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Inducing Heterochronic Shift in *Ciona Intestinalis*

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INDUCING HETEROCHRONIC SHIFT IN

CIONA INTESTINALIS

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ABSTRACT

Solitary and colonial ascidians show heterochrony in the development of adult organs. This project is a first step towards reproducing this heterochronic shift using *Ciona intestinalis* as a model and gaining insight into the number of genes involved in this process. In the first part of this thesis, an inducible transgene was constructed to reproduce the shift. The transgene was designed with a heat shock protein (HSP70) promoter to induce the expression of a gene at any time in development. A control transgene was constructed, but after two experiments, the transgene did not show the ability to be induced. With a possible mutation in the promoter, future experiments will address this issue and create a functional inducible transgene. The second part of this project describes the development of an easy and reliable protocol for inducing early metamorphosis. Potassium chloride (KCl) and heat shock were used to induce early metamorphosis in the solitary ascidian, *Ciona intestinalis*. KCl alone was unable to induce early metamorphosis. The heat shock experiments look to be a promising way to induce early metamorphosis. To create a reliable protocol, more trials need to be performed.

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CHAPTER 1

Introduction and Background

Solitary and colonial ascidians show evolutionary heterochrony in the development of adult organs. This thesis describes a first step towards reproducing this heterochronic shift and gaining insight into the number of genes in this process. Two steps were involved in the project: (a) construction of an inducible transgene; and (b) development of an easy and reliable protocol for inducing early metamorphosis. In this chapter, context is provided for Chapters 2 (construction of an inducible transgene) and 3 (protocol for early metamorphosis induction).

Heterochrony

Heterochrony, a major mode of morphological evolution, is the evolutionary change in developmental timing or rate (McNamara *et al.* 2012). It is generally separated into two types: paedomorphosis and peramorphosis. Paedomorphosis is a decrease in degree of development from ancestor to descendant, while peramorphosis is an increase in degree of development from ancestor to descendant. Each can be produced by different processes, involving the rates of development and/or the onset or offset of growth (McNamara *et al.* 2012). This project investigates the idea of predisplacement, the earlier onset of growth, in ascidians.

Ascidian Development

Ascidians, a type of tunicate, are sessile chordates. It had been proposed for some time (Romer, 1972), but was recently reaffirmed by phylogenomics (Dehal *et al.* 2002),

that tunicates are the closest living invertebrates to vertebrates. They start out as free-swimming larvae with a dorsal central nervous system and notochord. However, soon after the larval stage, they go through rapid metamorphosis becoming sessile adults, with a different body plan. The timing of this metamorphic event varies. It is between two ascidians, a solitary and a colonial, that a change in developmental timing is witnessed.

Both solitary and colonial ascidians (sexually reproducing) start out as free-swimming larvae and metamorphose into sessile adults. Their development can be seen as a two-part body plan consisting of somatic and visceral development (Romer, 1972). The visceral organ system consists of the gonads, gut, circulatory system and muscles and nerves associated with these organs. The visceral system is important for feeding and reproduction. The somatic organ system, important for movement and activity in the environment, is the central nervous system, sensory organs, notochord and locomotory muscles. The solitary ascidian, *Ciona intestinalis*, expresses genes at the larval stage for somatic structures and development of visceral organs at metamorphosis. In the colonial ascidian, *Didemnum vexillum*, both visceral and somatic organs develop at the larval stage, before it has undergone metamorphosis (Jeffery, 2007). Since coloniality is thought to have arisen at least three times independently from solitary ascidians (Zeng *et al.* 2005), this shift in development may be important for life as a colonial species. This difference seems to involve heterochronic shifts in gene activation which have led to different life histories for *C. intestinalis* and *D. vexillum* (Figure 1). This heterochronic shift is defined as predisplacement, a form of peramorphosis, or earlier onset of growth.

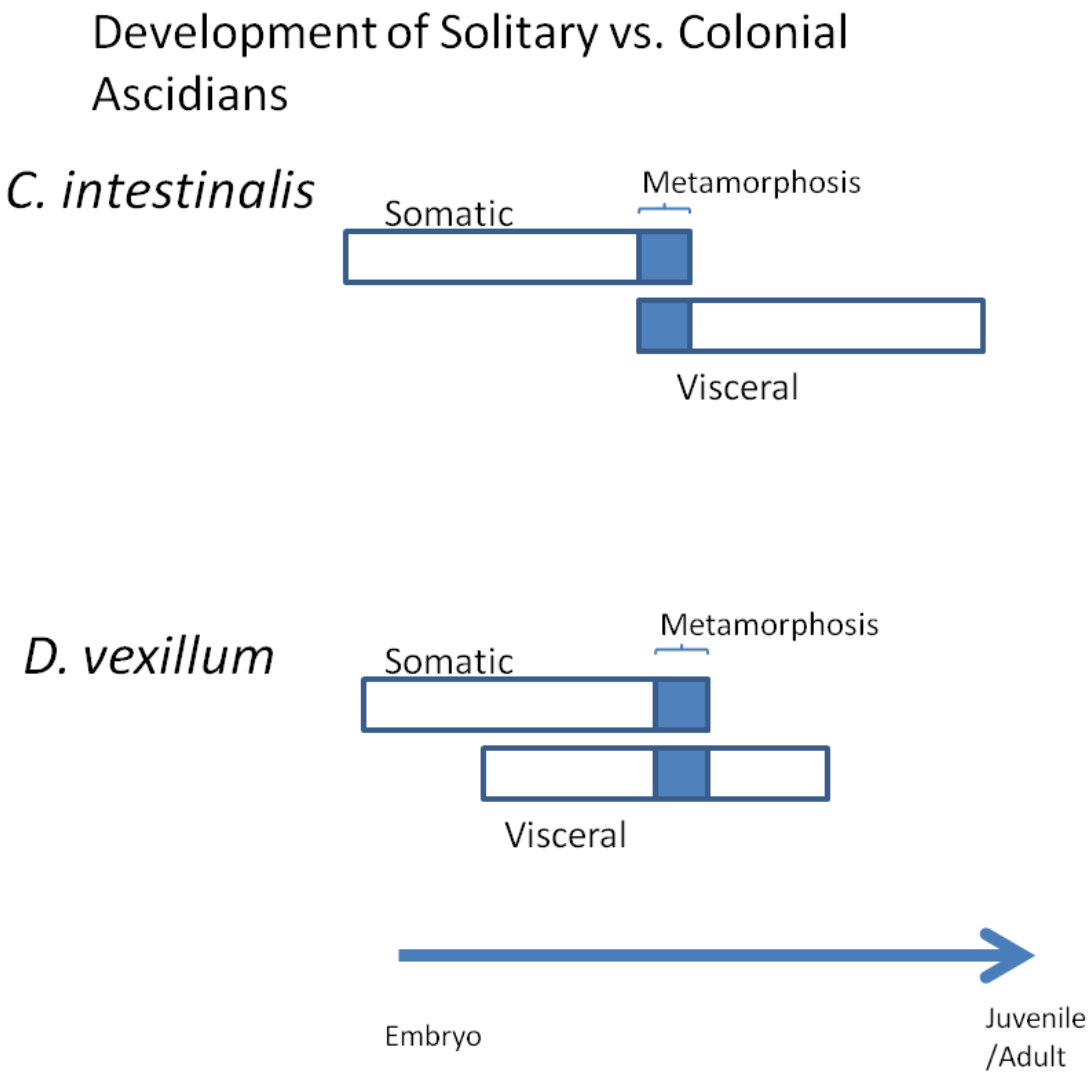


Figure 1. Development of solitary and colonial ascidians. Comparison of the developmental sequence for the solitary tunicate, *C. intestinalis*, and the colonial tunicate, *D. vexillum*. Development of visceral organs occurs before metamorphosis in *D. vexillum*, whereas the visceral organs do not start to develop until after metamorphosis in *C. intestinalis.* Based on an idea in Jeffery, 2007.

Ciona intestinalis vs *Didemnum vexillum*

Ciona intestinalis is a widely used model organism in developmental biology. Its genome, which has been fully sequenced, is small (~160 Mbp/haploid genome; Corbo *et al.* 2001) and is easily accessible on browsers like ANISEED (Ascidian Network for *In-situ* Expression and Embryological Data; Tassy *et al.* 2010). Its phylogenetic position makes it a good model organism for biomedical sciences (Stolfi *et al.* 2012). Its rapid development, the ability to obtain eggs and the ease of fertilization and manipulation (including transgenics) of the embryos have made it a choice model organism for developmental biology.

Less research has been done on *D. vexillum*, an invasive tunicate, and there is less accessibility of obtaining eggs and performing experiments. The developmental difference, however, makes it a good organism to contrast with *C. intestinalis*.

