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THE EFFECT OF ELEVATED TEMPERATURE ON LARVAL DEVELOPMENT OF *ACROPORA PALMATA*

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The effect of elevated Temperature on Larval Development of *Acropora palmata*

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Abstract: *Acropora palmata* thrived in the Caribbean and Florida but has suffered a great reduction in numbers over the past few decades. Temperature is the most prominent factor leading to coral decline. This experiment focuses on the effects of changes in temperature on *A. palmata* embryonic development. Larval development was observed via microscopic pictures. Pictures were taken of n=901 larval samples collected in August of 2008 and 2009. Ratios of larvae belonging to 7 developmental stages were recorded. High temperature (30 ℃ and 29 ℃) accelerated the larval development as larvae entered later developmental stages at an earlier time point. Low temperature (25 ℃ and 27 ℃) resulted in slowed larval development rate and delayed metamorphosis for settlement. Samples from different parentage also showed different patterns in development. Among them, batch 2 and individual cross x by 88 had the greatest fertilization rates and greatest larval surface areas. The strong effect of temperature on *A. palmata* larval development likely influences the dispersal, settlement and survival of the larvae.

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Introduction:

Acropora palmata is a major reef building coral species in Florida and the Caribbean. Its common name is Elkhorn coral because of its large antler-like branches. Since the 1980s, *A. palmata* has suffered a great decline in individual numbers (loss of >80%) and a reduction in its range. A great number of factors contribute to the decline of *A. palmata*. These factors include: hurricanes, increased predation, disease (White band disease), bleach and rising temperature (Bruckner, 2002). Adding to the problem is the fact that coral reefs do not grow well under environmental disturbances and show very poor recruitment after major destruction (Riegl, 2001).

In general, the influence of temperature on corals is rather complicated and versatile. Temperature can play either a positive or negative role. A study focusing on the bleaching events and sea surface temperature in Puerto Rico showed that the maximum sea surface temperature had increased by 0.7 ℃ during 1966-1995. This increasing temperature has been associated with large bleaching events and is a major cause of *A. palmata* decline since the 1980s (Winter, et al. 1998). Some studies have also shown that elevated temperature increases premature metamorphosis in water of coral larvae (Putnam, Edmunds & Fan, 2008). However, some studies also mentioned that *A. palmata* species are re-expanding their ranges northward as the temperature rises (Precht & Aronson, 2004). Therefore, based on these observations, one cannot draw the conclusion that increasing

temperature has only negative influences on adult *A. palmata*. Further, the effect of temperature on sexual reproduction is largely unknown. The aim of this study is to learn how temperature affects coral embryonic development and discover the ideal temperature for *A. palmata* embryonic development.

Temperature is closely associated with metabolic rates during early coral life stages. Studies on metabolic rate showed that a small increase in temperature will increase metabolic rate in coral species (Edmunds, 2005). Thus it is likely as a result, larvae incubated at higher temperature will deplete their stored nutrients more quickly than larvae incubated at lower temperature. Based on this, the main hypothesis is that higher temperature will accelerate embryonic development. We further hypothesize that an intermediate temperature will be most suitable for embryonic development at which both development rate and survival rate are high (O'Connor, et al. 2007). The results of this study will be presented in three parts: the fertilization rate, early development and late development of coral larvae. The results shall provide useful information for reef restoration projects in Caribbean where *A. palmata* is a major reef building species.

There are many studies conducted on *Acropora* embryonic development. Although the selected samples in this experiment are from *A. palmata*, the general development pattern for all *Acropora* species is similar (Randall & Szmant, 2009). Observations on *Acropora millepora* from a recent study (Ball, et al. 2002) show that the early stages (2 cells stage to 8 cell stage of cell division) take place in

the first 7-9 hours. After that, the cell division does not occur orderly; at around 13 hours cells form a flat bilayer embryo. The embryo in this stage is generally round but can also adapt irregular shapes. In the next stage (22-36 hours) gastrulation occurs and the embryo gradually becomes a sphere shape with an oral pore somewhere on the surface. At around 72 hours, the embryo starts to adapt a pear shape. The embryo becomes longer at around 96 hours. The larvae elongate and become spindle shaped. From this point on larvae begin to settle and metamorphose (Ball, et al. 2002). Normally for a larva to settle successfully, it needs to attach to a substrate via its aboral end (Hayashibara, et al. 1997). However, it is observed that some larvae metamorphose prematurely in seawater without actually attaching to a substrate. In this study, larvae were assigned and sorted into various developmental stages and the respective ratios of each stage were calculated. The respective ratio of these different stages provided information on the development rate of the larvae.

Elkhorn coral larvae were exposed to three temperatures and sampled over time. The null hypothesis is that the ratio of larvae in different developmental stages remains the same; the surface area remains the same; and the fertilization rate remains the same regardless of the temperature treatment or the batch and individual cross type. The alternative hypothesis is that higher temperature will cause larvae to enter later developmental stages earlier; therefore, having a higher ratio of more developed larvae. Higher temperature might also cause metamorphoses in water before settlement.

Materials and Methods:

Defining developmental stages:

A key point in the study is to rate the developmental status of larval sample via assigning each larva into developmental stages. Larvae undergo morphological and physiological changes as they grew. The start point of the whole process is the fertilization of an egg, and the end point is a settled and metamorphosed polyp. The many distinctive features larvae display during this process help us to break this developmental process down into several parts, or stages.

According to Randall and Szmant (2009), there are 17 distinctive developmental stages. Their developmental stages were derived from Okubo and Motokawa (2007) that broke down the developmental process into 15 stages. Both studies recognized the earlier developmental stages 1-15, the difference is that Randall and Szmant also recognized metamorphosed larvae and bifurcated larvae. The two additional stages recognized by Randall and Szmant occurred only at very late time points during and after metamorphosis (see introduction). In this study, 7 developmental stages were derived based on the developmental stages assigned in these two studies. Some stages were combined into one to reduce the number of dependent variables.

Stage 1: Pre-morula and morula stage:

This first stage includes all the early developmental stages of a larva up until the morula stage. This stage include the 2 cell stage, 4 cell stage, 8 cell stage, 16 cell stage and morula stage. The cell undergoes several divisions here and the cleavages are visible, resulting in clearly defined blastomeres. These blastomeres remain distinguishable in the next stage, the early prawn-chip stage. However, early pawn chip larvae are usually flat in shape. Therefore, any larva that is non-flat and has visible blastomeres should be categorized in this first stage.

Stage 2: Plate stage:

The plate stage refers to the several prawn chip stages described in Randall and Szmant (2009) and Okubo and Motokawa (2007). The prawn chip stages generally refer to larvae with flattened shape that have not started the gastrulation process yet. The larva remains a blastula in this stage. Unlike blastulas of other species that form a ball shape with blastocoels in the center, blastocoels are not formed in coral blastulas at this stage due to the flattened shape. The surfaces of larvae become smooth later in this stage so blastomeres are no longer visible.

Stage 3: Gastrulation stage:

In the third gastrulation stage larvae experience the most prominent visible morphological changes in early stages. According to Okubo and Motokawa (2007) study, the gastrulation begins with

larvae forming a blastocoel and the cells are starting to take a columnar shape. The larvae develop into a "dumbbell" shape and appear elongated at first. They have larger "heads" on either end and a slightly thinner "waist" in the middle. Then, one end of the larva invaginates to form a blastopore. The shape of the larvae resembles a bowl in this so called gastrulation process. At the very end of gastrulation, the larva turns into a spherical shape and the blastopore becomes invisible. Larvae may display an imperfect spherical shape before the gastrulation process is complete. Therefore, in this study, larvae displaying dumbbell shape, bowl shape, and imperfect spherical shape are all categorized into the gastrulation stage.

Stage 4: Round stage:

Visible morphological change slows down at the fourth round stage. As mentioned in the previous stage-description, larvae become spherical and the blastopore disappears at the end of gastrulation. Thus larvae in this stage look very similar to unfertilized eggs. Several physiological changes must occur for larvae to enter the next stage. Larvae develop cilia on the outer surface and the outer cells develop into a more distinguishable epidermis. So when the larva enters the next stage, there is a visible differentiated outer layer of cilia and epidermis. This process happens between 30 hours to 54 hours according to Okubo and Motokawa (2007) on *Acropora tenuis* and *Acropora*

digitifera. This time frame may differ with *A. palmata.* But nonetheless, larvae remain in this round egg like shape for approximately one day before entering the next stage.

Stage 5: Pear shape stage:

Just as the round shape larva is undergoing changes to form cilia and epidermis, the shape of the larva is slowly changing. It becomes elongated on one end and results in a trapezoid shape that resembles a pear. As mentioned before, larvae in this stage have a visible outer layer of cells. This feature distinguishes pear shape larvae from elongated larvae in earlier time points (they may look elongated during gastrulation from certain angles). In this stage, a visible "ring" structure appears in the center of the larva due to aggregation of ectoderm. But still, the most prominent and distinguishable features are the pear shape and the light ring of outer cells.

Stage 6: Spindle shape stage:

This stage shares many characteristics with the previous one. The shape of larva is now a spindle or bullet shape due to further elongation. The outer cell layer becomes more visually distinguishable at this stage. Okubo and Motokawa (2007) also talked about the formation of endodermal cell layer. The elongation is the lengthiest process of larval development. It starts at the

end of the round shape stage at around 54 hours and lasts until the larva finds a substrate to settle on or metamorphose prematurely, a process that can last as long as 150 hours for *A. tenuis and A.digitifera.*

Stage 7: Metamorphosed stage:

Some larvae metamorphose prematurely in the water column. Metamorphosis is an essential part of coral settlement. The coral transforms from a bilaterally symmetric larva into a radially symmetric polyp, resembling a flower.

Influence of temperature on the late development of *A. palmata* larvae:

A. palmata gametes were collected on 21st of August-2008 in Rincon, Puerto Rico. Egg-sperm bundles were collected from individual colonies by placing nets above them. Gametes produced by the colonies floated up and entered a cup at the top of the nets. Then the egg and sperm were separated and later crossed with gametes from another colony. Batches were produced by mixing equal amounts of sperm and eggs from each colony. Batch 1 and 3 were from colonies from House Beach. Batch 4 and 5 were from Bajo Gullardo.

Larvae were incubated at three different temperatures: 25 ℃, 27 ℃ and 30 ℃. They were sampled over time and preserved in a 4% formaldehyde seawater solution. In this experiment, samples studied included all four batches. Batch 1, 3, 4 and 5 were incubated in all three temperatures. The

report focuses only on those batch samples incubated at 25 ℃ and 27 ℃ because an insufficient amount of larvae incubated at 30 ℃ survived to later time points to be sampled. For each batch and temperature combination (e.g. Batch 1 incubated in 27 ℃), 4 replicates were created and raised in independent aquaria. Samples included in this study for late development were sampled at various time points that can be divided into four time periods ranging from 59 hours to 229 hours (Table 1).

Control samples (Secore) were collected during the Secore workshop in Puerto Rico, 2008 (see acknowledgement at the end of this report). Their data were included to provide a comparison with larvae incubated at a higher temperature for late development study. The Secore samples were incubated at 29.5 ℃.

A small amount of preserved larvae sample was extracted from each tube and transferred onto a slide. Images were then taken through a Carl-Zeiss Stereomicroscope Discovery V2.0. Most pictures were taken with a 14x or 11.3x magnification. The low magnifications were chosen and an average of 15 larvae was included in each picture. After the picture was taken, it was analyzed with the program Carl Zeiss AxiovisionLE 4.8. For each picture, area, radius and perimeter of the larvae were measured. In this report, only area results are presented.

