The evolutionary implications of alternative larval development in ascidians

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Abstract

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Considering traits that facilitate and inhibit evolutionary diversification can best be done with traits where the genetic and developmental basis is known. My PhD. research has focused on the evolutionary implications of larval development in molgulid ascidians. Ascidians or sea squirts, are a group of tunicates, which phylogenomic evidence suggests is sister-group to the vertebrates. Most ascidians develop as tadpole larvae that have an eyespot, gravity-sensing otolith, and a notochord, which converges and extends to form a tail that is used for dispersal. In molgulid ascidians, there have been 15 species described from at least four separate evolutionary origins that have lost the eyespot, otolith, and tail in their larval stage. Using transcriptome data for a species with normal tadpole larval development, a species with tailless larval development, and their hybrid, I have found that molgulid ascidians express genes normally expressed at metamorphosis early in development, and hypothesize this early expression is driving the tailless phenotype. Additionally, using molecular phylogenetic comparative methods in ascidians, I found species with the derived, tailless, less-dispersive larvae have lower speciation rates and smaller geographic ranges. Lower speciation rates in lineages with the derived, less dispersive larval development is in contrast to speciation rates estimated from molecular phylogenies in gastropods, and from fossil gastropods and echinoderms, and this difference suggests that the demography of species with different larval development may be important to consider.

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DEDICATION

to my partner in crime, Kathleen

Chapter 1

MOLGULA PUGETIENSIS IS A PACIFIC TAILLESS ASCIDIAN WITHIN THE ROSCOVITA CLADE OF MOLGULIDS

1.1 Abstract

The molgulid ascidians are a monophyletic group of tunicates that share specific morphological features, including a heart/kidney complex and hermaphroditic gonads found on both sides of the adult. The larval body plan, however, is quite plastic, with tailless species evolving at least four times independently within the molgulids. The Roscovita clade of molgulid ascidians includes three species that are found sympatrically in Roscoff, France, and includes both tailed and tailless species. Another species in this clade, *Molgula pacifica*, is a direct developing tailless molgulid ascidian within the Roscovita clade found on the outer-coast of Vancouver Island. *M. pacifica* larvae never hatch, and they undergo metamorphosis within the chorion. *Molgula pugetiensis* is a molgulid ascidian found in the San Juan Islands, but its development has not been reported. Here, we show that this species, belonging to the Roscovita clade, is a tailless ascidian species with larvae that hatch from the chorion before metamorphosis, similar to the well-described tailless *Molgula occulta* from Roscoff, France. Immediately after hatching, the tailless larvae develop ampullae and proceed to metamorphose into juveniles.

1.2 Introduction

Tunicates in the Molgulidae are a monophyletic group of solitary ascidians (Ascidiacea: Stolidobranchia) that are sister group to the families Styelidae and Pyuridae (Huber et al., 2000; Zeng and Swalla, 2005; Tsagkogeorga et al., 2009). Evidence from *18S rRNA* shows that the stolidobranch tunicates may also be closely related to the appendicularians, which include the pelagic oikopleurid tunicates that build mucous houses (Zeng and Swalla, 2005; Tsagkogeorga et al., 2009; Zeng et al., 2006). Molgulidae is an interesting group for research on the evolution of body plans because it contains species that have urodele, tailed, chordate larvae with notochord and muscle as well as closely related species with anural, tailless larvae that completely lack larval structures, including the sensory otolith and muscle and notochord in the tail (Swalla and Jeffery, 1990). Anural species can develop indirectly by hatching from the chorion before metamorphosis or directly by hatching from the chorion after metamorphosis. This loss of a tailed larva is likely to have happened multiple times evolutionarily (Hadfield et al., 1995; Tsagkogeorga et al., 2009; Zeng et al., 2006; Hadfield et al., 1995) and is tractable at a molecular level (Swalla and Jeffery, 1996; Jeffery et al., 1999).

Table 1.1: Selected anural and Pacific urodele mogulid characteristics

Species	**Tail?**	**Larval development**	**Adult habitat**	**Distribution**	**Reference**
Molgula tubifera	Urodele	Indirect	Algae, wood: attached	E. Atlantic wide	Berrill (1931)
M. cooperi	Urodele	Urodele	Sand, gravel: attached	N.E. Pacific	Berrill (1931)
M. verrucifera	Urodele	Indirect	Intertidal rock: attached	N.E. Pacific wide	Berrill (1931)
M. oculata	Urodele	Indirect	Sand: unattached	E. Atlantic wide	Berrill (1931)
M. occulta	Anural	Indirect	Sand: unattached	E. Atlantic wide	Lacaze-Duthiers (1877)
M. solenota	Anural	Indirect	Sand: unattached	E. Atlantic local	Berrill (1931)
M. macrosiphonica	Anural	Indirect	Sand: unattached	E. Atlantic wide	Lacaze-Duthiers (1877)
M. arenata	Anural	Indirect	Sand: unattached	W. Atlantic local	Berrill (1931)
M. robusta	Anural	Indirect	Sand: unattached	W. Atlantic local	Berrill (1931)
Bostrichobranchus digonas	Anural	Indirect	Sand: unattached	W. Atlantic wide	Swalla and Jeffery (1992) Rocha (2002)
B. pilularis	Anural	Indirect	Sand: unattached	W. Atlantic wide	Berrill (1931)
Eugyra arenosa	Anural	Indirect	Sand: unattached	E. Atlantic wide	Berrill (1931)
Molgula pugetiensis	Anural	Indirect	Sand: unattached	N.E. Pacific	This study
M. retortiformis	Anural	Direct	Rock: attached	Circumpolar	Bates (1995)
M. bleizi	Anural	Direct	Rock: attached	E. Atlantic local	Berrill (1931)
M. kolaensis	Anural	Indirect	Sand: unattached (?)	E.. Arctic local	Berrill (1931)
M. pacifica	Anural	Direct	Rock: attached	N.E. Pacific local	Bates and Mallett (1991)
M. tectiformis	Anural	Direct	Rock: attached	N.W. Pacific local	Tagawa et al. (1997)
M. provisionalis	Anural	Direct	Rock: attached	N.W. Atlantic local	Bates (1993)
M. oregonia	Anural	Direct	Rock: attached	N.E. Pacific	Ritter (1913)
M. regularis	Anural	Direct	Rock: attached	N.E. Pacific	Van Name (1945)

Within the molgulid ascidians, there are two monophyletic clades of the species that have been extensively studied, the Woods Hole clade and the Roscovita clade (Huber et al., 2000). The Woods Hole clade includes three sympatric, free-spawning species that can be found near Woods Hole, Massachusetts: *M. mahattensis* (tailed), *M. provisionalis* (tailless), and *M. arenata* (tailless). The Roscovita clade within the Molgulidae includes six species, three of which can be found in the mud flats off Roscoff, France: *M. oculata* (tailed), *M. occulta* (tailless), and *M. bleizi* (tailless) (Huber et al., 2000; Zeng and Swalla, 2005). Two of the other species, *M. pacifica* and *M. pugetiensis*, are found within the Northwest Pacific coast of the United States and Canada, while the tailed, brooding *M. citrina* is found on the Atlantic coast and in Europe (Huber et al., 2000). *M. citrina* and *M. echinosiphonica* have recently been synonymized to *M. citrina* (Shenkar and Swalla, 2010). The recent discovery of *M. citrina* in Alaska also suggests either that there has always been a circumpolar distribution or that it may be a recent invasion (Lambert et al., 2010). Here, we show that *Molgula pugetiensis*, first described by Herdman (Herdman, 1898) near Victoria, British Columbia, in 1898, is a tailless ascidian species found in the U.S. Pacific Northwest that hatches from the chorion before metamorphosing, similar to what has been reported for *M. occulta* (Swalla and Jeffery, 1990) and *M. arenata* (Whittaker, 1979).