Metamorphosis and *Ci-Meta1*

The dynamic changes ascidian larvae undergo during metamorphosis are complex, and little is known about the pathways involved (Nakayama-Ishirmura *et al*. 2009). According to Cloney (1982) and Nakayama-Ishirmura *et al*. (2009), there are ten basic events in metamorphosis. They include secretion of adhesives by the anterior of the trunk (papillae), retraction/regression of the papillae and tail, loss of the outer layer of the tunic, emigration of blood cells, rotation of organs, expansion of the tunic, retraction and phagocytosis of sensory vesicle and release of organ rudiments. Previous research focused on the papillae, which are important for sensing stimuli and are thought to be the control center for initiating metamorphosis (Figure 2; Satoh, 1995; Eri *et al.* 1999).

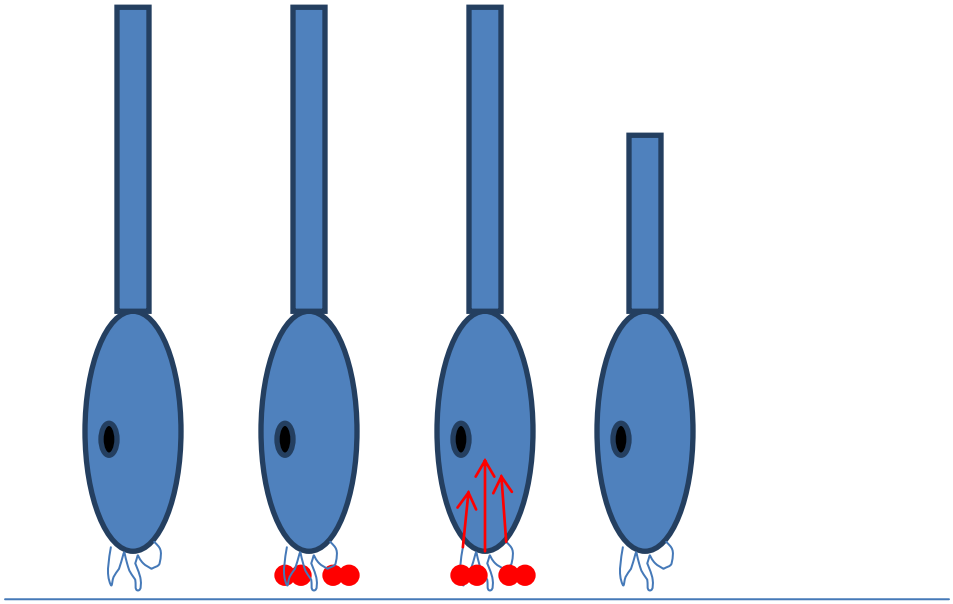


Figure 2. Theory of metamorphosis signaling in *C. intestinalis*. *C. intestinalis* attaches to a substrate, an initial cue for metamorphosis (red dots). This cue in turns releases a signal or activates a possible regulatory gene (arrows) initiating metamorphosis or formation of adult organ. Resorption of the tail is an initial sign of metamorphosis.

A number of possible genes are involved in *C. intestinalis* metamorphosis, including *Ci-Meta1, Ci-Meta2, Ci-Meta3, Ci-Meta4, Ci-Meta5,* and *Ci-Meta6,* all of which were identified by differential screening of larvae and juvenile cDNA (Nakayama et al. 2001; Nakayama et al. 2002). *Ci-Meta1* was of particular interest because of the protein Hemps, which was found to play a key role in metamorphosis in the ascidian *Herdmania curvata,* and is a probable ortholog of *Ci-Meta1*. Hemps induces metamorphosis in *H. curvata* and was seen to be localized in the anterior papillae, which are associated with signaling during metamorphosis (Eri *et al.* 1999).

Ci-Meta1 has a number of features similar to *Hemps* implicating it as an ortholog. Both contain epidermal growth factor (EGF) like repeats. *Ci-Meta1* contains 6 EGF-like repeats, possibly involved in receptor-ligand interaction (Appell *et al.* 1988) and 13 calcium-binding EGF- like repeats, possibly involved in protein-protein interaction (Rao *et al.* 1995). *In-situ* hybridization shows that *Ci-Meta 1* is also present in the anterior papillae of *C. intestinalis* (Fig. 4; Nakayama et al. 2001; Eri et al. 1999). This similarity to *Hemps* suggested that *Ci-Meta1* may be important in metamorphosis in *C. intestinalis,* possibly being a key regulator for early visceral gene development. *Ci-Meta 2* was also of interest because it is expressed in the anterior papillae, where the signal for metamorphosis is believed to originate. Ci-Meta2 is thought to encode a secreted protein and contains three thrombospondin repeats (Nakayama *et al.* 2001) involved in cell-cell to cell-matrix interactions, making it a possible signaling factor.

Inducible Transgene Construction

As we have seen, *Ci-Meta1* and *Ci-Meta2* are involved in regulating metamorphosis for *C. intestinalis* (Nakayama *et al*. 2001; Nakayama *et al*. 2002). With these genes, my goal was to construct an inducible transgene, which would allow the easy insertion of these genes, and over-express them in *C. intestinalis* before normal metamorphosis. Ultimately this step should provide insights into the number and type of genes involved in a heterochronic shift. I hypothesized that a small number of regulatory genes are involved in activating visceral organ development. By over-expressing one key gene, I expected that either partial or full reproduction of heterochrony would occur. An inducible transgene was designed to over-express genes of interest using the Gateway Recombination System® (GRS®; Figure 3). I describe this work in Chapter 2.

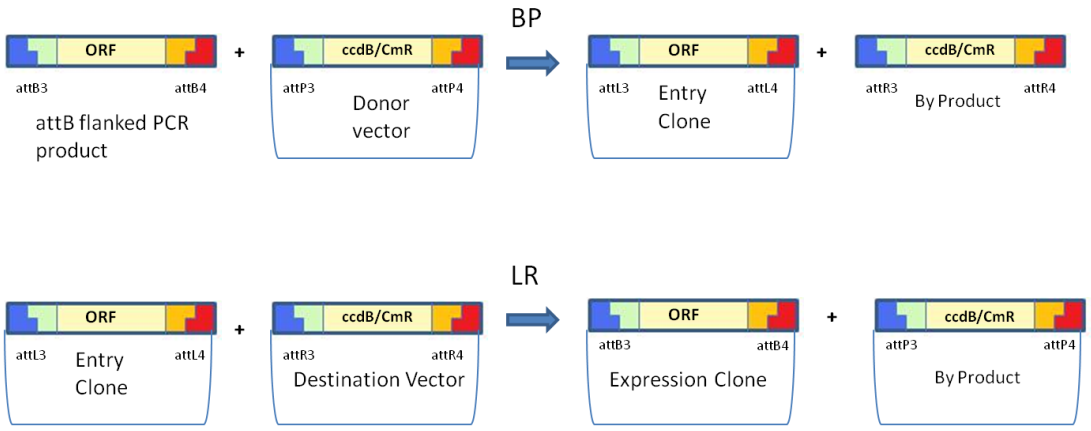


Figure 3**.** Overview of the Gateway® Recombination System. Diagram of the two major steps in the GRS®, the BP and LR reactions. In the BP reaction, the PCR product, with attB sites on either end, is recombined with the Donor Vector to create the Entry Clone, and in the LR reaction, the Entry Clone and Destination vector are recombined to create the Expression Clone. Based on Roure *et al.* 2007.

Inducing Early Metamorphosis

Ascidians can be induced to enter metamorphosis with potassium chloride, KCl, which depolarizes the nervous system membranes (Degnan *et a.l* 1997; Matsumura *et al.* 1999). Metamorphosis has been successfully induced by this method in the ascidians *Halocynthia roretzi*, *Herdmania momus*, and *Boltenia villosa* (Davidson *et al*. 2001; Degnan *et al.* 1997; Matsumura *et al*. 1999). Since inducing metamorphosis with KCl has proven feasible for these other ascidians, I hypothesized the same could be done with *C. intestinalis*. I describe this work in Chapter 3.

CHAPTER 2

Constructing an Inducible Transgene

An inducible transgene using a heat shock protein promoter was created to over-express a number of genes in *C. intestinalis*. The formation of the inducible transgene and possible explanations why it was not expressed in *C. intestinalis* are described below.

Introduction:

The inducible transgene contains four components: the heat shock protein (HSP70) promoter, the gene of interest, green fluorescent protein (GFP) and the GRS®. HSP70 is one of the major chaperone systems that is conserved in many organisms, including *C. intestinalis* (Wada *et al*. 2006). Previous research has shown that under a heat-stress treatment, *C. intestinalis* will induce HSP70 protein synthesis (Fujikawa *et al.* 2009). The HSP70 promoter has been successfully used to induce transgenes in species as diverse as *Drosophila* and sea-urchin embryos (McMahon *et al*. 1984). The *C. intestinalis* HSP70 promoter was chosen for this reason, since it can be easily induced during any time in development. GFP was chosen as a way to visualize that the transgene was induced.

