ELECTRONIC SUPPLEMENTARY MATERIAL 2/2

Temperature-dependent effects on fecundity in a serial broadcast spawning fish after whole-life high-CO_2 exposure

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Assessment of atresia in Atlantic silversides during a temperature, CO_2 experiment

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Introduction

Atresia is a normal, hormonal process of germ cell death and resorption in the female ovary as a response to energetic or environmental stressors (Tyler and Sumpter 1996; Blazer 2002; Lubzens et al 2010). If it occurs at high rates it can down regulate the number of advanced yolked oocytes, a measure of potential fecundity, so that the final number of eggs spawned, realized fecundity, is lower (McElroy et al. 2016; Mouchlianitis et al. 2019).

In the fecundity experiment that contrasted the effects of temperature and CO_2, Concannon et al. estimated potential fecundity, or standing stock fecundity, soon before first spawning in Atlantic silversides (*Menidia menidia*), from counts of whole oocytes. Here we assess if atresia was at a sufficient level during Concannon et al.'s experiments to down regulate this type of fecundity of the experimental females, particularly if it did so in some treatments but not others.

By examining a subset of ovaries, prepared by histology, we address a reviewer's concern that whole oocyte counts may have not recognized significant numbers of atretic cells that could have existed in one or more treatments, thereby influencing our comparisons of standing stock fecundity.

Methods

There were six tanks with a combination of two temperatures (17, 24°C) and two CO_2 levels (control, high). The data are pooled for some tanks, thereby restricting the analyses of percent atresia, a continuous variable called **Atresia.percent**, to the four different tank conditions, a categorical variable called **tank.cond**.

Although atresia can occur at different stages of oogenesis, in this experiment, Concannon et al. focused on the secondary stage of oocytes when they counted whole oocytes as one of their response variables. Here, however, this supplement reports on data taken from histology slides of a subset of those fish whose whole, yolked oocytes were counted. These oocyte counts from histology slides were used to determine if a proportion of advanced oocytes used to calculate the standing stock fecundity had significant levels of atresia that may have been unrecognizable from counts of whole oocytes.

Secondary germs cells were evaluated across the histology slide, as prepared with standard, wax mounts, and stained with hematoxylin and eosin. Each cell was categorized as atretic, possibly atretic, or not atretic. To be conservative in our conclusions,

Atresia.percent included both atretic as well as possibly atretic cells. Cells were also categorized by stage of atresia: alpha and beta. The alpha stage is the earliest stage of atresia, evident by fissures in the *zona pellucida* and degradation of the germinal vesicle and yolk granules (McElroy et al. 2016). Beta stage atresia showed more extensive degradation, such that no cytoplasmic features were recognizable; however, only one germ cell in beta atresia was observed, among the 5,321 germ cells examined, so beta atresia was not included in further analyses.

Data were entered into an spreadsheet and analyzed using RMarkdown (R version 4.0.5 (2021-03-31); The R Foundation for Statistical Computing).