1.3 Methods

A single individual of *Molgula pugetiensis* was collected in a dredge near Friday Harbor, Washington, in 2003 by the Evolution and Development of Metazoans course given at the University of Washingtons Friday Harbor Laboratories (FHL). Dr. Billie J. Swalla and Gretchen Lambert examined this adult specimen and found seven folds in the branchial basket; genomic DNA was then extracted and sequenced in the Swalla laboratory (Zeng et al., 2006) (GenBank accession number AY903920.1 for *18S rRNA*). Taxonomic and sequencing results showed that *M. pugetiensis* belongs in the Roscovita clade of seven-fold molgulid ascidians (Zeng and Swalla, 2005) first described in Huber et al. (2000). Even though the adult animal was gravid, with viable eggs and sperm, it did not self-fertilize. Three years later, in 2006, Dr. Bruno Pernet found a pair of *M. pugetiensis* in a dredge sample collected during Marine Invertebrate Zoology, another FHL summer course. The animals were returned to FHL, cross-fertilized by Dr. Swalla, and found to be another tailless species, like *M. arenata* and *M. occulta*, that hatches from its chorion before metamorphosis, in an anterior-to-posterior manner.

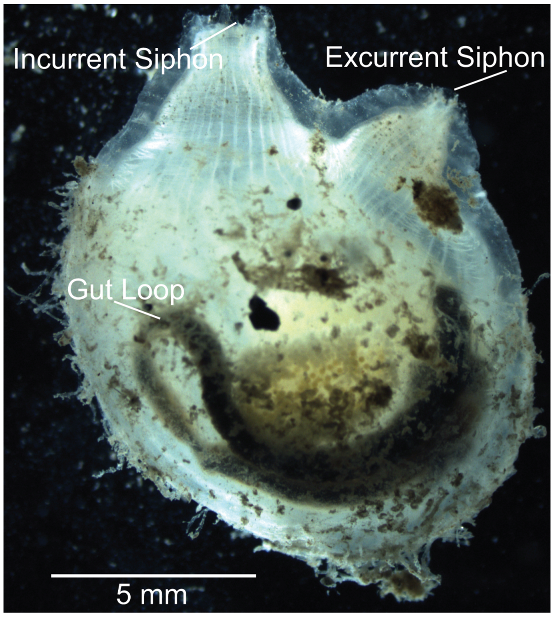

Figure 1.1: Morphology of adult *Molgula pugetiensis*. *M. pugetiensis* shows the typical molgulid ascidian morphology, with the hermaphroditic gonad visible within the gut loop on the right side of the animal. The incurrent and excurrent siphons are labeled. The scale bar is 5 mm.

M. pugetiensis was dissected and cultured at 12 °C in sea tables at FHL. *M. occulta* and *M. oculata* were dissected, cultured at room temperature (20 °C), and photographed in Roscoff, France, in 1999. In all three species, gonads were dissected with forceps from animals after the tunic had been removed: one gonad was kept for DNA analysis, and the other was dissected and fertilized. Eggs and sperm were loosened from the gonad by gentle pipetting with a glass Pasteur pipet. Sperm was removed from one dish and used to fertilize eggs of a second individual, and vice versa. About 50 embryos of *M. pugetiensis* were left to develop in 12 °C seawater until they hatched from the chorion. Embryos and hatched larvae of *M. pugetiensis* were photographed at FHL with a Nikon Eclipse E600 microscope with a Q Imaging camera from Photometrics.

M. pugetiensis was collected at a depth of about 1530 m in sand and shell hash. This environment is similar to that of other described indirect developing anural species (Berrill, 1931; Van Name, 1945). The type of larval development, habitat, and distribution of all known anural molgulids and some of the sympatric urodele species is summarized in Table 1.1. Molgulids with anural indirect development, like *M. pugetiensis*, have been found primarily in the northern Pacific and the northern Atlantic (Table 1.1). There is a single specimen of an arctic species, *M. kolaensis*, which was found to have brooding anural, indirect developing embryos (Table 1.1) (Van Name, 1945).

1.4 Results and Discussion

Figure 1.1 shows the external morphology of one of the adult *Molgula pugetiensis* collected near Friday Harbor Laboratories (University of Washington) in the U.S. Pacific Northwest. The adult is shown from the right side, where the gut loop is seen as a darkened line forming an S-shape before the anus loops out the atrial siphon. The elongated hermaphroditic gonad lies within the gut loop. This molgulid species looks remarkably similar to published photographs of *M. citrina*, one of the tailed, brooding species found in the Roscovita clade that is common to the eastern and western Atlantic and has been recently found off the Alaskan coast (Lambert et al., 2010).

Tailless molgulid ascidians frequently lack the extracellular space normally found between the egg and the follicle cells in tailed ascidian species, where the test cells reside as shown in Figure 1.2 (Swalla and Jeffery, 1990). *Molgula occulta*, a tailless molgulid ascidian that is found off the coast of Roscoff, France, has tightly flattened follicle cells, as seen in a hatching larva (Figure 1.2A), and had been reported earlier (Swalla and Jeffery, 1990). *M. pugetiensis* also lacks extracellular space in the chorion and has flattened follicle cells (Figure 1.2C). Figure 1.2 also compares hatching in *M. occulta*, which emerges from the chorion after 12 h (Figure 1.2A) and then develops four ampullae before immediately metamorphosing into a juvenile (Figure 1.2B). This species develops well at room temperature, or 20 °C. In *M. pugetiensis*, hatching also occurs in an anterior-to-posterior manner from the chorion, but time to hatching was 34 h at a much cooler temperature, 12 °C(Figure 1.2C). The follicle cells are flattened against the chorion (Figure 1.2C), similar to *M. occulta* (Figure 1.2A). *M. pugetiensis* also develops four ampullae before immediately metamorphosing into the adult form (Figure 1.2D). This is in stark contrast to *M. oculata*, a closely related tailed ascidian that has an otolith, a tail, and contains notochord and muscle cells (Figure 1.2E, F), also reported previously (Swalla and Jeffery, 1990). Note that the follicle cells are rounded up on the chorion (Figure 1.2E).