The first gene I inserted in the transgene was *Ci-MRFb*. This would act as a positive control, showing that the transgene is being induced. *Ci-MRFb* is a myogenic regulatory factor gene, which is involved in the muscle development of *C. intestinalis* and has previously been shown to cause ectopic muscle development when over-expressed in *C. intestinalis* embryos (Meedel *et al.* 2007). To insert the gene of interest into the transgene, the GRS® was used.

The inducible HSP70 transgene was successfully made with the positive control, *Ci-MRFb*. However, despite two attempts to express the transgene in *C. intestinalis* embryos, no induction was seen.

Materials and Methods:

In-Situ hybridization *Ci-Meta1* and *Ci-Meta2*

Using larval and juvenile cDNA (See Appendix I for protocol), partial sequences of both *Ci-Meta1* and *Ci-Meta2* were obtained. The primers used for *Ci-Meta1* included Meta1forward 2 and Meta1 reverse 1 (Table 3). A small piece of *Ci-Meta2* was obtained by Megan Walsh, an undergraduate student. Both *Ci-Meta1* and *Ci-Meta2* fragments were used to construct probes for *in-situ* hybridization to determine the normal expression pattern (Protocol in Appendix II).

Construction of the Destination Vector

The destination vector used, L1052A, was derived from a Gateway® clone (a gift of the P. Lemaire lab, called A10) which contained the GFP coding sequence. L1052A was first digested with XhoI and AsiSI(SgfI). HSP70 was amplified from *C. intestinalis* genomic DNA (gDNA). A large region was first amplified from gDNA using HSP70-11 primers (Table 1). From this product a smaller piece of HSP70 was amplified by nested PCR with HSP70-L forward 1 and reverse 1 (Table 1). The forward primer was designed with an AsiSI site at the 5'end and the reverse primer was designed with an XhoI site at the 5' end. This product was then cut with the same restriction enzymes, XhoI and AsiSI. DNA was purified using the Wizard® SV Gel and PCR Clean-Up System® from Promega

(Madison, WI). HSP70 and L1052A were then ligated together and transformed into ccdBr cells (Figure 4).

Gateway Construction

The myogenic regulatory gene, *Ci-MRFb*, was a gift from Thomas Meedel (e.g., Meedel, *et al.* 1997). Specific recombination sequences were incorporated into *Ci-MRFb* by PCR using CiMRF forward 1, CiMRF reverse 2 and 3 (Table 2). The PCR conditions were one cycle of 98°C for 30 s, followed by 32 cycles of 98°C for 7 s, 57°C for 15 s, 72°C for 90 s and a final extension of 5 m. PCR was performed with Phusion® polymerase (Thermo Fisher Scientific, Ipswich, MA). Once amplified, the DNA was excised from a gel and purified with the Wizard® SV Gel and PCR Clean-up System® (Promega, Madison, WI).

The PCR product, *Ci-MRFb*, was first recombined with pDONR-222. Fifty femtomoles (fmol) of each DNA was incubated with 1µl BP clonase (Thermo Fisher Scientific) and left overnight. This reaction was then transformed into competent cells, resulting in the CiMRFb Entry Clone. The entry clone was then recombined into the destination vector, L1052A- HSP70. Ten fmol of each DNA was incubated with 1µl of LR clonase and left overnight. This was then transformed into competent cells, resulting in the final expression clone (Figure 5). The entry clone and destination vector were sent for sequencing at the Rhode Island Genomics and Sequencing Center to verify the right clone had been obtained.

Table 1. HSP70 primer list. Below is the list of primers used to create the HSP70 promoter insert.

HSP70 Primers	
HSP70-11for	TATGACGCCACCATT GCCATCC
HSP70-11rev	GCCGTCCGTAATCTTCTAATTGCCC
HSP70-Lfor1	TTAGATGCGATCGC TGAGGATAAATACAGCGAC
HSP70-Lrev1	CCATGCTCGAG ATTCTTGTAATCTTCTGAATATCTA

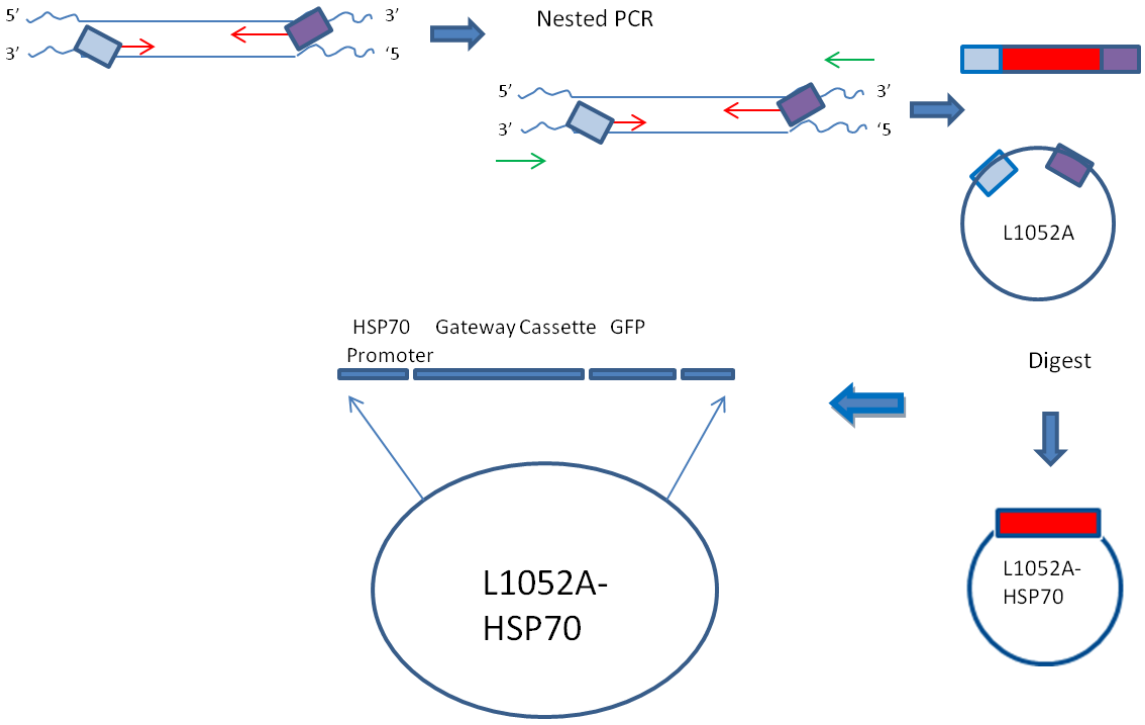


Figure 4. Destination vector construction. The figure above illustrates the basic design of the L1052A-HSP70 destination vector. Primers were designed with restriction sites for HSP70 on the ends of the sequence. A nested PCR was performed to obtain the correct HSP70 sequence. After obtaining the correct sequence, the product was digested and ligated into the vector, L1052A.

Table 2. *Ci-MRFb* primer list. Below is the list of primers used to create the *Ci-MRFb*insert.

Ci-MRFb primers	
CiMRF for1	GGGGACAAGTTTGTATAATAAAGTAGGCTAA GCAAATCCAGCCGGTAG
CiMRF rev2	GGGGACCACTTTGTATAGAAAAGTTGGGTAACAAACCAAACTAGACGC TCCC
CiMRF rev3	GGGGACCAACTTTGTATAGAAAAGTTGGGTACAA ACCAAACTAGACGCTCCC

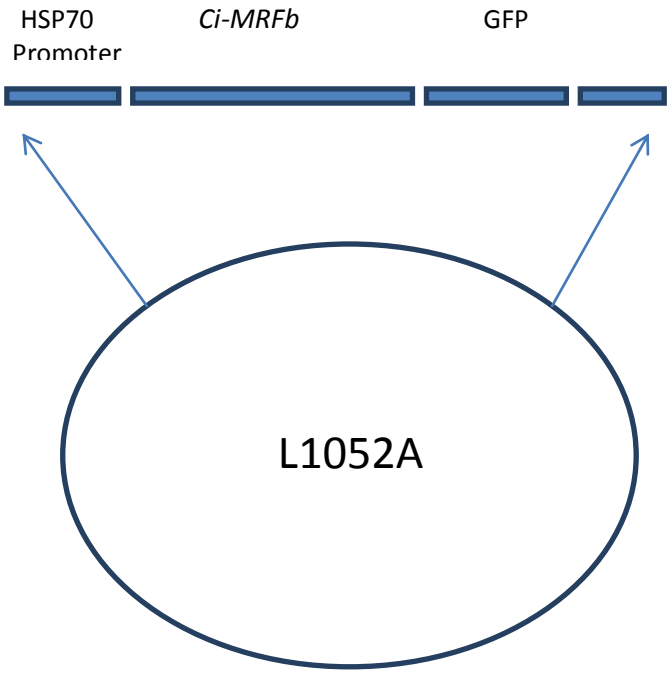


Figure 5. Expression *Ci-MRFb* clone. The diagram above shows the final expression clone construct. *Ci-MRFb* is flanked by the heat shock protein (HSP70) promoter and green fluorescent protein (GFP) coding sequence. L1052A was the initial vector used including both GFP and the Gateway® System.

