Dose-dependence and small-scale variability in responses to ocean acidification during squid, *Doryteuthis pealeii,* development

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Electronic Supplementary Materials

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Fig. S1. Acidification and egg culture system. Filtered, temperature controlled water from Vineyard Sound was piped into a header tank (behind PVC towers depicted) and flowed into PVC towers where airstones equilibrated water to the desired pCO_2 levels. Water exiting the chamber was split in a manifold (not depicted), which fed drip lines into the egg culture cups of each water bath.

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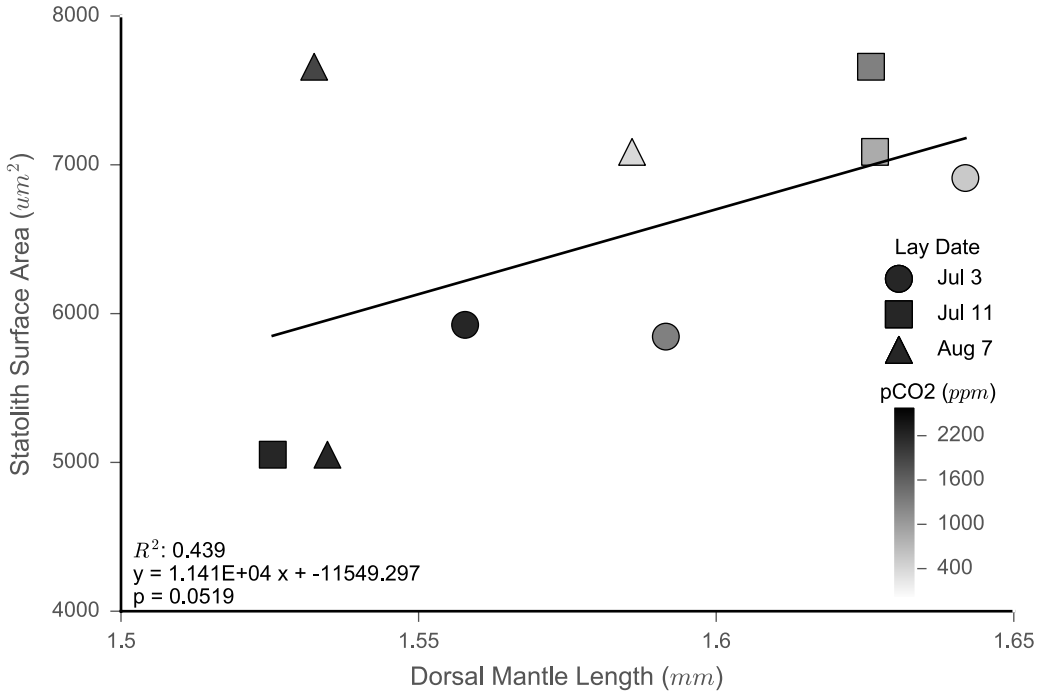

Fig. S2. Comparison of average statolith surface area and average mantle length of experimental paralarvae. Data are compiled within each treatment and all trials are plotted together. Symbols represent means, with shape corresponding to trial, and color corresponding to pCO_2 treatment (according to the color bar at right). The line depicts a linear regression, with R^2, equation, and p value reported in the lower left corner of the plot.

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Table S1. One-way ANOVAs of pCO_2 separated by metric and trial; significant p-values (< 0.05) in bold. Yolk volume measurements were log-transformed prior to statistical analysis. Compiled data is difference from trial mean.

	Mantle Length					**Yolk Sac Volume**				
Source	SS	df	*F*	p	Ω^2	SS	df	*F*	p	Ω^2
Jul 3										
pCO_2	0.573	2	20.98	**<0.001**	0.079	5.604	2	9.10	**<0.001**	0.038
Residual	6.305	462				125.9	409			
Jul 11										
pCO_2	1.035	2	38.27	**<0.001**	0.132	2.480	2	3.38	**0.035**	0.010
Residual	6.598	488				180.6	492			
Aug 7										
pCO_2	0.297	2	9.24	**<0.001**	0.032	0.703	2	2.19	0.113	0.005
Residual	7.867	490				76.52	477			
Compiled										
pCO_2	1.568	5	21.43	**<0.001**	0.066	4.243	5	3.02	**0.010**	0.007
Residual	21.11	1443				387.6	1381			

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Table S2. One-way ANOVAs of hatching date separated by metric, trial, and pCO_2 treatment; significant p-values (< 0.05) in bold. Yolk volume measurements were log-transformed prior to statistical analysis.

		Mantle Length					**Yolk Sac Volume**				
Trial	Source	SS	df	*F*	p	Ω^2	SS	df	*F*	p	Ω^2
	ESL Ambient										
	Date	0.071	5	1.219	0.303	0.007	1.396	5	0.788	0.560	-0.007
	Residual	1.816	156				52.772	149			
	1300 ppm										
Jul 3	Date	0.037	5	5.771	**<0.001**	0.141	9.624	5	10.429	**<0.001**	0.280
	Residual	1.756	139				21.224	115			
	2200 ppm										
	Date	0.153	5	2.168	0.061	0.036	6.870	4	6.616	**<0.001**	0.142
	Residual	2.145	152				34.007	131			
	850 ppm										
	Date	0.286	5	4.666	**<0.001**	0.096	9.343	5	6.727	**<0.001**	0.152
	Residual	2.038	166				42.780	154			
	1300 ppm										
Jul 11	Date	0.351	5	6.446	**<0.001**	0.135	1.878	5	1.290	0.271	0.009
	Residual	1.839	169				46.606	160			
	2200 ppm										
	Date	0.300	4	5.843	**<0.001**	0.119	8.603	5	3.926	**0.002**	0.080
	Residual	1.784	139				71.438	163			
	400 ppm										
	Date	0.169	5	2.202	0.057	0.036	3.813	5	5.519	**<0.001**	0.124
	Residual	2.382	155				21.141	153			
	1900 ppm										
Aug 7	Date	0.426	5	5.653	**<0.001**	0.125	1.214	5	1.430	0.216	0.010
	Residual	2.365	157				26.310	155			
	2200 ppm										
	Date	0.605	5	10.267	**<0.001**	0.215	4.229	5	6.575	**<0.001**	0.148
	Residual	1.920	163				19.812	154			

Table S3. One-way ANOVAs of culture cup separated by metric, trial, and pCO_2 treatment; significant p-values (< 0.05) in bold. Yolk volume measurements were log-transformed prior to statistical analysis.