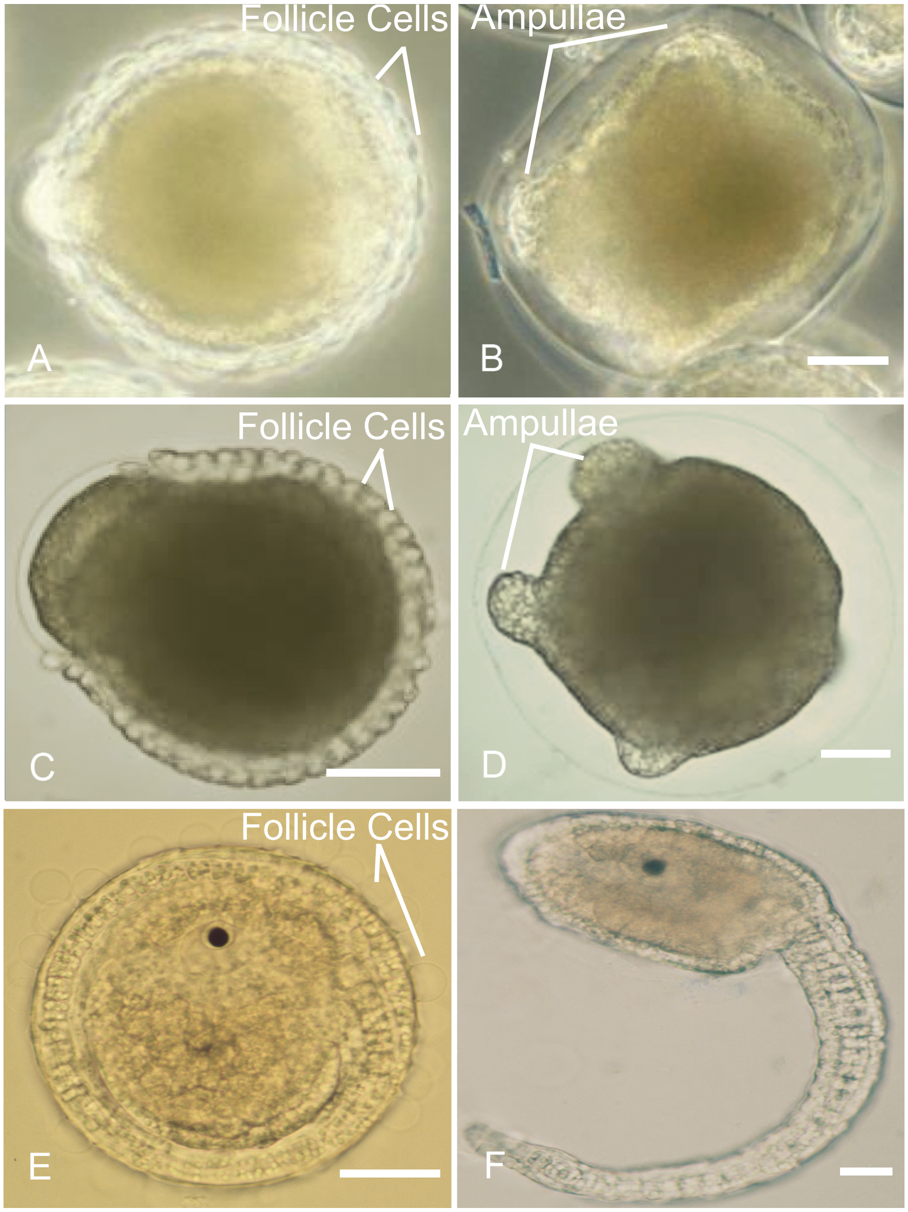

Figure 1.2: Hatching in the tailless ascidians *Molgula occulta* and *Molgula pugetiensis* and the tailed *M. oculata*. Larvae are oriented with anterior to the left, posterior to the right. *M. occulta*, a tailless ascidian from Roscoff, France, in the process of hatching from the chorion (A) and metamorphosing (B) with four primary ampullae forming. Follicle cells are flattened and tightly opposed to the chorion. *M. pugetiensis* also hatches from an anterior break in the chorion (C) and forms four primary ampullae immediately after hatching (D). The eggs and embryos of *M. pugetiensis* also have flattened follicle cells. In contrast, the closely related tailed species *M. oculata* develops within the chorion as a chordate tadpole larva, including a black pigment cell in the head that is part of the gravity-sensing otolith, and a tail containing notochord and muscle cells (E). This tailed species shows rounded follicle cells surrounding the chorion, as seen in most tailed species eggs and embryos. After hatching, the tail extends and the chordate tadpole larva swims for several hours (F) before settling, retracting its tail, and metamorphosing. The scale bar is 40 μm.

M. pugetiensis development is described here for the first time as having anural, indirect larval development. Follicle and test cell differentiation, hatching from the chorion, adult habitat, development of four ampullae immediately after hatching, and the relatively short timing of larval development are characteristics similar to those of the well-studied tailless molgulid *M. occulta* (Table 1.1) (Swalla and Jeffery, 1990). Both of these species hatch from the chorion before metamorphosis and then, shortly after hatching, develop ampullae and metamorphose directly into the adult form. This is in contrast to direct anural larval development as seen in *M. pacifica* and *M. provisionalis*, where the larvae do not hatch out of the chorion before metamorphosis. Instead, the fertilized eggs attach to substrate immediately and develop through to metamorphosis as sessile embryos and juveniles (Swalla and Jeffery, 1990; Bates and Mallett, 1991). Although the eggs, embryos, and juveniles looked remarkably similar to those of *M. occulta* found in France, the timing of hatching was delayed, with developmental timing more similar to the Pacific Northwest *M. pacifica*. This is likely due to the water temperature, with the seawater in the northeast Pacific at approximately 9–12 °C in contrast to the room temperature (20 °C) development for *M. occulta*. Both of the Pacific species, *M. pugetiensis* and *M. pacifica*, are members of the Roscovita clade and would probably develop much more quickly if the eggs and embryos were heat-tolerant.

Phylogenetic analyses of the Molgulidae show that urodele development is likely to have been lost at least four times independently (Huber et al., 2000; Zeng and Swalla, 2005; Hadfield et al., 1995). The tailed urodele larva is probably the ancestral mode in Molgulidae, because tailed urodele larval development is highly conserved across the Ascidiacea and the tadpole larva has been lost in only two other non-mogulid taxa, in another stolidobranch ascidian family, the Styelidae (Millar, 1954, 1962). Therefore, outgroup analyses strongly support the existence of an ancestral tailed tadpole larva for the Molgulidae. Within the Roscovita clade, at least two independent origins of tail loss have been documented in closely related species (Jeffery et al., 1999). In anural developing species *M. occulta* and *M.*

bleizi, independent *larval muscle actin* pseudogenes have been found (Jeffery et al., 1999); the insertions and deletions leading to pseudogene formation were not shared between the two closely related tailless species, supporting the hypothesis that they evolved independently from a tailed ancestor. Table 1.1 also lists the larval development (if known) and distributions of all the described molgulid taxa on the Pacific coast of North America. *M. pugetiensis* has been only sporadically reported since first being described in 1898 (Herdman, 1898). Several molgulid species have few documented locations along the Pacific coast; these include *M. cooperi*, *M. oregonia*, and *M. regularis*. Although none of these species have yet been sequenced, the six folds in their branchial baskets would suggest that all of them may be more closely related to the Woods Hole *Molgula* clade. The urodele, or tailed, species *M. verrucifera* that has been found in southern California has seven folds and will likely be another member of the Roscovita clade (Berrill, 1931). Therefore, future work examining the molecular phylogenetic and developmental status of these species will shed light on the evolution of larval development in the Molgulidae and speciation in the Pacific Ocean.

Anural larvae are unable to swim because of their tail loss. They lack the gravity-sensing otolith, notochord, and *larval muscle actin*, so after hatching out of the chorion, these larvae are unable to swim or orient them-selves and are thus completely ineffective at any active dispersal (Swalla and Jeffery, 1990). It is a conundrum, then, how these species, which would seem incapable of dispersal, are able to establish stable breeding populations, sometimes even distantly located from their sister species. Most of the described anural species are found in sandy habitats unattached to any substratum as adults (Table 1.1). However, two anural, direct-developing species (*M. bleizi* and *M. pacifica*; both in the Roscovita clade) can be found attached to hard substrates, including the roof of rock caves inside blowholes formed by wave action and hollowed out rocks in the very low intertidal (Berrill, 1931; Young et al., 1988).

Since sandflats contain both urodele and anural species, this environment has been proposed to allow for the independent loss of the urodele larva in molgulids (Berrill, 1931). A novel hypothesis to account for the loss of active dispersal potential is that many of these species have been found in northern latitudes with relatively large tidal exchanges that allow for enough passive dispersal to relax selection on the genes that control active swimming traits (Huber et al., 2000). This hypothesis could be tested by comparing the gene flow of sympatric closely related species like the urodele *M. oculata* and the anural, indirect *M. occulta*.

Larval development has been described for only a small fraction of the nearly 150 known species of *Molgula* (Shenkar et al., 2013). A total of 14 species have been reported with anural larval development, and we report the 15th anural *Molgula* species (Table 1.1). The questions of how speciation occurred in the Roscovita clade and how many times anural development evolved in Molgulidae may become clearer as knowledge on the development of more species becomes available.

Chapter 2

DEVELOPMENTAL MODE INFLUENCES DIVERSIFICATION IN ASCIDIANS

2.1 Abstract

Ascidian species (Tunicata: Ascidiacea) usually have tailed, hatching tadpole larvae. In several lineages, species have evolved larvae that completely lack any tail tissues and are unable to actively disperse. Some tailless species hatch, but some do not hatch before going through metamorphosis. We show here that ascidian species with the highest speciation rates are those with the largest range sizes and tailed hatching larval development. We use methods for examining diversification in binary characters across a posterior distribution of trees, and show that mode of larval development predicts geographic range sizes. Conversely, we find species with the least dispersive larval development (tailless, non-hatching) have the lowest speciation rates and smallest geographic ranges. Our speciation rate results are contrary to findings from sea urchins and snails from the fossil record, and we suggest this difference could be due differences in the transition from feeding to non-feeding larval development in sea urchins and snails compared to hatching tailed to non-hatching tailless larval development in ascidians, which may have impacted the microevolutionary dynamics of these species differently.

