

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

Curtis A. Provencher

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# 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 nest 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 RNeasy 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 Biosystems™). 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 chambers set up at a northern (Harvard
#Forest, MA) and southern site (Duke Forest, NC) warming ground 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, baseline cycle
#9-15), RIN(RNA integrity), cDNA(ng/uL), vol cDNA and H2O(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 20130702 20...
##  $ 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 ...
## $ Isolation.Date : int 20150811 20150731 20150814 20150813 20150813 20150730 20150814 20...
## $ 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 =
    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

```
# 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

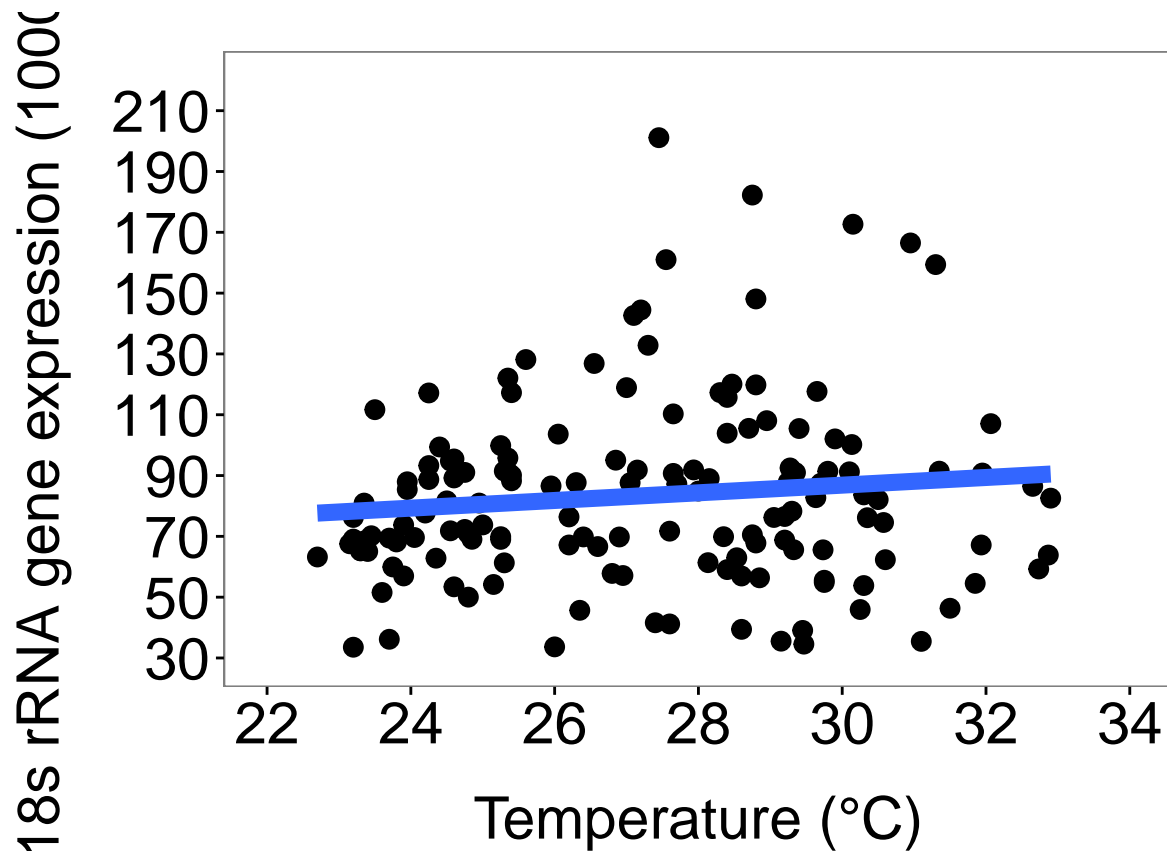
```
hkg.mod1 <- lm((1000/warm$CT_18s) ~ baittemp.ave * Site + RIN_Value, data = warm)
summary(hkg.mod1)
```

```
##
## 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
```

```
# 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).
```

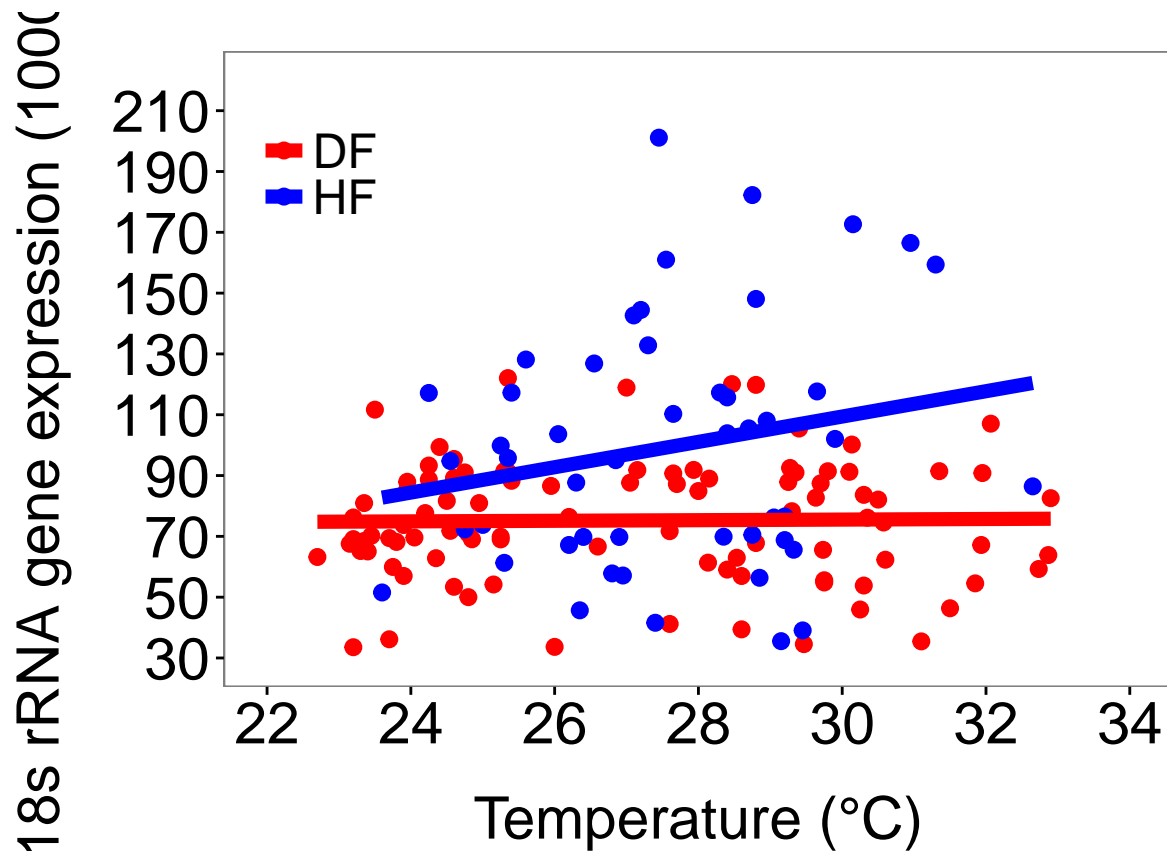