The Data

There were 36 females with gonad histology slides of the ovary. Six were from tank A (17° C, control CO_2), 8 from tank B (17°C, high CO_2), 11 from tanks C, G (24°C, high CO_2), and 11 from tanks D, H (24°C, control CO_2). An average of 147.8 germ cells were counted and categorized per female (range: 69-248).

```
library (tidyverse); library (readxl)
dat.mat <- read_excel('Atresia_Updated.xlsx', sheet = 'Sheet1') %>%
  rename(Atresia.percent = `% Atresia`) %>%
  mutate ( tank.cond = paste0(Temp, CO2)) %>%
  arrange (tank.cond)
str(dat.mat) # examine the structure of the data matrix

## tibble [36 x 12] (S3: tbl_df/tbl/data.frame)
##  $ Fish ID        : chr [1:36] "A242" "A243" "A255" "A262" ...
##  $ Tank           : chr [1:36] "A" "A" "A" "A" ...
##  $ Temp           : num [1:36] 17 17 17 17 17 17 17 17 17 17 ...
##  $ CO2            : chr [1:36] "control" "control" "control" "control" ...
##  $ Alpha          : num [1:36] 0 0 1 0 1 0 0 0 3 0 ...
##  $ Beta           : num [1:36] 0 0 0 0 0 0 0 0 0 0 ...
##  $ Could be alpha : num [1:36] 0 1 1 0 2 0 1 0 1 0 ...
##  $ Could be beta  : num [1:36] 0 0 0 0 0 0 0 0 0 0 ...
##  $ Total          : num [1:36] 219 110 185 243 183 72 162 181 183 191 ...
##  $ Atresia.percent: num [1:36] 0 0.909 1.081 0 1.639 ...
##  $ Notes          : chr [1:36] NA NA NA NA ...
##  $ tank.cond      : chr [1:36] "17control" "17control" "17control" "17cont
rol" ...

head(dat.mat) # a quick peek at the first few rows of data

## # A tibble: 6 x 12
##   `Fish ID` Tank   Temp CO2   Alpha  Beta `Could be alpha` `Could be beta`
Total
##   <chr>     <chr> <dbl> <chr> <dbl> <dbl>            <dbl>           <dbl>
<dbl>
## 1 A242      A        17 cont~     0     0                0               0
219
## 2 A243      A        17 cont~     0     0                1               0
```

```
110
## 3 A255      A        17 cont~     1     0               1               0
185
## 4 A262      A        17 cont~     0     0               0               0
243
## 5 A269      A        17 cont~     1     0               2               0
183
## 6 A280      A        17 cont~     0     0               0               0
72
## # ... with 3 more variables: Atresia.percent <dbl>, Notes <chr>,
## #   tank.cond <chr>
```

Results

At an individual level, atresia was low. Over half the fish had no atresia (19 of 36 fish), and all but one had < 5% atresia (an outlier had 11.8% atresia in the high temperature control tank).

```
table (signif(dat.mat$Atresia.percent, 2))

##
##    0 0.55 0.62 0.91  1.1  1.4  1.6  1.7  2.2  2.4    3  3.9    4  4.7   12
##   19    1    1    1    3    1    1    1    1    1    1    1    1    2    1

table (round(dat.mat$Atresia.percent, 1))

##
##    0  0.5  0.6  0.9  1.1  1.4  1.6  1.7  2.2  2.4    3  3.9    4  4.7 11.8
##   19    1    1    1    3    1    1    1    1    1    1    1    1    2    1
```

At a group level, the average percent atresia in all four tank conditions was low < 3%, even < 1% in two of the tank conditions.

```
library(doBy)
summaryBy(Atresia.percent~tank.cond, data=dat.mat, FUN=c(mean, sd), na.rm=TRU
E)

## # A tibble: 4 x 3
##   tank.cond Atresia.percent.mean Atresia.percent.sd
##   <chr>                    <dbl>              <dbl>
## 1 17control                0.605              0.705
## 2 17high                   1.36               1.54
## 3 24control                2.61               3.59
## 4 24high                   0.324              0.776
```

These point estimates of **Atresia.percent** were not statistically different from zero based on the range of confidence limits for each **tank.cond** (here the intercept value refers to the tank condition of 17°C, control CO_2).

```
  confint(glm(dat.mat$Atresia.percent~dat.mat$tank.cond))
```

```
##                                  2.5 %   97.5 %
## (Intercept)                -1.1485556 2.358394
## dat.mat$tank.cond17high    -1.5629753 3.076284
## dat.mat$tank.cond24control -0.1700496 4.189661
## dat.mat$tank.cond24high    -2.4603403 1.899371
```

