5) +
    scale_color_manual(name = "", values = c("red", "blue")) + scale_x_continuous(limits = c(22,
    34), breaks = seq(22, 34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(30,
    220), breaks = seq(30, 220, 20), "18s rRNA gene expression (1000/CT)")
```

```
## Warning: Removed 91 rows containing non-finite values (stat_smooth).
```

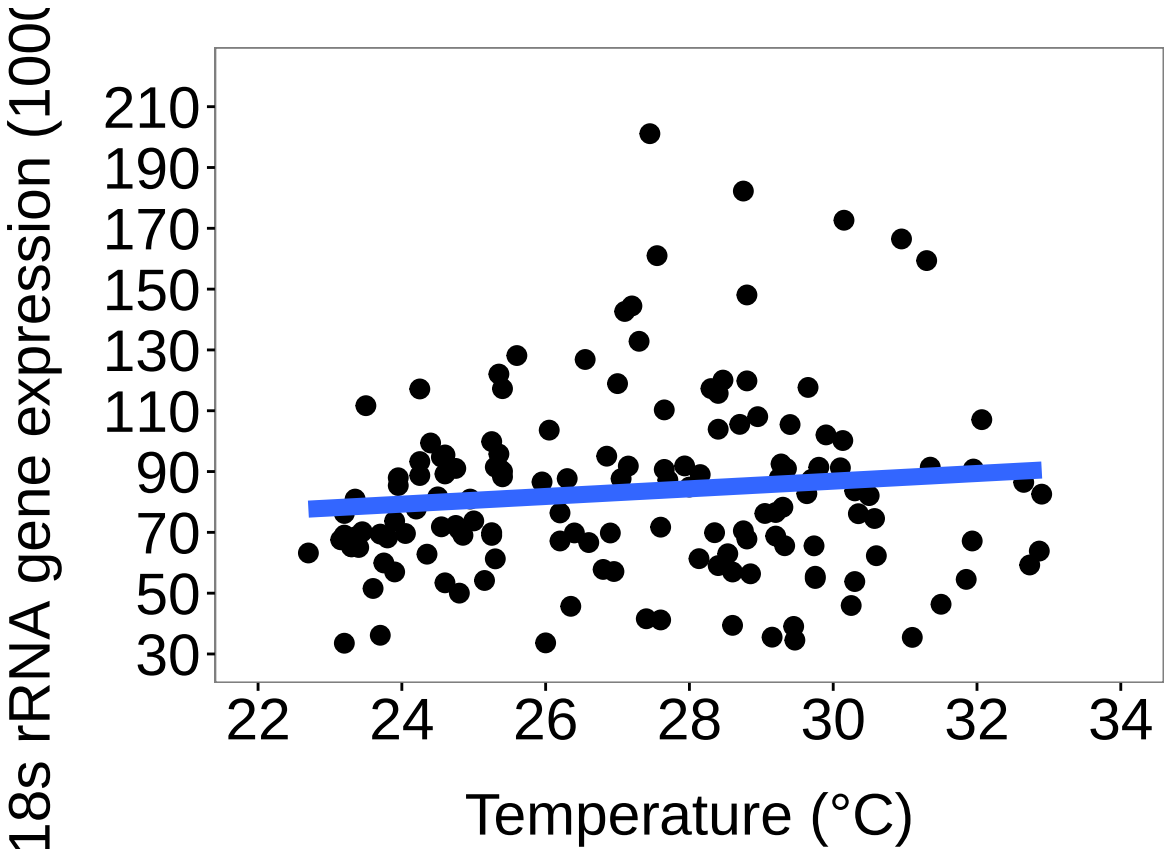
```
## Warning: Removed 91 rows containing missing values (geom_point).
```




```
# 18s expression overall
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_18s))) + geom_point(size = 3) +
    T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
    values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
    34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(30, 220), breaks = seq(30,
    220, 20), "18s rRNA gene expression (1000/CT)")
```

```
## Warning: Removed 91 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 91 rows containing missing values (geom_point).
```



Evaluating actin

```
hkg.mod2 <- lm(warm$CT_actin ~ baittemp.ave * Site + RIN_Value, data = warm)
summary(hkg.mod2)
```

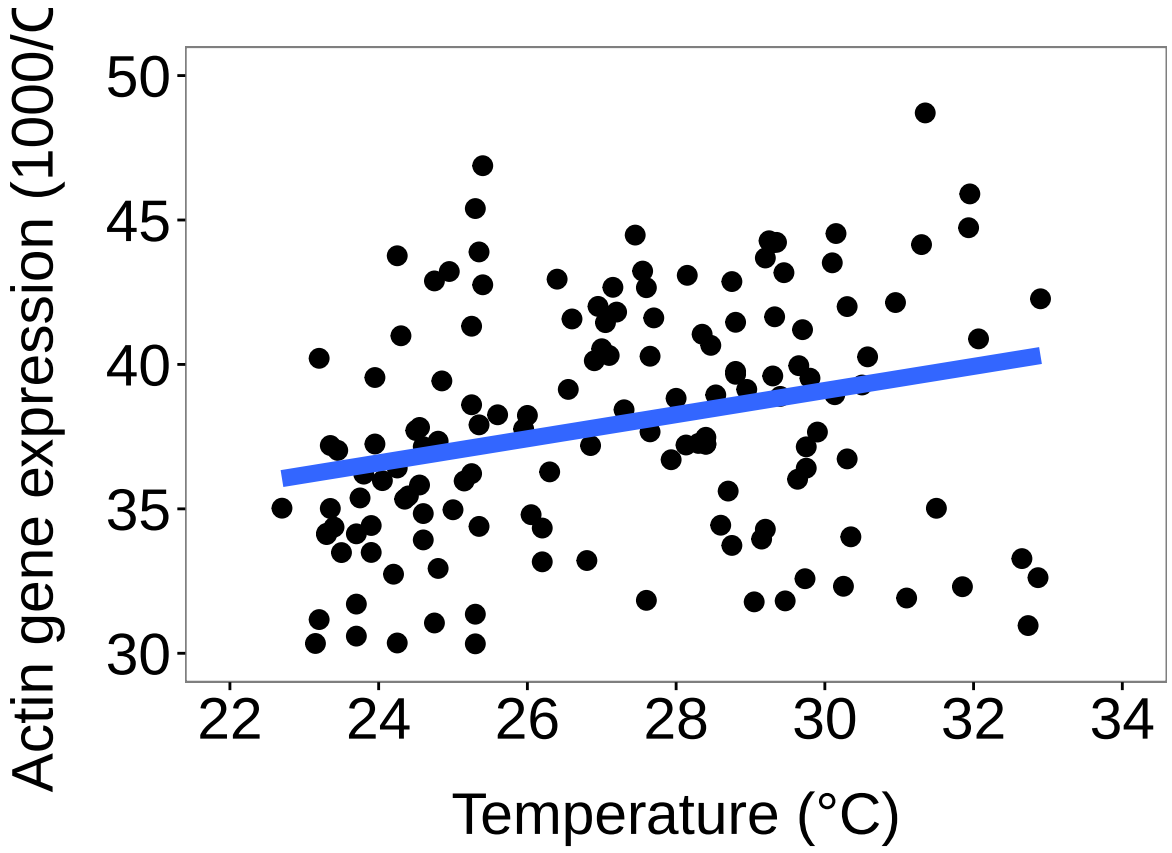
```
##
## Call:
## lm(formula = warm$CT_actin ~ baittemp.ave * Site + RIN_Value,
##     data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -6.4509 -2.1856 -0.2958  1.8374  8.3133
##
## Coefficients:
##                     Estimate Std. Error t value Pr(>|t|)
## (Intercept)          32.5982     2.8733  11.345   <2e-16 ***
## baittemp.ave         -0.1820     0.1103  -1.650    0.101
## SiteHF                5.9039     7.3849   0.799    0.425
## RIN_Value            -0.1938     0.1349  -1.437    0.153
## baittemp.ave:SiteHF  -0.2005     0.2677  -0.749    0.455
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
```