Fertilization & Dechorionation

Eggs were removed from 2-4 *C. intestinalis* adults and washed with filtered sea water. A small quantity of sperm was taken from each animal and placed with the eggs for fertilization. After 5 minutes, embryos were washed with filtered sea water (FSW) and placed in 10ml of seawater containing 1% thioglycolate (pH 10). They were then transferred to 8ml of 1% thioglycolate and 200µl of 100 mg/ml Pronase E for 2 – 5 minutes, until the chorions fell off (monitored with dissecting microscope; Vierra *et al.* 2012). Finally, embryos were washed multiple times with FSW.

Electroporation

250 µl of dechorinated embryos in seawater were placed in a 0.4 cm electroporation cuvette with 350 µl of 1 M mannitol and 50 µg of the desired plasmid in 100µl of water, then pulsed for 90 msec at 30 V using an ECM 830 electroporator (BTX; Holliston, MA). Transformed embryos were placed in FSW with antibiotics (2% of 1:1 penicillin/streptomycin) in gelatin-coated petri dishes to grow in an 18°C incubator.

Heat Shock

Half of the embryos that had been electroporated with the L1052A-*CiMRFb* expression clone and half of the control embryos were placed in a 28°C incubator at gastrula stage (4.5-5 hours post-fertilization) for one hour. Embryos were then placed back at 18°C and left to grow until the late tail bud stage (Hotta *et al.* 2007).

Analysis

Embryos were analyzed for over-expression of the transgene by looking for fluorescence from GFP, and by acetycholinesterase (ACHE) histochemistry (see Appendix II for protocol). No GFP expression and no over-expression of muscle development was seen by ACHE staining (Figure 7).

Results:

In-situ hybridization results indicate the strongest expression of both *Ci-Meta1* and *Ci-Meta2* in the anterior papillae (Figure 6). Due to the tunic, there was a lot of background staining. An additional protocol (Appendix II) was created to effectively remove the tunic without harming the embryo.

Two separate electroporation experiments using the *Ci-MRFb* expression clone resulted in neither GFP expression nor over-expression of muscle development (Figure 7). The majority of the ACHE staining was seen in the tail, which normally contains muscle at the larval stage. It was expected that over-expression of muscle would be seen in the trunk of the larva.

Sequencing results showed *Ci-MRFb* and GFP to be in frame with each other. However, the sequence results for HSP70 showed a possible mutation in the start codon (Figure 8).

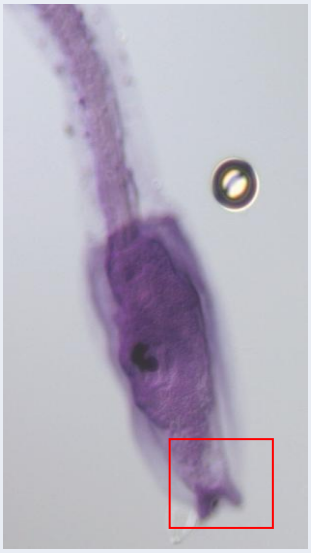
In-situ Hybridization Results		
Control	*Ci-Meta1*	*Ci-Meta2*
		

Figure 6. *In*-situ hybridization results. The figure above shows *in-situ* hybridization results using *Ci-Meta1* and *Ci-Meta2* probes. The left image is a control *C. intestinalis* larva which shows no expression of *Ci-Meta1* or *Ci-Meta2*. The center image is a preparation using a *Ci-Meta1* probe, showing the darkest expression in the anterior palps. The right image shows results using a *Ci-Meta2* probe, also showing the darkest expression in the anterior palps. Red boxes indicate regions of expression.

Control-AChE Experimental-AChE

Figure 7. ACHE staining. Above are the results from the ACHE staining, showing both the control and experimental conditions. Dark brown staining resulting from muscle expression shows the majority of staining in the tail (normal). No abnormal muscle development is seen.

Discussion:

In-situ hybridization

Expression of both *Ci-Meta1* and *Ci-Meta2* in the anterior papillae is consistent with the idea that both are key genes involved in regulating metamorphosis. This is in agreement with previous research in the asicidan *B. villosa* in which an EGF signaling factor, *cornichon,* was also found in the anterior papillae, and is associated with the initiation of metamorphosis (Davidson *et al.* 2001). Trying to obtain the full length sequence for *Ci-Meta1* resulted in a large amount of troubleshooting due to EGF-like repeats which resulted in a ladder-like product. A variety of primers were designed to overcome this problem (Table 3), but were unsuccessful. Future research will focus on obtaining the full-length sequence. Full-length *Ci-Meta2* was obtained from the *C. intestinalis* Gene Collection Release I (Satou *et al*. 2002).

Inducible Transgene

There was a mutation in the start of the coding sequence of the HSP70 promoter. This mutation is not present in the native heat shock elements (Figure 8), which are important for the function of HSP70; this may have accounted for both the absence of GFP fluorescence and the over-expression of muscle.

Even though there is a mutation in the promoter, this may not be the reason why it was not expressed. Previous research has shown up-regulation of HSP70 in *C. intestinalis* only at the larval stage (Fujikawa *et al.* 2009). Thus, it is possible that transcription factors needed to induce the HSP70 promoter may not be present early in the development of *C. intestinalis*. *In-situ* hybridization or RT-PCR can be performed to find

out if the transcript is being made and is present, but is not translated. This could clarify if there was a problem with the promoter. Another way to find out if the HSP70 promoter is inducible early in development is by constructing a transgene with only the HSP70 promoter and GFP gene. This could be electroporated into embryos at different times in development to determine whether or when it is induced after introduction in this way.

Another factor may be the temperature at which the embryos were heat shocked. Since there is no standard protocol for inducing the HSP70 promoter in *C. intestinalis*, it is possible that a higher temperature is needed for inducing the promoter. This could also be explored by using the transgene design of only the HSP70 promoter and GFP gene, and placing the embryos at higher temperatures to see if this results in induction.

Even though the control transgene failed to work, I believe this is the first step in creating an efficient inducible transgene that will be useful for future experiments. Once this transgene shows the ability to be induced in *C. intestinalis* embryos, *Ci-Meta1* and *Ci-Meta2* , as well as other genes, could be easily swapped in and lead to understanding more about the heterochronic shift in ascidians.

Table 3. *Ci-Meta1* primers. Listed below are the primers used in attempts to obtain the full length *Ci-Meta1* sequence.

Meta1 for2	CTACACTGGAAATGGTTTAGTTTGC
Meta1 rev1	ACAGTTGTGGTATCGGTAACAGC
Meta1 for5	ATAAAGTAGGCTATGAAGACWTCAATTGTGCTCGC
Meta1rev5	GAAAAGTTGGGTTGTCACGGTAGTCATGGC
Meta1 for6	ATAAAGTAGGCTATGATTGTGCTCGCYGGCCTCG
Meta1 rev6	GAAAAGTTGGGTGGTAGTCATGGCAACAGTT
Meta1 for7	ATAAAGTAGGCTCAGAAAAA ATGAGACCAAACGTATGTGGTTC
Meta1 for9	AATTGTGCTCGCTGGCCTCG
Meta1 rev8	TGTCACGGTAGTCATGGC
Meta1 for6 kozac	ATAAAGTAGGCT CAGAAAAAATGGCTATGATTGTGCTCGCYGGCCTCG
Meta1 for7 ext	ACAAGTTTGTATAATAAAGTAGGCT CAGAAAAAATGAGACCAAACGTATGTGGTTC
Meta1 rev6 ext	ACCAACTTTGTATAGAAAAGTTGGGT GGTAGTCATGGCAACAGTT
Meta1 for8 ext	ACAAGTTTGTATAATAAAGTAGGCT CAGAAAAAATG ATTGTGCTCGCTGGCCTC
Meta1 rev7 ext	ACCAACTTTGTATAGAAAAGTTGGGT CTCTTTAAGTGCAAATGTCACG

Figure 8. Results of HSP70 BLAST search. Above are the results from a BLAST search with the query representing the sequence from the transgene and the subject being the known HSP70 sequence. The purple boxes represent the heat shock elements and the red box indicates the mutation. The transcriptional start site is not highlighted in this figure, but is found within 25 base pairs of the TATA box (query sequence 224-229).

CHAPTER 3

Inducing Early Metamorphosis

Inducing metamorphosis early in ascidian development promises to be an asset in studying the genetic pathways involved in metamorphosis, as well as knowing the timing of competence for different ascidians. Although many ascidians can be induced with potassium ions, for *C. intestinalis,* heat shock appears to be the best inducer that I tested.