```
# 18s expression by site
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_18s), colour = factor(Site))) +
  geom_point(size = 2.5) + T + geom_smooth(method = "lm", se = FALSE, size = 2.5) +
  scale_color_manual(name = "", values = c("red", "blue")) + scale_x_continuous(limits = c(22,
34), breaks = 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).
```





### Evaluating actin

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

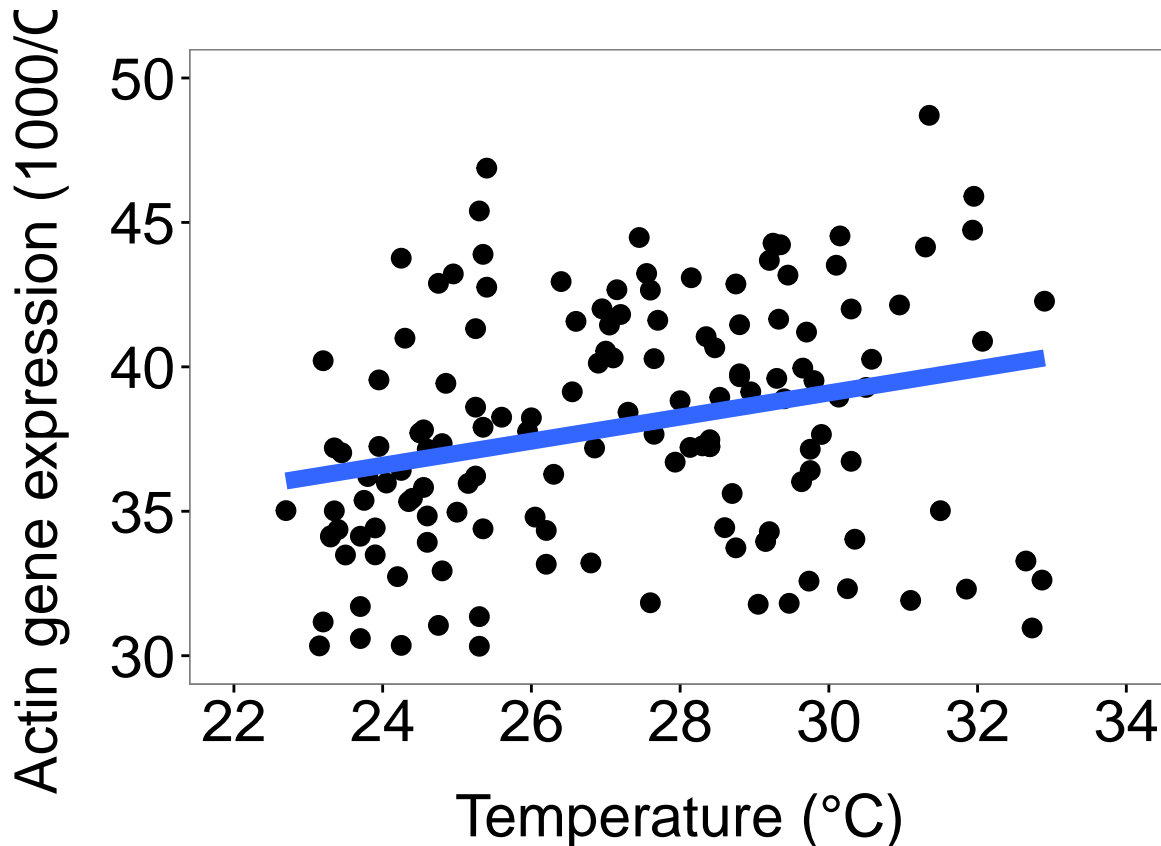
```
##
## Call:
## lm(formula = warm$CT_actin ~ baittemp.ave * Site + RIN_Value,
##     data = warm)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -6.4509 -2.1856 -0.2958  1.8374  8.3133
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)    32.5982     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
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).
```

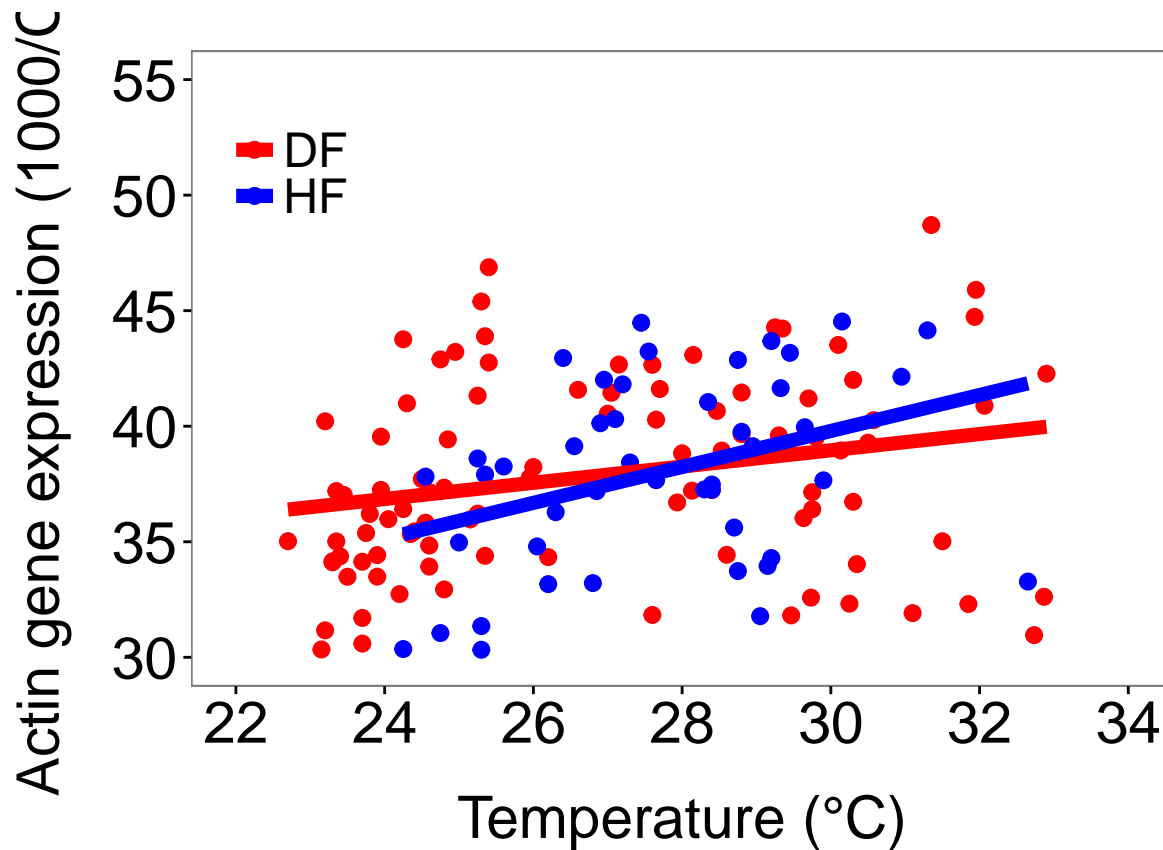
```
## Warning: Removed 96 rows containing missing values (geom_point).
```