		Mantle Length					**Yolk Sac Volume**				
Trial	Source	SS	df	*F*	p	Ω^2	SS	df	*F*	p	Ω^2
	ESL Ambient										
	Cup	0.113	2	5.059	**0.007**	0.048	7.538	2	12.286	**<0.001**	0.127
	Residual	1.774	159				46.629	152			
	1300 ppm										
Jul 3	Cup	0.258	2	9.851	**<0.001**	0.109	0.452	2	0.877	0.419	-0.002
	Residual	1.863	142				30.396	118			
	2200 ppm										
	Cup	0.033	2	1.113	0.331	0.001	0.323	2	0.530	0.590	-0.007
	Residual	2.265	155				40.553	133			
	850 ppm										
	Cup	0.201	2	8.014	**<0.001**	0.075	0.804	2	1.231	0.295	0.003
	Residual	2.123	169				51.318	157			
	1300 ppm										
Jul 11	Cup	0.048	2	1.937	0.147	0.011	1.323	2	2.287	0.105	0.015
	Residual	2.142	172				47.161	163			
	2200 ppm										
	Cup	0.148	2	5.380	**0.006**	0.057	6.832	2	7.745	**<0.001**	0.074
	Residual	1.936	141				73.210	166			
	400 ppm										
	Cup	0.289	2	10.105	**<0.001**	0.102	2.038	2	6.938	**0.001**	0.070
	Residual	2.262	158				22.915	156			
	1900 ppm										
Aug 7	Cup	0.437	2	14.853	**<0.001**	0.145	1.392	2	4.209	**0.017**	0.038
	Residual	2.354	160				26.131	158			
	2200 ppm										
	Cup	0.068	2	2.283	0.105	0.015	0.503	2	1.677	0.190	0.008
	Residual	2.458	166				23.539	157			

Table S4. Counts of staged, failed embryos and hatchling paralarvae, compiled by treatment. Failed embryos were staged as early (embryonic stages 1 - 16), middle (17 - 26), and late (27 - 30). G-tests of hatching distributions are reported with significant p-values ($p < 0.05$) in bold. Since all G-tests were significant, exponents of the p-value are listed to compare degrees of significance.

Treatment pCO_2	Early	Middle	Late	Hatched	Percent Hatched	Source	*G*	df	p	exp
Jul 3										
ESL Ambient	149	9	0	948	85.71	ESL x 1300	162.857	3	**<0.001**	-35
1300 ppm	13	0	2	1117	98.67	ESL x 2200	32.510	3	**<0.001**	-07
2200 ppm	87	0	2	993	91.77	1300 x 2200	67.514	2	**<0.001**	-15
						All	171.110	6	**<0.001**	-34
Jul 11										
850 ppm	25	6	0	814	96.33	850 x 1300	19.027	2	**<0.001**	-05
1300 ppm	44	33	0	940	92.43	850 x 2200	51.866	2	**<0.001**	-12
2200 ppm	26	61	0	796	90.15	1300 x 2200	15.649	2	**<0.001**	-04
						All	56.021	4	**<0.001**	-11
Aug 7										
400 ppm	10	7	20	835	95.76	400 x 1900	13.566	3	**0.003**	-03
1900 ppm	10	27	16	799	93.89	400 x 2200	13.902	3	**0.003**	-03
2200 ppm	13	11	4	922	91.77	1900 x 2200	18.520	3	**<0.001**	-04
						All	30.544	6	**<0.001**	-05

1. Morphometric analysis of statoliths

1.1a *Statolith morphometrics protocol & R code*

The following R (Version 3.3.3) code was implemented within an R Notebook (StatolithMorphometrics.rmd; available at github.com/czakroff/Statoliths) within RStudio (Version 1.0.136 for Mac OS X). It provides the method for analyzing the silhouetted statolith (black statolith on white background) JPEG's produced in Adobe Photoshop (as described in the main text). This program requires a CSV of metadata with at least the following columns (presumably there will be other relevant sample data as well):

1. Path (path in your system to folder containing the silhouetted images)
2. PicName (image names/ID's of your samples)

   Note: The program is written to access a metadata CSV where the PicName column refers to the original SEM TIFF image. This is why the "#Rename Files" block removes the ".tif" and adds "_BW.jpg." This step can be removed if the PicName column in the metadata refers directly to your silhouetted JPEG's.

The program accesses the silhouetted statolith JPEG's provided by the metadata CSV to build Outline objects using the Momocs package (Bonhomme et al., 2013), which are then analyzed for basic morphometrics as well as the additional metric of rugosity (internal angle variance) developed as part of this manuscript. A few notes on the operation of this code:

- The coo_alignxax method horizontally flips the alignment of the statoliths (they are set with dome pointing right and toward the top of the image and wing pointing left during Photoshop processing). It doesn't affect the outcome, but is important to note.
- The Momocs package contains a number of additional, more complex methods for visualizing and analyzing the shape of objects (elliptical fourier analysis, for instance), which may be worth adding to your analysis depending on the questions of your study.
- The 150 point resolution was determined through an assessment of a sample of 50 test shapes (Fig. S3) and is discussed in detail following the code.

```
---
title: "Statolith Morphometrics"
output: html_notebook
---

This R notebook contains the basic methods to read in and analyze the morphometrics of squid statoliths using the Momocs package (Bonhomme et al., 2013). In addition, the code for calculating the rugosity of the statolith edge (the variance of the internal angles of the outline) is included. The program uses a metadata CSV file (you must supply the PATH to this file) to access the paths and filenames of your image samples (silhouetted JPEGs: black statolith on white background) and then processes the outlines through Momocs to get the basic morphometrics (area, rectangularity, circularity, length, width, and length:width) as well as the rugosity and then outputs this data to
```

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a CSV containing an appended version of the original metadata table (you must supply the PATH and name of this file).

More on Momocs can be found at:
Bonhomme, V., Picq, S., Gaucherel, C. and Claude, J. (2013). Momocs: outline analysis using R. J. Stat. Softw. 56, 1–24.