```
## Residual standard error: 3.054 on 139 degrees of freedom
##   (93 observations deleted due to missingness)
## Multiple R-squared:  0.07745,    Adjusted R-squared:  0.0509
## F-statistic: 2.917 on 4 and 139 DF,  p-value: 0.02355
```

```
# overall actin expression by temp
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_actin))) + geom_point(size = 3) +
    T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
    values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
    34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(30, 50), breaks = seq(30,
    50, 5), "Actin gene expression (1000/CT)")
```

```
## Warning: Removed 96 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 96 rows containing missing values (geom_point).
```



Evaluating gapdh

```
hkg.mod3 <- lm(warm$CT_gapdh ~ baittemp.ave * Site + RIN_Value, data = warm)
summary(hkg.mod3)
```

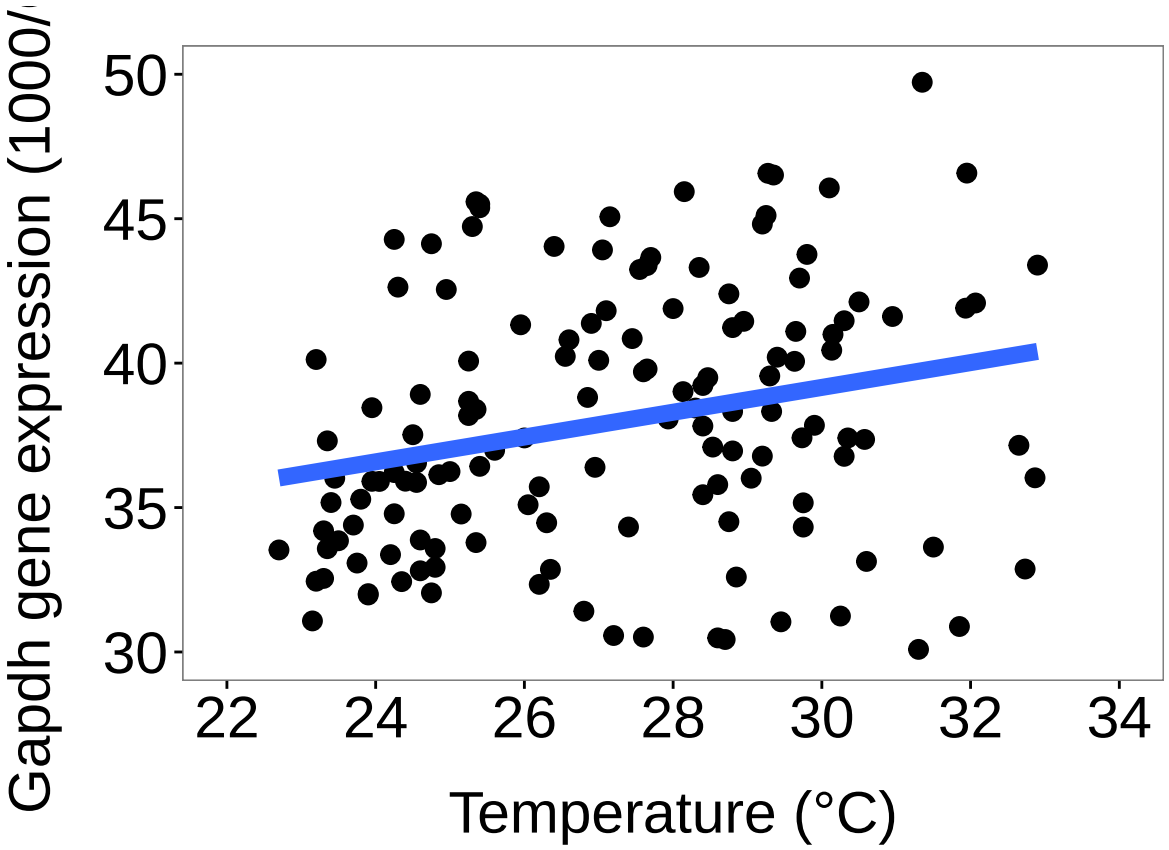
```
##
## Call:
```

```
## lm(formula = warm$CT_gapdh ~ baittemp.ave * Site + RIN_Value,
##     data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -5.6014 -2.6920 -0.2269  2.2440  7.7893
##
## Coefficients:
##                     Estimate Std. Error t value Pr(>|t|)
## (Intercept)          35.1678     3.0838  11.404   <2e-16 ***
## baittemp.ave         -0.2891     0.1183  -2.445   0.0158 *
## SiteHF               -5.3337     7.6499  -0.697   0.4869
## RIN_Value            -0.1868     0.1417  -1.318   0.1898
## baittemp.ave:SiteHF   0.2370     0.2774   0.854   0.3944
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 3.16 on 136 degrees of freedom
##   (96 observations deleted due to missingness)
## Multiple R-squared:  0.09225,    Adjusted R-squared:  0.06556
## F-statistic: 3.455 on 4 and 136 DF,  p-value: 0.01008
```

```
# overall gapdh expression by temp
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_gapdh))) + geom_point(size = 3) +
    T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
    values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
    34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(30, 50), breaks = seq(30,
    50, 5), "Gapdh gene expression (1000/CT)")
```

```
## Warning: Removed 98 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 98 rows containing missing values (geom_point).
```



Statistics

CT values themselves served as the measure of gene expression with 18s rRNA and actin serving as internal controls.

Hsp83 regression models

```
########################################################## hsp83 regresssion model

# MODEL WITH 18s- temp effect, site effect, and site by temp interaction
hsp83.mod <- lm(CT_83 ~ baittemp.ave * Site + RIN_Value + CT_18s, data = warm)
summary(stepAIC(hsp83.mod, direction = "backward"))
```

```
## Start:  AIC=270.33
## CT_83 ~ baittemp.ave * Site + RIN_Value + CT_18s
##
##                     Df Sum of Sq     RSS    AIC
## <none>                             875.75 270.33
## - RIN_Value          1     18.94  894.69 271.37
## - baittemp.ave:Site  1     29.54  905.29 273.04
## - CT_18s             1    928.48 1804.24 370.97