```
# actin expression by site
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_actin), 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,
  55), breaks = seq(30, 55, 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
## ---
```

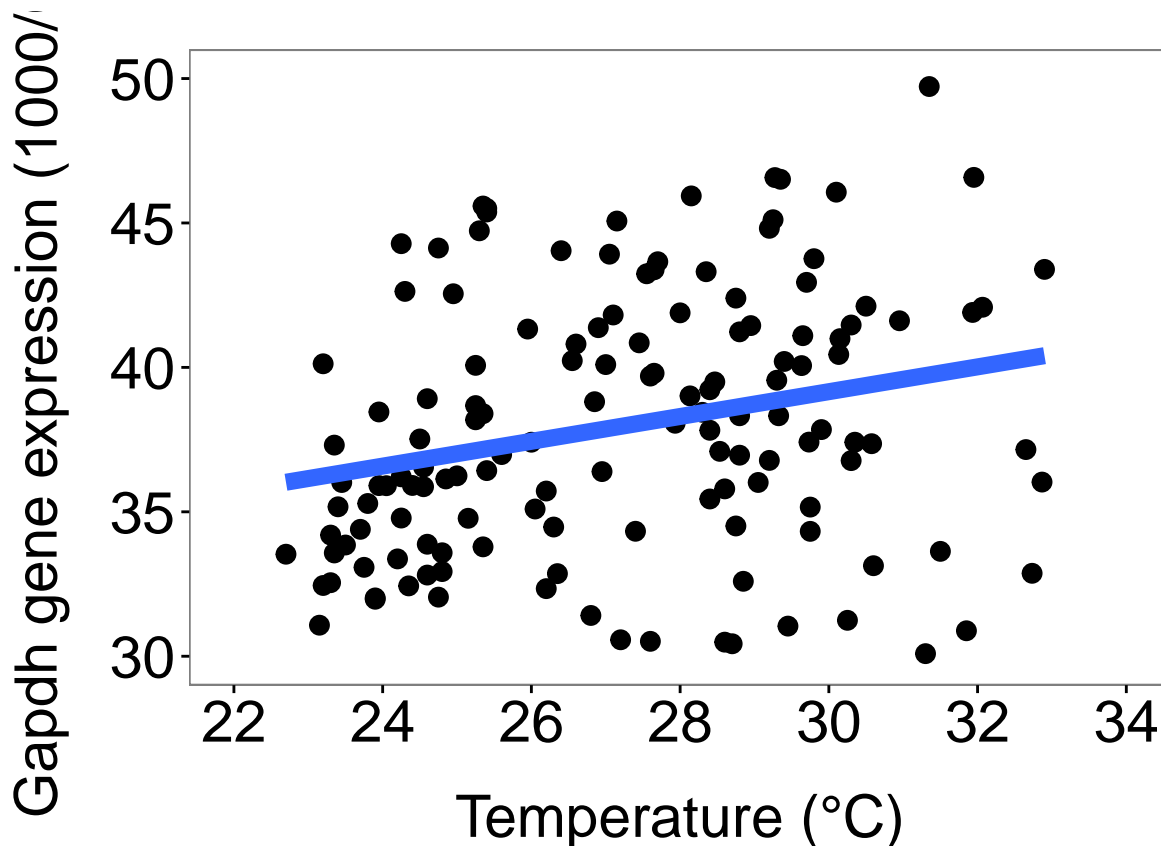
```
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 3.16 on 136 degrees of freedom
## (96 observations deleted due to missingness)
## Multiple R-squared:  0.09225,    Adjusted R-squared:  0.06556
## F-statistic: 3.455 on 4 and 136 DF,  p-value: 0.01008
```

```
# overall gapdh expression
```

```
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).
```

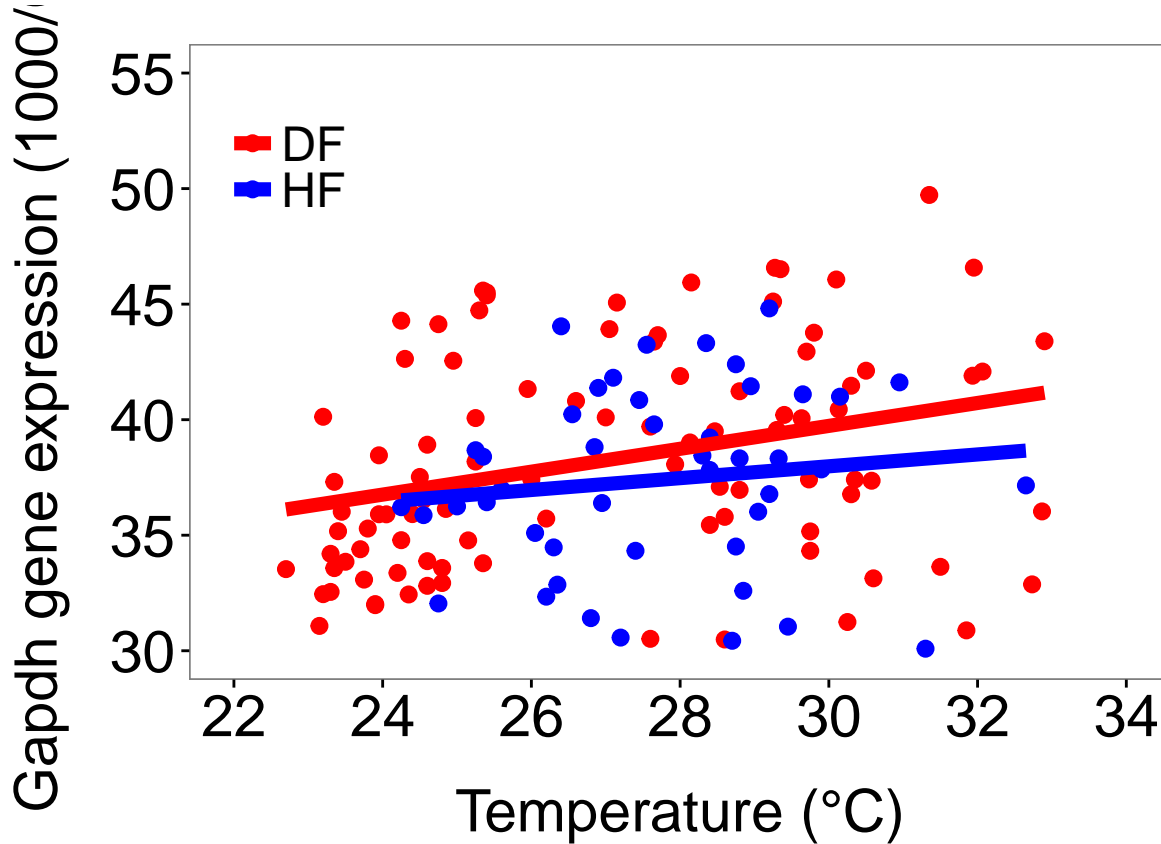


```
# gapdh expression by site
```

```
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_gapdh), 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,
  55), breaks = seq(30, 55, 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
```

```
length(resid(hsp83.act.mod))
```

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

```
# Visualize hsp83 model par(mfrow=c(2,2))
# plot(stepAIC(hsp83.mod,direction='backward')) par(mfrow=c(1,1))

# Construct model to control for HKG
warm83 <- subset(warm, warm$CT_83 != "NA")
dim(warm83)
```

```
## [1] 144 25
```

```
warm83_2 <- subset(warm83, warm83$CT_18s != "NA")
dim(warm83_2)
```

```
## [1] 143 25
```

```
res.mod1 <- lm(CT_83 ~ CT_18s, data = warm83_2)
summary(res.mod1)
```

```
##
## Call:
## lm(formula = CT_83 ~ CT_18s, data = warm83_2)
##
## Residuals:
```

```
##      Min      1Q  Median      3Q      Max
## -8.3786 -2.7258 -0.5027  2.4044  9.1961
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept) 20.81144    0.78070  26.658  <2e-16 ***
## CT_18s      0.51944    0.05353   9.704  <2e-16 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 3.265 on 141 degrees of freedom
## Multiple R-squared:  0.4004, Adjusted R-squared:  0.3962
## F-statistic: 94.16 on 1 and 141 DF,  p-value: < 2.2e-16

## test
## test1<-lm(CT_83~baittemp.ave*Site+RIN_Value+CT_18s,data=warm83);summary(test1)
warm83_2$hsp83.resids <- scale(resid(res.mod1), center = TRUE) * -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>                        469.92 180.29
## - 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
```

```
# Visualizing hsp70 regression model par(mfrow=c(2,2))
# plot(stepAIC(hsp70.mod,direction='backward')) par(mfrow=c(1,1))

# Construct model to control for HKG and then plot the residuals as a
# function of temperature
```

```
warm70 <- subset(warm, warm$CT_70 != "NA")
dim(warm70)
```

```
## [1] 145 25
```

```
warm70_2 <- subset(warm70, warm70$CT_18s != "NA")
dim(warm70_2)
```

```
## [1] 144 25
```

```
# Getting residuals
res.mod2 <- lm(CT_70 ~ +CT_18s, data = warm70_2)
# resid(resid.mod2);length(resid(resid.mod2))
warm70_2$hsp70_resids <- scale(resid(res.mod2), center = TRUE) * -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
```

```
# visualizing hsp40 regression model par(mfrow=c(2,2))
# plot(stepAIC(hsp40.mod,direction='backward')) par(mfrow=c(1,1))