Version 1.1 written by Casey Zakroff (czakroff@whoi.edu) May 3 2018
in R Version 3.3.3 on Mac. Code and protocols available at:
https://github.com/czakroff/Statoliths

###Step 1
Turn on X11! (Required for running in Mac)

###Process statoliths for outlines
```{r}
#Add Momocs to your active library
library(Momocs)
```

```{r}
#Load in statolith metadata (add your own path & filename)
data <- read.csv('path/filename.csv') #read in metadata CSV
path <- as.character(data$Path) #read path column for statolith silhouettes
samples <- as.numeric(length(path)) #number of samples
```

```{r}
#Set Pixel to Micrometer Ratio
cf <- 6 #conversion factor = 6px/um for my data. Depends on magnification of your images (but should all be the same).
```

```{r}
#Rename Files
x <- c() #temporary array for PicNames
for (i in data$PicName){
  p <- substring(i, 1, nchar(i)-4) #put String in p, but remove ".tif" from SEM image filename
  p <- paste(p, '_BW.jpg', sep = "") #adds silhouette JPEG file ending
  x <- c(x, p) #add to temporary array
}

data$PicName <- x #reassign to PicName column in image metadata
```

```{r}
#Write Statolith Outlines
statolith <- import_jpg(paste(path,data$PicName, sep = "")) #read in all statolith images
stato <- Out(statolith,factor(data$pCO2)) #build Outline objects
```
```{r}
#Optional: view statolith outlines
panel(stato)
```

```{r}

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```

```
#Reorganize Statoliths
stato_align <- coo_alignxax(stato) #align along x-axis
stato_align <- coo_center(stato_align) #center all outlines
stato_align <- coo_slidedirection(stato_align, "N") #place start point of outline at top
```

```{r}
#Optional: view all aligned statoliths superimposed
stack(stato_align)
#Or view as panel
#panel(stato_align)
```

###Basic Morphometrics
```{r}
#Get basic morphometrics
mets <- measure(stato_align, coo_area, coo_circularity, coo_rectangularity) #measure area, circularity, and rectangularity
df <- data.frame(matrix(unlist(mets), nrow = samples, byrow = F)) #store in temporary dataframe
lw <- coo_lw(stato_align) #measure length and width
df$X4 <- NULL #remove extraneous column
df$X1 <- (df$X1)/(cf^2) #convert area to micrometers, overwrite in dataframe
df$Length <- lw[1,]/cf #convert length to micrometers, add to dataframe
df$Width <- lw[2,]/cf #convert width to micrometers, add to dataframe
df$LWRatio <- df$Length/df$Width #calculate length/width ratio, add to dataframe
names(df) <- c("Area","Circularity","Rectangularity", "Length", "Width", "LWRatio") #rename columns
```

```{r}
#Add morphometric data to metadata
len <- length(data)
for (i in c(1:length(df))){
  data[len+i] <- df[i]
}
```

###Statolith Rugosity

```{r}
#Functions for statolith rugosity (internal angle variance) calculation

#Calculate the angle between two vectors (in radians)
angleCalc <- function(M,N){
  abs(atan2(N[2],N[1]) - atan2(M[2],M[1]))
}

#Check the calculated angle (in degrees) is the internal angle (toward center, so less than 180 degrees)
checkAngle <- function(cent, p1, p2, p3, theta){
  p4 <- c(((p1[1]+p3[1])/2),((p1[2]+p3[2])/2)) #calculate point half way between endpoints (n and n+2)
  d1 <- sqrt((p2[1]-cent[1])^2+(p2[2]-cent[2])^2) #distance from centroid to original mid point (n+1)
  d2 <- sqrt((p4[1]-cent[1])^2+(p4[2]-cent[2])^2) #distance from centroid to halfway point
  if (d1 == d2){ #if distances are equal, then its a 180 degree line
    theta <- 180.0
  } else if(d1 > d2){ #if mid point (n+1) farther away than calculated halfway point, then angle is internal
    theta <- theta
  } else { #if calculated halfway point farther away than mid point (n+1), then angle is external
```

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```
    theta <- 360-theta #subtract from 360 degrees to get internal angle
  }
}
```

```{r}
#Set outline resolution
res <- 150 #number of points
statoSam <- coo_sample(stato_align, res) #sample outlines with new resolution
```

```{r}
#Get the position of the centroids of each statolith outline
center <- coo_centpos(stato_align)
```

```{r}
#Get the position of the centroids of each statolith outline
s <- statoSam[h]
```

```{r}
#Calculate internal angle variances
iAngVar <- c() #empty array for results
for(h in c(1:length(statoSam))){
  s <- statoSam[h] #pull statolith outline
  s <- s[[1]] #pull list of outline points
  angle <- c() #empty array for angles
  for (i in c(1:res)){
    x1 <- s[i,1] #pull xpos of nth point
    y1 <- s[i,2] #pull ypos of nth point
    if (i < res-1){ #for most point along the outline
      x2 <- s[(i+1),1] #pull xpos of n+1th point
      x3 <- s[(i+2),1] #pull xpos of n+2th point
      y2 <- s[(i+1),2] #pull ypos of n+1th point
      y3 <- s[(i+2),2] #pull ypos of n+2th point
    } else if (i == res-1) { #but for the penultimate point on the outline
      x2 <- s[(i+1),1] #pull xpos of n+1th point
      x3 <- s[1,1] #pull xpos of first point on the outline
      y2 <- s[(i+1),2] #pull ypos of n+1th point
      y3 <- s[1,2] #pull ypos of first point on the outline
    } else { #and for the last point on the outline
      x2 <- s[1,1] #pull xpos of first point on the outline
      x3 <- s[2,1] #pull xpos of second point on the outline
      y2 <- s[1,2] #pull ypos of first point on the outline
      y3 <- s[2,2] #pull ypos of second point on the outline
    }
    xdiff1 <- x2-x1 #line adjacent to first line
    xdiff2 <- x3-x2 #line adjacent to second line
    ydiff1 <- y2-y1 #line opposite to
    ydiff2 <- y3-y2
    a <- c(xdiff1,ydiff1) #adjacent and opposite of first line
    b <- c(xdiff2,ydiff2) #adjacent and opposite of second line
    tempAngle <- abs(180-(angleCalc(a,b)*180/pi)) #get angle and convert to degrees
    tempAngle <- checkAngle(center[h,],c(x1,y1),c(x2,y2),c(x3,y3),tempAngle) #check/convert to internal angle
    angle <- c(angle, tempAngle) #store angle
```