##
```

```
## Call:
## lm(formula = CT_83 ~ baittemp.ave * Site + RIN_Value + CT_18s,
##     data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -9.3026 -1.6463  0.0008  1.7405  6.0676
##
## Coefficients:
##                      Estimate Std. Error t value Pr(>|t|)
## (Intercept)          37.03806    2.50690  14.774  < 2e-16 ***
## baittemp.ave         -0.58013    0.09333  -6.216 5.84e-09 ***
## SiteHF               13.95924    5.88264   2.373   0.0190 *
## RIN_Value            -0.19240    0.11220  -1.715   0.0887 .
## CT_18s                0.51854    0.04318  12.008  < 2e-16 ***
## baittemp.ave:SiteHF -0.45808    0.21388  -2.142   0.0340 *
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 2.538 on 136 degrees of freedom
##   (95 observations deleted due to missingness)
## Multiple R-squared:  0.6474, Adjusted R-squared:  0.6344
## F-statistic: 49.93 on 5 and 136 DF,  p-value: < 2.2e-16
```

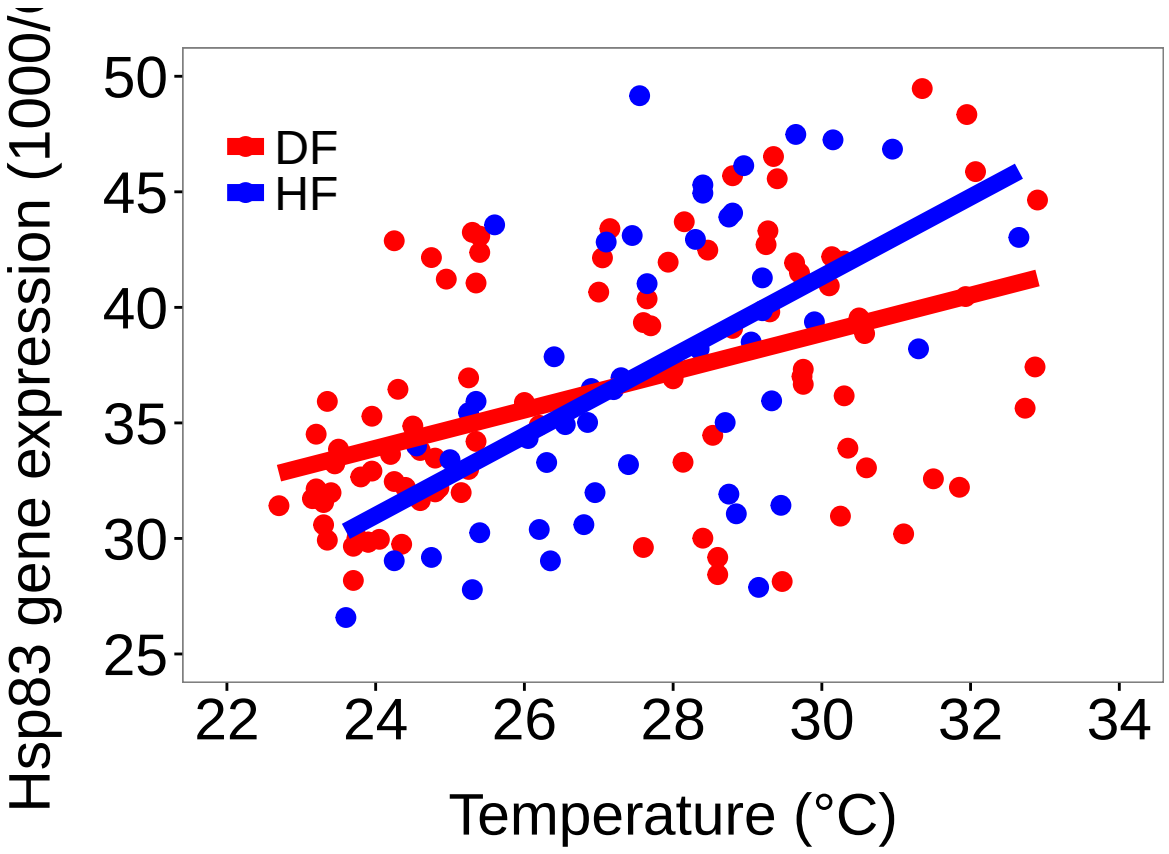
```
# MODEL WITH ACTIN- no interaction or site effect seen
hsp83.act.mod <- lm(CT_83 ~ baittemp.ave * Site + RIN_Value + CT_actin, data = warm)
summary(stepAIC(hsp83.act.mod, direction = "backward"))
```

```
## Start:  AIC=262.63
## CT_83 ~ baittemp.ave * Site + RIN_Value + CT_actin
##
##                      Df Sum of Sq     RSS    AIC
## - baittemp.ave:Site  1      3.60  847.12 261.22
## <none>                            843.53 262.63
## - RIN_Value          1     20.35  863.88 263.95
## - CT_actin           1    822.57 1666.10 355.24
##
## Step:  AIC=261.22
## CT_83 ~ baittemp.ave + Site + RIN_Value + CT_actin
##
##                Df Sum of Sq     RSS    AIC
## - Site          1      4.05  851.17 259.89
## <none>                       847.12 261.22
## - RIN_Value     1     25.20  872.32 263.30
## - baittemp.ave  1    151.40  998.52 282.08
## - CT_actin      1    831.94 1679.07 354.32
##
## Step:  AIC=259.89
## CT_83 ~ baittemp.ave + RIN_Value + CT_actin
##
##                Df Sum of Sq     RSS    AIC
## <none>                       851.17 259.89
## - RIN_Value     1     29.35  880.52 262.60
## - baittemp.ave  1    155.18 1006.35 281.17
```

```
## - CT_actin      1    828.27 1679.44 352.35

##
## Call:
## lm(formula = CT_83 ~ baittemp.ave + RIN_Value + CT_actin, data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -4.6598 -1.6331 -0.1078  1.4837  8.6187
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  18.95951    3.20824   5.910 2.65e-08 ***
## baittemp.ave -0.43831    0.08835  -4.961 2.07e-06 ***
## RIN_Value    -0.23484    0.10884  -2.158   0.0327 *
## CT_actin      0.81018    0.07069  11.462  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 2.511 on 135 degrees of freedom
##   (98 observations deleted due to missingness)
## Multiple R-squared:  0.6239, Adjusted R-squared:  0.6155
## F-statistic: 74.65 on 3 and 135 DF,  p-value: < 2.2e-16
```

```
# hsp83 plot by site
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_83), colour = factor(Site))) +
    geom_point(size = 3) + T + geom_smooth(method = "lm", se = FALSE, size = 3) +
    scale_color_manual(name = "", values = c("red", "blue")) + scale_x_continuous(limits = c(22,
    34), breaks = seq(22, 34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(25,
    50), breaks = seq(25, 50, 5), "Hsp83 gene expression (1000/CT)")
```

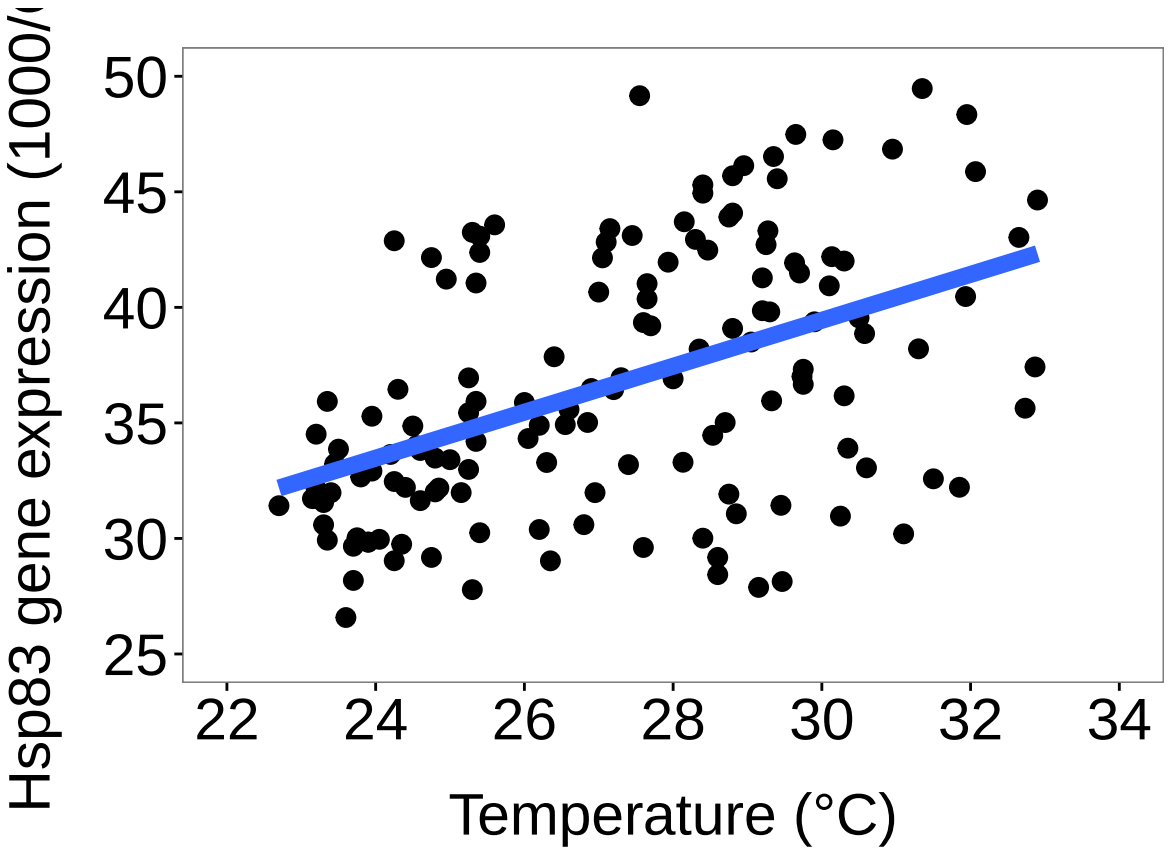
```
## Warning: Removed 93 rows containing non-finite values (stat_smooth).