# Construct model to control for HKG and then plot the residuals as a
# function of temperature

warm40 <- subset(warm, warm$CT_40 != "NA")
dim(warm40)
```

```
## [1] 139 25
```

```
warm40_2 <- subset(warm40, warm40$CT_18s != "NA")
dim(warm40_2)
```

```
## [1] 138 25
```

```
# getting residuals
res.mod3 <- lm(CT_40 ~ +CT_18s, data = warm40_2)
# resid(resid.mod2);length(resid(resid.mod2))
warm40_2$hsp40_resids <- scale(resid(res.mod3), center = TRUE) * -1
```

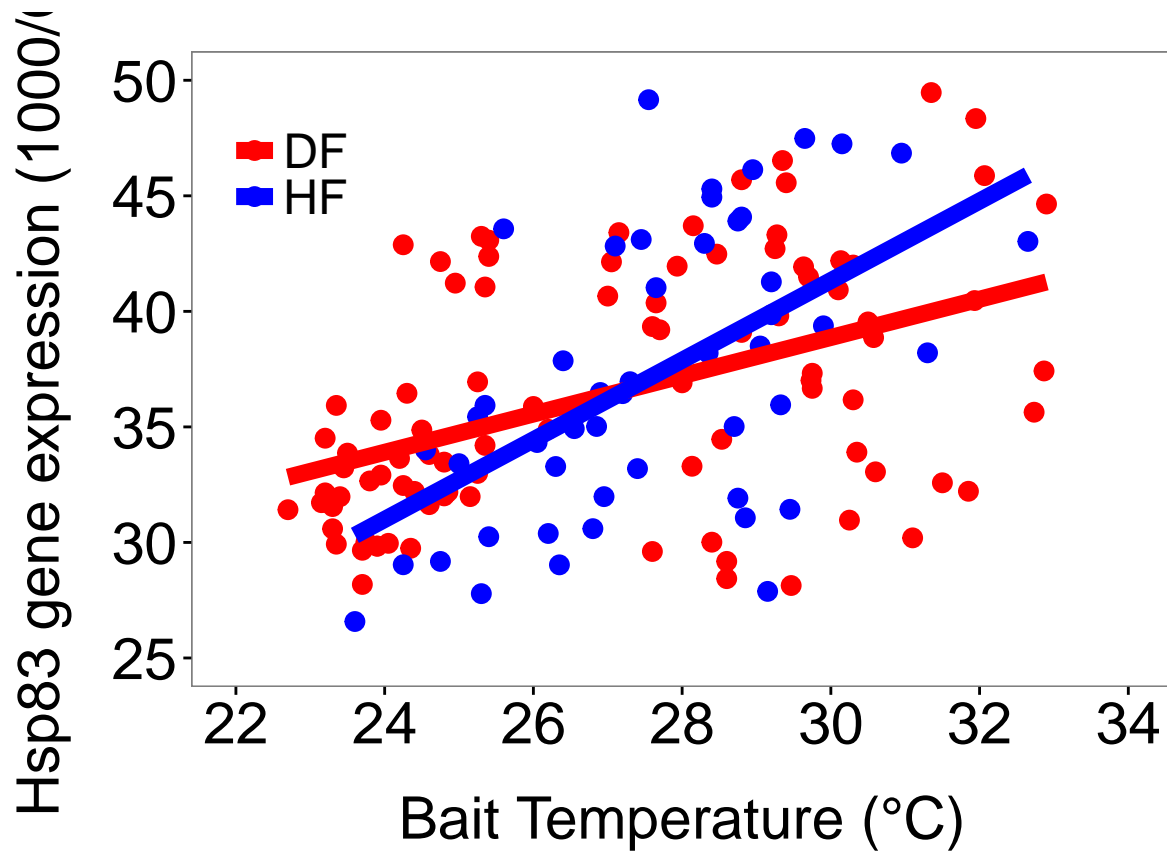
## Plotting regression models

### *Hsp83* expression as a function of bait temperature

```
# 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), "Bait 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).
```

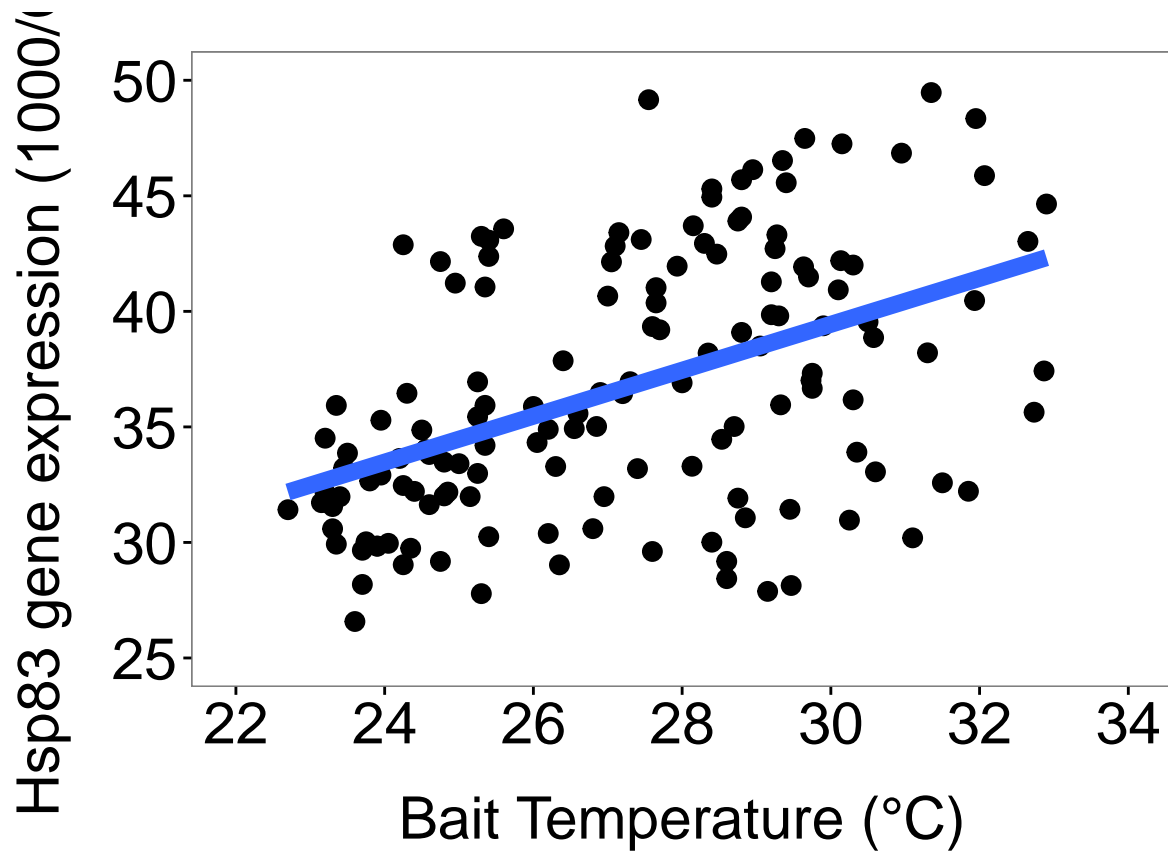
```
## Warning: Removed 93 rows containing missing values (geom_point).
```