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```
  }
  iAngVar <- c(iAngVar, var(angle)) #calculate variance of internal angles and add to array
}
```

```{r}
#Add Internal Angle Variance column to data
data$iAngVar <- iAngVar
```

###Output Data
```{r}
#Write results CSV (add your own path and filename)
write.table(data, file = 'path/filename_results.csv')
```

1.1b *Internal angle variance: Resolution assessment*

Two metrics were developed as a potential quantification of statolith edge rugosity:

- Variance of the internal angle of the outline
- Variance of the angle of lines tangent to the outline

A sample set of 50 shapes (Fig. S3), drawn by CZ, was assessed with both metrics at a range of outline resolutions (50, 100, 150, 250, 350, and 450 points). The five highest and five lowest ranking shapes were assessed for each metric at each resolution. Produced outlines of shapes were also visually compared to their originals to determine maintenance of shape integrity in the resulting outline. Three series of shapes were also assessed separately for their progression within each metric and resolution. These were (shapes within series are listed from most rugose to least):

- Circle-studded squares (CSS)
  - Size 1 [Fig. S3 #39]
  - Flattened Size 1 [Fig. S3 #40]
  - Size 2 [Fig. S3 #38]
  - Size 3 [Fig. S3 #37]
- Triangle-studded rectangles (TSR)
  - Size 1 [Fig. S3 #33]
  - Size 2 [Fig. S3 #34]
  - Size 3 [Fig. S3 #35]
  - Flattened Size 3 [Fig. S3 36]
- Corals
  - Coral 4 [Fig. S3 #47]
  - Coral 3 [Fig. S3 #46]
  - Coral 2 [Fig. S3 #45]
  - Coral 1 [Fig. S3 #44]

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A subset of the results of these analyses is presented in Tables S5 and S6. The data demonstrated that internal angle variance better represented rugosity (complexity/variability of the outline) while tangent angles represented the “sharpness” or “pointiness” of a shape (e.g. shapes #12 and #15 ranked very highly in tangent angle variance despite not being particularly rugose; Fig. S3). The 150-point outline resolution maintained shape integrity and produced the best results in both metrics, and the internal angle variance results best fit the concept of rugosity at this resolution, so these were used for the statolith analysis described in the main text.

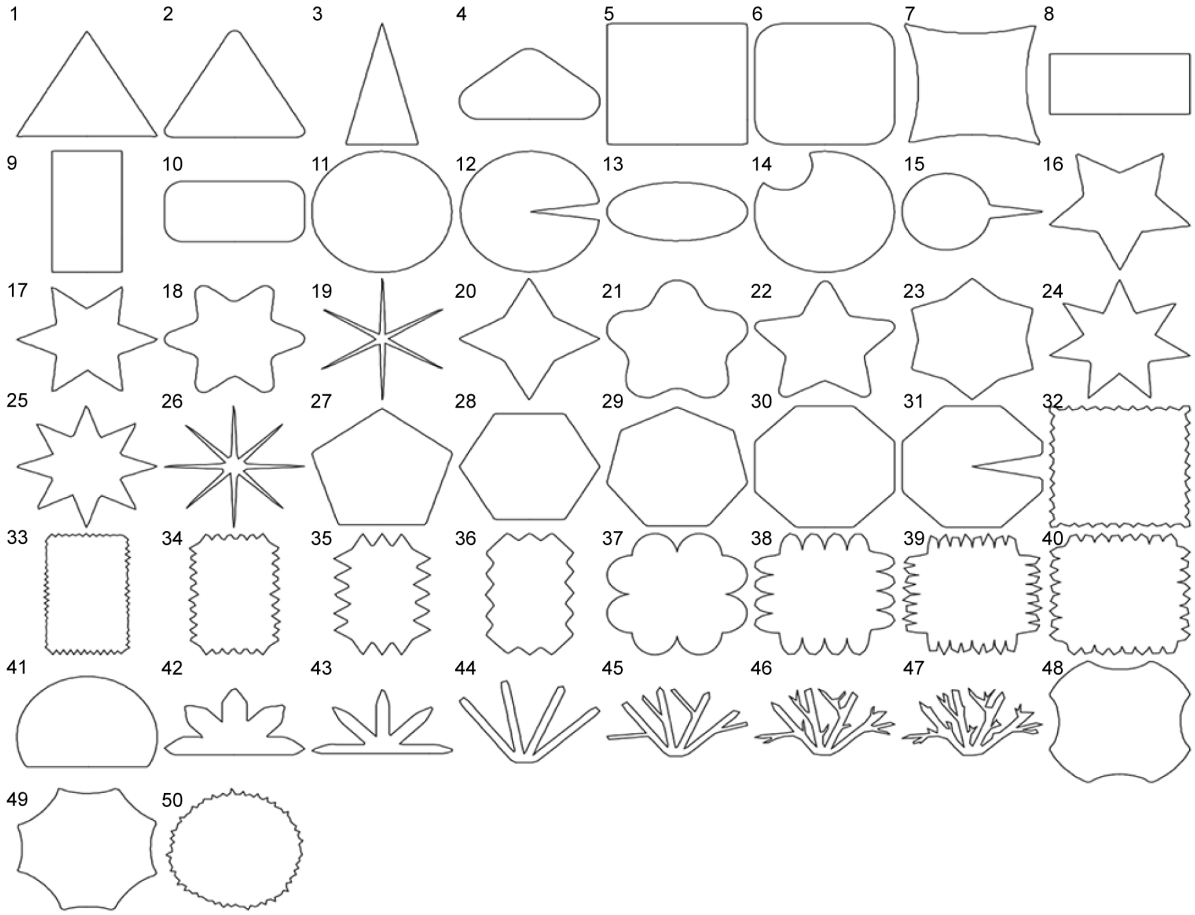

Fig. S3. Sample set of shapes for assessment of rugosity metrics and resolution (outlined here at 150 points). Shape outlines presented here were produced using the panel() method of the Momocs R Package. CZ superimposed the numbers for reference

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Table S5. Subset of shape testing data at three of six point resolutions, showing the top 5 and bottom 5 shapes determined by each metric. The datasets were assessed to determine which metric at which resolution best captured and quantified edge variability of the shapes.

		Internal Angle Variance			Tangent Angle Variance		
Resolution	Rank	Shape Name	#	Value	Shape Name	#	Value
50 pt	1	Coral 4	47	11040.1	Circle-studded square, Size 1	39	0.043
	2	Coral 3	46	9976.5	Circle-studded square, Flat Size 1	40	0.020
	3	Circle-studded square, Size 4	38	7915.6	Coral 4	47	0.013
	4	8-pointed star	26	6244.1	Circle-studded square, Size 2	38	0.010
	5	Circle-studded square, Size 1	39	6117.3	Coral 3	46	0.008
	46	Cut circle / Brain coral	41	107.3	Oval	13	1.4E-4
	47	Rounded square	06	94.3	Rounded Square	06	1.3E-4
	48	Flat rounded triangle	04	83.9	Flat rounded triangle	04	1.3E-4
	49	Oval	13	66.1	Rounded rectangle	10	7.5E-5
	50	Circle	11	7.1	Circle	11	3.6E-6
150 pt	1	Circle-studded square, Size 1	39	7636.1	Circle-studded square, Size 1	39	0.013
	2	Triangle-studded rectangle, Size 1	33	6991.3	Coral 4	47	0.007
	3	Coral 4	47	6748.1	Circle-studded square, Flat Size 1	40	0.006
	4	Circle-studded square, Flat Size 1	40	5581.5	Coral 3	46	0.005
	5	Spiky circle	50	4517.3	8-pointed star	26	0.005
	46	Octagon	30	67.5	Flat rounded triangle	04	1.1E-4
	47	Cut circle / Brain coral	41	65.6	Rounded square	06	1.1E-4