2.2 Introduction

Marine species have evolved modes of larval development that differ in dispersal potential. Thorson asserted that the primary advantage of swimming larvae for sessile or sedentary marine invertebrates is increased dispersal capabilities (Thorson, 1950). However, an alternative hypothesis suggests that planktonic larval development may have evolved in some lineages as an adaptation for escaping from benthic predators (Strathmann, 2007). Nevertheless, studies of sister-species in divergent metazoan phyla have shown that greater dispersal potential in feeding larvae often positively correlates with higher rates of gene flow between populations (Hellberg, 2009).

Studies explicitly examining the relationship between larval development and geographic range in extant marine metazoan species have found a positive correlation between larval dispersal potential and geographic range (Emlet, 1995; Kohn, 2012). Marine invertebrates species with higher dispersal potential positively correlate with longer species durations, and larger geographic ranges (Hansen, 1978, 1983; Jablonski, 1986; Jeffery et al., 2003). Fossil snails and urchins with the derived non-planktonic or less dispersive larval phase show higher speciation rates than planktonic species (Hansen, 1978, 1983; Jablonski, 1986; Jeffery et al., 2003; Duda and Palumbi, 1999). In fossil studies, examining the evolution of larval development has often not been done in a phylogenetic framework (Hansen, 1978, 1983; Jablonski, 1986), which may overestimate trait effects.

Most of the 3000 described ascidians (Tunicata: Ascidiacea) develop as non-feeding tadpole larvae that swim for a short period (hours to days), then settle, and metamorphose into sessile, filter-feeding adults (Shenkar and Swalla, 2011). The Styelidae and Molgulidae show at least five independent origins of tailless larval development (Huber et al., 2000). Some tailless species develop indirectly by hatching from the chorion before metamorphosis and some hatch from the chorion only during metamorphosis (Maliska and Swalla, 2010; Tagawa et al., 1997).

Likelihood models have been developed recently for examining how discrete characters influence diversification rates inferred from incompletely sampled phylogenies (Maddison et al., 2007; FitzJohn et al., 2009; FitzJohn, 2012). Using a phylogeny of 45 ascidian species in the Molgulidae and Styelidae, and current distribution records of these species to estimate geographic range sizes, we find that species with less dispersive larval development (tailless non-hatching species) have lower speciation rates and smaller geographic ranges.

Table 2.1: Tailless ascidian species described in Molgulidae and Styelidae their habitat, and larval characteristics.

Species	**Habitat**	**Larval Hatching?**	**Brooding?**	**Self-Fertile?**	**In Study?**	**Reference**
Bostrichobranchus digonas	Sand	Hatching	Yes	?	Yes	Swalla and Jeffery (1992)
B. pilularis	Sand	Hatching	Yes	?	No	Berrill (1931)
Eugyra arenosa	Sand	Hatching	No	?	Yes	Berrill (1931)
M. arenata	Sand	Hatching	No	?	Yes	Berrill (1931)
M. bleizi	Rock	Non-hatching	Yes	?	Yes	Berrill (1931)
M. ellistoni	Rock	Non-hatching?	Yes	?	No	Kott (1985)
M. kolaensis	Sand	Hatching	Yes	?	No	Swalla and Jeffery (1992)
M. macrosiphonica	Sand	Hatching	No	?	No	Lacaze-Duthiers (1877)
M. occulta	Sand	Hatching	No	?	Yes	Lacaze-Duthiers (1877)
M. pacifica	Rock	Non-hatching	No	?	Yes	Bates and Mallett (1991)
M. provisionalis	Rock	Non-hatching	No	Yes	Yes	Bates (1993)
M. pugetiensis	Sand	Hatching	No	No	Yes	Maliska and Swalla (2010)
M. retortiformis	Rock	Non-hatching	No	?	Yes	Bates (1995)
M. robusta	Sand	Hatching	No	?	No	Berrill (1931)
M. solenota	Sand	Hatching	?	?	No	Berrill (1931)
M. tectiformis	Rock	Non-hatching	No	No	Yes	Tagawa et al. (1997)
Pelonaia corrugata	Sand	Hatching	No	No	Yes	Millar (1954)
Polycarpa tinctor	Sand	Hatching	Yes	?	No	Millar (1962)

2.3 Methods

The two-state binary-state speciation and extinction model (BiSSE), uses likelihood methods to calculate the probability that a group of extant species would have evolved as observed, given a particular model of the characters effect (Maddison et al., 2007). The BiSSE model uses six parameters: speciation rates in state 0 and state 1, extinction rates in state 0 and state 1, and character transition rates in state 0 and state 1. Maddison et al. (2007) describe how this model is put into a likelihood framework, "The underlying likelihood model would allow speciation and extinction rates to depend on the character state of a lineage at each point in time and allow the character state to change. Inferences about speciation and extinction rates as a function of character state could then be made based on their likelihood: the probability of observing the data (the phylogeny and the current character states) given proposed values for the rate parameters." Further, the BiSSE method estimates ancestral character states while taking into account the effect of that ancestral character state on speciation and extinction using this transition rate.

These calculations work from the tips to the root. Going back in time, these calculations describe the probability that a lineage with state 0 or 1 at a certain time before the node of a clade would evolve into a clade with species that have states 0 or 1. They usually do this by slicing through the tree from the tips to the root in very small time intervals until the analysis is completed at the root of the tree. Once equations are derived for the change in the probability over the change in time, the time intervals are shrunk by using the definition of a derivative to obtain differential equations describing the change in these probabilities (Maddison et al., 2007). By integrating these differential equations along the branches, this method then solves for the overall probability of the data given the BiSSE model with a likelihood score. The MuSSE then introduces more parameters from new discrete characters (FitzJohn et al., 2009). This score then allows someone to compare and contrast different models where, for example, transitions from one character to another are set to 0 or extinction rates are set to be equal using AIC.

Table 2.2: Tailed ascidian species described in Molgulidae and Styelidae their habitat, and larval characteristics.

Species	**Habitat**	**Larval Tailed?**	**Brooding?**	**Self-Fertile?**	**Reference**
Botrylloides leachi	Rock	Yes	Yes	?	Berrill (1931)
B. planus	Rock	Yes	Yes	?	Zeng et al. (2006)
B. primigenus	Rock	Yes	Yes	?	Van Name (1945)
B. schlosseri BS19 E	Rock	Yes	Yes	?	Berrill (1931)
B. schlosseri BS31 D	Rock	Yes	Yes	?	Berrill (1931)
B. schlosseri BS8 A	Rock	Yes	Yes	?	Berrill (1931)
B. violaceus	Rock	Yes	Yes	No	Brown and Swalla (2007)
Botrylloides diegensis	Rock	Yes	Yes	?	Van Name (1945)
Cnemidocarpa finmarkiensis	Rock	Yes	No	?	Bishop et al. (2001)
C. humilis	Rock	?	?	?	
Dendrodoa aggregata	Rock	Yes	Yes	?	Berrill (1931)
D. grossularia	Rock	Yes	Yes	?	Berrill (1931)
Distomus variolosus	Rock	Yes	Yes	?	Berrill (1948)
Metandrocarpa taylori	Rock	Yes	Yes/No	?	Watanabe and Lambert (1973)
Molgula citrina	Rock	Yes	Yes	?	Berrill (1931)
M. complanata	Rock	Yes	Yes	?	Berrill (1931)
M. manhattensis	Rock	Yes	No	No (Lacaze-Duthiers, 1877)	Berrill (1931)
M. occidentalis	Rock	Yes	No	Yes (Lacaze-Duthiers, 1877)	Van Name (1945)
M. oculata	Rock	Yes	No	Yes (Lacaze-Duthiers, 1877)	Berrill (1931)
M. socialis	Rock	Yes	No	?	Berrill (1931)
Polyandrocarpa tincta	Rock	Yes	Yes	?	Berrill (1931)
P. zorritensis	Rock	Yes	Yes	?	Vazquez and Young (1996)
Polycarpa aurata	Rock	?	?	?	
P. mytilerga	Rock	?	?	?	
P. papillata	Rock	Yes	No	?	Zeng et al. (2006)
P. pomaria	Rock	Yes	Yes/No	?	Zeng et al. (2006)
Polyzoa opuntia	Rock	Yes	Yes	?	Van Name (1945)
Stolonica socialis	Rock	Yes	Yes	?	Berrill (1948)
Styela gibsii	Rock	Yes	No	?	Young and Chia (1985)
S. montereyensis	Rock	No	Yes	?	Young and Braithwaite (1980)
S. plicata	Rock	Yes	No	?	Thiyagarajan and Qian (2003)
Symplegma reptans	Rock	Yes	Yes	?	Sugimoto and Nakauchi (1974)
S. rubra	Rock	Yes	Yes	?	Monniot and Monniot (1997)
S. viride	Rock	Yes	Yes	?	Berrill (1931)