## Warning: Removed 93 rows containing missing values (geom_point).
```




```
# hsp83 plot overall
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_83))) + geom_point(size = 3) +
    T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
    values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
    34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(25, 50), breaks = seq(25,
    50, 5), "Hsp83 gene expression (1000/CT)")
```

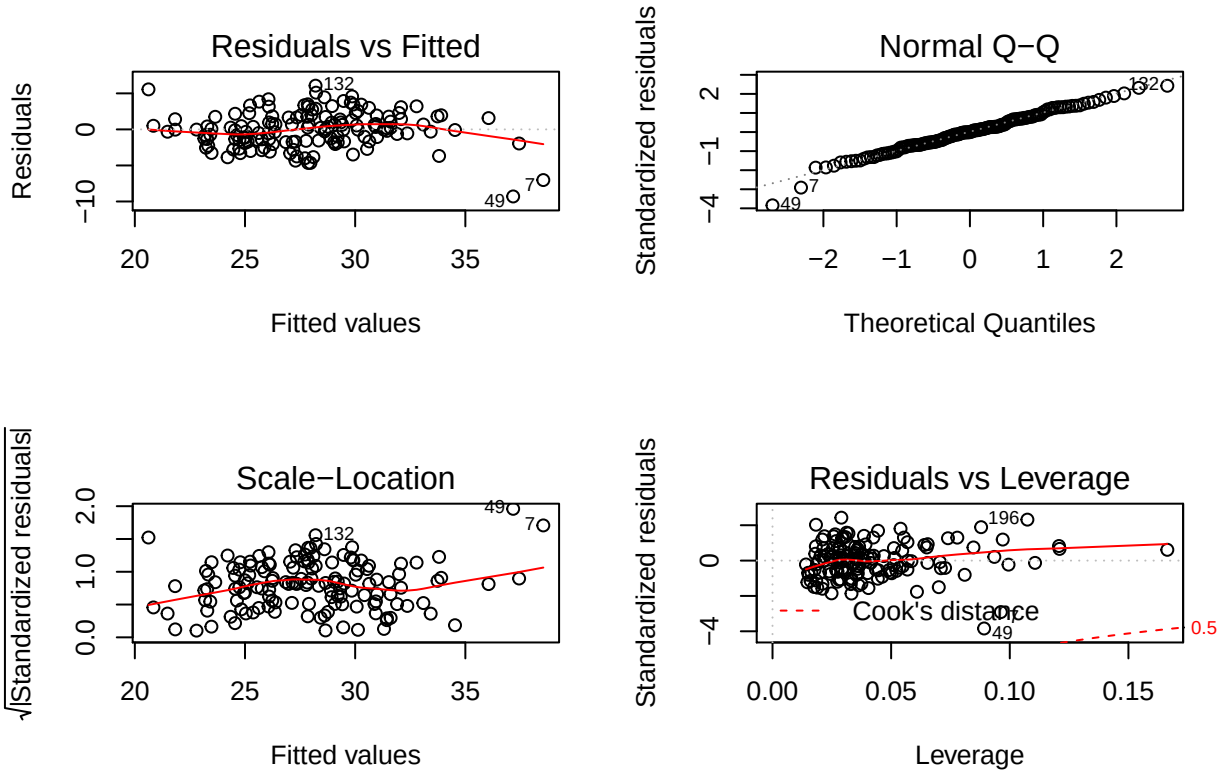
```
## Warning: Removed 93 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 93 rows containing missing values (geom_point).
```




```
# visualize hsp83 model
par(mfrow = c(2, 2))
plot(stepAIC(hsp83.mod, direction = "backward"))
```

```
## Start:  AIC=270.33
## CT_83 ~ baittemp.ave * Site + RIN_Value + CT_18s
##
##                     Df Sum of Sq     RSS    AIC
## <none>                             875.75 270.33
## - RIN_Value          1     18.94  894.69 271.37
## - baittemp.ave:Site  1     29.54  905.29 273.04
## - CT_18s             1    928.48 1804.24 370.97
```




```
par(mfrow = c(1, 1))
```

Hsp70 regression models

```
########################################################## hsp70 regression model

# MODEL WITH 18s- no site effect seen
hsp70.mod <- lm(CT_70 ~ baittemp.ave * Site + RIN_Value + CT_18s, data = warm)
summary(stepAIC(hsp70.mod, direction = "backward"))
```

```
## Start:  AIC=180.29
## CT_70 ~ baittemp.ave * Site + RIN_Value + CT_18s
##
##                       Df Sum of Sq    RSS    AIC
## - baittemp.ave:Site  1      5.85 469.76 180.08
## <none>                            463.92 180.29
## - RIN_Value          1     21.22 485.14 184.69
## - CT_18s             1    399.29 863.21 267.09
##
## Step:  AIC=180.08
## CT_70 ~ baittemp.ave + Site + RIN_Value + CT_18s
##
##                Df Sum of Sq    RSS    AIC
## - Site          1      1.13 470.89 178.43
## <none>                      469.76 180.08
## - RIN_Value     1     27.86 497.63 186.32
```

```
## - baittemp.ave  1     96.64 566.41 204.84
## - CT_18s        1    402.52 872.29 266.58
##
## Step:  AIC=178.43
## CT_70 ~ baittemp.ave + RIN_Value + CT_18s
##
##                Df Sum of Sq    RSS    AIC
## <none>                      470.89 178.43
## - RIN_Value     1     29.48 500.37 185.11
## - baittemp.ave  1     97.88 568.78 203.43
## - CT_18s        1    439.40 910.30 270.68


##
## Call:
## lm(formula = CT_70 ~ baittemp.ave + RIN_Value + CT_18s, data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -6.3410 -1.2981  0.1183  1.2881  4.6334
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  30.81485    1.68181  18.322  < 2e-16 ***
## baittemp.ave -0.34274    0.06376  -5.375 3.15e-07 ***
## RIN_Value    -0.23261    0.07886  -2.950  0.00373 **
## CT_18s        0.34885    0.03063  11.389  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 1.841 on 139 degrees of freedom
##   (94 observations deleted due to missingness)
## Multiple R-squared:  0.5975, Adjusted R-squared:  0.5888
## F-statistic: 68.78 on 3 and 139 DF,  p-value: < 2.2e-16
```