In order to describe the development rate of the larvae between 59 hours and 229 hours, the number of larvae that reached pear shape stage, spindle shaped stage and metamorphosed stage (stages

5-7) were counted and expressed as the ratio of each respective stage in each picture. In order to compare different samples, four time periods (59-81 hours, 90-116 hours, 131-161 hours and 186-223 hours) that divide larvae samples taken over a certain amount of incubation time were defined. Table 1 shows all 4 time periods and the number of observed larvae in each time range. The four time periods were named as A, B, C and D respectively.

Two way ANOVA tests were performed to compare the means of stage 5 and stage 6 larval ratios and larval surface area. Factors were: 1) Batch number (4 levels: 1, 3, 4 and 5) 2) temperature (2 levels: 25 ℃ and 27 ℃) and 3) sampling time (4 levels: time periods in table 1, A, B, C and D). ANOVA analyses were conducted with two factors each time (batch number and temperature, sampling time and temperature). A t-test comparing the ratio of metamorphosed larvae incubated under two temperatures was also carried out. This t-test was performed with data from the last time period D only. The statistic program used is SPSS 18 (IBM, Somers)

The surface area data of 2008 was transformed in order to pass normality test. A histogram showed that the data was skewed to the right. A logarithmic transformation of the data was therefore performed. A similar transformation was carried out for the ratio of metamorphosed larvae. Due to many zero values in the data, (2 + n) was used instead of n in the logarithmic transformation (n = ratio of the metamorphosed larvae).

Influence of temperature on fertilization and early development of *A. palmata* larvae:

The similar procedures of sample collection in August-2008 were conducted in 11th of August 2009. However there were some differences. For the 2009 samples, the larvae were incubated under 27 ℃, 29 ℃ and 30 ℃ degrees respectively and sampled over 3-5 days (7.5 hours after incubation to 124 hours after incubation). The data from 2008 contained samples incubated up to 8 or 9 days, but they lacked the collection from earlier time periods.

In the 2009, 4 batches were created (batches 2, 3, 4 and 5). Batch 2 was created on August 12th, 2009, al other batches were born on August 13th, 2009. Individual crosses were also included in the study. There were 6 individual crosses: x by 88, x by j, x by 78, j by 88, 78 by j, and 78 by 88 (egg donor by sperm donor). The individual crosses were all created the on August 12th, 2009. As in 2008, there were 4 replicates for each batch/temperature or cross/temperature combination.

The 2009 samples yielded data on early larval development and fertilization rates. Larvae were categorized into the first five developmental stages discussed above. Surface area data was also included.

Fertilization rates were calculated by counting larvae that showed visible division from 1.5 to 7.5 hours and dividing this number by the total number of larvae plus eggs from the picture. Batch 2 results were separated from the other three batch samples because they were created on different days.

It is worth noting that gametes were mixed and incubated for one hour at 27 degrees before being distributed to different temperatures for incubation.

Data analysis for the early developmental rate included a mixed model ANOVA, with a covariance structure that included batch by temperature nested within replication:

Replication (Batch*Temperature)

In the case of individual crosses, the covariance structure included cross type (cross) by temperature nested within replication:

Replication (Cross*Temperature)

The main effects of batch, temperature and the 3-way interaction of time batch and temperature were tested.

For the surface area data, two-way ANOVA tests were performed. One test was performed with temperature and batch as factors while another test was performed with time and temperature as factors.

Results:

Figure 1 shows the developmental stages defined in the Material and Methods section. Stages 1 to 5 were counted to determine the embryonic development rate of *A. palmata* for the early development experiment. Stages 5 to 7 were counted for the late development experiment.

Fertilization rate:

Figure 2 provided a time comparison of fertilization rates between 1.5 hours and 5 hours of batch 2 sample. Batch 2 was the only sample that had enough early time point samples to make this time comparison.

In Figure 2, the fertilization rate increased slightly from 1.5 hours to 5 hours at all three temperatures. This observation was supported by a two way ANOVA test conducted to compare the factors time and temperature ($F = 8.737$, $p < 0.05$). The fertilization rate varied slightly among the 3 temperatures but showed no statistical difference ($F = 3.494$, $p = 0.052 > 0.05$). However, the p value was very close to 0.05. The two higher temperatures had a slightly higher fertilization rate. But there was no significant interaction between temperature and time ($F = 0.186$, $p = 0.832 > 0.05$).

Although fertilization rate varied very little from one temperature to another within the same batch, there was a significant difference among batch samples (Figure 3, 2-way ANOVA: F-temperature = 0.707, $p = 0.502 > 0.05$, F-batch = 18.655, $p < 0.001$). Tukey test showed that all four batches were significantly different from

each other in fertilization rate. The marginal means of fertilization rates for batches were: 0.868 (batch 2), 0.318 (batch 3), 0.446 (batch 4), 0.586 (batch 5).There was no significant interaction between the two factors ($F = 0.863$, $p = 0.499 > 0.05$).

The fertilization rate had similar pattern in individual cross samples (Figure 4). According to the Two-way ANOVA test results, only the types of individual crosses influenced the fertilization rate significantly ($F = 22.042$, $p < 0.001$), temperature had no main effect ($F = 0.131$, $p = 0.877 > 0.05$) and there was no significant interaction of cross and temperature ($F = 0.301$, $p = 0. 979 > 0.05$). The highest fertilization rate came from cross x by 88 with a marginal mean of 0.557, while the lowest fertilization rate came from cross 78 by 88 with marginal mean of 0.051. Thus the fertilization rate of cross x by 88 was 10 times higher of that from cross 78 by 88. Other than 78 by 88, cross 78 by j and cross x by 78 both had fertilization rate lower than 0.1 (marginal means: 78 by j: 0.063, x by 78: 0.058).

The overall fertilization rate of all batch samples was 0.555. It was much higher compared to the overall fertilization rate of all individual cross samples, which was 0. 222.

Influence of temperature on early development:

Early development of larvae was also significantly influenced by temperature difference as well as individual cross type (figures 5A-D and figures 6A-F).

At higher temperatures (29℃ and 30℃), most larvae had developed beyond stage 1 at 9.5 hours, while a few larvae still remained in this stage at 27 ℃ (ratio at stage 1 around 0.107). This difference of larval ratio at stage 1 caused by temperature was statistically significant. ($F= 26.674$, $p< 0.05$) There was no significant difference of samples from different batches in this stage ($F= 0.397$, $p= 0.756$). There was no significant interaction of time, temperature and batch factors ($F= 0.732$, $p= 0.634$).

The individual cross sample showed the same main effect of temperature for larval ratio of stage 1. ($F= 69.462$, $p< 0.001$). There was a significant difference caused by different cross type as well ($F= 4.112$, $p= 0.003< 0.05$). Cross78 by j had larval ratio (0.021) at stage 1 significantly lower than the ratio from other crosses. The overall average for larval ratio at stage 1 for individual crosses was 0.1432. Most of the stage 1 larvae from individual crosses were found at 7.5 hours after fertilization (marginal mean: 0.289), only a few remained in stage 1 at 13.5 hours (marginal mean: 0.004). There was a significant time by temperature by batch interaction for stage 1 larval ratio ($F= 8.613$, $p< 0.001$).

Larvae at 29℃ showed the highest ratio at the second stage (Plate stage) (marginal mean: 0.432), larvae at 30℃ showed the smallest ratio (marginal mean: 0.371). This difference by temperature was statistically significant. ($F= 5.605$, $p= 0.005< 0.05$) Larvae from different batches showed no statistical significant difference ($F= 0.106$, $p = 0.956$). Most larvae developed out of this

stage at 26 hours with only 0.006 of larvae remaining in this stage at that time. There was no significant time by temperature by batch interaction ($F= 1.306$, $p= 0.214$).

Temperature however, did not show a main effect for the larval ratio at stage 2 for individual crosses ($F= 0.344$, $p= 0.709$). The marginal means for the three temperatures varied slightly around the overall mean of larval ratio of this stage (0.4009). There was a significant main effect for cross types ($F= 14.7$, $p< 0.001$). Cross 78 by j had significantly lower larval ratio at stage 2 (0.103) while cross x by j had significantly higher larval ratio at stage 2 (0.614). Larval ratio for stage 2 was high at 7.5 hours (marginal mean: 0.447) and 13.5 (marginal mean: 0.489) hours after fertilization, but it dropped significantly at 16.5 hours (marginal mean: 0.181). There were no larvae in stage 2 at 27 hours after fertilization in the individual cross samples. There was a significant time by temperature by cross type interaction ($F= 4.894$, $p<0.001$).

When looking at the ratio of larvae in the first two stages, the focus was on the first few time points (5 hours to 16.5 hours after fertilization) as most larvae developed beyond that stage in later time points. But starting from the third stage, the gastrulation stage, the developmental stages became more integrated through the timeline. The third stage is observed from 9.5 hours to 50.5 hours in batch samples and 13.5 hours to 48 hours in individual cross samples. For batch samples, the greatest difference in larval ratio of stage 3 from various temperatures existed between 27 ℃ (marginal mean:

0.331), and the two higher temperatures (marginal means: 0.143 for 29 ℃ and 0.150 for 30 ℃). ($F= 69.656$, $p< 0.001$). Different batch samples showed slight variation in their larval ratios at this stage, but overall main effect of batch is not significant ($F= 2.326$, $p= 0.091$). The greatest amount of stage 3 larvae for batch samples were found around 26 hours after fertilization (marginal mean: 0.494), at 50 hours, only 0.058(marginal mean) of larvae remained in stage 3. There was a significant time by temperature by batch interaction ($F= 12.391$, $p< 0.001$).

For individual cross samples, cross type had a main effect on the larval ratio of stage 3 ($F= 8.154$, $p< 0.001$). Crosses 78 by j and 78 by 88 had significantly higher larval ratio of stage 3 compared to other crosses (marginal means: 0.561 for 78 by j, 0.497 for 78 by 88), cross x by j had significantly lower ratio of stage 3 (marginal mean: 0.297). The main effect of temperature on the larval ratio of stage 3 was also significant ($F= 8.656$, $p< 0.001$). Larvae incubated at 27 ℃ had higher ratio of stage 3 (marginal mean: 0.437) than larvae incubated at 29 ℃ and 30 ℃ (marginal means: 0.366 for 29 ℃ and 0.381 for 30 ℃). The highest larval ratio was found at 16.5 hours (marginal mean: 0.693), it declined gradually afterwards. 48 hours after fertilization, there were 0.107(marginal mean) of larvae still in stage 3. There was a significant time by temperature by cross type interaction ($F= 13.191$, $p< 0.001$).

Sample from various batches showed no significant difference in the larval ratio of stage 4, the round stage ($F= 1.450$, $p= 0.245$). Stage 4 also had a greater spread out through time compared to the first two stages (26 hours to 74.5 for batch samples, 27 hours to 124 hours for individual cross samples). At 26 hours, 0.5 of the larvae from batch samples were already in stage 4. The ratio increased and the greatest amount of larvae from stage 4 was found at 50.5 hours for batch samples (marginal mean: 0.873). The larval ratio of stage 4 decreased afterwards and at 74.5 hours 0.41 of the larvae remained in stage 4. The larval ratio of stage 4 varied significantly across temperatures as well ($F= 9.209$, $p= 0.001$), larvae incubated at 27 ℃ had significantly lower ratio at stage 4 (marginal mean: 0.61) when compared to larvae incubated at 29 ℃ and 30 ℃ (marginal means: 0.697 for 29 ℃ and 0.676 for 30 ℃). There was a significant interaction of time by temperature by batch ($F= 13.823$, $p< 0.001$).

Cross 78 by 88 died off at 38.5 hours, there was no data for this cross at stage 5. Cross 78 by j died off at 16.5 hours, and there was no data for this cross at stage 4 and 5.