	48	Flat rounded triangle	04	27.5	Cut circle / Brain coral	41	1.0E-4
	49	Rounded square	06	25.9	Rounded rectangle	10	6.7E-5
	50	Rounded rectangle	10	22.4	Circle	11	9.3E-7
350 pt	1	Triangle-studded rectangle, Size 1	33	3397.7	Circle-studded square, Size 1	39	0.006
	2	Circle-studded square, Size 1	39	3126.6	8-pointed star	26	0.004
	3	Coral 4	47	2879.2	6-pointed star	19	0.004
	4	Circle-studded square, Flat Size 1	40	2270.5	Coral 3	46	0.004
	5	Spiky circle	50	2159.9	Coral 4	47	0.003
	46	Tall rectangle	9	64.4	Octagon	30	7.6E-5
	47	Square	5	48.5	Cut circle / Brain coral	41	6.6E-5
	48	Long rectangle	8	48.3	Rounded rectangle	10	5.9E-5
	49	Octagon	30	39.8	Spiky circle	50	5.5E-5
	50	Rounded rectangle	10	34.8	Circle	11	4.5E-7

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Table S6. Subset of shape series data showing results for circle-studded squares (CSS), triangle-studded rectangles (TSR) and coral sets at three outline resolutions. Both metrics produced the correct progression in all three series at 150 points.

		Internal Angle Variance			Tangent Angle Variance		
Resolution	Rank	CSS	TSR	Coral	CSS	TSR	Coral
50 pt	1	Size 2, #38	Size 3, #35	4	Size 1, #39	Size 1, #33	4
	2	Size 1, #39	Size 2, #34	3	Flat Size 1, #40	Size 2, #34	3
	3	Flat Size 1, #40	Flat Size 3, #36	2	Size 2, #38	Size 3, #35	2
	4	Size 3, #37	Size 1, #33	1	Size 3, #37	Flat Size 3, #36	1
150 pt	1	Size 1, #39	Size 1, #33	4	Size 1, #39	Size 1, #33	4
	2	Flat Size 1, #40	Size 2, #34	3	Flat Size 1, #40	Size 2, #34	3
	3	Size 2, #38	Size 3, #35	2	Size 2, #38	Size 3, #35	2
	4	Size 3, #37	Flat Size 3, #36	1	Size 3, #37	Flat Size 3, #36	1
350 pt	1	Size 1, #39	Size 1, #33	4	Size 1, #39	Size 2, #34	3
	2	Flat Size 1, #40	Size 2, #34	3	Size 2, #38	Size 3, #35	4
	3	Size 2, #38	Size 3, #35	2	Flat Size 1, #40	Size 1, #33	2
	4	Size 3, #37	Flat Size 3, #36	1	Size 3, #37	Flat Size 3, #36	1

1.2 *Average pixel intensity variance protocol & MATLAB code*

The following MATLAB code provides the method for analyzing statolith surface variance (StatoSurfVar) by calculating the average pixel intensity variance across five user-placed analysis boxes. This code uses the cut PNG statolith images created in Adobe Photoshop (as described in the main text). The path to and the filenames of those images must be saved in a text file in the MATLAB folder entitled 'ssvFiles.txt', with the path as the first line and each individual image filename on its own subsequent line.

The code is run with a String that acts to label the analysis run (e.g. dataset or date of analysis) and uses this to name the output tables. Once the code is running, it will present the first image in the provided list as a MATLAB figure, which you can then click on five times in order to place the pixel variance analysis boxes. The boxes will then appear on the image and you will be prompted to accept them (by clicking 'Yes' or 'No' in the pop-up box) before the program will move on to the next image. The intent is that these boxes are placed haphazardly, but this can result in overlap or capturing surface contaminants within the boxes, so this interface allows you to iteratively place the boxes as desired to fit the sample.

Once you have accepted the squares on the last image in your dataset, the program will output the centroids of all squares of all images to a CSV file entitled with your 'runName' and the variance data to a CSV file entitled 'runName_results'. The final MATLAB Figure will not automatically close, but the program will still have completed. JPEG's of all images with the analysis squares superimposed are output during the program's operation. It is recommended to move all of these to a subfolder named with

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your ‘runName’ because they can get substantive with high sample sizes and if a sample is rerun the original JPEG will be overwritten.

The ‘runName_results.csv’ file has proxy titles of “Variance_#_#’ (if you can fix this, please do). Otherwise manually rename “Variance_1_1’ as ‘Statolith ID’, ‘Variance_2_1 - 5’ as ‘Box 1 - 5 Variance’, and ‘Variance_3_1’ as ‘Average Variance’.