```
# MODEL WITH ACTIN- site effect seen
hsp70.act.mod <- lm(CT_70 ~ baittemp.ave * Site + RIN_Value + CT_actin, data = warm)
summary(stepAIC(hsp70.act.mod, direction = "backward"))
```

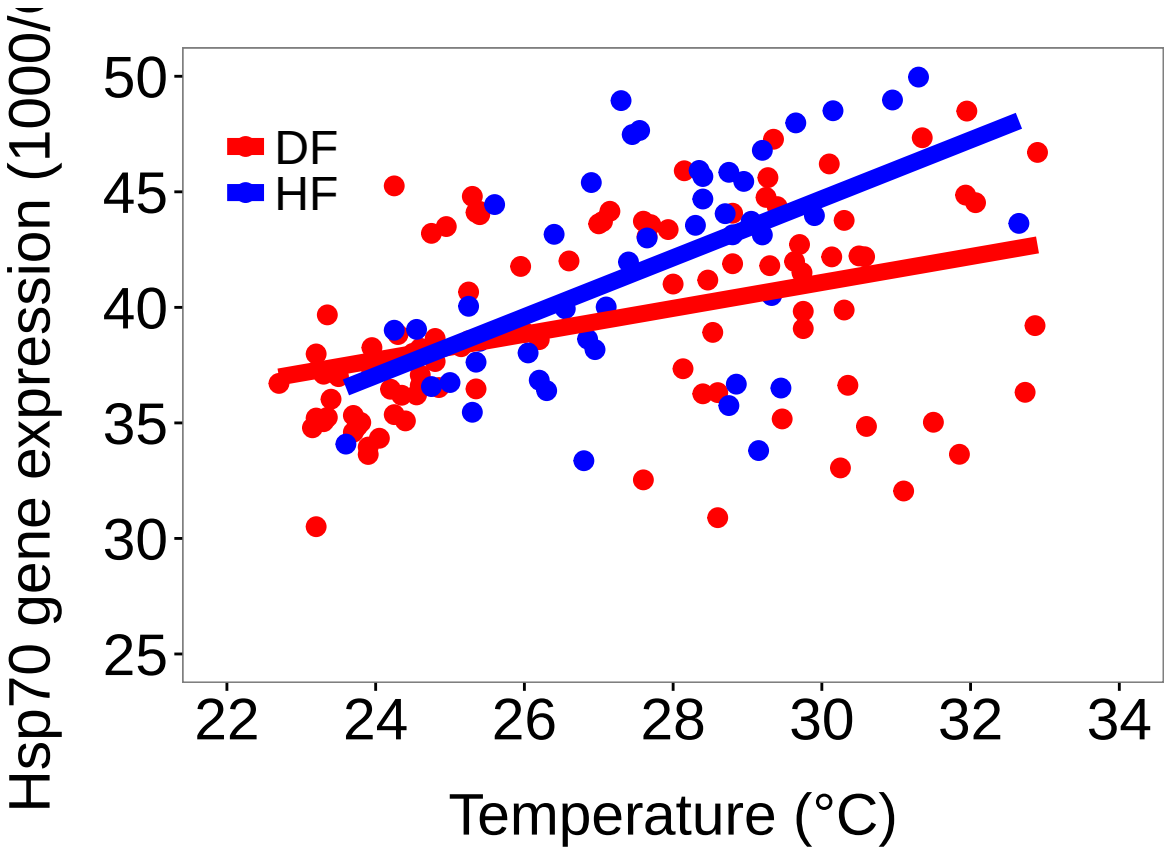
```
## Start:  AIC=141.21
## CT_70 ~ baittemp.ave * Site + RIN_Value + CT_actin
##
##                     Df Sum of Sq    RSS    AIC
## - baittemp.ave:Site  1      0.33 352.87 139.34
## <none>                           352.53 141.21
## - RIN_Value          1     23.19 375.72 148.19
## - CT_actin           1    437.84 790.37 253.05
##
## Step:  AIC=139.34
## CT_70 ~ baittemp.ave + Site + RIN_Value + CT_actin
##
##                Df Sum of Sq    RSS    AIC
## <none>                      352.87 139.34
## - RIN_Value     1     25.47 378.34 147.17
```

```
## - baittemp.ave  1     30.12 382.99 148.89
## - Site          1     45.32 398.19 154.38
## - CT_actin      1    440.69 793.55 251.61

##
## Call:
## lm(formula = CT_70 ~ baittemp.ave + Site + RIN_Value + CT_actin,
##     data = warm)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -4.2540 -0.9640 -0.2234  0.7384  6.2752
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  15.89305    2.05893   7.719 2.27e-12 ***
## baittemp.ave -0.19317    0.05669  -3.407 0.000863 ***
## SiteHF       -1.23852    0.29633  -4.180 5.20e-05 ***
## RIN_Value    -0.22154    0.07071  -3.133 0.002118 **
## CT_actin      0.59060    0.04532  13.033  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 1.611 on 136 degrees of freedom
##   (96 observations deleted due to missingness)
## Multiple R-squared:  0.6794, Adjusted R-squared:  0.6699
## F-statistic: 72.04 on 4 and 136 DF,  p-value: < 2.2e-16
```

```
# hsp70 model by site
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_70), colour = factor(Site))) +
    geom_point(size = 3) + T + geom_smooth(method = "lm", se = FALSE, size = 3) +
    scale_color_manual(name = "", values = c("red", "blue")) + scale_x_continuous(limits = c(22,
    34), breaks = seq(22, 34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(25,
    50), breaks = seq(25, 50, 5), "Hsp70 gene expression (1000/CT)")
```

```
## Warning: Removed 93 rows containing non-finite values (stat_smooth).
```

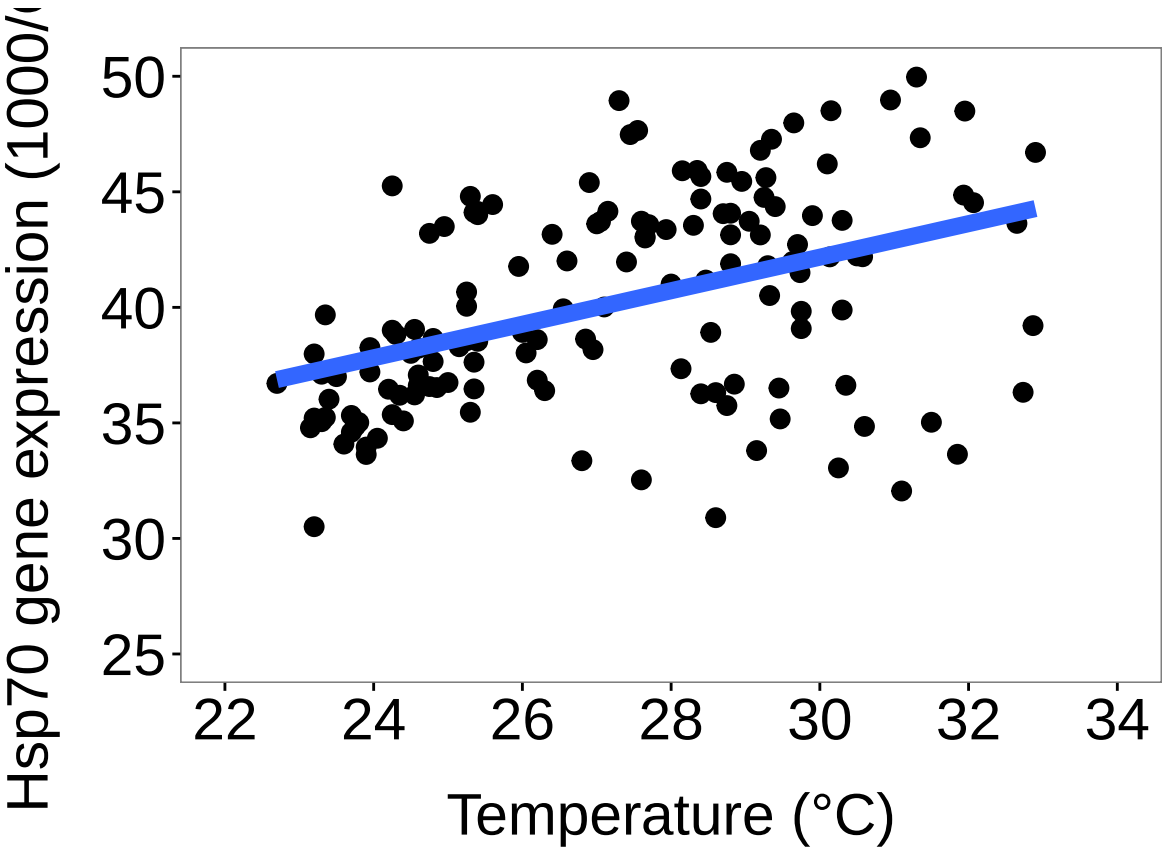
```
## Warning: Removed 93 rows containing missing values (geom_point).
```