Trees were generated from 1840 bp of *18S rRNA* in Genbank for 45 ingroup taxa of Styelidae and Molgulidae from Genbank and from the phlebobranch outgroup, Ciona intestinalis. Sequences were aligned using MAFFT v. 6 (Katoh and Toh, 2008). We used a

GTR + Γ + I substitution model selected using the Akaike Information Criterion (AIC) (Akaike, 1974) in MrModeltest v. 2.3 (Nylander, 2004). Because ascidians have a scanty fossil record (Fedonkin et al., 2012), we used an uncorrelated, relaxed-clock, phylogenetic estimation in BEAST (Drummond and Rambaut, 2007) for 100 million generations. Table 2.1 contains species with tailless hatching, and tailless non-hatching larval development. We were not able to use certain tailless species due to tissue being unavailable (Table 2.1). The development of 31 of 34 tailed hatching species used in phylogeny reconstruction can be found in Table 2.2. For the three species where larval developmental information was not available, we assumed they were tailed hatching species because all other species in their genera used in the analyses have tailed hatching larval development.

Brooding and self-fertility were other characters scored in Tables 2.1 and 2.2 for species used in these analyses. We define brooding as an embryo developing and hatching into the larval (hatching species) or juvenile (non-hatching) within the brood chamber or atrial siphon of the parent. We tested to see if there were differences in diversification rates between brooding and non-brooding species in BiSSE and found the null model of no difference in diversification rate between brooders and non-brooders to have the lowest AIC score in 100 of 100 posterior trees sampled. Since ascidians are hermaphroditic, self-fertility is the ability for an individual to be able to fertilize an egg with its own sperm. Since one needs to usually do fertilizations to discover this, this information is lacking for many of the species in our analyses (Table 2.1; Table 2.2). Data on tailed and tailless larval development and hatching and non-hatching tailless larvae (Table 2.1; Table 2.2), were used to examine how these categorical traits influence speciation rates using a binary-state speciation and extinction (BiSSE) and multi-state speciation and extinction model (MuSSE) (Maddison et al., 2007; FitzJohn et al., 2009) in the R package diversitree (FitzJohn, 2012). The BiSSE framework compared models where diversification differed between tailed and tailless larval development, hatching and non-hatching as well as the combination of these characters in the MuSSE framework.

AIC (Akaike, 1974) was used to test between different models on the consensus tree in BEAST and 100 posterior trees. We compared only speciation rates in the BiSSE and MuSSE analyses because simulation studies have shown extinction rates are difficult to estimate assuming a birth-death model of speciation and extinction as in BiSSE and MuSSE (Rabosky, 2010). The skeletal trees incomplete sampling method was used to account for the missing taxa in our tree when estimating rates using BiSSE and MuSSE (FitzJohn et al., 2009). Extinction rates were set to be the same rates, given the potential for misestimation and parameter correlation. However, it is possible that if extinction rates were widely divergent between species with different larvae, then our model would not be an adequate description of the process, and our inferences may be misled. While we acknowledge this as a possibility, given how notoriously unreliable estimates of extinction rates are (Rabosky, 2010), and that estimates derived from molecular phylogenies tend to be very low (often close to 0), we do not think this is a likely scenario.

We estimated geographic range sizes for all 45 species of styelid and molgulid ascidians using OBIS and GBIF (http://www.obis.com; http://www.gbif.org). Many species in the Molgulidae and Styelidae have been introduced into non-native native habitat (Lambert et al., 2010), making native geographic ranges difficult to assess. We took phylogeographic studies for invasive species into account for assessing native range sizes by using what the authors concluded were the most conservative estimate of native range sizes for *Botryllus schlosseri*, *Molgula manhattensis*, and *Styela partita* (Bock et al., 2012; Haydar et al., 2011; Pineda et al., 2011). For the rest of the species, we removed outilers from the latitude points, because most ranges had a large coastal North-South distribution. We made a conservative estimate of geographic range size by taking the interquartile range of the second and third quartile of the latitude coordinate for each species and kept all latitude points that were within the difference of the second quartile and interquartile range and the sum of the third quartile and the interquartile range. The corresponding longitude point for each record was also kept (Figure 2.1, Figure 2.2).

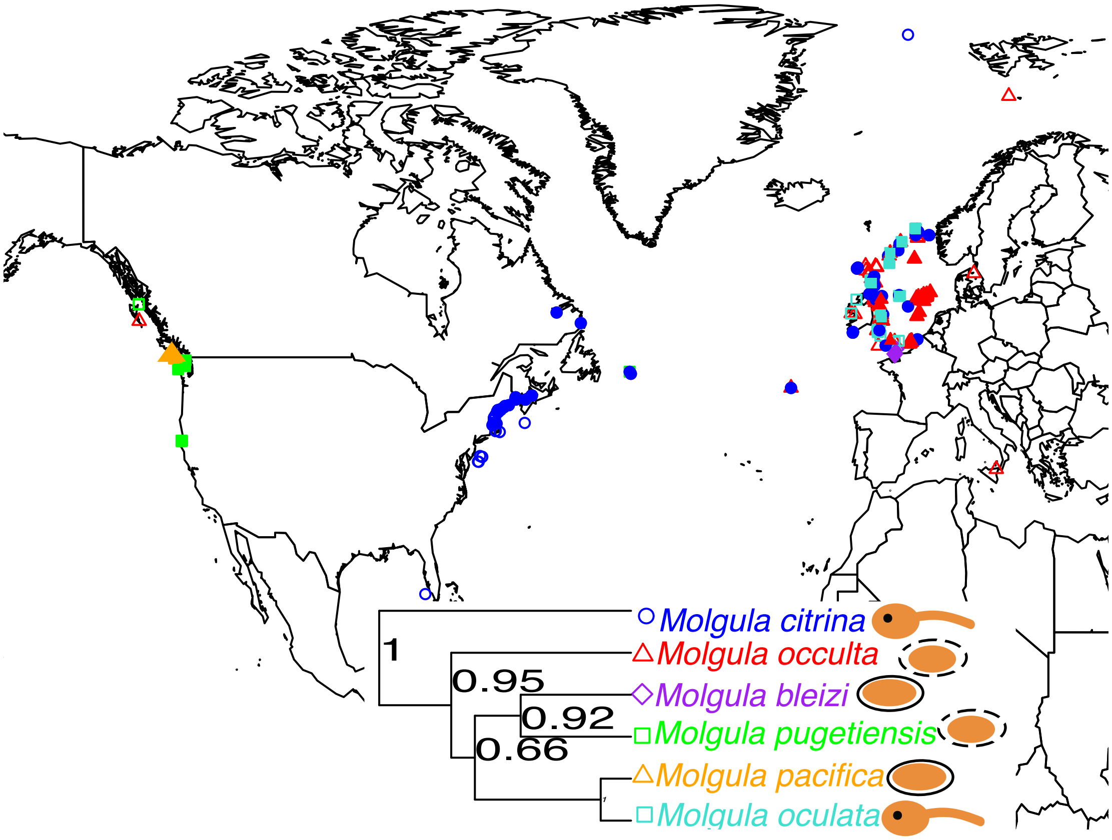

Figure 2.1: Range size estimates for the Roscoff clade of molgulids. Distribution points with open circles were removed as outliers from the range estimation. Branches to the left of the species names show the phylogenetic relationships from 18S rRNA shown otherwise in Figure 2.4. Cartoon shows the larval development (hatching or non-hatching in dotted or non-dotted capsules).