```
# 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), "Bait Temperature (°C)") + scale_y_continuous(limits = c(25, 50),
  breaks = seq(25, 50, 5), "Hsp83 gene expression (1000/CT)")
```

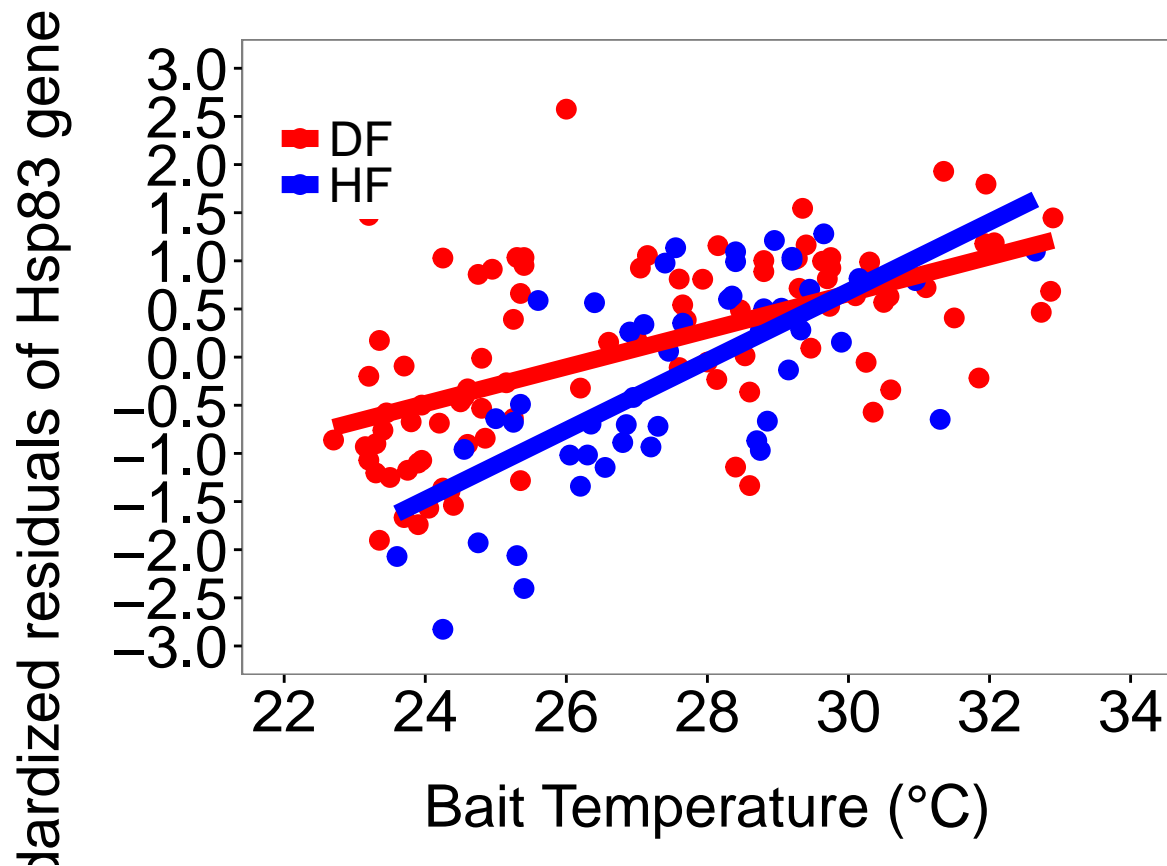
```
## Warning: Removed 93 rows containing non-finite values (stat_smooth).
```

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



```
# hsp 83 plot controlling for HKG (18s)- standarized residuals of hsp83
hsp83.plot <- ggplot(warm83_2, aes(x = baittemp.ave, y = (hsp83.resids), 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), "Bait Temperature (°C)") + scale_y_continuous(limits = c(-3,
3), breaks = seq(-3, 3, 0.5), "Standardized residuals of Hsp83 gene expression")

hsp83.plot
```



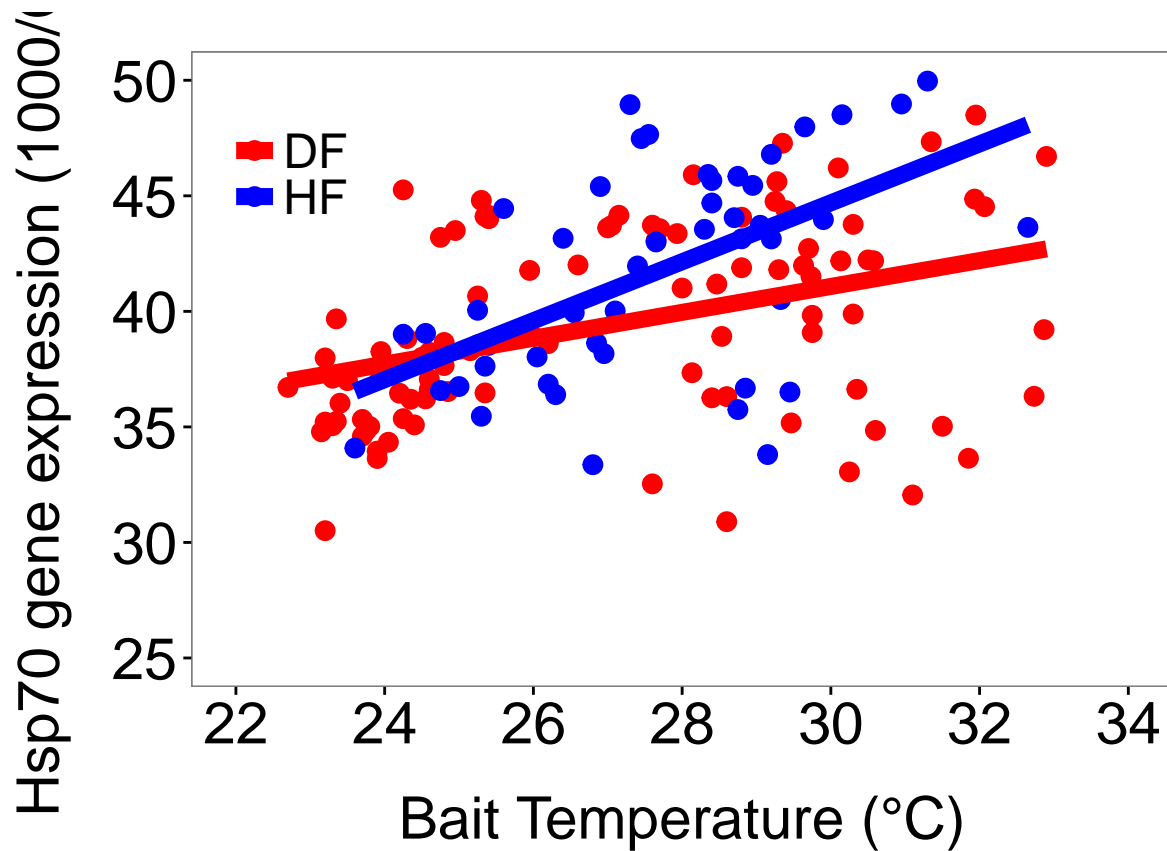
*Hsp70* expression as a function of bait temperature