```
function StatoSurfVar(runName)
%{StatoSurfVar(String) analyzes average pixel variance among five
%user-defined boxes on a statolith image. Per run (named by the input
%String runName), StatoSurfVar takes a file path (line 1) and list of image
%file names (remaining lines [separated by \n]) stored in ssvFiles.txt and
%prompts the user to define the centroids of the 100 px x 100 px analysis
%boxes by clicking the image five times. The program will then display the
%analysis boxes and prompt the user to confirm them before continuing with
%the analysis. StatoSurfVar outputs .jpg images of the input images with
%the boxes drawn on top, a .csv containing the centroid positions of the
%five boxes for all input images, and a .csv with the resultant average
%variances. Note: sID assumes the creator's naming convention and should be
%redefined based on how you ID/how you want to ID your statolith images.
%
%Version 1.1 written by Casey Zakroff (czakroff@whoi.edu) May 2 2018
%in MATLAB version 2016b on Mac. Code and protocols available at:
%https://github.com/czakroff/Statoliths
%}
%% Read file list
fileID = fopen('ssvFiles.txt','r'); %open your path/data list
formatSpec = '%s';
files = textscan(fileID,formatSpec,'Delimiter',{'\n'}); %read it
fclose(fileID); %close it
path = files{1}{1}; %file path is the first line of ssvFiles.txt
results = cell(length(files{1})-1,1); %array for the results
%% Image processing loop
for i = 2:(length(files{1})) %from the line after the path to the end
%% Read and display image
    filename = files{1}{i}; %read in filename of an individual .png
    if isempty(filename) %deals with multiple empty lines at end of list
        results(length(results)) = [];
        break;
    end
    sID = filename(1:length(filename)-8); %Alter this based on your ID's
    im = imread(strcat(path,filename)); %read in image
    imshow(im); %display image
%% Build squares on image
    while(1)
        sqrCents = int32(ginput(5)); %reads 5 user clicks for centroids
        sqrPos = [];
        for j = 1:5 %draw 5 100px squares with input centroids
            pos = [sqrCents(j,:)-50,100,100];
            sqrPos = cat(1, sqrPos, pos);
        end
        sqIm = insertShape(im, 'Rectangle', sqrPos); %draw squares on image
        imshow(sqIm); %show image with squares
        %prompt user
        choice = menu('Accept Analysis Squares?','Yes','No');
        if choice == 1 %end loop if user chooses 'Yes'
            break;
        end
```

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```
    end
    imwrite(sqIm, strcat(sID,'_gradSqr.jpg')); %output image with squares
%% Build centroid table
    if i == 2 %build initial table for centroids of 5 squares
       T = table([string(sID);string(sID);string(sID);string(sID);...
           string(sID)],[1;2;3;4;5],sqrCents);
    else %expand table for each additional image processed
       for j = 1:5
            T(height(T)+1,:) = {string(sID),j,sqrCents(j)};
       end
    end
%% Calculate variance of individual boxes
    v = zeros(1,5); %array to store variance values
    for j = 1:5
        sqr = im((sqrCents(j,2)-50):(sqrCents(j,2)+49),...
            (sqrCents(j,1)-50):(sqrCents(j,1)+49)); %subset image
        x = reshape(sqr, [1,10000]); %reshape pixel values as array
        v(j) = var(double(x)); %get variance of pixel value array
    end
    avgVar = mean(v); %calculate mean variance across boxes
    results{i-1} = {sID,v,avgVar}; %add variances and mean to results table
%% Write square centroid table
    T.Properties.VariableNames = {'StatolithID' 'BoxNumber' 'SqrCentPos'};
    writetable(T,strcat(runName,'.csv'));
end
%% Write results table
res = cell2table(results); %convert 2D array to table
res.Properties.VariableNames = {'Variance'}; %label (needs improvement)
writetable(res,strcat(runName,'_results.csv'));
end
```

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2. Egg number covariate analyses

Dorsal Mantle Length

As stated in the manuscript, egg number shows a weak correlation as a continuous covariate with dorsal mantle length (Fig. S4). Assessing all data, there is only a weak, non-significant (LR, $P = 0.34$) trend in the baseline, low pCO_2 treatments, but a significant trend across treatments (LR, $P < 0.05$). These trends are skewed in the data by what appears to be an outlier point (although data is very limited related to the population). When this point (Jul 11/~100 eggs) is removed, the linear relationship between dorsal mantle length and egg number is consistently strong, even when normalized for the shifting baseline state between clutches/trials (Fig. S4).

The slope of the relationship between DML and egg number appears relatively consistent across CO_2 treatment bins, suggesting what may be a broadly independent covariate given more data. However, an ANOVA on our data demonstrated significant interactions between egg number and pCO_2 in all cases, indicating significantly different slopes (Tables S7, S8). Across trials, egg number was a consistently significant factor in determining paralarvae mantle length and significantly interacted with pCO_2 treatment, date, and cup (the last, unsurprising as it functionally is a categorical representation of the same factor in our experimental system; Table S7). In the data compiled across trials, egg number and trial showed the strongest impact on differences in paralarvae mantle length across pCO_2 treatments, demonstrating the major influence of season, parentage, and clutch on the state of the paralarvae, although pCO_2 was still a significant influence as well (Table S7).

Yolk Volume

A cursory examination of yolk volume and egg number also suggests a potential relationship, however it is clear in the raw data that this trend is strongly driven by trial differences (Fig. S4). When the data is normalized for shifting baselines between trials, there is still a trend of increasing yolk volume with increasing egg number, but it is non-significant (LR, $P > 0.05$) and highly variable (Fig. S4). More data would be needed to see if this relationship holds for the population, but would be worthwhile to collect, as it suggests egg capsules with more eggs are broadly more invested in by mothers and may fare better.

Despite the relative weakness in the correlation between yolk volume and egg number in the regression plots, the statistical models indicate that egg number and pCO_2 significantly interact to impact paralarval yolk volume (Table S8). Within trials, the interactions of egg number and pCO_2 with both hatching date and cup consistently showed significant impacts on yolk volume (Table S7). When compiled across trials, egg number and pCO_2 (as a factor of trial) show independent effects on yolk volume (Table S7).