Introduction:

Some of the ascidians for which artificial induction of metamorphosis has been studied in the past include *Boltenia villosa, Halocythia roretzi* and *Herdmania momus.* For these, researchers have found that metamorphosis can be induced by the use of potassium ions (Degnan *et al.* 1996; Davidson *et al.* 2001; Matsumura *et al.* 1999). I chose to study this phenomenon because potassium induction has not been studied in *C. intestinalis* and, as there is no standard protocol for inducing early metamorphosis, this could prove useful for future research in the lab.

Potassium ions are thought to depolarize the membrane of excitable cells (Matsumura *et al.* 1999). The membrane potential change acts to initiate metamorphosis. The critical cells responsive to the potassium ions are the papillae sensory neurons which in turn signal the anterior palps to release another signal for metamorphosis to begin (Degnan *et al.* 1997; Eri *et al.* 1999; Jackson *et al.* 2002). Different ascidians respond differently to different potassium concentrations. For the ascidian, *H. momus*, researchers observed induction of metamorphosis with 10-75mM potassium ions (Degnan *et al.* 1997). For *H. roretzi*, researchers found concentrations of 20mM and 50mM potassium chloride were able to induce metamorphosis, while 30mM suppressed metamorphosis

(Matsumura *et al.* 1999). For *B. villosa*, 50mM potassium was used to induce metamorphosis (Davidson *et al.* 2001).

Based on this previous research, I hypothesized that *C. intestinalis* could also be induced with potassium chloride (KCl) and a simple protocol could be created. Results from my studies indicated a lack of early induction by KCl, but success with heat shock.

Materials and Methods:

Collection and Experiment

Eggs collected from 2-3 *C. intestinalis* were fertilized by adding a small drop of sperm from two of the animals and leaving them to fertilize for 5 min. Embryos were then washed with FSW and placed in petri dishes to incubate at 17-18°C until hatching, approximately 18 hrs. Within one hour of hatching, embryos were separated into dishes containing 30mM KCl, or 40 mM KCl, or FSW. These concentrations were based on published research (Matsumura *et al.*1999), as well as an introductory experiment that showed 50 mM KCl delayed metamorphosis in *C. intestinalis* (data not shown). Between 20 and 40 embryos were placed in small petri dishes for each solution and experiments were carried out in triplicate (3 samples in FSW, 3 in FSW with 30 mM KCl, 3 in FSW with 40 mM KCl). Embryos were judged to be undergoing metamorphosis if tail resorption had begun (Matsumura *et al.* 1999). The same embryos in each dish were counted for metamorphosis every 2 hr post hatching up until 12 hr, and then again at 24 hr post hatching. Each concentration series was repeated three times (three experiments with three samples).

The last two trials included a separate heat shock treatment along with the KCl treatments. For the heat shock treatment, 20-30 embryos were placed in 3 dishes of FSW (similar to the control) and these three samples were placed in an incubator with a 10°C increase between 4 and 6 hr post hatching. After two hours they were placed back at the original temperature.

Analysis

Data from trials 1 and 2 (trials 1, 2 and 3 were KCl treatments) were combined for analysis. Trial 3 (Appendix III) was not included in the analysis since the data were statistically different from previous trials and may have been the result of unhealthy embryos. Trials 4 and 5, which included the heat shock treatments, were also combined to analyze the effect of heat shock. Averages were calculated from the three replicates for each trial; in addition, the averages for combined trials 1 and 2 and for combined trials 4 and 5 were calculated separately.

A one-way ANOVA was performed to analyze the significance of percent metamorphosis data at 24 hr post hatching using the statistical program JMP (SAS Institute Inc., Cary, NC). A p-value of less than 0.05 was considered statistically significant.

Results:

Tables 4 and 5 represent the raw results for the first two trials, showing the number of *C. intestinalis* undergoing metamorphosis for each treatment and each replicate. For both trials, induction began at 8 hr post hatching for 0 mM, 30 mM, and 40 mM KCl treatments. These two trials were combined to find the average percent metamorphosis for each hour post hatching (Figure 9). Both 30 mM and 40 mM KCl resulted in a higher average percentage of metamorphosis from 8-24 hr post hatching; however, these results were not found to be statistically different from the control treatment ($p=0.0622$) (Figure 10).

Tables 6 and 7 represent the raw data for trials 4 and 5, showing the number of *C. intestinalis* undergoing metamorphosis for each treatment and each replicate. The heat shock treatment induced metamorphosis early in trial 4, but not in trial 5. These two trials were combined to find the average percent metamorphosis for each hour post hatching (Figure 11). Heat shock had the highest average percentage of metamorphosis throughout the trial. Due to this, the treatments were statistically different from the control ($p=0.0064$) (Figure 12).

Table 4 . Trial 1 early induction data. Embryos were examined every 2 hr for metamorphosis (tail resorption). There were three replicates for the trial. All embryos (~20-40 per petri dish) came from the same fertilization.

Trial 1									
Replicate #1				#2			#3		
KCL conc.	0mM	30mM	40mM	0mM	30mM	40mM	0mM	30mM	40mM
2 hrs post	0/36	0/24	0/27	0/36	0/42	0/25	0/36	0/21	0/32
4 hrs post	0/36	0/24	0/27	0/36	0/42	0/25	0/36	0/21	0/32
6 hrs post	0/36	0/24	0/27	0/36	0/42	0/25	0/36	0/21	0/32
8 hrs post	2/36	1/24	2/27	2/36	1/42	3/25	2/36	0/21	6/32
10 hrs post	7/36	2/24	6/27	7/36	5/42	8/25	7/36	3/21	7/32
12 hrs post	9/36	5/24	7/27	9/36	9/42	10/25	9/36	5/21	11/32
24 hrs post	10/36	5/24	7/27	10/36	9/42	12/25	10/36	6/21	12/32

Table 5. Trial 2 early induction data. Embryos were counted every 2 hr for metamorphosis (tail resorption). There were three replicates for the trial. All embryos (~10-20) came from the same fertilization.

Trial 2									
Replicate #1				#2			#3		
KCL conc.	0mM	30mM	40mM	0 mM	30mM	30mM	0 mM	30mM	40mM
2 hrs post	0/12	0/11	0/15	0/15	0/15	0/15	0/12	0/16	0/18
4 hrs post	0/12	0/11	0/15	0/15	0/15	0/15	0/12	0/16	0/18
6 hrs post	0/12	0/11	0/15	0/15	0/15	0/15	0/12	0/16	0/18
8 hrs post	0/12	1/11	2/15	0/15	2/15	2/15	0/12	3/16	3/18
10 hrs post	1/12	1/11	4/15	1/15	5/15	5/15	0/12	3/16	3/18
12 hrs post	2/12	4/11	4/15	2/15	5/15	5/15	0/12	4/16	4/18
24 hrs post	2/12	7/11	7/15	2/15	10/15	10/15	4/12	7/16	5/18

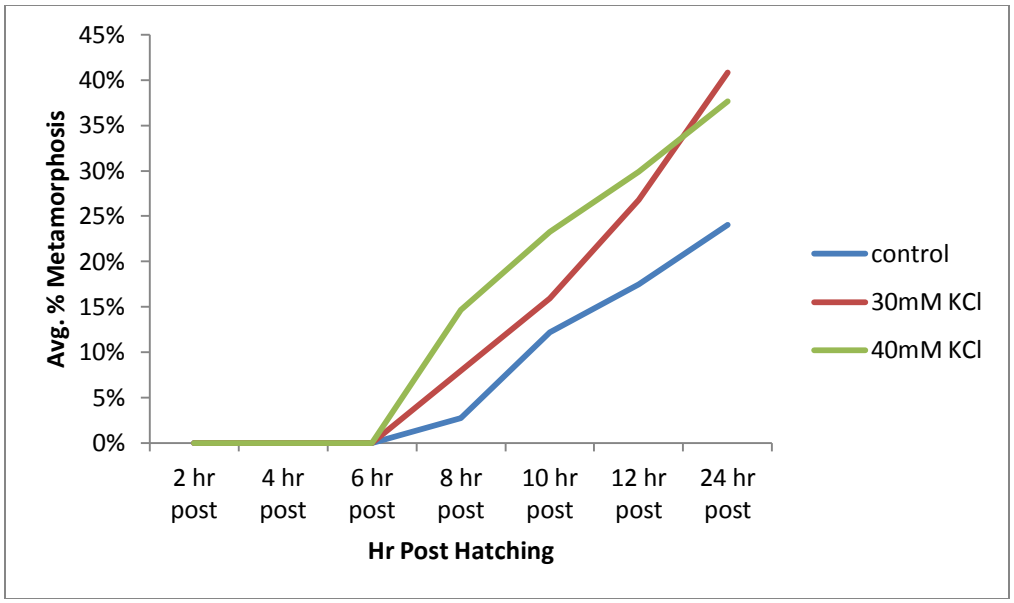


Figure 9. Trials 1 and 2 average metamorphosis over time. The above graph represents the average percent metamorphosis of trial 1 and 2 combined. Embryos from all treatments started metamorphosis between 8 and 10 hr post hatching.