After removing outlier occurrence points, range sizes were estimated in two different ways: using a greatest line between two species occurrences or the ellipsoid area of all occurrences (Figure 2.2) while also taking into account the curvature of the Earth. These estimations of geographic range are commonly used (Gaston, 1996). We estimated ranges in R using the packages maptools (Lewin-Koh, 2012) and geosphere (Hijmans et al., 2012). If a species was only described from one locality, they were given a range of 1 km for the greatest line distance and ellipsoid area. If a species was described from two localities, then the geographic range from the greatest line distance was used for the ellipsoid area.

We then tested to see if either binary or additively larval traits were a predictor of geographic range size using Bayesian phylogenetic mixed models (BPMM) in the R package

MCMCglmm (Hadfield and Nakagawa, 2010). The posterior probability of different models were estimated using a MCMC approach, running the chains for ten million iterations with a one million iteration burn-in.

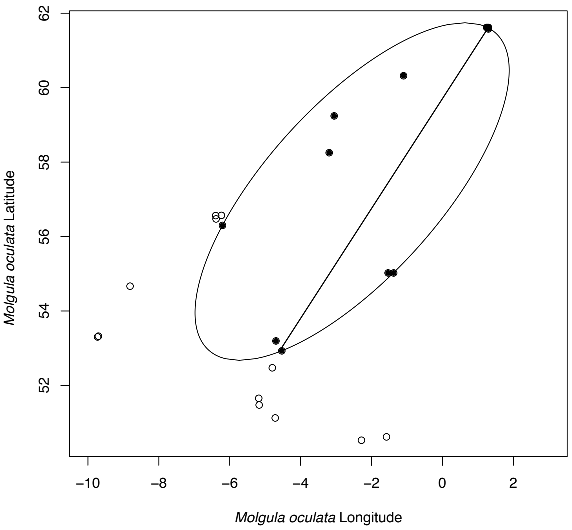

Figure 2.2: Example of distribution points removal for distribution records for *Molgula oculata*. Open circles are points removed to make a conservative estimate of range size. We took a rhumbline distance, or the longest straight-line distance, and the ellipsoidal area for all 45 species used in this study. We also took into account the curvature of the Earth in these range estimates.

In the initial studies presenting the methods for BiSSE, the authors cautioned using these methods on a tree of less than several hundred taxa because these methods could falsely reject a null hypothesis of no difference in diversification on a tree with fewer taxa (Maddison et al., 2007). In order to explore if our results have potentially introduced Type-I error, we simulated data for 100 trees of 23, 45, 90, and 450 tips using the diversitree package. We simulated these trees using the normal distributions of speciation, extinction, and transition rates derived from our BiSSE results on the Molgulidae. We simulated trees using mean parameters found across our 100 tree BiSSE analyses of tailed and tailless species. We simulated trees with parameters where speciation, extinction, and transition rates for state 0 were drawn from a normal distribution with means of 0.25, 0.167, and 0.125, respectively. We used parameters where speciation, extinction, and transition rates for state 1 were drawn from a normal distribution with means of 0.1, 0.22, and 0.167, respectively. We simulated trees of 23, 45, 90, and 450 tips using the same speciation, extinction, and transition rates for state 0 and 1 (where state 0 was at the root), to test if tree size impacted diversification rate estimates. We then did this for 100 different rates.

2.4 *Results and Discussion*

All 100 trees show higher speciation rates in tailed species compared to tailless species (Figure 2.3A), and 99/100 trees show higher speciation rates in hatching species compared to non-hatching species (Figure 2.3B).

Table 2.3: Model comparison using AIC for BiSSE and MuSSE analyses. The best-fit model from the BEAST consensus tree is in bold and the number of 100 posterior trees that agree with the best-fit model from the consensus tree are labeled. Td - Tailed; Tl - Tailless; H - Hatching; NH - Non-Hatching; SI - State Independent; SD - State Dependent; q - transition.

Td vs. Tl		**H vs. NH**		**Interaction**		**Td+H vs. Tl+H vs. Tl+NH**	
Model	AIC	Model	AIC	Model	AIC	Model	AIC
SD	**-212.08**	SD	-223.82	SI	-174.58	SD	-183.72
SD-qTl-Td	-209.38	**SD-qNH-H**	**-226.12**	SD	-145.94	SD-q21	-186.84
		SD-qH-NH	**-225.58**	**Interaction**	**-191.26**	SD-31	-188.30
						SD-q21, q31	-190.43
						SD-q21, q32	**-192.38**
# trees agree	80/100	# trees agree	61/100	# trees agree	100/100	# trees agree	54/100

We tested to see if a combination of our two sets of binary larval traits (tailed and taillessness, hatching and non-hatching) is better for assessing speciation rates using a recently developed test (FitzJohn, 2012). All 100 trees show a better AIC fit for a model where speciation is estimated as a combination of these binary traits (Table 2.3). 94/100 trees and 100/100 trees show higher speciation rate estimates in the interaction model when compared to speciation rate estimates for tailed and tailless alone, or for hatching and non-hatching alone, respectively. 100/100 posterior trees show the highest speciation rates in tailed hatching species when compared to tailless hatching, and tailless non-hatching species. Tailless hatching species show higher speciation rates when compared to tailless non-hatching species in 65/100 posterior trees.

Mean rhumbline range sizes for all tailed, hatching species used in the study were 2425 ± a standard error (s.e.) of 1201 km. Range sizes were 2164 km ± s.e. 1038 km, and 201.4 ± 239.9 km for tailless, hatching, and tailless, non-hatching species, respectively . For rhumbline range sizes (Figure 2.2), we found species with hatching larval development to