Embryonic Survival and Hatching Time

Neither embryonic survival (measured as percent hatched) nor hatching time (measured as time in days to 50% hatching) showed consistent or significant correlations with egg number. Embryonic survival broadly shows decreases with increasing egg number, but these wash out when differences are normalized for trial differences (Fig. S4). The data across treatments

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suggests that if a capsule is not entirely successful (< 95% hatched) then increasing egg number may exacerbate losses (Fig. S4). As increasing egg number increases the number of oxygen consumers, it may be that embryonic survival is more driven by oxygenation state in our system. Our data may then represent the result of variability in oxygenation due to both the experimental system and the egg capsule.

Hatching time shows decreases in the raw data, with a significant trend across all treatments (LR, $P = 0.01$), but this again is clearly driven by trial differences (Fig. S4). When normalized for shifting baselines between trials, no effect of egg number on hatching time is seen, although the slope of response appears to vary between low (slight increase) and high (decrease) pCO_2 treatment bins (Fig. S4).

Fig S4. Relationships between metrics and number of eggs per capsule (next page). Data are presented as raw data (left two columns) and normalized (right two columns: "Lowest pCO_2" [550, 850, 400 ppm] by treatment mean, "All Treatments" by trial mean). Hatching time is presented here as the time (in days) to 50% hatching calculated from the cumulative hatching curves presented in Fig. 7 of the manuscript. Symbols represent means, shape represent egg clutch lay date/trial (circle = Jul 3, square = Jul 11, triangle = Aug 7), color represents binned pCO_2 treatments (light gray = low [550, 850, 400 ppm], medium gray = mid [1300, 1300, 1900 ppm], dark gray = high [2200 ppm]; bins were used instead of a gradient for simplicity). Yolk volume means are back calculated from logarithmic scale in the raw data, but not for the differences used in the normalized data. Lines represent linear regressions; significant p values ($\alpha = 0.05$) are marked by an asterisk. Black regression line and statistics represent all data points. The gray dashed regression line and statistics in the "Dorsal Mantle Length" row have removed the 'Jul 11/~100 egg' data point as an outlier.

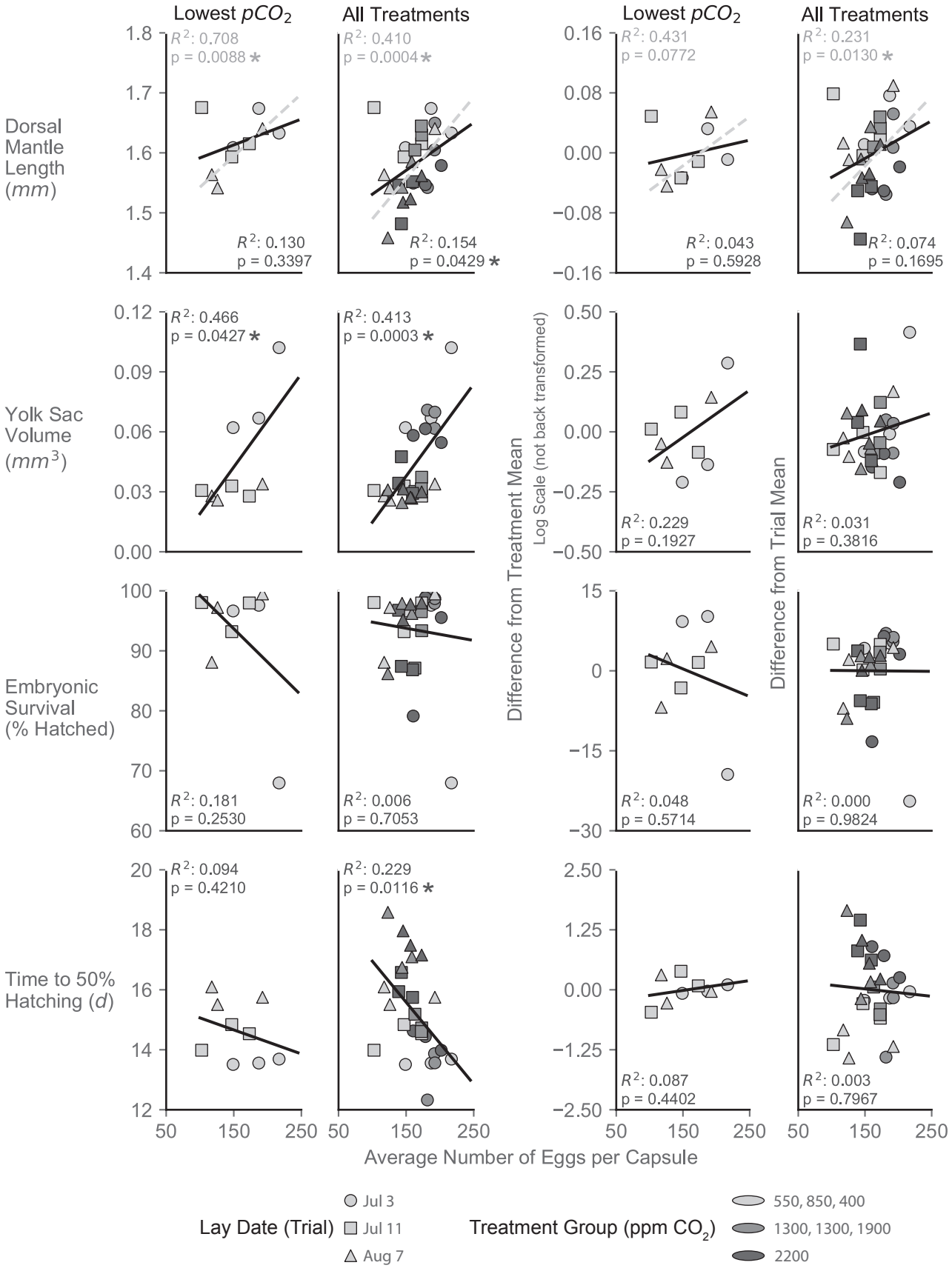

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Table S7. Type II nested ANOVAs for individual trials and compiled data (normalized by difference from trial mean) of both mantle length and (log-transformed) yolk volume. Egg number is included as an independent continuous covariate. Significant p values (α = 0.05) in bold; '<<' indicates a negative exponent of 50 or greater.