For individual cross sample, the distribution of larvae in stage 4 had similar pattern compared to the batch samples. At 27 hours, 0.489 of the larvae were in stage 4. The ratio increased to 0.884 at 48 hours. The ratio decreased afterwards. Temperature also had a main effect with the larval ratio at stage 4 ($F= 26.998$, $p< 0.001$). Similar to batch samples, individual cross samples incubated at 27 ℃

had significantly lower ratio at stage 4 when compared to the other two temperatures (marginal means: 0.513 for 27 ℃, 0.596 for 29 ℃ and 0.608 for 30 ℃). Unlike the batch samples where batch had no significant effect, cross type had a significant main effect for the individual cross samples ($F= 6.449$, $p= 0.001$). Crosses x by 78, 78 by 88 and j by88 had significantly higher larval ratio of stage 4 when compared to crosses x by 88 and x by j (marginal means: 0.608 for x by 78, 0.590 for 78 by 88, 0.679 for j by 88, 0.522 for x by 88 and 0.503 for x by j). There was a significant time by temperature by cross type interaction ($F= 5.043$, $p< 0.001$).

For batch samples, all three temperatures were significantly different at the fifth stage, the pear shape stage ($F= 56.507$, $p< 0.001$). Overall, larvae at 27 ℃ had the lowest ratio (marginal mean: 0.138). Larvae at 29 ℃ had medium ratio (marginal mean: 0.384). Larvae at 30 ℃ had the highest ratio (marginal mean: 0.496). There was no significant difference among samples from various batches ($F= 2.028$, $p= 0.127$). Across time, stage 5 larvae appeared at 50.5 hours with a very low ratio (marginal mean: 0.07), but increased to a significantly higher ratio at 74.5 hours (marginal mean: 0.59). There was a significant time by temperature by batch interaction ($F= 4.365$, $p< 0.001$).

For individual cross samples, temperature also had a significant main effect with larval ratio of stage 5 ($F= 47.586$, $p< 0.001$). Although larval ratios of stage 5 at 29 ℃ and 30 ℃ were both higher than the ratio at 27 ℃; the ratios at 29 ℃ and 30 ℃ did not vary significantly (marginal means: 0.251

for 27 ℃, 0.602 for 29 ℃ and 0.598 for 30 ℃). The time distribution of stage 5 larvae was similar in individual cross samples when compared to batch samples. Stage 5 larvae started to appear at 48 hours (marginal mean: 0.011), the ratio increased to 0.869 at 124 hours. Cross type was also a significant main effect for larval ratio of stage 5 ($F= 5.409$, $p= 0.003$). Cross j by 88 had significantly lower larval ratio of stage 5 (marginal mean: 0.303) when compared to other crosses. In contrast, cross x by j had significantly higher larval ratio of stage 5 (marginal mean: 0.599). There was a significant time by temperature by cross type interaction ($F= 6.791$, $p<0.001$).

In general, higher temperatures had lower ratio of larvae in the first 2 stages while higher ratio in the third and fifth stages. Samples from different batches did not show significant differences in larval ratios of various stages. On the other hand, samples from different individual crosses showed significant differences in larval ratios of various stages.

Figures 7A and 7B showed the surface area data of larvae from batch samples during early development. In general, larvae raised at higher temperatures (30 ℃: marginal mean: 347327μm^2) still showed slightly higher surface area than larvae raised at lower temperatures (27 ℃, marginal mean: 318828 μm^2). The main effect of temperature from two-way ANOVA test was significant ($F= 3.898$, $p= 0.022< 0.05$). The effects of time and batch on surface areas were also statistically significant. Larvae showed largest surface area at the second time point at 9.5 hours (marginal mean = 487645

μm^2), the surface area stayed at a constantly level around 300000 μm^2 afterwards. ($F= 58.265$, $p < 0.001$) Larvae in batch 2 had significantly greater surface areas (mean = 392971 μm^2) than larvae in all other three batches (around 310000 μm^2) ($F = 17.319$, $p < 0.001$). There is no significant time by temperature by batch interaction ($F= 0.610$, $p= 0.946$).

Figures 8A and 8B showed the surface area data from individual cross samples. Like for the batch samples, there was also a significant main effect of temperature on surface area for individual cross samples ($F= 7.908$, $p< 0.001$). In this case, surface area of larvae incubated at 29 ℃ (marginal mean: 328855 μm^2) was significantly lower than the surface area of larvae incubated at 27 ℃ and 30 ℃ (marginal means: 348345μm^2 for 27 ℃ and 355488 μm^2 for 30 ℃). Surface area for individual cross samples changed over time in a similar pattern compared to batch samples, having second largest area at the second time point (at 13.5 hours, marginal mean: 389295 μm^2). However this was surpassed and the greatest surface area occurred at the last time point (at 124 hours, marginal mean: 390995 μm^2). Thus time also had a main effect of surface area for individual cross samples ($F= 9.115$, $p< 0.001$). The surface area also varied greatly from one individual cross to another ($F= 19.338$, $p< 0.001$). Crosses x by 88 and x by j had significantly greater surface areas (marginal means: 373958 μm^2 for x by 88 and 371263 μm^2 for x by j). Crosses x by 78 and j by 88 had surface area in the middle range (marginal means: 332030 μm^2 for x by 78 and 341348 μm^2 for j by 88). Crosses 78 by j and 78 by 88 had

smallest surface areas when compared to other crosses (marginal means: 308177 μm^2 for 78 by j and 291546 μm^2 for 78 by 88). There is a significant time by temperature by cross type interaction (F= 1.549, p= 0.012< 0.05).

Influence of temperature on late development:

The larval ratios from 3 late developmental stages at 4 time periods of all batch samples as well as the surface area of larvae samples are shown in Figures 9A-9D.

In figure 9-A, pear shaped larval ratio varied greatly from time to time (F-time = 17.967, p < 0.001). Larvae reached the pear stage earlier at 27 ℃ compared to 25 ℃ and as a result, the difference of pear shaped larvae ratio of two temperatures was greatest at time period C. This was confirmed by a two-way ANOVA comparison of pear shaped larval ratio with temperature and time points as factors (F-temperature x time period = 3.996, p < 0.05). There was a significant interaction between time and temperature. Tukey test showed time periods Cs had a significant difference of means between two temperatures (p < 0.001) while there was no difference during other time periods. Overall the difference between the means of pear shaped larval ratio from two temperatures was insignificant (F-temperature = 1.848, p = 0.176).

At 27 ℃ larvae first reached spindle shaped stage at time period B (figure 9-B). There's an increasing number of larvae reaching this stage until time period D for both temperatures (F-time = 23.139, p < 0.001).

For spindle shaped larval ratios, the temperature and time interactions were rather consistent (F-temperature x time period = 2.292, $p > 0.05$) with larvae incubated at 27 ℃ having a higher ratio overall. Therefore, unlike the previous case, temperature played a significant role in altering the means of the spindle shaped larval ratios by elevating the ratio at 27 ℃ (F-temperature = 10.840, $p < 0.001$).

Metamorphosed larvae (figure 9-C) first appeared at time period C and increased significantly at time period D (up to about 28% of larvae, F-time = 47.037, $p < 0.001$). Both temperatures had similar ratios for metamorphosed larvae (F-temperature = 0.625, $p > 0.05$).

The surface area of the larvae (figure 9-D) increased with time at both temperatures (two-way ANOVA using logarithmically transformed data F-time = 19.549, $p < 0.001$). The surface area of larvae was significantly higher at 27 ℃ than at of 25 ℃ (F-temperature = 5.242, $p < 0.05$). The temperature induced variations were consistent at all four time periods (F-temperature x time period = 0.939, $p = 0.423$, no significant time vs temperature interaction). Therefore, temperature played a role in determining larval size by slightly increasing the surface area.

The surface area of the SECORE sample were much lower than that of the batch samples that were either incubated at 25 ℃ or 27 ℃ (Table 2) (F-SECORE vs. Batch = 67.293, $p < 0.001$). The ratio of pear shaped larvae was also significantly lower in SECORE samples (F-SECORE vs. Batch = 6.465, $p < 0.05$). There was

no significant difference between ratios of spindle shaped larvae. The ratio of metamorphosed larvae was much higher in SECORE samples compared to batch samples (F-SECORE vs. Batch = 11.611, $p < 0.05$).

In the following, the batches were combined by site to investigate site effects (House beach: batch 1 and batch 3and Bajo Gullardo: batch 4 and batch 5, Figures 10A-10D).

Batch 1/3 and 4/5 generally showed similar trends through time. However, Batch 4/5 had much higher ratio of pear shaped larvae than batch 1/3 at time point D at 25 ℃ (Figure 10A, F-batch number = 9.502, $P < 0.001$). Other than that, temperature had induced significant variation among batches for the pear shaped larval ratio (F-temperature x batch number = 3.962, $P = 0.009$). For spindle shaped larval ratios, batch number did not play a significant role in causing the variations (Figure 10B, F-batch numbers = 2.403, $P = 0.070 > 0.05$). It appeared that Batch 1/3 had much higher ratio of metamorphosed larvae than batch 4/5but this was not supported by statistics (Figure 10C, F-batch = 2.200, $P = 0.091$). However, larvae from different batches had significant variations in surface area (Figure 10D, F-batch = 4.938, $P = 0.003$).

Discussion:

Fertilization Rate:

Since gametes were mixed and fertilized before they were applied to different temperatures, temperature must be excluded as a factor influencing fertilization rate in this experiment. This was corroborated in statistical tests. However, temperature can play an important role in determining the date in which colonies release gametes. In fact, aquariums have been trying to manipulate spawning via controlling temperature. (Petersen, Falcato, Gilles & Jones, 2007).

Interestingly, different batch samples showed a significant variation in fertilization rate (figure 3). Specifically batch 2 had the highest fertilization rate while batch 3 had the lowest. This seemed to be a consistent pattern in several other results. Batch 2 had the highest ratio in the fifth developmental stage while batch 3 had the lowest. Batch 2 also had the greatest surface area among the four batches. Therefore, these results suggest that certain individual cross or parent combination can outperform others in early development; and that fertilization rate is a great indicator as to which cross will perform the best later on. This trend is again confirmed in the individual cross data. The two crosses that had the highest fertilization rates (figure 4), crosses x by 88 and x by j, also had greater surface areas and higher larval ratios in the fifth developmental stage. The two crosses with low fertilization rate, 78 by j and 78 by 88, did not survive past 40 hours after fertilization. The other cross that had low

fertilization rate, x by 78 however, did survive longer than those other two crosses. This association of fertilization rate, larval size and development may be significant; it implies that parentage significantly affects the survival rate of larvae. One explanation for this observation is the maternal effect that links the fertilization success to the quality of the eggs being fertilized. Larger eggs can be considered more mature and provide more nutrition (Beiring & Lasker, 2000). This might explain why higher fertilization rate was associated larger sized larvae.

In the study of Beiring and Lasker (2000), they also discussed how colony size may influence the production of eggs. For the species *Plexaura flexuosa,* larger colony size increased the volume and amount of the eggs produced and released during spawning. Due to this, a great amount of sexual reproduction in the natural environment was actually achieved by only a small proportion of colonies in the population (Beiring & Lasker, 2000). This may help to explain why certain individual crosses had very low fertilization rates. The donor colonies may not have been large or healthy enough to produce high quality gametes.

The difference in fertilization rates between batch samples and individual cross samples implied the importance of genetic diversity during sexual reproduction of *A. palmata*. Batch samples that have more genetically diverse gametes had higher fertilization rates compared to individual cross

samples. Individual coral genotypes might differ in their compatibilities and thus genetic diversity likely plays an important role in coral sexual reproduction.