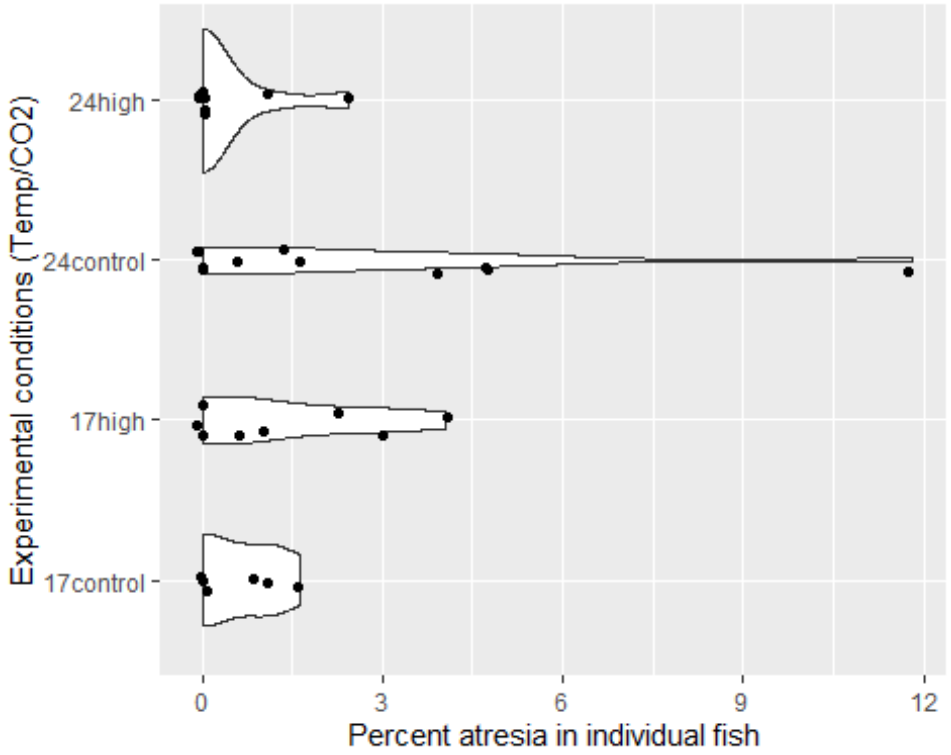
This conclusion is supported by the more formal general linear model: **Atresia.percent** = b0(17 Control) + b1(17 High) + b2(24 Control) + b3(24 High) + e_i. Here, the intercept value, b0, estimates a percent atresia in the 17 control **tank.cond** that was not different than zero (P = 0.50), and the adjusted estimates for the other treatments, b1-3, did not differ from b0 (P > 0.08).

```
  summary(glm(dat.mat$Atresia.percent~dat.mat$tank.cond))

##
## Call:
## glm(formula = dat.mat$Atresia.percent ~ dat.mat$tank.cond)
##
## Deviance Residuals:
##     Min       1Q   Median       3Q      Max
## -2.6147  -1.0024  -0.3244   0.8244   9.1830
##
## Coefficients:
##                            Estimate Std. Error t value Pr(>|t|)
## (Intercept)                  0.6049     0.8946   0.676   0.5038
## dat.mat$tank.cond17high      0.7567     1.1835   0.639   0.5272
## dat.mat$tank.cond24control   2.0098     1.1122   1.807   0.0802 .
## dat.mat$tank.cond24high     -0.2805     1.1122  -0.252   0.8025
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## (Dispersion parameter for gaussian family taken to be 4.802355)
##
##     Null deviance: 186.09  on 35  degrees of freedom
## Residual deviance: 153.68  on 32  degrees of freedom
## AIC: 164.41
##
## Number of Fisher Scoring iterations: 2
```

To visualize all of this, by **tank.cond**, the following plots show the specific points (black dots) and the general shape of the distribution of these points as outlined by an ad hoc violin shape.

```
dat.mat %>%
    ggplot(aes(x=tank.cond, y=Atresia.percent)) +
  geom_violin() +
  geom_jitter(shape=16, width = 0.1, height = 0.1) +
  labs(x="Experimental conditions (Temp/CO2)", y="Percent atresia in individu
al fish") +
  coord_flip()
```



Summary and recommendations

Atresia was low and not significantly different than 0 percent in all tank conditions. It was also not significantly different between the control and treatment tanks at either temperature. No change to whole oocyte counts was made to adjust for these low levels of atresia.

References

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