```
# hsp70 plot overall
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_70))) + geom_point(size = 3) +
    T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
    values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
    34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(25, 50), breaks = seq(25,
    50, 5), "Hsp70 gene expression (1000/CT)")
```

```
## Warning: Removed 93 rows containing non-finite values (stat_smooth).
```

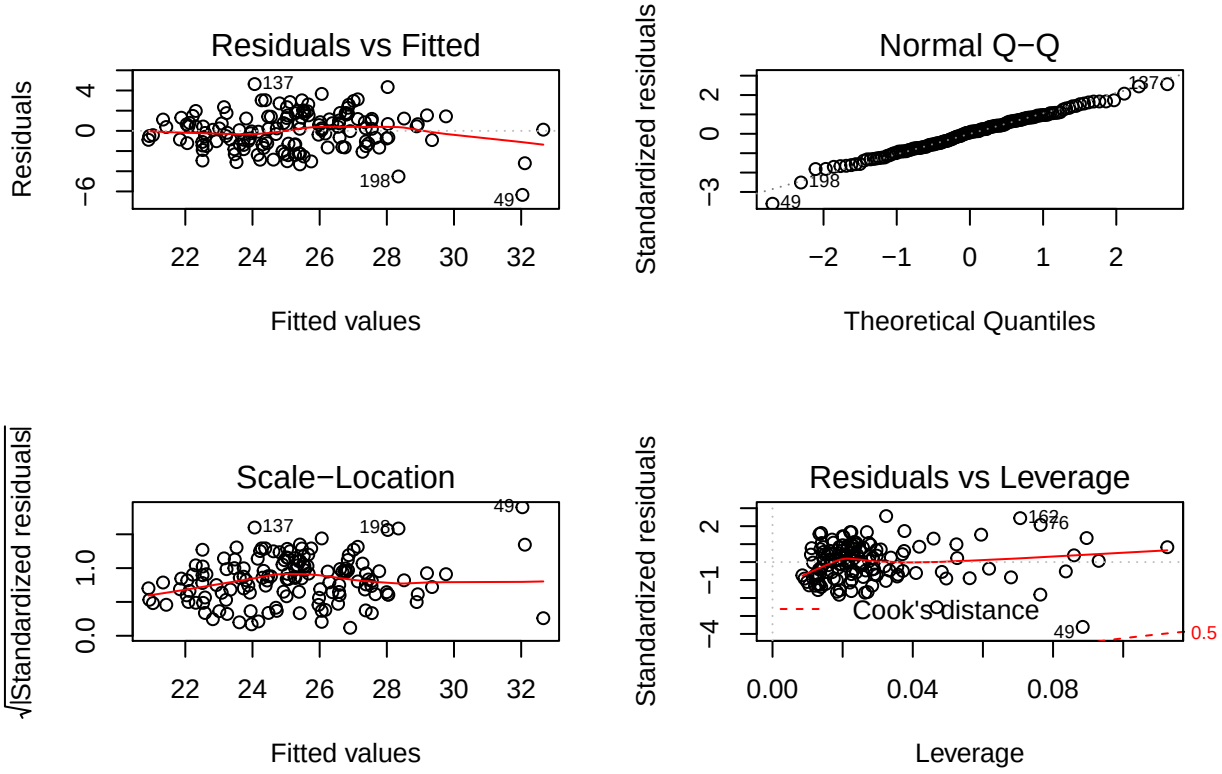
```
## Warning: Removed 93 rows containing missing values (geom_point).
```




```
# visualizing hsp70 regression model
par(mfrow = c(2, 2))
plot(stepAIC(hsp70.mod, direction = "backward"))
```

```
## Start:  AIC=180.29
## CT_70 ~ baittemp.ave * Site + RIN_Value + CT_18s
##
##                      Df Sum of Sq    RSS    AIC
## - baittemp.ave:Site  1      5.85 469.76 180.08
## <none>                           463.92 180.29
## - RIN_Value          1     21.22 485.14 184.69
## - CT_18s             1    399.29 863.21 267.09
##
## Step:  AIC=180.08
## CT_70 ~ baittemp.ave + Site + RIN_Value + CT_18s
##
##                Df Sum of Sq    RSS    AIC
## - Site          1      1.13 470.89 178.43
## <none>                       469.76 180.08
## - RIN_Value     1     27.86 497.63 186.32
## - baittemp.ave  1     96.64 566.41 204.84
## - CT_18s        1    402.52 872.29 266.58
##
## Step:  AIC=178.43
## CT_70 ~ baittemp.ave + RIN_Value + CT_18s
##
```

```
##                Df Sum of Sq    RSS    AIC
## <none>                      470.89 178.43
## - RIN_Value     1     29.48 500.37 185.11
## - baittemp.ave  1     97.88 568.78 203.43
## - CT_18s        1    439.40 910.30 270.68
```




```
par(mfrow = c(1, 1))
```

Hsp40 regression models

```
########################################################## hsp40 regression model

# MODEL WITH 18s- site trend seen
warm.40 <- subset(warm, warm$CT_40 != "NA" & RIN_Value != "NA")
hsp40.mod <- lm(CT_40 ~ baittemp.ave * Site + RIN_Value + CT_18s, data = warm.40)
summary(stepAIC(hsp40.mod, direction = "both"))
```

```
## Start:  AIC=103.19
## CT_40 ~ baittemp.ave * Site + RIN_Value + CT_18s
##
##                      Df Sum of Sq    RSS    AIC
## - baittemp.ave:Site   1      2.73 269.29 102.58
## <none>                            266.56 103.19
## - RIN_Value           1      4.24 270.80 103.35
## - CT_18s              1    380.13 646.69 222.61
##
```

```
## Step:  AIC=102.59
## CT_40 ~ baittemp.ave + Site + RIN_Value + CT_18s
##
##                     Df Sum of Sq    RSS    AIC
## <none>                           269.29 102.58
## + baittemp.ave:Site  1      2.73 266.56 103.19
## - Site               1      6.05 275.34 103.63
## - RIN_Value          1      6.08 275.37 103.64
## - baittemp.ave       1     61.69 330.98 128.84
## - CT_18s             1    385.85 655.14 222.39