Unfortunately, diversity is often threatened or reduced in unfavorable environments. Baums, Miller and Hellburg (2006) stated in their study that lower phenotypic diversity is found in marginal environments. This lowers the chance of finding a compatible sexual partner. Furthermore, Gilmour (1999) stated that reefs experiencing high level of sedimentation tend to have lower species diversity. They also have lower growth rates that reduce colony size. This in turn would reduce the reproductive input of these colonies (Beiring & Lasker, 2000).

Early Development:

Temperature played a significant role in early embryo development of *A. palmata*. In general higher temperatures increased the developmental rate. The largest developmental rate difference occurred between 27 ℃ and the two higher temperatures (29 ℃ and 30 ℃, figures 5A-D, 6A-F). However in stage 5, the pear shape stage, larvae incubated at 30 ℃ had a higher ratio than samples from both lower temperatures at around 70 hours after fertilization. Larvae from 27 ℃ entered the 5^{th} stage very slowly while larvae from 29 ℃ developed into this stage faster. At around 120 hours after fertilization, larvae from 29 ℃ and 30 ℃ had similar ratios in stage 5. This stage is very important in *A. palmata* embryogenesis because as cilia develope on the larval surface (Randall & Szmant, 2009), larvae also

experience a boost in swimming speed at around 100 to 120 hours (unpublished data from Baums lab, collected by S. J. Giri). The significance is that larvae entering the pear shape stage would gain advantages in swimming speed as well as development. The advantage in swimming speed would help larvae travel further in search of a settlement substrate. The advantage in development would allow larvae to enter the next spindled stage faster so they can be morphologically ready for settlement. While a higher temperature may benefit larvae dispersal, it may also limit it by depleting the biological fuel stored in larvae at a faster rate. Further study must be carried out to determine the overall effect of rising temperature on larval dispersal. Dispersal of larvae is related to reef resilience and recovery and it is a key component in the connectivity pattern of reefs in a local environment (Underwood, Smith, van Oppen & Gilmour, 2009). The study on effect of temperature on embryogenesis is a key link to the effect of temperature on larval dispersal.

Larval development pattern was significantly different for samples from different individual crosses, but was not significantly different for samples from different batches. The diversity of the batch samples might have evened out the differences in individual crosses. Negri, Marshall and Heyward's study in 2007 on *A. millepora* reported greater amount of aberrations above 30 ℃. A point of interest would be whether samples from different individual crosses have different amount

of aberrations during high heat stress and how should that be associated with fertilization rate and larval size. Studies in the future should therefore include temperature treatments above 30 ℃.

Late Development:

The observations supported the hypothesis that higher temperature induces higher developmental rate in late development as well (figures 9A-9D). While the pear shaped larval ratio was less influenced by temperature, the higher temperature increased the rate at which larvae entered spindle shaped stage. This could be a benefit to coral larvae because they would spend less time in the open water and develop into the spindled stage earlier. The spindle shaped stage is the stage where larvae are ready for settlement (Ball, et al. 2002). Therefore, if sea water temperature does not exceed a certain critical level, higher temperature can accelerate the developmental process and allow earlier settlement. Munday, et al, (2009) mentioned that the faster developmental rate induced by higher temperature is also beneficial when it comes to increasing reef connectivity. But if the temperature becomes too high (30-31 ℃), the plank tonic larval duration (O'Connor, et al. 2007) would decrease and therefore limit larval dispersal. Further, high temperatures are associated with increased larval mortality rates though such rates were not measured in this study. Bassim and Sammarco reported in 2003 that *Diploria strigosa* larvae had 50% to 70% greater mortality at 30-32 ℃ sea water temperature (Bassim & Sammarco, 2003). This is consistent with the studies of Munday, et al (2009), and

O'Connor, et al (2007), as these authors also concluded that a very high temperature (>29 ℃) will reduce the dispersal and mobility of larvae. Putnam, et al (2008), suggested that a temperature as high as 29 ℃ reduced the percentage of swimming larvae by 81% (Putnam, et al, 2008). A major consequence of a very high temperature (>29 ℃) is the increase in the ratio of prematurely metamorphosed larvae. Further, high temperature might also increase post-settlement mortality (Nozawa & Harrison, 2007).

While pre-settlement mortality rates were not assessed directly here, larval size might serve as a proxy. Larval size was very similar at lower temperatures (25 ℃ and 27 ℃) but decreased significantly at 29 ℃ (Table 2). We suggest here that increased metabolic rate depleted the resources stored in the larvae and caused the shrink in size. While in this study, spawning parents had experienced the same temperatures prior to the onset of the experiment, larval size can also be influenced by the reproductive effort of the adults. A study (Kojis, 1986) done in the Great Barrier Reef in Australia showed that colonies in lower latitudes where temperature is higher spent less effort in each reproductive cycle although they have more reproductive cycles each year. Bleaching events associated with higher temperature can also lower the coral's reproductive efforts because energy is invested in regeneration activities (Henry & Hart, 2005). Therefore, the individual history of the parent colonies (sickness, age) could potentially decrease larval size by reducing gamete sizes.

The ANOVA test results suggested a slight increasing trend of surface area. This observation can be explained by the appearance of metamorphosed larvae. The change in shape could geometrically cause an increase in surface area. One observation quite interesting from our studies is that the larvae incubated at 27 ℃ actually showed a greater surface area when compared to the larvae incubated at 25 ℃. Since larvae incubated at 27 ℃ did not show a significantly higher ratio of metamorphosed larvae at time point D, the greater surface area could indicate a better development condition when compared to the other temperature.

In conclusion, higher temperature increased the development rate of larvae while it may decrease their survival rate. High temperature does not just allow larvae to reach the spindle shaped stage faster, but also causes premature metamorphosis. However even the lower temperature did not seem to prevent premature metamorphosis entirely.

In terms of survival rate, barely any larvae were sampled at later time points for 30 ℃ because of poor survival rate and possibly poor sampling methods. The larvae incubated at 29 ℃ at the last time point had the greatest number of metamorphosed larvae and smallest larval size. Both indicate a decrease in fitness and could lead to lowered survival rate.

Observations show that 27 ℃ is the best incubation temperature during embryonic development for *A. palmata*. Larvae incubated at this intermediate temperature had largest sizes, fast development

rate to ready the larvae for settlement and low incidents of premature metamorphosis. Larvae incubated at 27 ℃ have an advantage over larvae incubated at 25 ℃ for higher speed of development and advantage over 29 ℃ for less premature metamorphosis and greater larval size.

Our hypothesis was supported by the results. Temperature caused larvae to enter later developmental stages earlier and higher temperature had a significant inductive effect on premature larval metamorphosis.

The batch samples had great variation within the group. ANOVA test results showed that at 25 ℃, batch 4 was significantly different from batch 1 and 5 and at 27 ℃, batch 3 was significantly different from batch 1 and 4 for pear shaped larvae. Figures 10B-D also showed that they had great variation in other ratios. Although batch 1 and 3, 4 and 5 were collected at respectively the same locations and created in the same fashion, a significant difference occurred between 1/3 and 4/5. This could be a result of local adaptation to different conditions the two batches were sampled from.

In future studies, a longer incubation time could be set up to further investigate the temporal effects on coral larvae. Further, genotyping of individual larvae could help to determine the parentage of larvae in batch cultures to see if certain individuals stand out among others. This will help us to study what role competition plays to alter the effects of temperature on larvae.

Conclusion:

This report shows that higher temperature not exceeding 30 ℃ can be beneficial for *A. palmata* as it increases developmental rate, allowing larvae to enter the pear shaped stage earlier and acquire higher swimming speed earlier. This would eventually benefit their settlement and dispersal. In later development (after 150 hours), a lower temperature is more beneficial because it reduces the rate of premature metamorphosis. The optimal temperature pattern for development may vary in other coral species. Therefore it is very important study the effect of temperature pattern for early coral development in coral restoration projects. Optimal temperature pattern should increase both the development rate and success of settlement of coral larvae.

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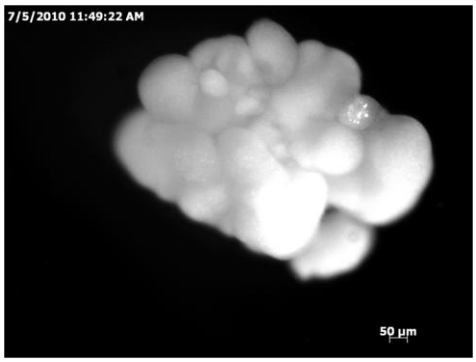
Tables and Figures:

Table 1: Number of Larvae for different time periods, temperatures and batch numbers. *Given are the sample sizes analyzed for each sampling period (A – D), temperature treatment (25 ℃ and 27 ℃) per batch (batch 1/3 and 4/5).*

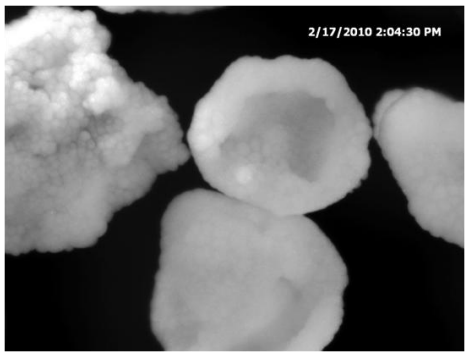
Temperature	25 ℃	25 ℃	27 ℃	27 ℃
Batch number	1/3	4/5	1/3	4/5
A(59-81 hours)	20	121	45	350
B(90-116 hours)	134	121	136	214
C(131-161 hours)	280	92	205	276
D(186-223 hours)	192	36	89	38

Table 2, Mean of ratios of 3 developmental stages and surface area for SECORE samples in comparison with batch samples, *Ratio of metamorphosed larvae only had time points C and D because time points A and B did not contain any metamorphosed larvae, the cells contain mean values ± standard deviations.*

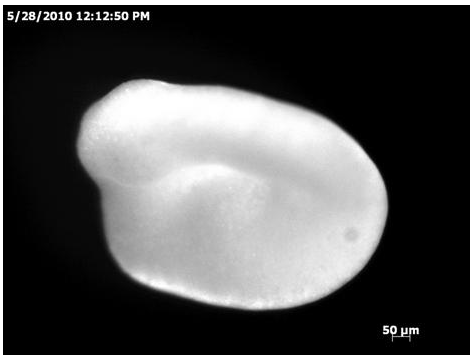
Source	Surface area (m^2)	Ratio of pear shape	Ratio of spindle shape	Ratio of metamorphosed
SECORE	2.62E-07 ±8.51E-08	0.38 ±0.27	0.26 ±0.33	0.40 ±0.15
BATCHES	3.83E-07 ±5.54E-08	0.53 ±0.24	0.17 ±0.22	0.06 ±0.17