	Mantle Length				**Yolk Sac Volume**			
Source	SS	df	*F*	*p*	SS	df	*F*	*p*
Jul 3								
pCO_2	$-8.686*10^{-13}$	2	$-3.687*10^{-11}$	1.000	$8.260*10^{-15}$	2	$2.765*10^{-12}$	1.000
pCO_2 : Date	3.298	15	18.663	**<0.001**	0.097	15	4.336	**<0.001**
pCO_2 : Cup	5.899	6	83.462	**<<0.001**	0.016	6	1.731	0.112
Egg Number	507.5	1	43,082	**<<0.001**	1.481	1	991.4	**<<0.001**
Egg Number : pCO_2	0.368	2	15.634	**<0.001**	$3.453*10^{-6}$	2	$1.156*10^{-3}$	0.999
Egg Number : pCO_2 : Date	0.286	15	1.616	0.062	0.103	15	4.589	**<0.001**
Egg Number : pCO_2 : Cup	4.450	6	62.960	**<<0.001**	0.035	6	3.951	**<0.001**
Residual	5.030	427			0.563	377		
Jul 11								
pCO_2	$-9.320*10^{-12}$	2	$-4.175*10^{-10}$	1.000	$-1.169*10^{-14}$	2	$-8.596*10^{-12}$	1.000
pCO_2 : Date	9.271	15	55.373	**<<0.001**	0.023	15	2.287	**<0.001**
pCO_2 : Cup	0.152	6	2.267	**0.036**	$-2.268*10^{-4}$	6	-0.056	1.000
Egg Number	482.6	1	43,223	**<<0.001**	0.127	1	187.4	**<0.001**
Egg Number : pCO_2	0.011	2	0.515	0.598	$6.778*10^{-3}$	2	4.984	**<0.001**
Egg Number : pCO_2 : Date	9.572	15	5.717	**<<0.001**	0.017	15	1.661	0.056
Egg Number : pCO_2 : Cup	4.812	6	7.185	**<<0.001**	0.026	6	6.463	**<0.001**
Residual	5.068	454			0.310	456		
Aug 7								
pCO_2	$-4.320*10^{-11}$	2	$-1.802*10^{-9}$	1.000	$2.316*10^{-15}$	2	$7.880*10^{-12}$	1.000
pCO_2 : Date	1.284	15	7.143	**<0.001**	$8.847*10^{-3}$	15	4.013	**<0.001**
pCO_2 : Cup	0.388	6	-5.393	1.000	$-1.135*10^{-3}$	6	-1.287	1.000
Egg Number	158.8	1	13,254	**<<0.001**	0.050	1	342.5	**<<0.001**
Egg Number : pCO_2	0.314	2	13.108	**<0.001**	$2.179*10^{-4}$	2	0.741	0.477
Egg Number : pCO_2 : Date	0.348	15	1.934	**0.019**	$3.761*10^{-3}$	15	1.706	**0.047**
Egg Number : pCO_2 : Cup	3.838	6	53.376	**<0.001**	$4.667*10^{-3}$	6	5.292	**<0.001**
Residual	5.441	454			0.065	441		
Compiled Data								
Trial	0.002	2	0.065	0.937	328.1	2	$2.227*10^{5}$	**<<0.001**
Trial : pCO_2	21.08	15	120.8	**<<0.001**	1373	15	$1.243*10^{5}$	**<<0.001**
Trial : pCO_2 : Date	425.0	306	119.3	**<<0.001**	-0.433	306	-1.920	1.000
Trial : pCO_2 : Cup	0.033	36	0.078	0.999	$6*10^{-6}$	36	$2.345*10^{-4}$	1.000
Egg Number	0.209	1	17.98	**<0.001**	32.22	1	43,739	**<<0.001**
Egg Number : Trial	302.3	2	12,987	**<<0.001**	-0.026	2	-17.74	1.000
Egg Number : Trial : pCO_2	67.91	15	389.0	**<<0.001**	-34,307	15	$-3.105*10^{6}$	1.000
Egg Number : Trial : pCO_2 : Date	590.7	306	165.9	**<<0.001**	0.218	306	0.969	0.325
Egg Number : Trial : pCO_2 : Cup	92.10	36	219.8	**<<0.001**	0.026	36	0.969	0.325
Residual	15.54	1335			0.939	1274		

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Table S8. Type II ANOVAs of pCO_2, with egg number as an independent covariate, separated by metric and trial. Significant p-values (< 0.05) in bold. Yolk volume measurements were log-transformed prior to statistical analysis.

	Mantle Length					**Yolk Sac Volume**				
Source	SS	df	*F*	p	Ω^2	SS	df	*F*	p	Ω^2
Jul 3										
pCO_2	0.501	2	19.008	**<0.001**	0.070	0.027	2	7.466	**<0.001**	0.070
Egg Number	0.060	1	4.558	**0.033**	0.007	0.040	1	22.087	**<0.001**	0.007
Egg Number : pCO_2	0.199	2	7.540	**<0.001**	0.025	0.019	2	5.101	**<0.01**	0.025
Residual	6.047	459				0.743	406			
Jul 11										
pCO_2	1.108	2	41.903	**<0.001**	0.140	0.008	2	5.014	**<0.01**	0.016
Egg Number	0.074	1	5.563	**0.019**	0.008	0.001	1	1.747	0.187	0.001
Egg Number : pCO_2	0.115	2	4.351	**0.013**	0.011	0.006	2	3.798	**0.023**	0.011
Residual	6.409	485				0.367	489			
Aug 7										
pCO_2	0.390	2	13.368	**<0.001**	0.044	$3.27*10^{-4}$	2	0.983	0.375	0.000
Egg Number	0.591	1	40.523	**<0.001**	0.070	$6.12*10^{-4}$	1	3.676	0.056	0.005
Egg Number : pCO_2	0.169	2	5.774	**<0.01**	0.017	0.002	2	7.345	**<0.001**	0.026
Residual	7.107	490				0.079	474			
Compiled										
pCO_2	2.060	5	29.702	**<0.001**	0.086	0.186	5	39.483	**<0.001**	0.111
Egg Number	0.245	1	17.677	**<0.001**	0.010	0.055	1	58.470	**<0.001**	0.033
Egg Number : pCO_2	0.772	5	11.125	**<0.001**	0.031	0.099	5	21.045	**<0.001**	0.058
Residual	19.93	1437				1.296	1375			

3. References

Bonhomme, V., Picq, S., Gaucherel, C. and Claude, J. (2013). Momocs: outline analysis using R. *J. Stat. Softw.* **56**, 1–24.