##
## Call:
## lm(formula = CT_40 ~ baittemp.ave + Site + RIN_Value + CT_18s,
##     data = warm.40)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -6.5247 -0.8178  0.0043  0.9718  3.4569
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  33.13150    1.42408  23.265  < 2e-16 ***
## baittemp.ave -0.29707    0.05402  -5.499 1.91e-07 ***
## SiteHF        0.45083    0.26181   1.722   0.0874 .
## RIN_Value    -0.11216    0.06497  -1.726   0.0866 .
## CT_18s        0.38146    0.02774  13.753  < 2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 1.428 on 132 degrees of freedom
##   (1 observation deleted due to missingness)
## Multiple R-squared:  0.6583, Adjusted R-squared:  0.648
## F-statistic: 63.59 on 4 and 132 DF,  p-value: < 2.2e-16
```

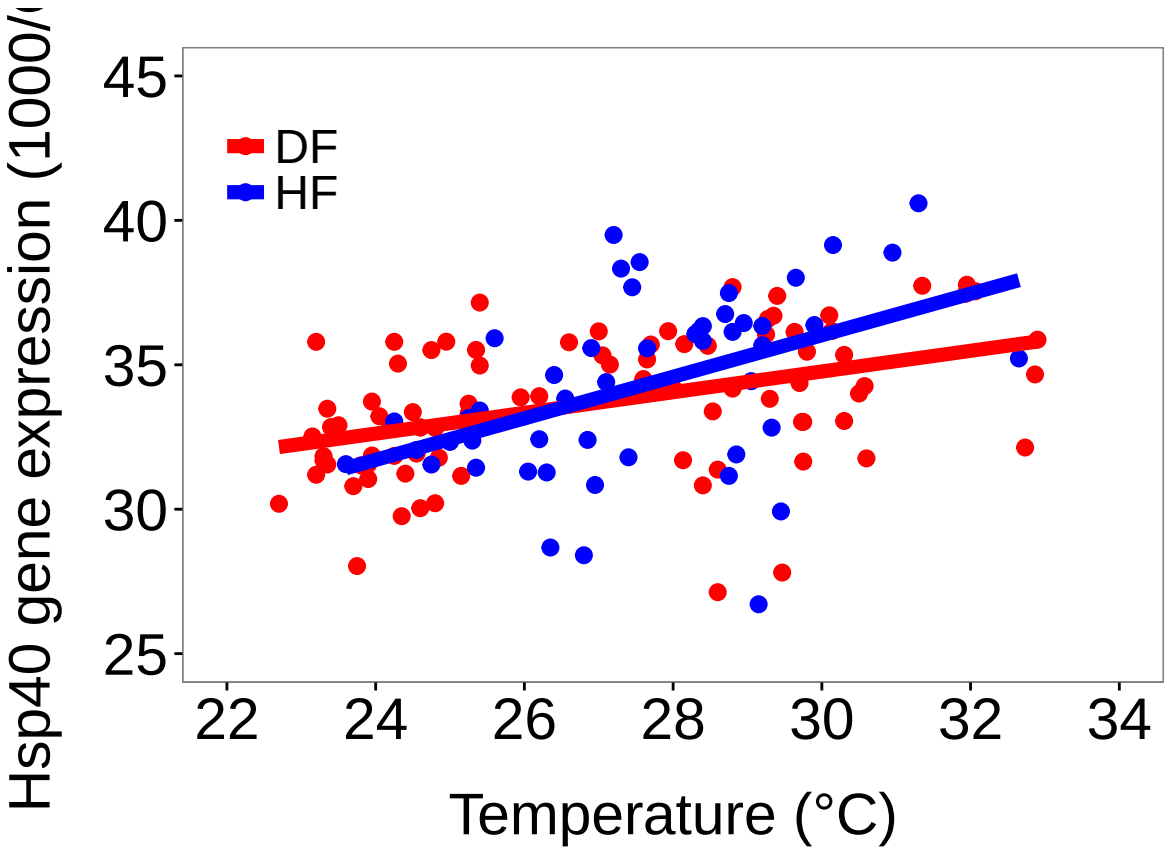
```
# MODEL WITH ACTIN- no site trend
hsp40.act.mod <- lm(CT_40 ~ baittemp.ave * Site + RIN_Value * CT_actin, data = warm.40)
summary(stepAIC(hsp40.act.mod, direction = "both"))
```

```
## Start:  AIC=151.23
## CT_40 ~ baittemp.ave * Site + RIN_Value * CT_actin
##
##                       Df Sum of Sq    RSS    AIC
## - baittemp.ave:Site    1    2.1527 375.30 150.00
## - RIN_Value:CT_actin   1    2.6446 375.79 150.18
## <none>                             373.15 151.23
##
## Step:  AIC=150.01
## CT_40 ~ baittemp.ave + Site + RIN_Value + CT_actin + RIN_Value:CT_actin
##
##                       Df Sum of Sq    RSS    AIC
## - RIN_Value:CT_actin   1    2.4303 377.73 148.87
## - Site                 1    5.2677 380.57 149.87
```

```
## <none>                              375.30 150.00
## + baittemp.ave:Site   1    2.1527 373.15 151.23
## - baittemp.ave        1   26.4943 401.80 157.15
##
## Step:  AIC=148.87
## CT_40 ~ baittemp.ave + Site + RIN_Value + CT_actin
##
##                      Df Sum of Sq    RSS    AIC
## - RIN_Value           1     4.058 381.79 148.30
## <none>                              377.73 148.87
## - Site                1     6.214 383.95 149.06
## + RIN_Value:CT_actin  1     2.430 375.30 150.00
## + baittemp.ave:Site   1     1.938 375.79 150.18
## - baittemp.ave        1    24.461 402.19 155.28
## - CT_actin            1   195.408 573.14 202.74
##
## Step:  AIC=148.3
## CT_40 ~ baittemp.ave + Site + CT_actin
##
##                     Df Sum of Sq    RSS    AIC
## <none>                             381.79 148.30
## + RIN_Value          1     4.058 377.73 148.87
## - Site               1     7.463 389.25 148.90
## + baittemp.ave:Site  1     3.092 378.70 149.21
## - baittemp.ave       1    42.535 424.33 160.46
## - CT_actin           1   195.992 577.78 201.82


##
## Call:
## lm(formula = CT_40 ~ baittemp.ave + Site + CT_actin, data = warm.40)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -2.8941 -1.0927 -0.3345  0.7751  7.4378
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  24.78894    2.39421  10.354  < 2e-16 ***
## baittemp.ave -0.22872    0.06010  -3.806 0.000217 ***
## SiteHF       -0.50693    0.31801  -1.594 0.113352
## CT_actin      0.42062    0.05149   8.169 2.38e-13 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 1.714 on 130 degrees of freedom
##   (4 observations deleted due to missingness)
## Multiple R-squared:  0.4593, Adjusted R-squared:  0.4468
## F-statistic: 36.81 on 3 and 130 DF,  p-value: < 2.2e-16
```