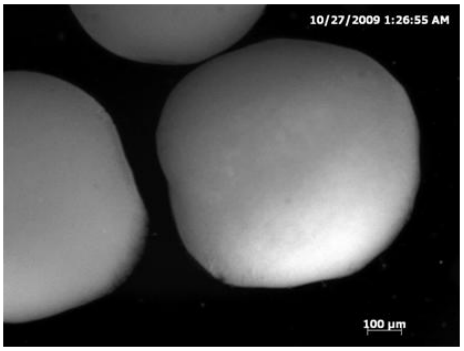
Stage 1



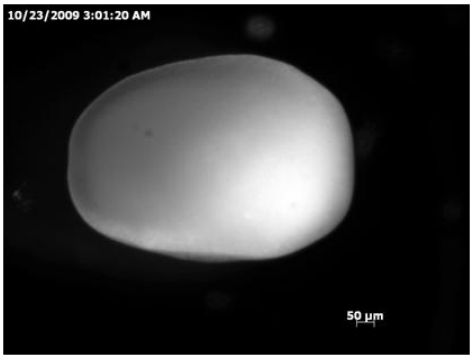
Stage 2



Stage 3



Stage 4



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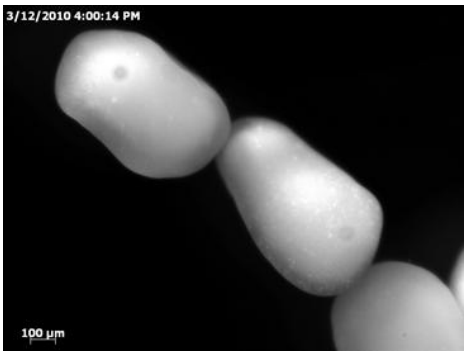
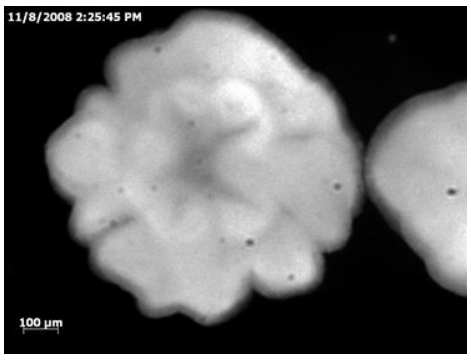


Stage 6



Stage 7

Figure 1, Developmental stages 1-7 of *A. palmata* larval development: *Stage 1: Pre-morula and morula stage, Stage 2: Plate stage, Stage 3: Gastrulation stage, Stage 4: Round stage, Stage 5: Pear shape stage, Stage 6: Spindle shape stage, and Stage 7: Metamorphosed Stage. A scale is given at the bottom of the graphs. Larvae ranged in diameter from 500µm to 700µm.*

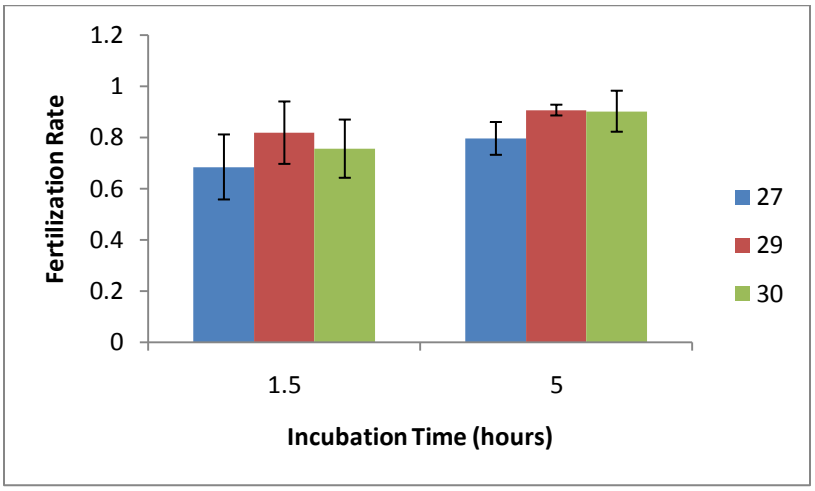


Figure 2, Fertilization Rate Under 3 Temperatures vs Time, *different colored bars represent average fertilization rate from 4 replicates from different temperatures, the error bars are the standard deviations.*

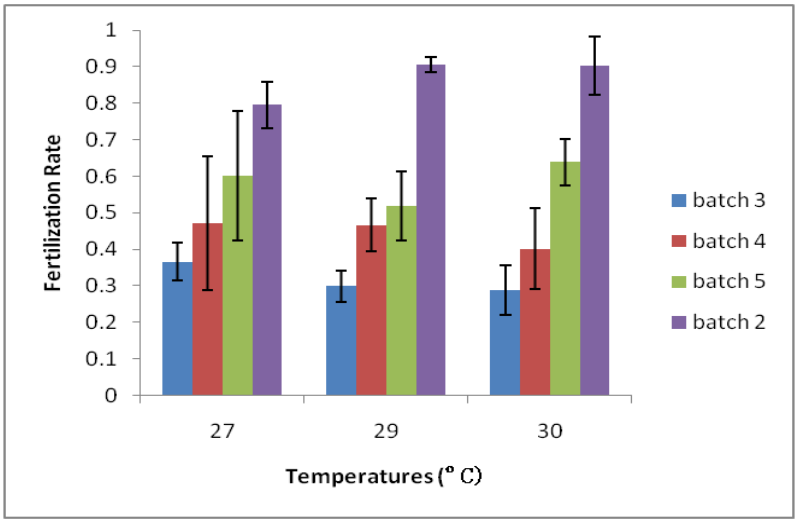


Figure 3, Fertilization Rate of Batch Samples 2, 3, 4 & 5 after 5 Hours of Incubation vs. Temperature, *in this figure the x – axis represents the 3 different temperatures in which larvae were incubated, different bars represent the average fertilization rate of 4 replicates from different batch samples.*

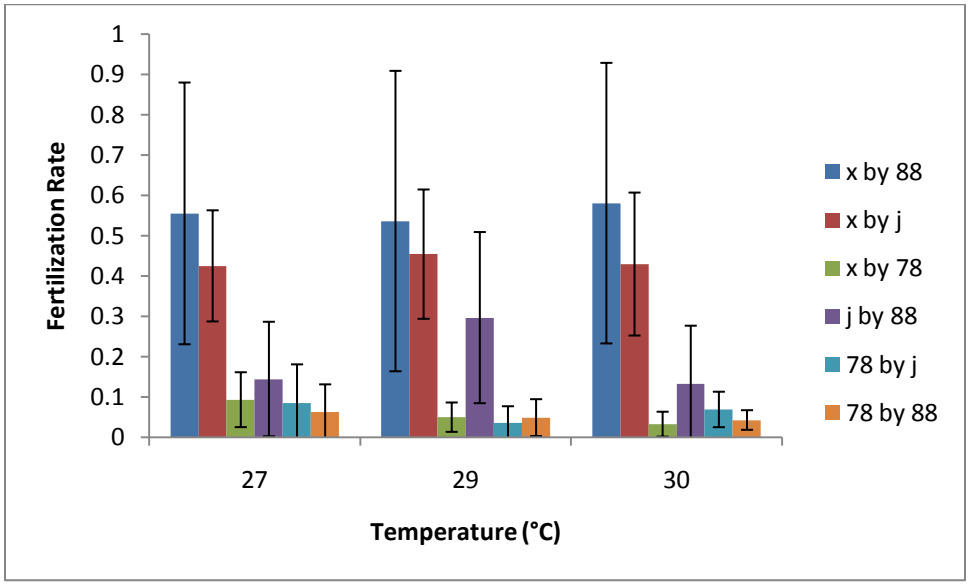
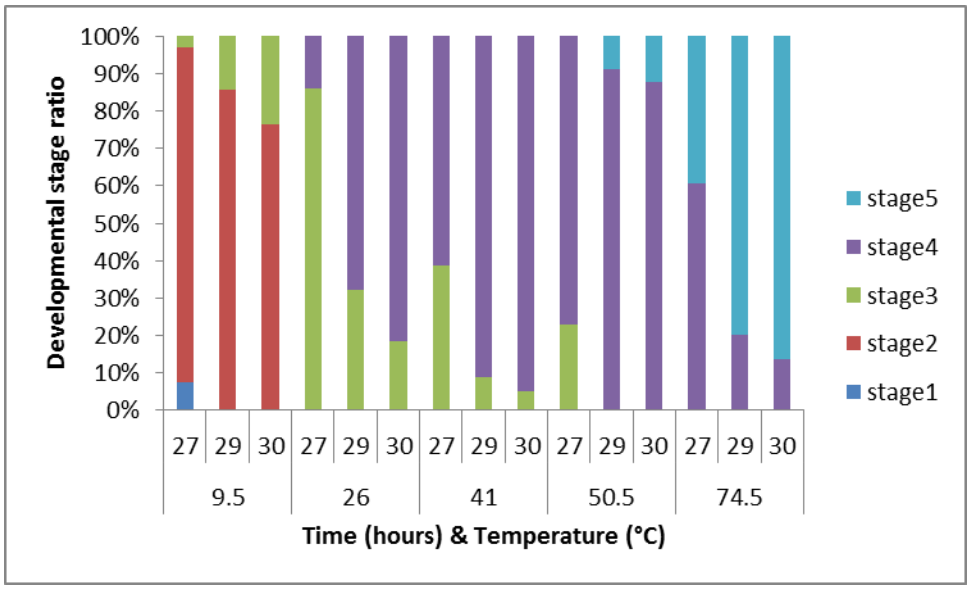
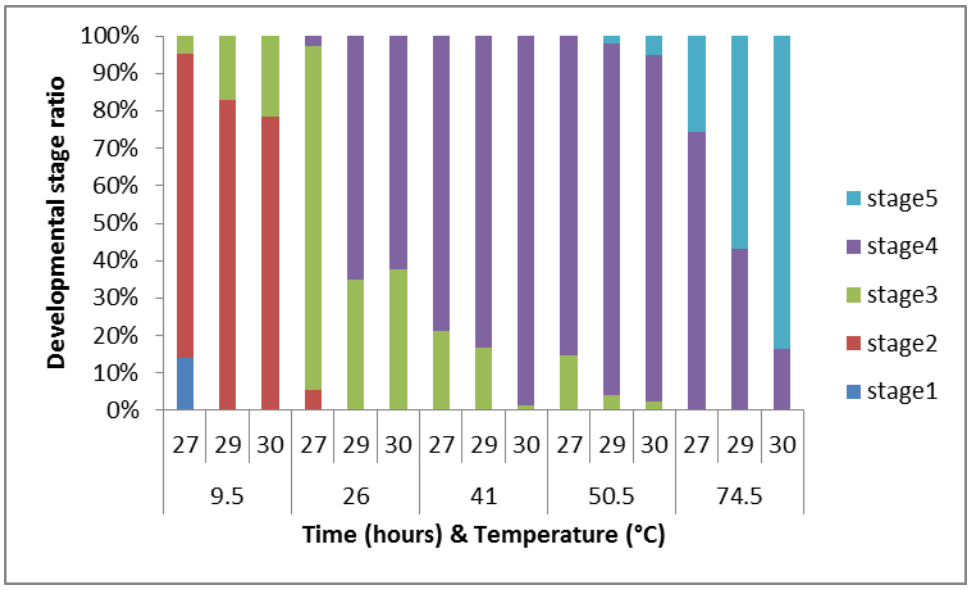


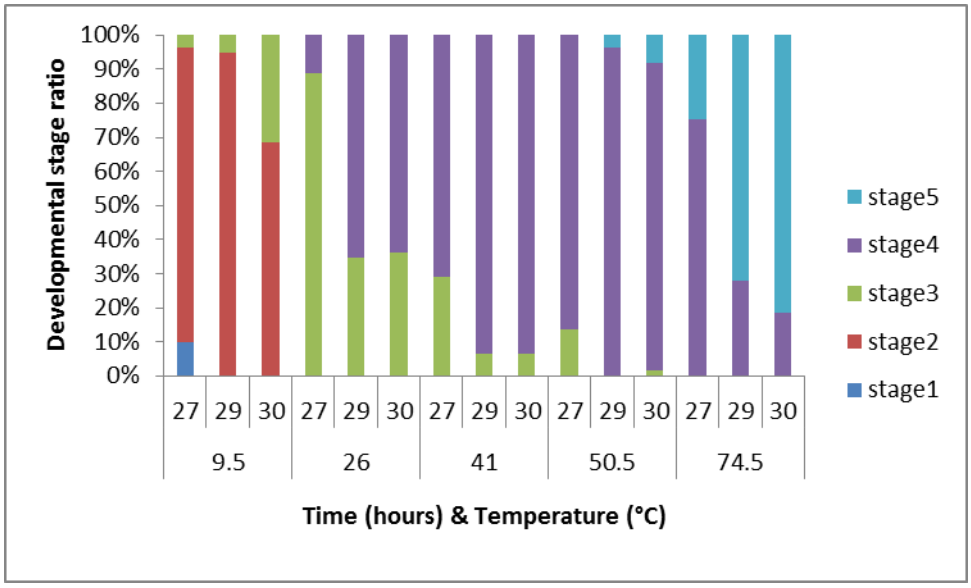
Figure 4, Fertilization Rates for Individual Crosses vs. Temperature, *error bars represent standard deviations.*