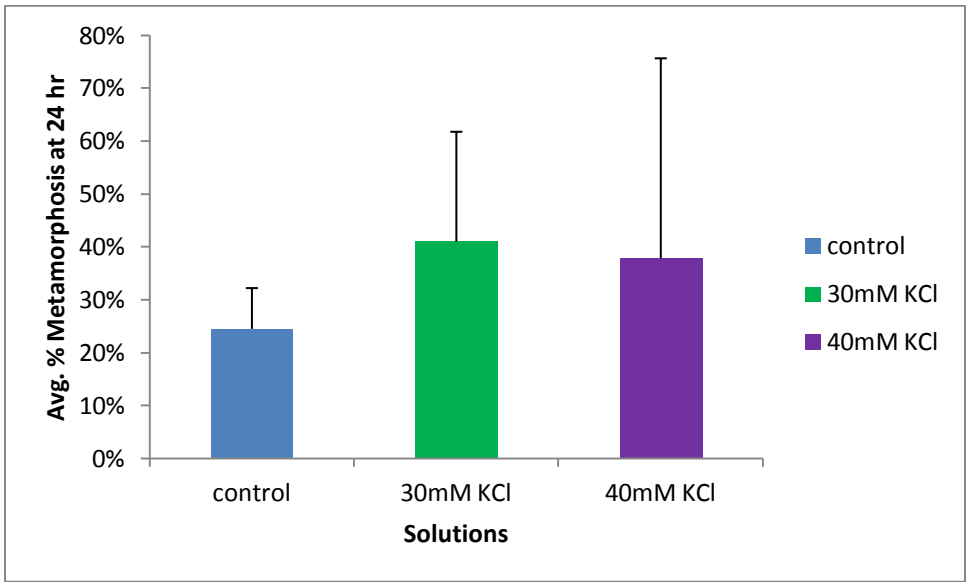


Figure 10. Trials 1 and 2 average metamorphosis at 24 hr. The graph shows the average percent of metamorphosis for each treatment in trials 1 and 2 combined at 24 hr post hatching. Using a one-way ANOVA (JMP), no statistical significance was found ($F_{1,17}$= 4.02, P=0.0622) for the treatment.

Table 6. Trial 4 early induction data. Embryos were counted every 2 hr for metamorphosis (tail resorption). There were three replicates for the trial. All embryos (~15-30) came from the same fertilization.

Trial 4												
Replicate #1					#2				#3			
KCL	0mM	30mM	40mM	HS	0mM	30mM	40mM	HS	0mM	30mM	40mM	HS
2 hr post	0/17	0/24	0/20	0/10	0/19	0/27	0/21	0/15	0/16	0/19	0/24	0/17
4 hr post	0/17	0/24	0/20	0/10	0/19	0/27	0/21	0/15	0/16	0/19	0/24	0/17
6 hr post	0/17	0/24	0/20	3/10	0/19	0/27	0/21	5/15	0/16	0/19	0/24	5/17
8 hr post	0/17	1/24	2/20	3/10	1/19	0/27	2/21	6/15	3/16	0/19	0/24	5/17
10 hr post	5/17	2/24	3/20	4/10	3/19	2/27	2/21	10/15	3/16	1/19	1/24	8/17
12 hr post	5/17	2/24	3/20	4/10	3/19	3/27	4/21	10/15	5/16	2/19	1/24	8/17
24 hr post	7/17	2/24	5/20	7/10	7/19	3/27	4/21	13/15	5/16	3/19	2/24	15/17

Table 7. Trial 5 early induction data. Embryos were counted every 2 hr for metamorphosis (tail resorption). There were three replicates for the trial. All embryos came from the same fertilization.

Trial 5												
#1					#2				#3			
KCL	Control	30mM	40mM	HS	Control	30mM	40mM	HS	Control	30mM	40mM	HS
2 hr post	0/30	0/36	0/40	0/28	0/25	0/38	0/43	0/38	0/15	0/42	0/40	0/34
4 hr post	0/30	0/36	0/40	0/28	0/25	0/38	0/43	0/38	0/15	0/42	0/40	0/34
6 hr post	7/30	8/36	11/40	19/28	6/25	2/38	13/43	15/38	3/15	10/42	17/40	10/34
8 hr post	12/30	9/36	11/40	19/28	12/15	5/38	15/43	20/38	4/15	11/42	19/40	15/34
10hr post	27/30	13/36	11/40	19/28	17/25	11/38	21/43	20/38	7/15	16/42	24/40	18/34
12 hr post	27/30	13/36	13/40	19/28	20/25	11/38	21/43	20/38	8/15	16/42	24/40	18/34
24 hr post	28/30	23/36	25/40	24/28	23/25	12/38	36/43	33/38	9/15	23/42	24/40	32/34

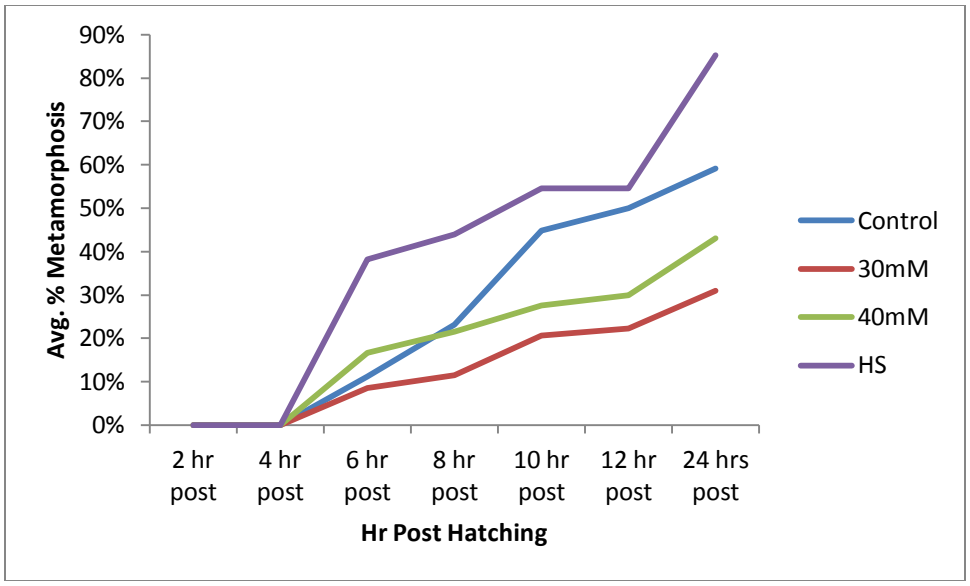


Figure 11. Trials 4 and 5 average metamorphosis over time. The above graph represents the average percent metamorphosis for trials 4 and 5 combined. Embryos from all treatments started metamorphosis between 6 and 8 hr post hatching.

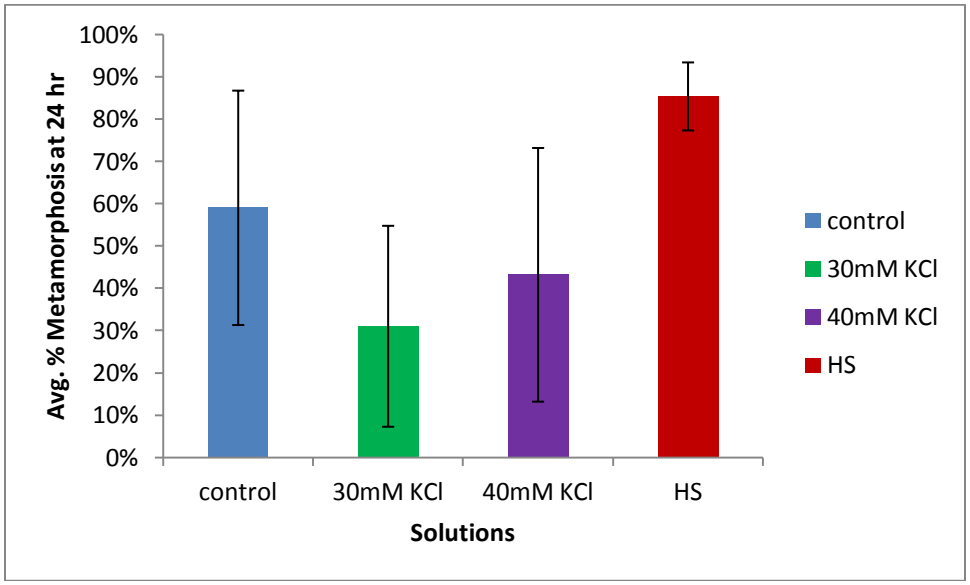


Figure 12. Trials 4 and 5 average metamorphosis at 24 hr. The above graph shows the average percent metamorphosis for each treatment in trials 4 and 5 combined at 24 hr post hatching. Using a one-way ANOVA (JMP), statistical significance was found between the control and the heat shock treatments ($F_{1,23}$=9.0736, P=0.0064).