```
# hsp40 plot by site
ggplot(warm.40, aes(x = baittemp.ave, y = (1000/CT_40), colour = factor(Site))) +
    geom_point(size = 2.5) + T + geom_smooth(method = "lm", se = FALSE, size = 2.5) +
    scale_color_manual(name = "", values = c("red", "blue")) + scale_x_continuous(limits = c(22,
```

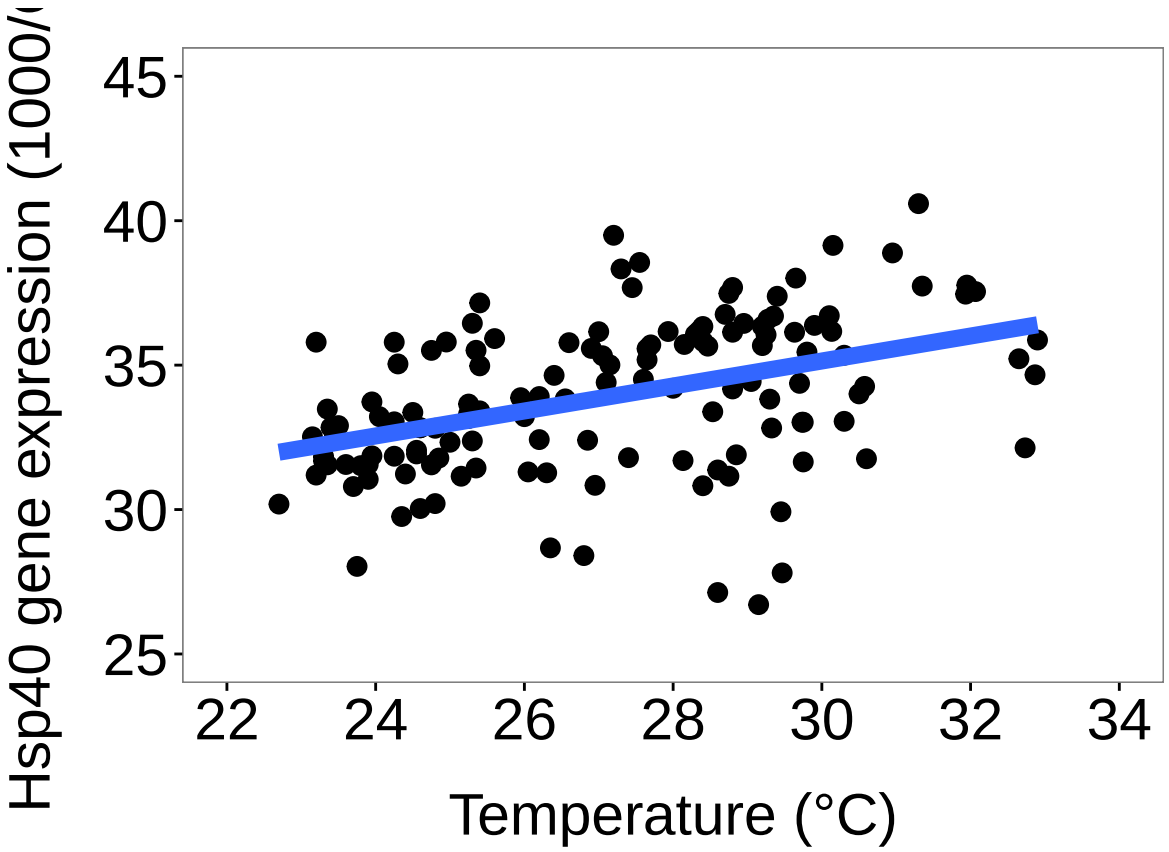
```
    34), breaks = seq(22, 34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(25,
    45), breaks = seq(25, 45, 5), "Hsp40 gene expression (1000/CT)")
```




```
# hsp40 plot overall
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_40))) + geom_point(size = 3) +
    T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
    values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
    34, 2), "Temperature (°C)") + scale_y_continuous(limits = c(25, 45), breaks = seq(25,
    45, 5), "Hsp40 gene expression (1000/CT)")
```

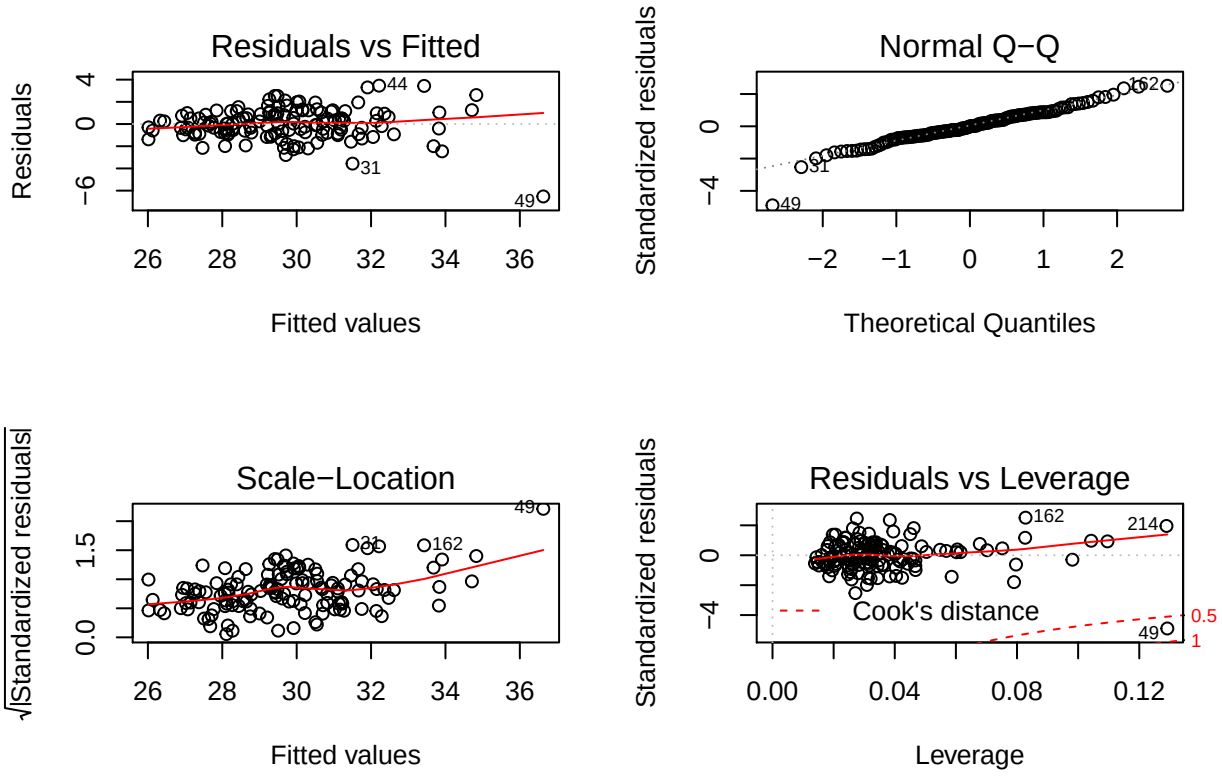
```
## Warning: Removed 98 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 98 rows containing missing values (geom_point).
```




```
# visualizing hsp40 regression model
par(mfrow = c(2, 2))
plot(stepAIC(hsp40.mod, direction = "backward"))
```

```
## Start:  AIC=103.19
## CT_40 ~ baittemp.ave * Site + RIN_Value + CT_18s
##
##                     Df Sum of Sq    RSS    AIC
## - baittemp.ave:Site  1      2.73 269.29 102.58
## <none>                           266.56 103.19
## - RIN_Value          1      4.24 270.80 103.35
## - CT_18s             1    380.13 646.69 222.61
##
## Step:  AIC=102.59
## CT_40 ~ baittemp.ave + Site + RIN_Value + CT_18s
##
##                Df Sum of Sq    RSS    AIC
## <none>                      269.29 102.58
## - Site          1      6.05 275.34 103.63
## - RIN_Value     1      6.08 275.37 103.64
## - baittemp.ave  1     61.69 330.98 128.84
## - CT_18s        1    385.85 655.14 222.39
```




```
par(mfrow = c(1, 1))
############################################################
```

```
sessionInfo()
```

```
## R version 3.2.3 (2015-12-10)
## Platform: x86_64-apple-darwin13.4.0 (64-bit)
## Running under: OS X 10.11.1 (El Capitan)
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## attached base packages:
## [1] stats     graphics  grDevices utils     datasets  methods   base
##
## other attached packages:
## [1] formatR_1.2   MASS_7.3-45   tidyr_0.4.1   ggplot2_2.0.0 dplyr_0.4.3
## [6] plyr_1.8.3
##
## loaded via a namespace (and not attached):
##  [1] Rcpp_0.12.3      digest_0.6.8     assertthat_0.1   grid_3.2.3
##  [5] R6_2.1.2         gtable_0.1.2     DBI_0.3.1        magrittr_1.5
##  [9] scales_0.3.0     evaluate_0.7.2   highr_0.5        stringi_1.0-1
## [13] lazyeval_0.1.10  rmarkdown_0.7    tools_3.2.3      stringr_1.0.0
## [17] munsell_0.4.2    yaml_2.1.13      parallel_3.2.3   colorspace_1.2-6
## [21] htmltools_0.2.6  knitr_1.10.5
```