```
# 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), "Bait 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).
```

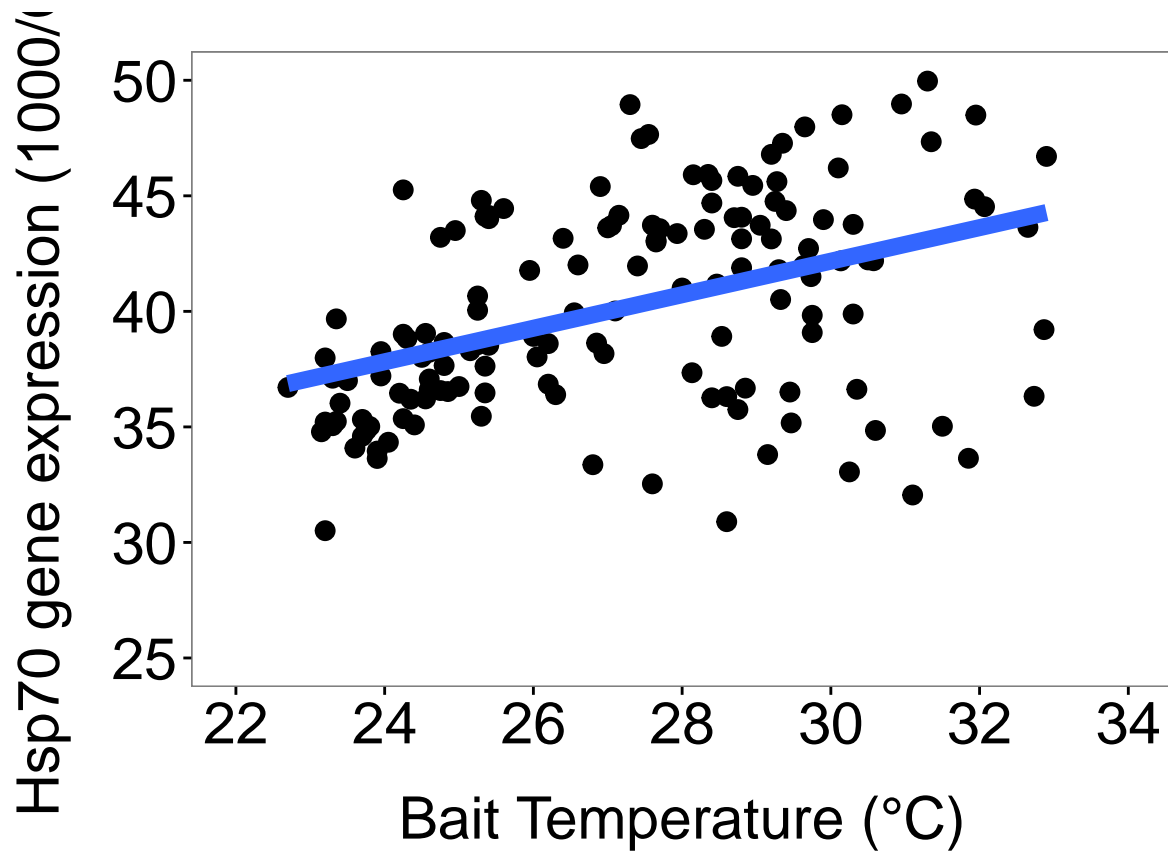




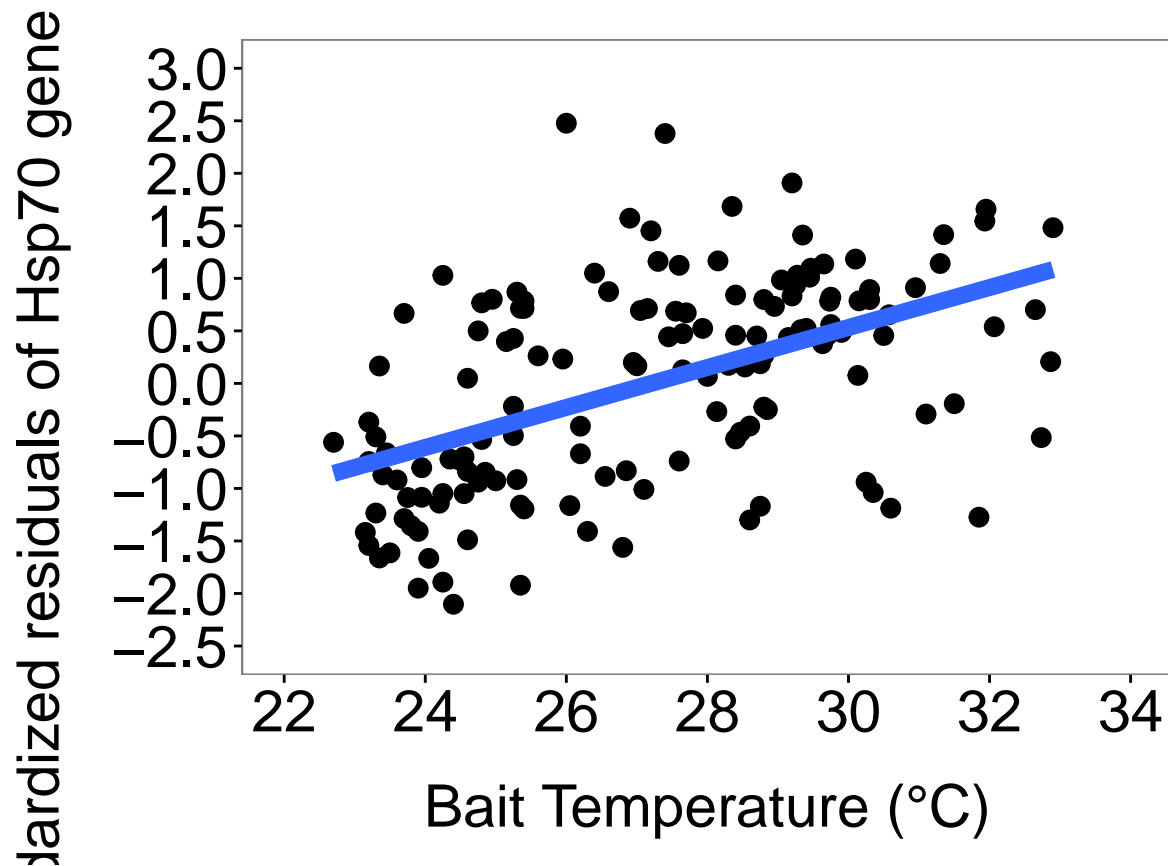
```
# hsp70 plot overall
ggplot(warm, aes(x = baittemp.ave, y = (1000/CT_70))) + geom_point(size = 3) +
  T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
  values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
  34, 2), "Bait Temperature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

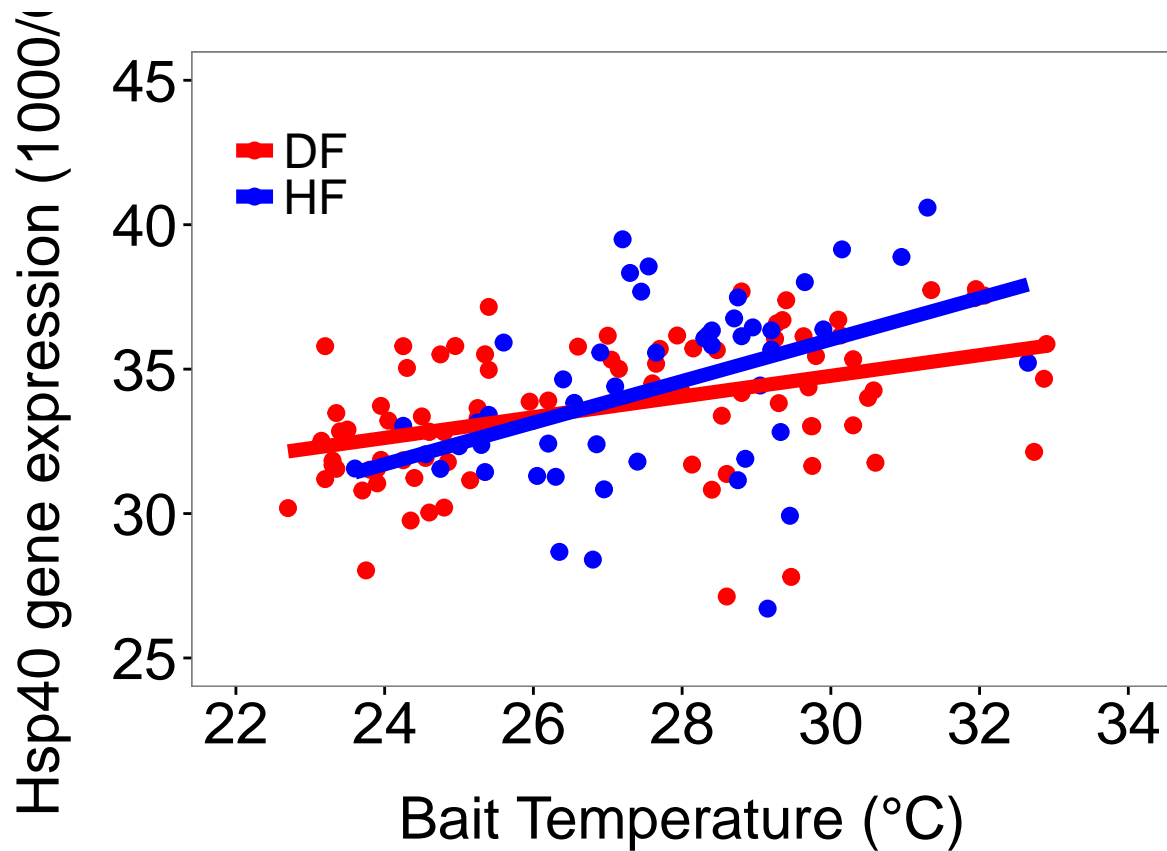


```
# hsp70 plot controlling for HKG (18s)- standarized residuals of hsp70
hsp70.plot <- ggplot(warm70_2, aes(x = baittemp.ave, y = hsp70_resids)) + geom_point(size = 3) +
  T + geom_smooth(method = "lm", se = FALSE, size = 3) + scale_color_manual(name = "",
  values = c("red", "blue")) + scale_x_continuous(limits = c(22, 34), breaks = seq(22,
  34, 2), "Bait Temperature (°C)") + scale_y_continuous(limits = c(-2.5,
  3), breaks = seq(-3, 3, 0.5), "Standardized residuals of Hsp70 gene expression")
hsp70.plot
```