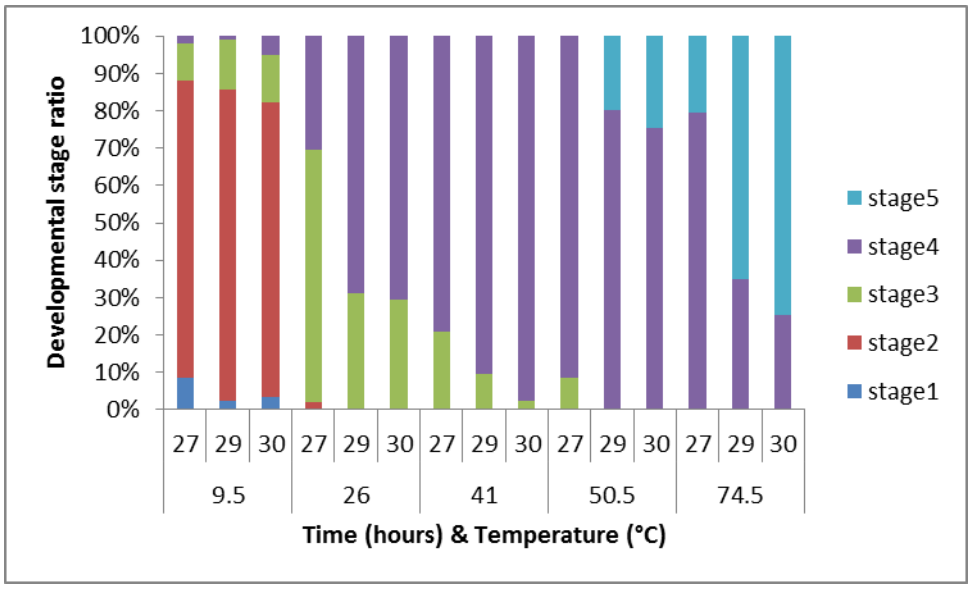
5A-Batch 2



5B-Batch 3

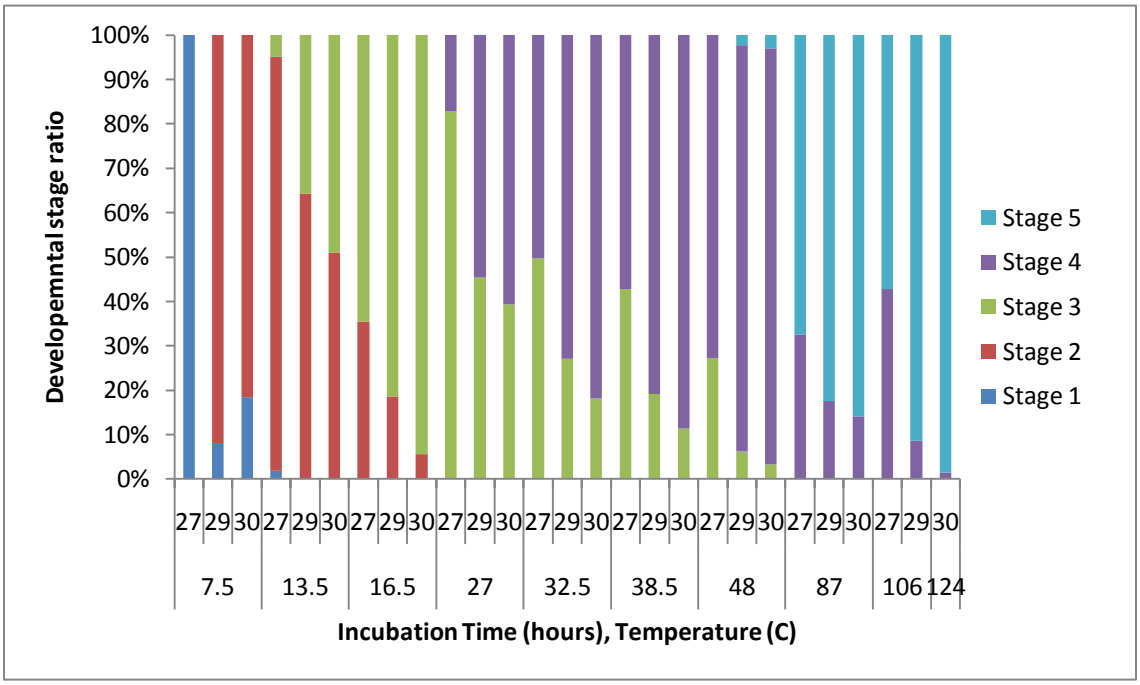


5C-Batch 4

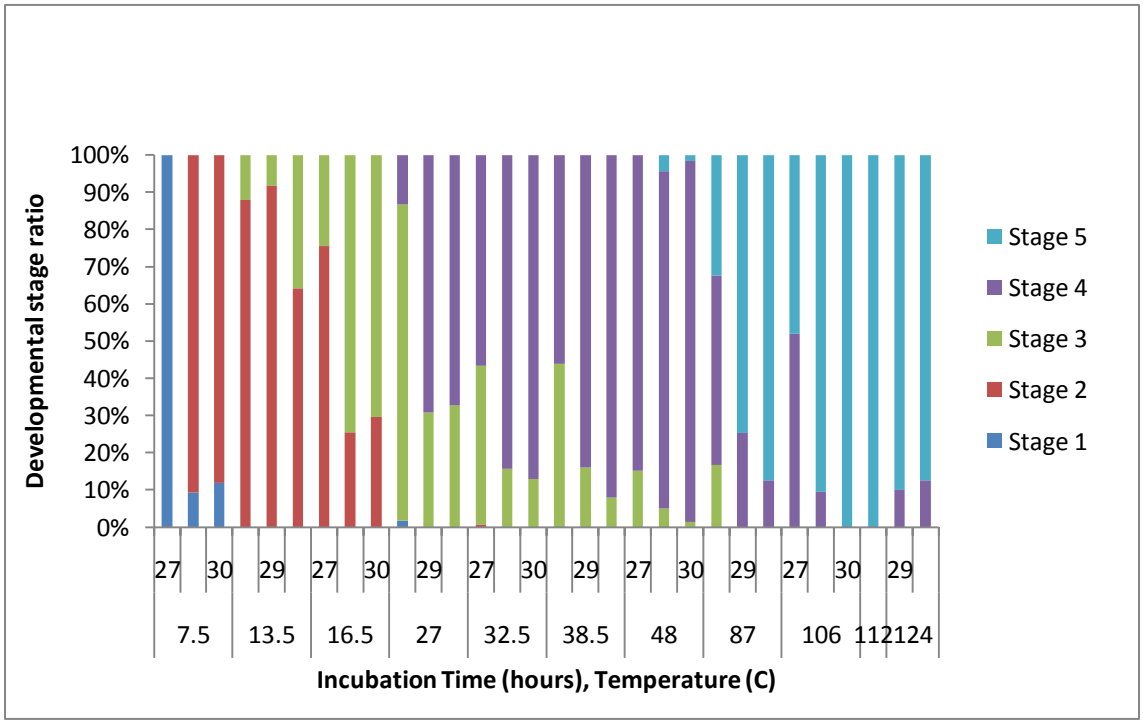


5D-Batch 5

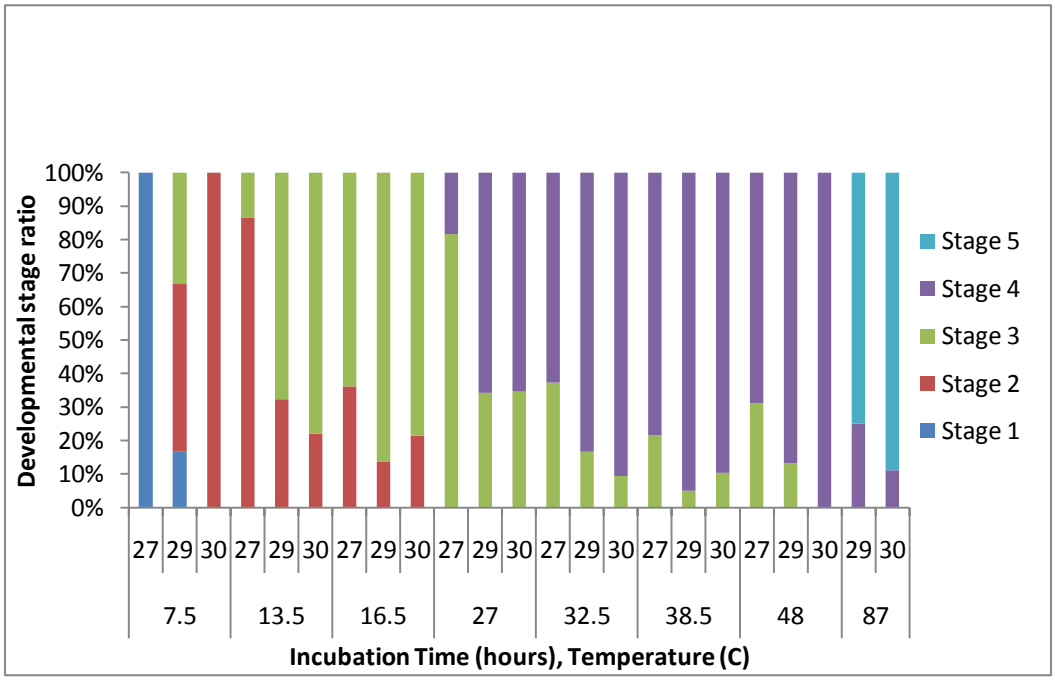
Figures 5A-5D, Developmental Stage Ratio for Batch Larval Samples, *A through D correspond to batches 2 to 5, the y-axis consists of the ratios of developed larvae in each of the stages, the x-axis is divided by time points and then temperature.*