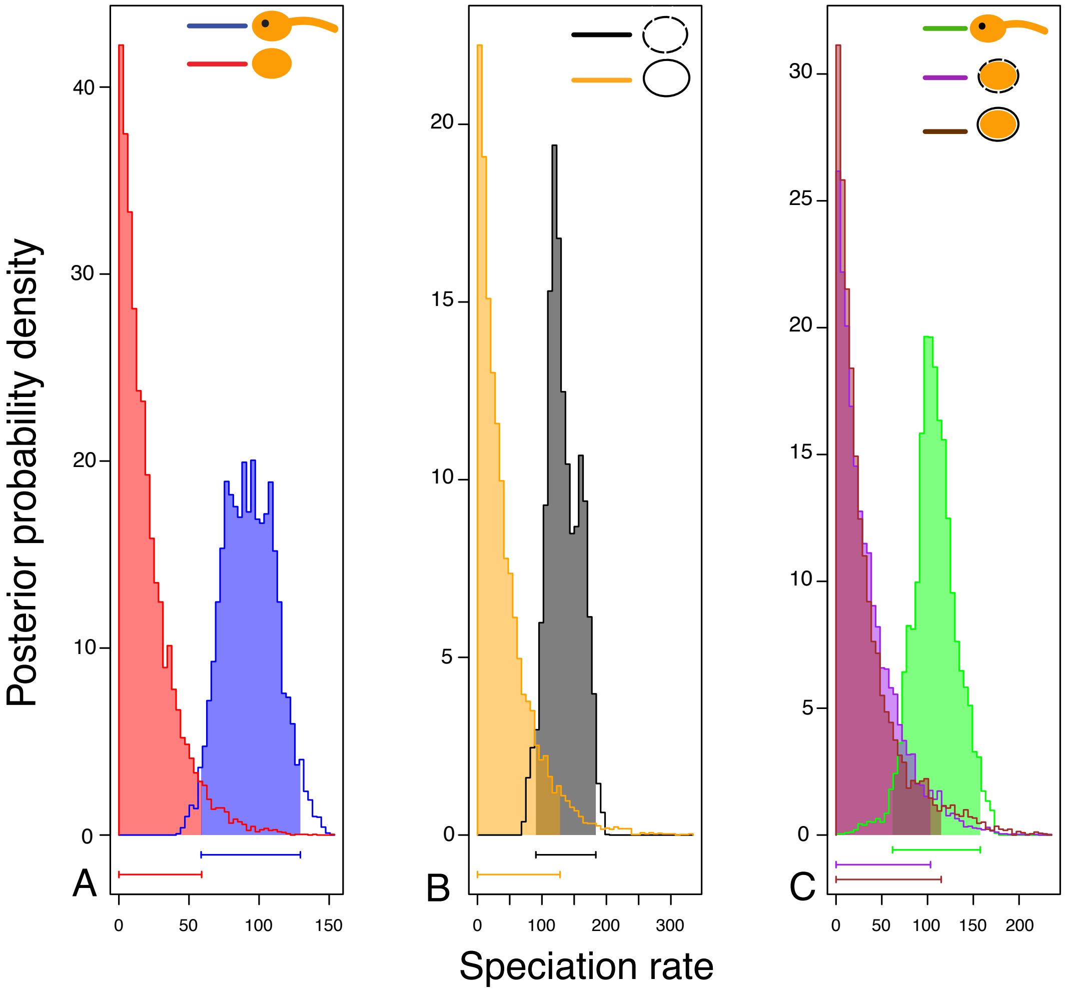

Figure 2.3: Speciation rates (in speciation events per substitution per site) for different larval character states using BiSSE and MuSSE. A) speciation rates from BiSSE analyses of tailed and tailless species; B) speciation rates from BiSSE analyses of hatching and non-hatching species; C) MuSSE model for species with tailed hatching, tailless hatching, and tailless non-hatching larval development. The line below each speciation rate distribution is the 95% credible interval. Species with tailed larvae have a tail and eyespot, tailless species that hatch have a dotted circle, and tailless species that do not hatch have a solid circle.

have larger range sizes than species with non-hatching larval development ($\beta = 2.51$ ± s.e. 2.12; p = 0.0212). We found similar results for total range sizes comparing species with hatching and nonhatching larvae ($\beta = 5.066$ ± s.e. 2.56; $p < 0.001$). We found mean range sizes for species with tailed hatching larval development to be the largest, mean range sizes for species with tailless hatching larval development to be intermediate, and mean range sizes for species with tailless non-hatching larval development to be the smallest ($\beta = 4.80$ ± s.e. 3.41; p = 0.0067). Mean ellipsoid range sizes for all tailed, hatching species used in the study were 3.070 X 10^6 ± 2.711 X 10^6 km^2. Range sizes were 2.638 X 10^6 ± s.e. 2.322 X 10^6 km^2, and 3.883 X 10^4 ± 4.772 X 10textsuperscript4 km^2 for tailless, hatching, and tailless, non-hatching species, respectively. Figure 2.4 shows the BEAST consensus tree with larval development and geographic ranges for each species (Figure 2.4). We used a Bayesian Phylogenetic Mixed Model (BPMM) (Rabosky, 2010) to estimate if larval development is a predictor of range size. For ellipsoid range sizes (Figure 2.2), we found tailed larval development is significantly positively correlated when compared to tailless larval development ($\beta = 3.33$ ± s.e. 3.97; p=0.040). Hatching larval development is significantly positively correlated with range size when compared to non-hatching larval development ($\beta = 6.05$ ± s.e. 3.13; p=0.0048). Tailed hatching larval development is significantly positively correlated with range size when compared to tailless hatching, and tailless non-hatching larval development ($\beta = 5.30$ ± s.e. 5.26; p = 0.049).

Our results demonstrate that species with tailed, hatching larval development have higher speciation rates than ascidian species with tailless hatching, and tailless non-hatching larval development (Figure 2.3), based on the available phylogeny (Figure 2.4). Data for 45 of an estimated 762 described species in the Molgulidae and Styelidae were used, but we did use an incomplete sampling method to take this missing data into account (FitzJohn et al., 2009). Nevertheless, the small sample of species could bias our results. We also have to be cautious that the phylogeny used for this study may not be the true species tree due to the coalescent process (Liu et al., 2009). However, most clades are reflective of taxonomic and morphological relationships (Van Name, 1945), and are in agreement with a phylogeny we inferred on a subset of taxa at *18S* and *28S rRNA* genes. We accounted for low support for species relationships in our phylogeny by comparing BiSSE and MuSSE analyses across 100 posterior trees.

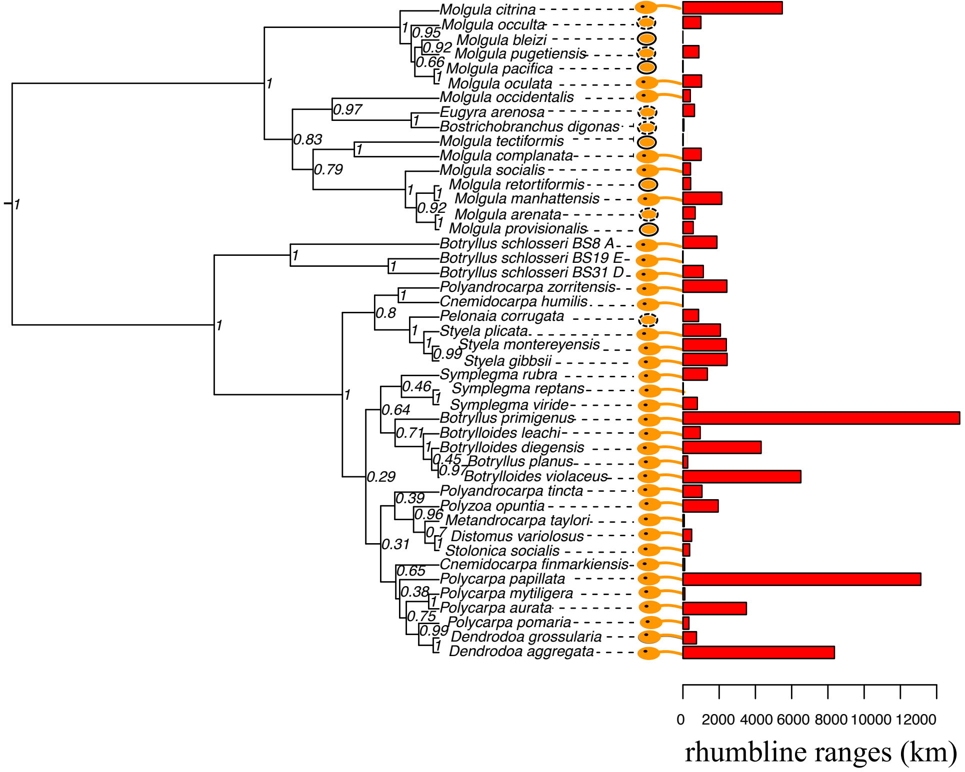

Figure 2.4: Ultrametric phylogeny of molgulid and styelid ascidians from a consensus BEAST tree. The lower axis bar graph shows range size for each species in km. Species with tailed larvae have a tail and eyespot, tailless species that hatch have a dotted circle, and tailless species that do not hatch have a solid circle.

A simulation study examining the ability to detect differences in rates of speciation, extinction and character transitions found there to be a decrease in the ability to detect the true simulated differences using BiSSE when phylogenies were moderately sized (Davis et al., 2013). However, low power should tend to reduce our ability to detect differences between parameters, rather than exacerbate them. We have found that when we simulate trees with a 2.5 times difference in speciation rates between the ancestral and the derived state, decreases in trees of 23 species (77/100 trees). However, our tree size of 45 tips is enough to detect similar results in large trees (95/100 trees with 45 tips show higher speciation rates in the ancestral character compared to 98/100 trees with 450 tips; Figure 2.5). Therefore, having a tree of 45 species will be large enough to obtain reliable estimates of diversification between two character states in a tree. Figure S4 also suggests that there is a larger deviation from the true rates under which the trees were simulated for trees of 23 species (Figure 2.6). While this analysis did not take into account the 'incomplete sampling' method or investigate different values between speciation, extinction, and transition rates, further simulation studies will need to be done to disentangle the effect of these methods on power.