Discussion:

The KCl induction experiment showed a variety of results, but failed to support the hypothesis that it would induce early metamorphosis of *C. intestinalis*. Although in the first two trials, both 30mM and 40mM KCl treatments had a higher average percent metamorphosis, this was not statistically significant relative to controls (Figures 9 and 10). In trials 4 and 5, a heat shock condition was added, which was successful in inducing early metamorphosis (Figures 11 and 12). The variation in the data between the trials could have been due to the condition of the animals. Trial 3, which is reported in Appendix II, displayed few embryos undergoing metamorphosis after the KCl treatments, but a high percent for the control. This contradicted the other four experiments and may have been due to the condition of the animals. Mortality and abnormal development were rarely seen throughout the experiments and thus I conclude that they played a minor role in the variation in the data.

One reason why early metamorphosis induction might not have been observed could be because the animals were placed in the KCl solutions before they were competent.[1] The time of *C. intestinalis* larval competence is not yet known. The larvae were placed in the KCl solution within one hour of hatching in the hope that when they did become competent a substance that could induce them would be already present.

Another possible explanation for the failure of KCl to induce early metamorphosis is that the KCl treatments had a higher percent of potassium than originally expected. Seawater alone contains about 400ppm potassium ions (Lenntech,

[1] "Competence" is defined as the larval ability to respond to external signals when they come into contact with a signal to start induction (Degnan *et al.* 1997). For example, *H. momus* becomes competent 3 hours after hatching.

2014), which should not result in a significant increase in overall concentration. Although a higher ion concentration may result in lower metamorphosis rates, previous research has shown ascidians responding to 75mM KCl treatments (Degnan *et al* 1997).

Two heat shock treatments induced early metamorphosis. This result was statistically significant at 24 hours post hatching. Previous research has shown that heat shock could induce metamorphosis in *C. intestinalis* (Kroiher, *et al.* 1992). Using heat shock as the standard protocol could give us a better idea on how early metamorphosis could be induced. In future experiments, the embryos could be heat shocked at 2 and/or 3 hours post hatching. This would give us a better indication of how early they can be induced, as well as a known time frame for when *C. intestinalis* becomes competent.

To really gain a better understanding of the effect of both KCl and heat shock on *C. intestinalis* embryos, more trials need to be done. Based on the results, KCl does not seem to induce metamorphosis early, having a higher number of embryos undergoing metamorphosis over a 24-hour span. Heat shock treatments indicated the ability to induce early metamorphosis, as well as having a higher number of organisms metamorphose over the 24-hour span. These results indicate heat shock may be the most reliable method to use.

APPENDIX I

Additional Projects

Blood Vascular System, Growing up *C .intestinalis,* and Stripping Tunics

This project began by examining the formation of the blood vascular system in *C.intestinalis*. *C. intestinalis* does not start developing visceral organs until metamorphosis has started. Therefore the vasculature does not start to form until after metamorphosis has begun. This made the first step in the project growing up *C. intestinalis* to the juvenile stage. Because there is already substantial literature on culturing *C. intestinalis*, this was a fairly routine procedure. To grow up *C. intestinalis,* at least 2-3 animals were fertilized and placed in 150 by 25mm petri dishes. A diatom culture was prepared and added to the petri dishes about 3 days after fertilization (Appendix II). The dishes were replaced with new filtered seawater, and the animals were fed about 1ml culture every two days. This protocol was a modified version from Joly *et al.* 2007 and Chiba *et al.* 2004. Once juveniles had developed, we attempted to express reporter transgenes for Vegf, Fli1, and Fli2, proteins expected to be involved in the formation of the vascular system (Appendix II), as well as perform in-situ hybridization. In conducting these experiments, it was determined that the tunic created background problems while staining. The procedure in place for stripping the tunics was not very effective and left the embryos either damaged or with the majority of the tunic remaining. Over the course of this project, a fairly easy protocol was developed to efficiently remove the tunic from the larvae, but there is still work to be done for the juveniles (Figure 13). Because of technical difficulties the gene expression studies were put on hold.

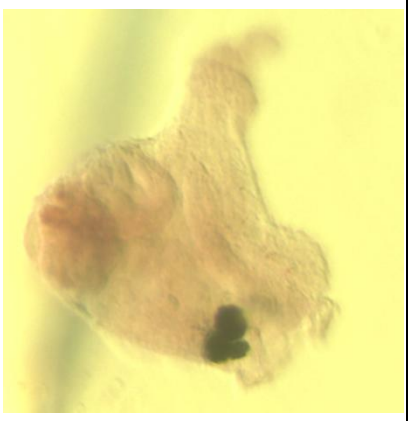
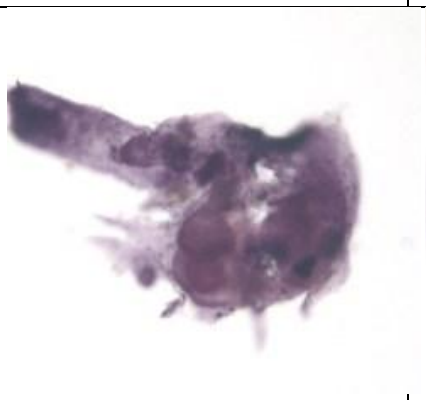
Control Juvenile	*Meta1 In-situ*: Juvenile	*Meta 1 In-situ*: Larvae
		

Figure 13. Tunic troubleshooting. This image shows the problems encountered with *in-situ* hybridization. The first image shows a control juvenile, the second image is of a juvenile that has gone through the *in-situ* hybridization process using the probe *Meta1*. The third image shows a larva which has also gone through the *in-situ* hybridization process and the *Meta1* probe has been used. The destruction of the embryo and the interference of the tunic are shown above.

APPENDIX II

Additional Protocols

Animal Collection:

Ciona intestinalis was collected off docks in Snug Harbor, Rhode Island and kept in an 18°C tank.

Diatom Culture Protocol:

In autoclaved 1L flasks, 200ml of filtered seawater was added along with 37µl of micro-algae grow solution (Florida Aqua Farms Inc.) or at a concentration of 186µl/L. Half a culture tube (~1.5ml) of diatoms was added to each flask (courtesy of the Jenkins Lab). Cultures were aerated by pumping air through an in-line filter. The flasks were then placed on top of an orbital shaker. (modified from Joly *et al.* 2007 and Chiba *et al.* 2004.)

Removing Tunics from fixed *Ciona* Larvae

Fixed embryos stored in ethanol were rehydrated with 70% ethanol, 50% ethanol, 30% ethanol, and then rinsed twice with distilled water. The embryos were then washed twice for 5 minutes with PTw (1x phosphate buffer saline (PBS) + 0.1% Tween 20). They were then incubated in 20µg/ml proteinase K (source) for 40 minutes at 37°C. The tube was flash vortexed fifty times and left to incubate in the proteinase K solution for another 30–60 minutes and then vortexed another fifty times. In that time frame, tunics were checked periodically to see if they came off. Once the tunics were removed, the embryos were washed twice in PTw for 5 minutes and then post-fixed in 8% para-formaldehyde for 30 minutes at room temperature. The embryos were either washed in PTw as performed previously or dehydrated in 100% ethanol and stored at -20°C.

Ascidian Antibody Staining:

Fixed embryos were rehydrated in 75% ethanol, 50% ethanol, 30% ethanol, and then rinsed twice with PTw. Permeabilization was done by washing the embryos with water and then 1 ml of cold acetone for 5 minutes. They were then washed through a series of water, PTw, PBTT1(1x PBS, 0.4% Triton X-100; 0.2% Tween 20) and then incubated in PBTT1 with rocking for 30 minutes. The embryos were then washed twice with PBT and incubated in 1% Normal Goat Serum (NGS)/PBT (source) with rocking for 1 hour at room temperature. Primary antibody (1:10 dilution; monoclonal antibody from mouse, Invitrogen) in PBT/NGS was added and was incubated at room temperature for an hour or at 4°C overnight. The primary antibody was washed out 4x for 20 minutes each in PBT. Secondary antibody (1:500 dilution; goat anti-mouse horseradish peroxidase conjugated, Invitrogen) in PBT was added and left to incubate at room temperature for an hour. The secondary antibody was washed out 1x for 5 minutes in PBT and 1x for 20 minutes in PBT. Samples were then washed 2x for 20 minutes in PBST (PBS/0.1% Triton X-100). Because the secondary antibody was horseradish peroxidase (HRP) conjugated, 300µl of diaminobenzidine (DAB, 10mg/ml) replaced the last wash. 3% hydrogen peroxide (final 0.3%) was added to start the reaction and was monitored from 5 minutes to 1 hour. The reaction was stopped by washing the embryos 3x w/PBST.