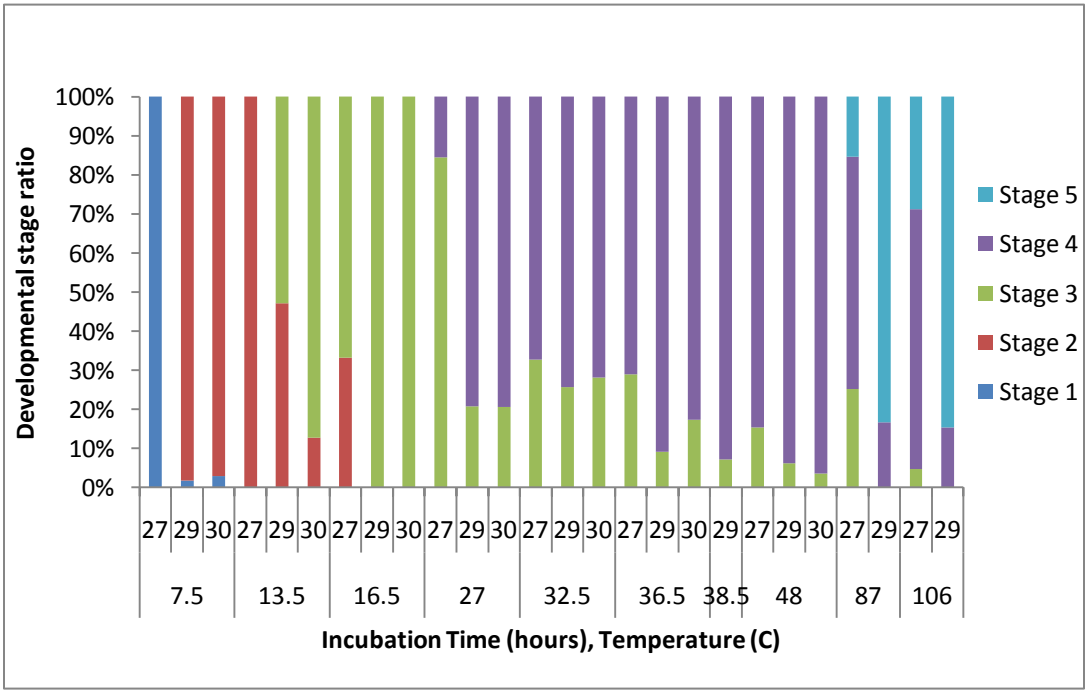
6A-x by 88



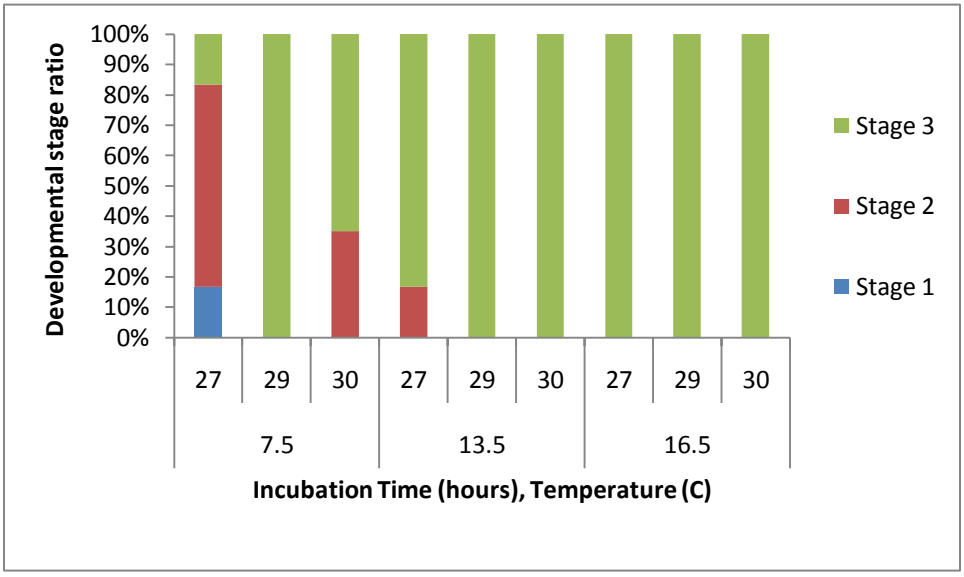
6B-x by j



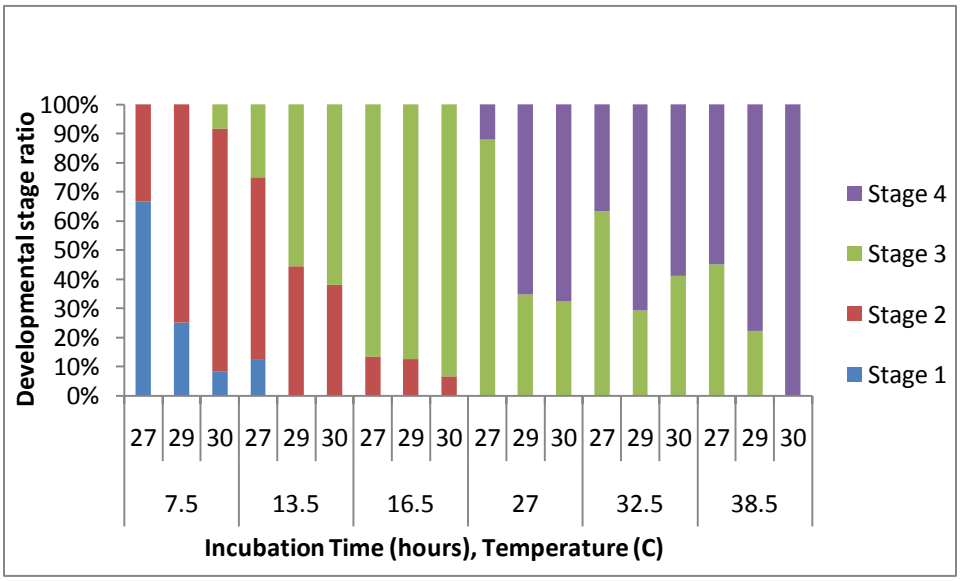
6C-x by 78



6D-j by 88

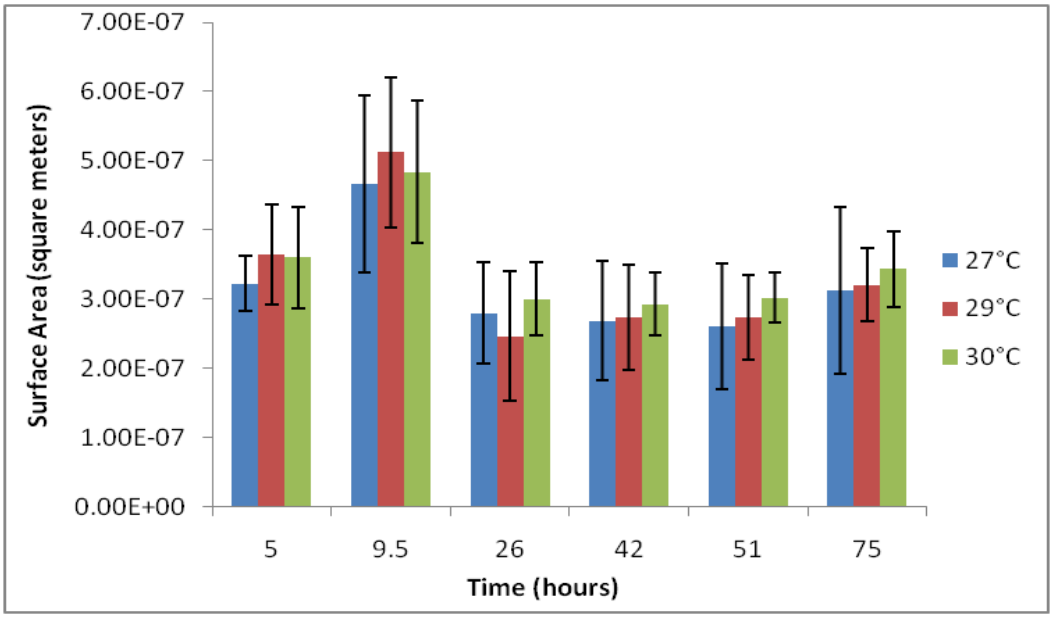


6E-78 by j

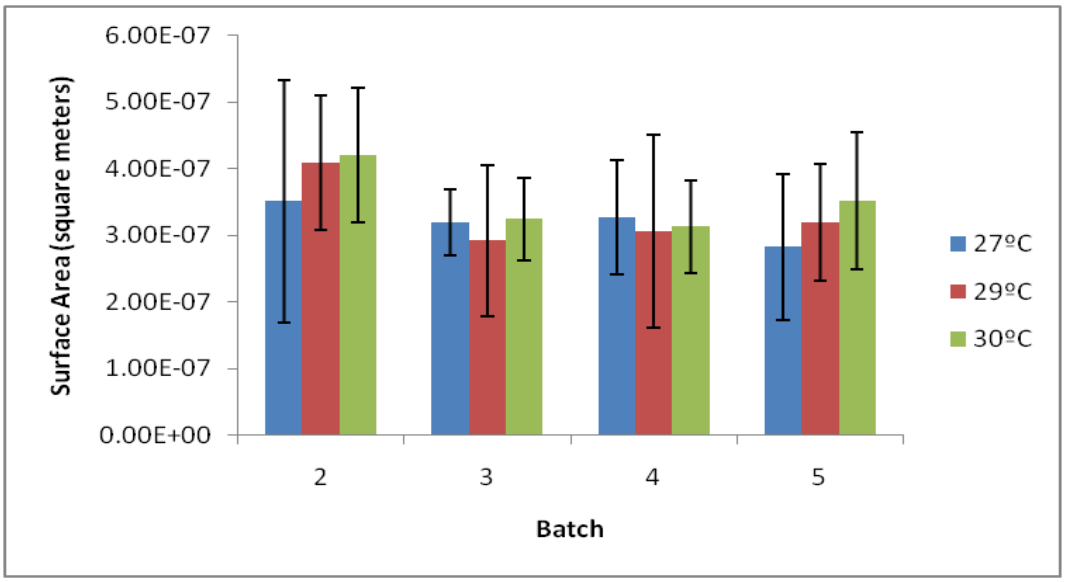


6F-78 by 88

Figures 6A-6F, Larval Ratio in Various Developmental Stages for Individual Crosses, *A through F correspond to individual crosses from x by 88 to 78 by 88, the y-axis consists of ratio of developed larvae in each of the stages, the x-axis is divided by time points and then temperature.*