*Hsp40* expression as a function of bait temperature

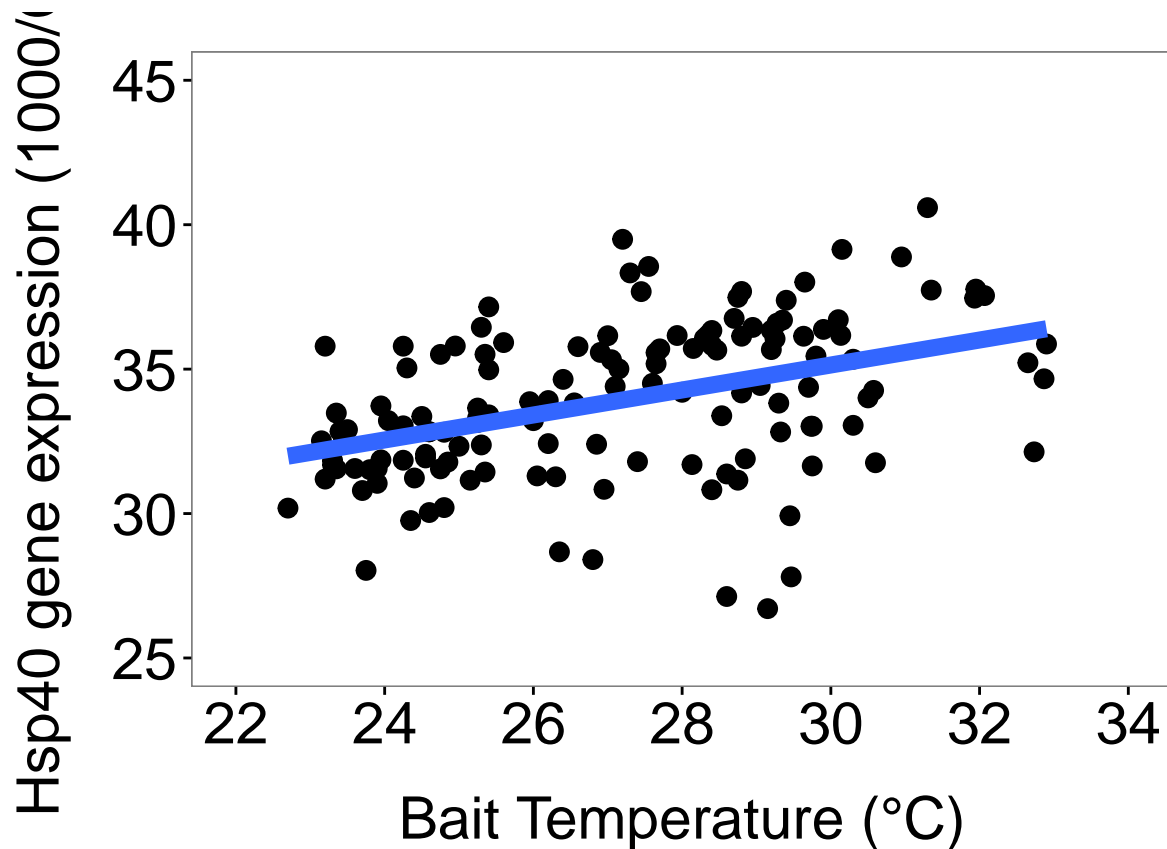
```
# hsp40 plot by site
ggplot(warm.40, aes(x = baittemp.ave, y = (1000/CT_40), colour = factor(Site))) +
  geom_point(size = 2.5) + T + geom_smooth(method = "lm", se = FALSE, size = 2.5) +
  scale_color_manual(name = "", values = c("red", "blue")) + scale_x_continuous(limits = c(22,
34), breaks = seq(22, 34, 2), "Bait 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), "Bait 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).
```

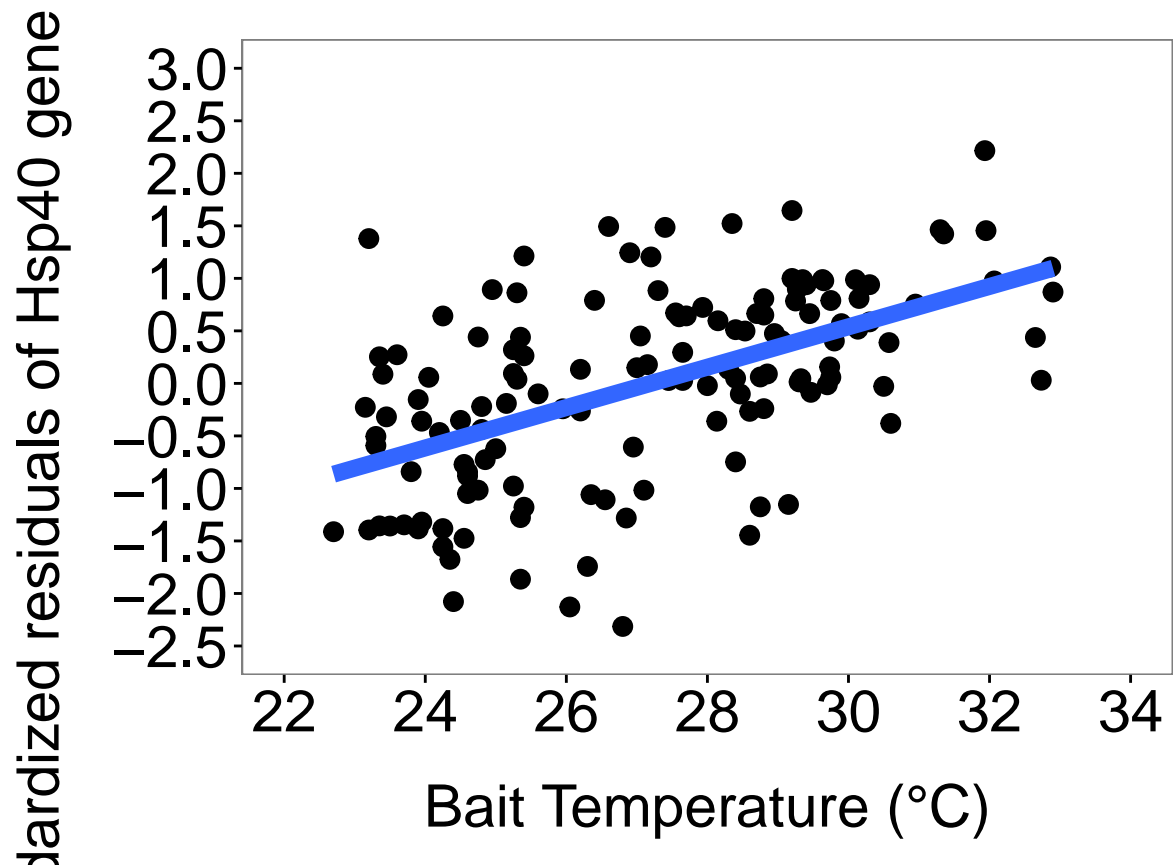
```
## Warning: Removed 98 rows containing missing values (geom_point).
```



```
# hsp40 plot controlling for HKG (18s)- standarized residuals of hsp40
hsp40.plot <- ggplot(warm40_2, aes(x = baittemp.ave, y = hsp40_resids)) + 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), "Bait Temperature (°C)") + scale_y_continuous(limits = c(-2.5,
  3), breaks = seq(-3, 3, 0.5), "Standardized residuals of Hsp40 gene expression")
hsp40.plot
```

```
## Warning: Removed 2 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 2 rows containing missing values (geom_point).
```