In-situ Hybridization:

Fixed embryos stored in ethanol were rehydrated with 75% ethanol, 50% ethanol, 30% ethanol and then washed 3x with PTw (1x phosphate buffer saline + 0.1% tween

20). The tunic was then stripped using the protocol described above. Embryos were then placed in a mini column (Mobicol, M1002) with a 10 um filter, and washed twice with 1% triethanolamine in PTw. They were then washed twice for 5 minutes in 0.25% acetic acid in 1% triethanolamine/PTw solution (1.3 µl acetic acid per 0.5ml of 1% triethanolamine). Embryos were then washed 3x with PTw. Embryos were pre-hybridized for 1 hour at 60°C in hybridization (hybe) buffer (50% formamide, 5x SSC pH4.5, 0.1% Tween 20, 50ug/ml heparin, 50 ug/ml yeast RNA, 2x Denhardt's solution, and 50 ug/ml herring sperm DNA which was denatured and added just before use). After the hour pre-hybridization period 50 ng of riboprobe (Ci-meta1 and Ci-meta2 probes , see riboprobe synthesis section) was added to per ml of hybe solution which was heated at 80-90°C for 8 minutes. Embryos were hybridized overnight at 60°C. The following day the embryos were washed 2x with hybe buffer at the hybridization temperature for 5 minutes and 1x for 30 minutes. This was then followed by a series of washes for 20 minutes at 60°C: 75% hybe/25% 2x SSC/0.1% tween, 50% hybe/50% 2x SSC/0.1% tween, 25% hybe/75% 2x SSC/0.1% tween/ 100% 2x SSC/0.1% tween, 3x in 0.1x SSC/ 0.1% Tween. Embryos were then washed for 10 minutes at room temperature (RT) in each of the following: 75% 0.1x SSC/ 0.1% Tween/ 25% PTw, 50% 0.1x SSC/ 0.1 tween/ 50% PTw, 25% 0.1x SSC/ 0.1% tween, 75% PTw, 2x in 100% PTw. Detection was done by washing the embryos 2x for 10 minutes and 1 hour with rocking in 2% non-fat dry milk in PTw. Alkaline phosphatase conjugated anti-digoxygenin antibody (Roche) was then added (1:5000 dilution) and incubated at RT for 90 minutes with rocking or overnight at 4°C without rocking. The antibody was then washed out 3x for 5 minutes each in 2% block/PTw and 6x for 15 minutes each in 0.1% BSA/ PTw. Embryos were then washed 2x for 5 minutes

each with AP Buffer (100mM NaCl, 25mM $MgCl_2$, 100mM Tris pH 9.5, 0.1% Tween 20). 750µl of 2x Substrate Solution (6.6µl/ml NBT, nitro-blue tetrazolium chloride and 5µl/ml of BCIP, 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt in AP Buffer; Roche). Embryos were checked periodically until stained, which could take a few minutes to a few days depending on the probe. When the stain was fully developed, the substrate solution was washed out 4x for 5 minutes each in PTw. Embryos can be stored at 4°C.

cDNA protocol:

Embryos were collected and stored at -80°C. They were then resuspended in 1ml Tri reagent® (Molecular Research Company, Inc.) and homogenized in a 1.5 ml microcentrifuge tube with a plastic pestle (Kontes). 500µl of 1-bromo-3-chloropropane was added and left to incubate at room temperature for 2-15 minutes. Once centrifuged for 15 minutes at 12,000 g, the upper aqueous phase was transferred to a new tube, washed with 100% isopropanol, stored at room temperature for 5-10 minutes and then centrifuged for 8 minutes. The supernatant was removed and the pellet was washed in 75% ethanol and centrifuged. The ethanol was removed and the pellet was air dried for 3-5 minutes and dissolved in 11µl nuclease free water.

Riboprobe Synthesis:

1 µg of template DNA was mixed with 10x transcription buffer, 10mM ATP, 10 mM CTP, 10mM GTP, 10mM UTP, 3.5mM labeled UTP, 0.5µl RNAsin (source?) and 2µl of designated enzyme (T7, T3, or SP6). The reaction was mixed and incubated for 2-4 hours at 37°C. Two µl of RNAse-free DNAse I (New England BioLabs) was added to the

reaction and incubated at 37°C for 15 minutes. 1.1µl of 100mM EDTA was added and the reaction heated to 75°C for 10 minutes. 20µl of RNAse-free water was added to the reaction and the resulting riboprobe was checked for size on a 1% agarose gel.

Acetlycholinesterase Staining:

Embryos were fixed in 2% paraformaldehyde in PTw for 30 min. They were then rinsed 4x in 0.1M sodium phosphate buffer, pH 6.5. Fresh staining medium (10mg of acetylthiocholine iodide (source) in 6.5 ml of 0.1 M sodium phosphate, 0.5 ml 0.1 M sodium citrate, 1 ml 30 mM cupric sulfate, 1 ml water and 1ml 5 mM potassium ferricyanide) was added to the embryos. They were incubated for 2-4 hr at 37°C, checking periodically for staining. Once stained, embryos were rinsed with PTw.

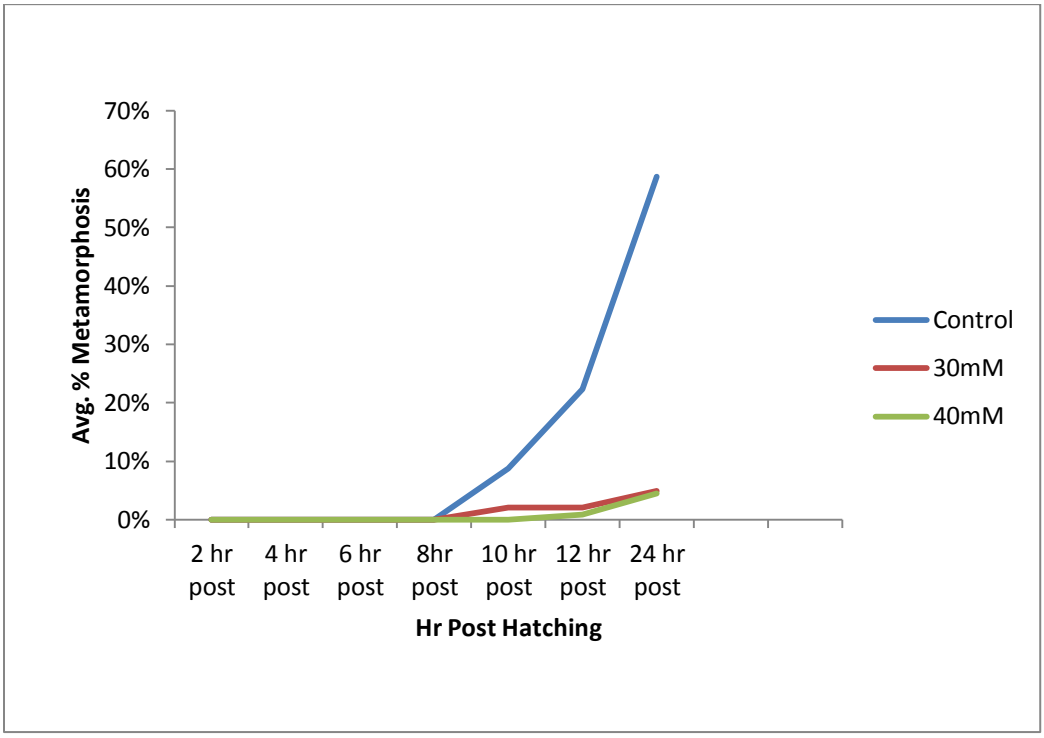
APPENDIX III

Supplementary Data

Table 8 . Trial 3 early induction collection data. Embryos were counted every 2 hr for metamorphosis (tail resoprtion). There were three replicates for the trial. All embryos (~20-40) came from the same fertilization.

Trial 3									
Replicate #1				#2			#3		
KCL conc.	0mM	30mM	40mM	0mM	30mM	40mM	0mM	30mM	40mM
2 hrs post	0/19	0/35	0/37	0/20	0/36	0/33	0/18	0/29	0/20
4 hrs post	0/19	0/35	0/37	0/20	0/36	0/33	0/18	0/29	0/20
6 hrs post	0/19	0/35	0/37	0/20	0/36	0/33	0/18	0/29	0/20
8 hrs post	0/19	0/35	0/37	0/20	0/36	0/33	0/18	0/29	0/20
10 hrs post	2/19	1/35	0/37	2/20	0/36	0/33	1/18	1/29	0/20
12 hrs post	3/19	1/35	1/37	8/20	0/36	0/33	2/18	1/29	0/20
24 hrs post	12/19	3/35	2/37	17/20	1/36	1/33	5/18	1/29	1/20

Figure 14. Trial 3 average metamorphosis over time. The graph represents the average percent metamorphosis. Embryos in all treatments started metamorphosis between 8 hr and 10 hr post hatching.



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