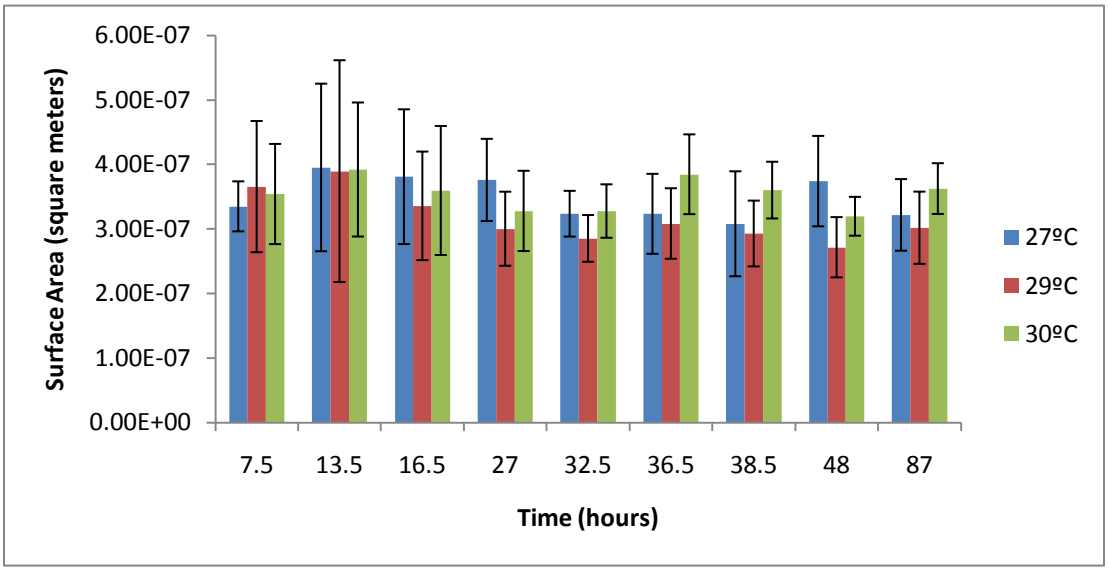


7A

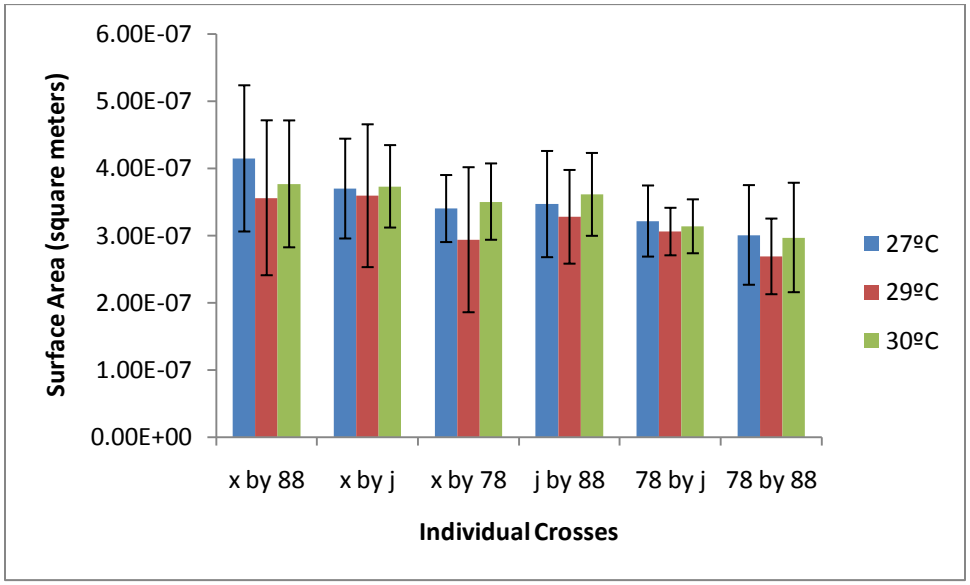


7B

Figures 7A and 7B, Surface Area of Larvae from Batch Samples, *7A illustrates the surface areas of larvae at various time points while 7B illustrates the surface areas of larvae from various batches. Error bars are standard deviations.*

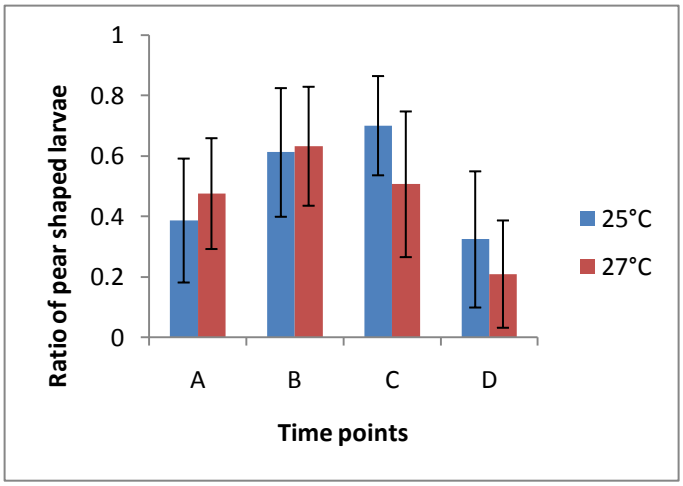


8A

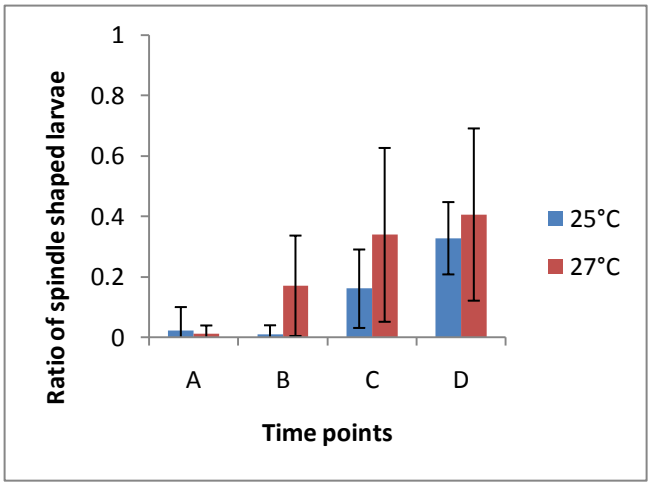


8B

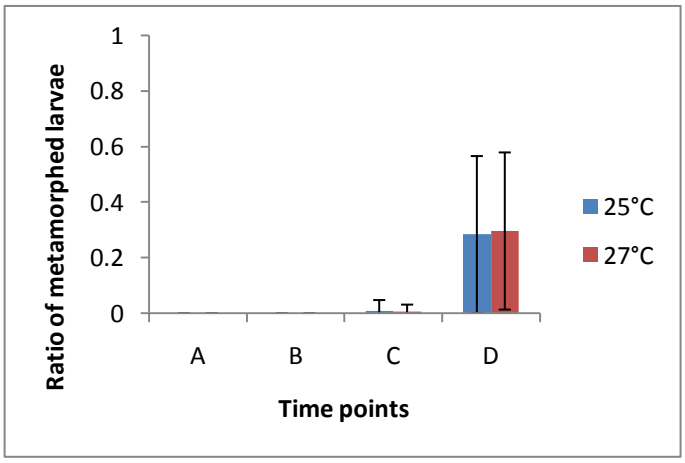
Figures 8A and 8B, Surface Area of Larvae from Individual Crosses, *8A illustrates the surface areas of larvae at various time points while 8B illustrates the surface areas of larvae from various individual crosses. Error bars represent standard deviations.*



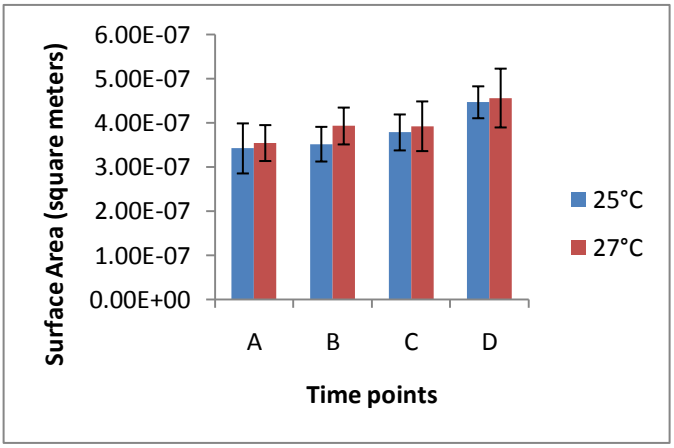
A



B

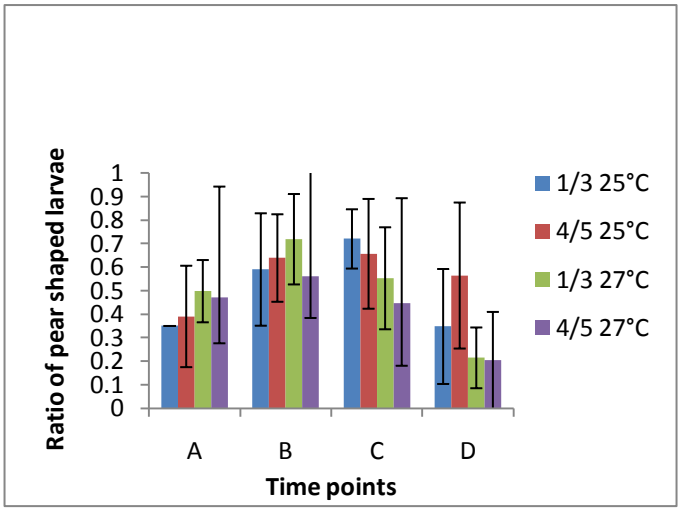


C

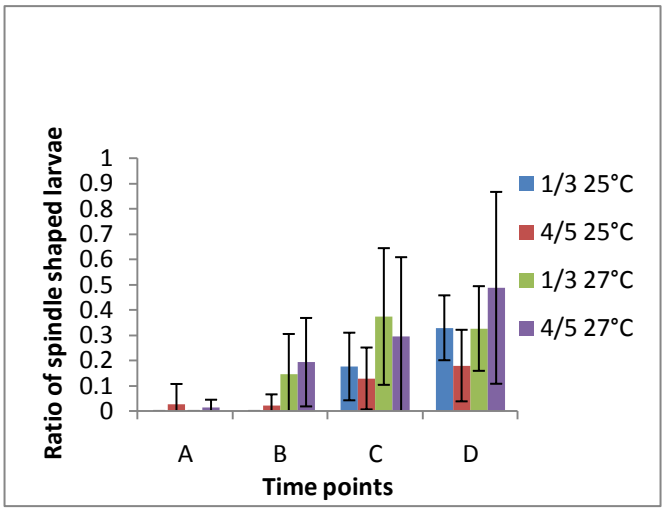


D

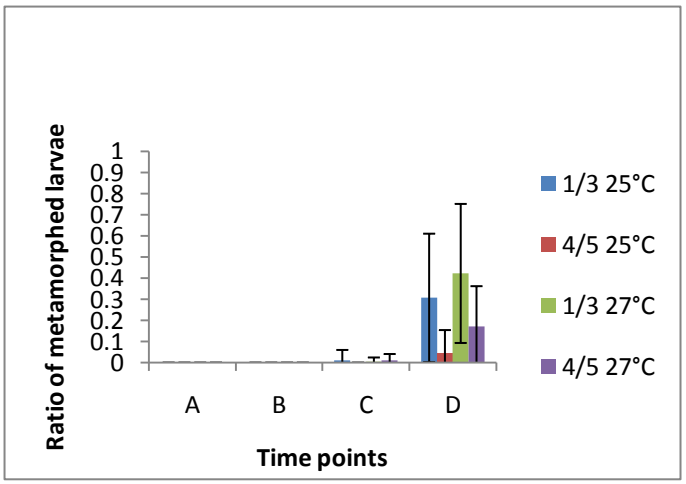
Figures 9A-9D: Ratio of 3 late larval developmental stages and surface area versus incubation time of all batch samples. *The time points correspond to the time ranges in table 1 in. The error bars are standard deviations. Each panel (A, B and C) represents a different development stage of the larvae (A – pear shaped, B – spindle shaped, C metamorphosed) while D represents the surface area.*



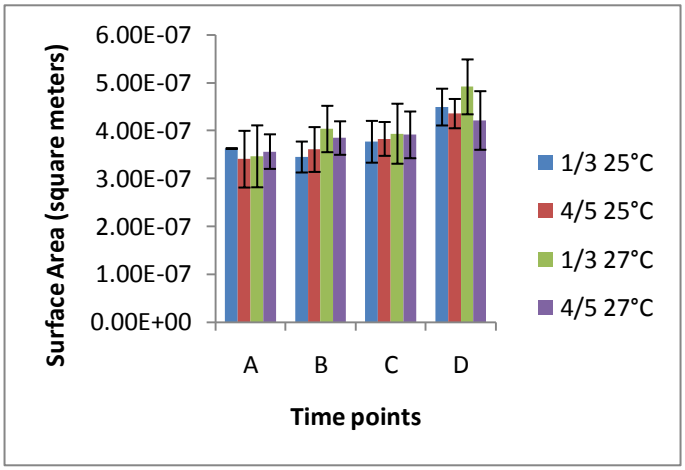
A



B



C



D

Figure 10A-10D, Ratios of 3 late developmental stages and surface area of larvae of batch 1/3 and 4/5, *the time points are correspondent with the time ranges in table 1 in methods section. The error bars are standard deviations. Each panle (A, B, C or D) represents a different measurement of the larvae.*

November 15th, 2010

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