Three panel figures

```
library(gridExtra)
```

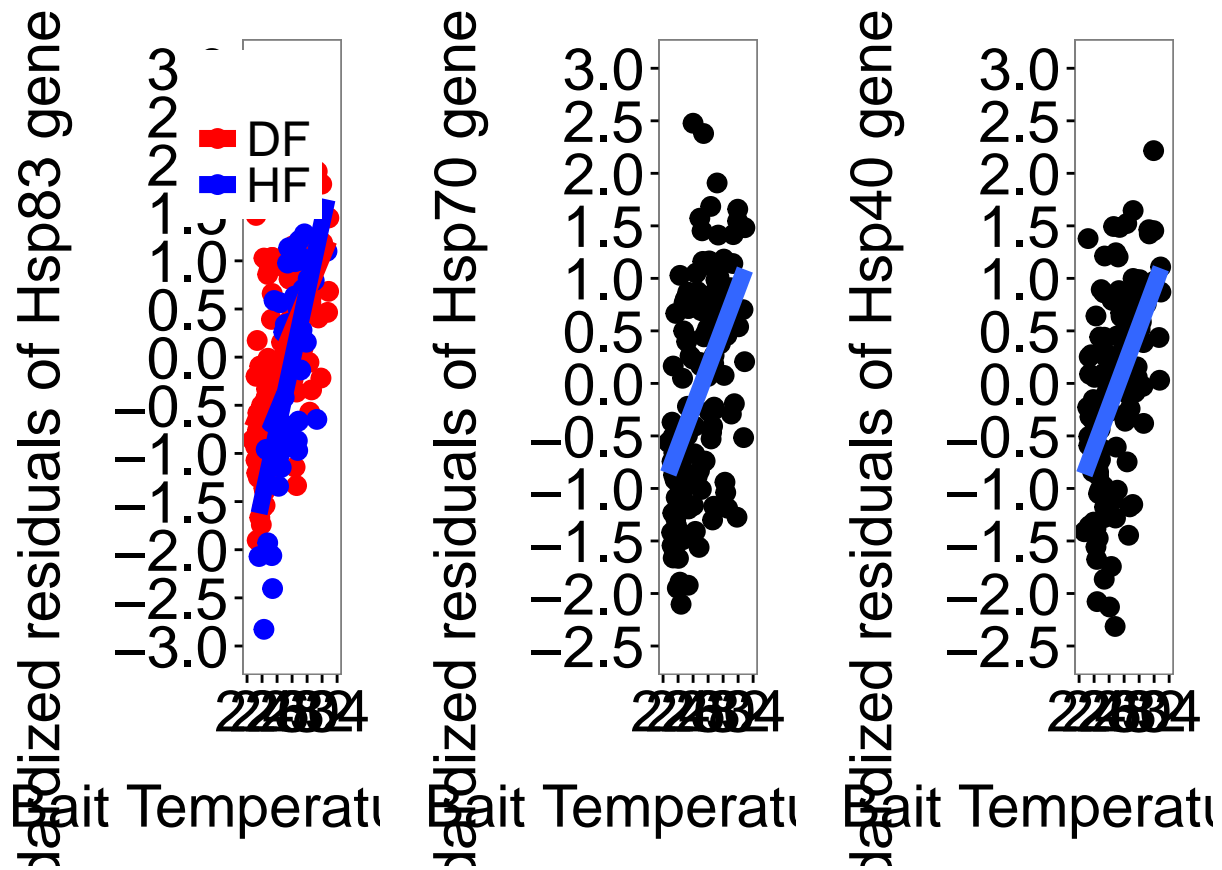
```
## Warning: package 'gridExtra' was built under R version 3.2.4
```

```
##
## Attaching package: 'gridExtra'
##
## The following object is masked from 'package:dplyr':
##
##   combine
```

```
grid.arrange(hsp83.plot, hsp70.plot, hsp40.plot, ncol = 3)
```

```
## Warning: Removed 2 rows containing non-finite values (stat_smooth).
```

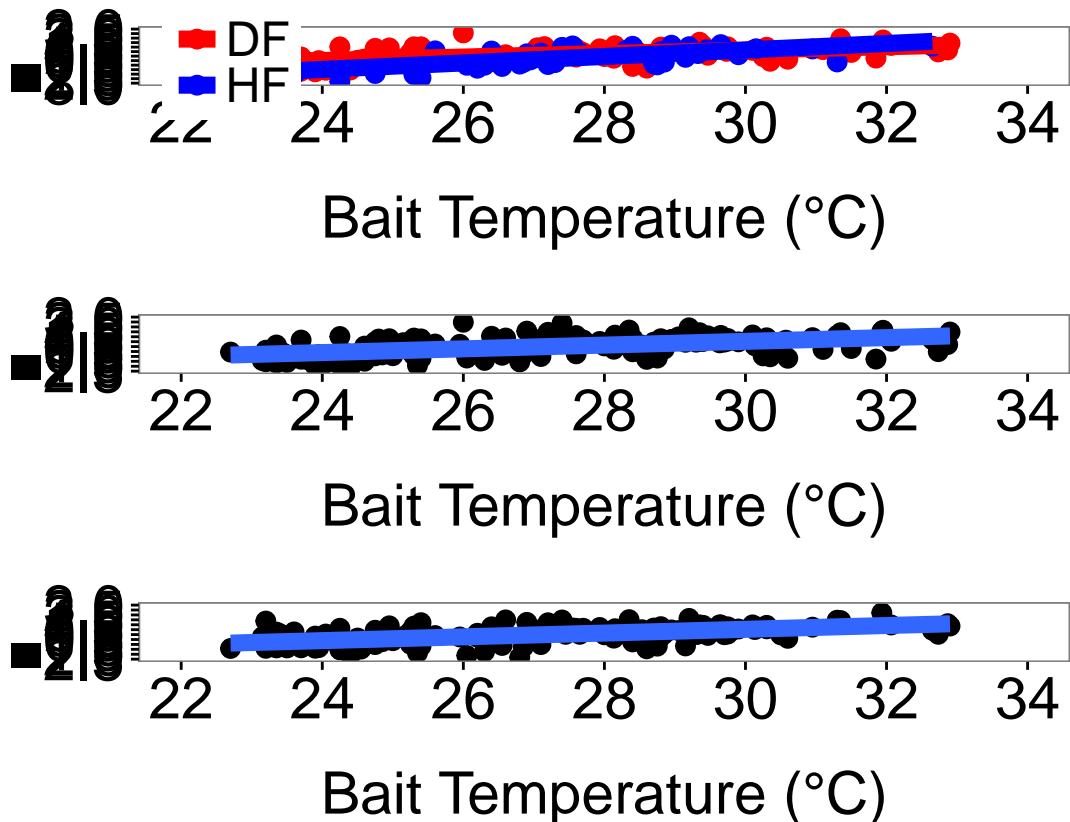
```
## Warning: Removed 2 rows containing missing values (geom_point).
```



```
grid.arrange(hsp83.plot, hsp70.plot, hsp40.plot, nrow = 3)
```

```
## Warning: Removed 2 rows containing non-finite values (stat_smooth).
```

```
## Warning: Removed 2 rows containing missing values (geom_point).
```



```
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] gridExtra_2.2.1 formatR_1.2  MASS_7.3-45  tidyr_0.4.1
## [5] ggplot2_2.0.0  dplyr_0.4.3  plyr_1.8.3
##
## loaded via a namespace (and not attached):
## [1] Rcpp_0.12.3      knitr_1.10.5    magrittr_1.5    munsell_0.4.2
## [5] colorspace_1.2-6 R6_2.1.2        stringr_1.0.0   highr_0.5
## [9] tools_3.2.3      parallel_3.2.3  grid_3.2.3      gtable_0.1.2
## [13] DBI_0.3.1        htmltools_0.2.6 yaml_2.1.13     lazyeval_0.1.10
## [17] assertthat_0.1   digest_0.6.8    evaluate_0.7.2  rmarkdown_0.7
## [21] stringi_1.0-1    scales_0.3.0
```