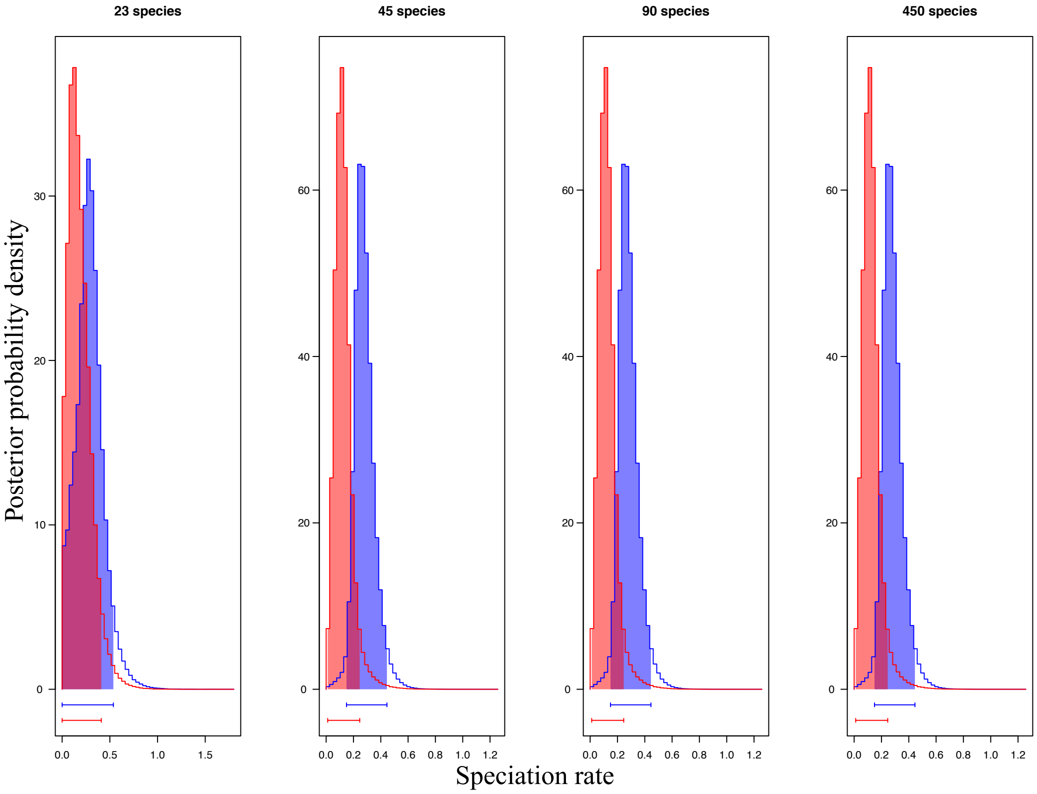

Figure 2.5: BiSSE power analysis results. MCMC analyses were run for 10000 iterations with 1000 iteration burn-in and concatenated across 100 datasets for four different tree sizes.

We believe that tailed, hatching species cannot evolve from a tailless ancestor because there is molecular evidence to show pseudogenes are formed in proteins critical for swimming in multiple tailless species (Swalla et al., 1999). It is still unclear, however, why these derived modes of larval development have evolved multiple times (Figure 2.4). Species selection may be acting to maintain tailed, hatching larval development in molgulid and styelid ascidians; this argument was made to explain higher diversification rates in plant species with ancestral self-incompatibile reproduction than in species with derived self-compatible reproduction (Goldberg et al., 2010).

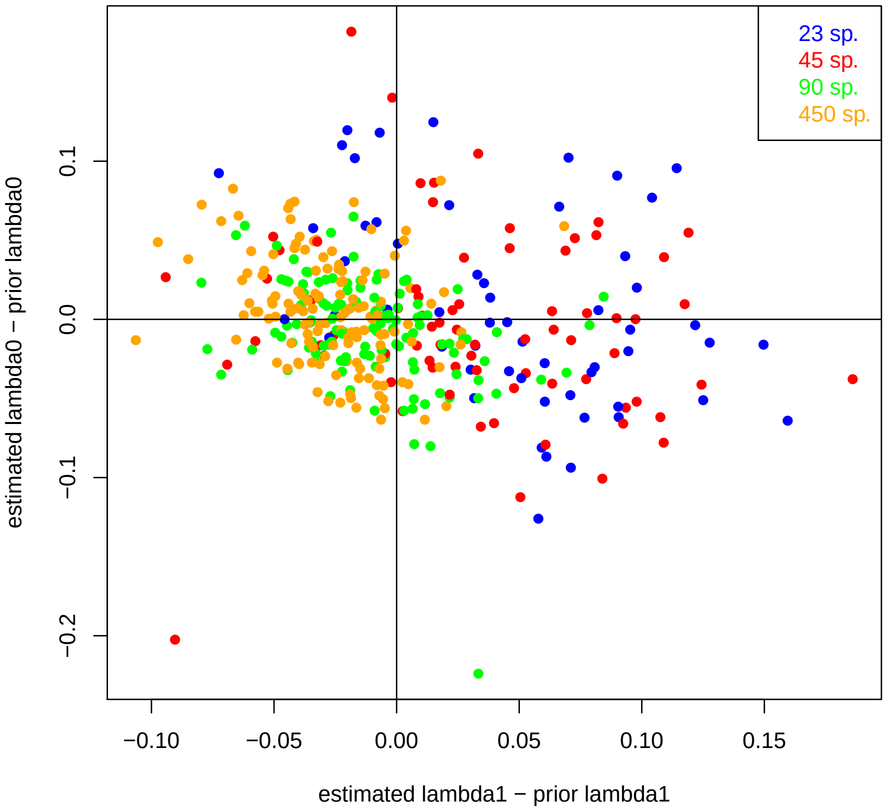

Figure 2.6: Power analysis results for trees with 23, 45, 90, and 450 species. The plot shows the difference between the estimated speciation rate and true speciation rate under which the speciation rate was simulated. The y-axis shows character state 0 and the x-axis shows character state 1. Expected estimates that were perfect would equal 0 on both axes.

Our results showing lower speciation rates in the derived tailless and non-hatching species, which also have significantly smaller geographic ranges, is contrary to the findings of snails and urchins in the fossil record where derived species with smaller geographic ranges have higher speciation rates (Hansen, 1978, 1983; Jablonski, 1986; Jeffery et al., 2003). These contrary results may also be due to unforeseen issues with comparing speciation rates estimated from fossil data and molecular phylogenies. It is also possible that these contrary results could be due to comparing speciation rates for groups with alternative larval development that are fundamentally different. The transition from feeding to non-feeding larval development in fossil snails and sea urchins may fundamentally affect dispersal and diversification dynamics in different ways than the loss of the tail and hatching in ascidians.

While these transitions to a less dispersive larval mode decrease range sizes in all of the groups compared, range size may not predict speciation dynamics entirely. The findings of lower speciation rates in derived tailless and non-hatching styelid and molgulid ascidian species could also be influenced by the population dynamics of these species with alternative modes of larval development. Some simulation studies support lower speciation rates in species with ecological and geographically patchy distributions (Gavrilets and Vose, 2005; Birand et al., 2012). Species with non-hatching tailless larval development typically have patchy distributions and are found in very specific locations. For example, *Molgula pacifica* is found in high wave action “blow-holes” (Young et al., 1988).

Stanleys work on burrowing bivalves predicted a log-normal relationship between mean population size and speciation rate (Stanley, 1986). This curve would predict at very low speciation rates, small population sizes, and we suggest that this could be the case for tailless non-hatching molgulids (Stanley, 1986). This rationale was used to describe species with small average population sizes, going through a higher rate of extinction than speciation, so “many entire species are dying out, then few small populations representing incipient species will be able to blossom into full-fledged species” (Stanley, 1979). While we are unable to reliably estimate extinction rates using our methods (Rabosky, 2010), more work will be needed to understand the population dynamics of species with different modes of development in ascidians and other marine invertebrates.

Chapter 3

MOLGULID ASCIDIANS HAVE A RADICAL HETEROCHRONIC SHIFT IN THE METAMORPHIC GENE NETWORK

3.1 Abstract

Transcriptome and genome data offer new approaches to examine the origin and evolution of the chordate body plan. We study chordate body plan evolution in ascidians by comparing two closely related species with radically different larval body plans–the tailed *Molgula oculata* and the tailless *M. occulta*. Embryos of tailed *M. oculata* develop 40 notochord cells that converge and extend to form the notochord in the center of the tadpole larvas tail. Muscle cells flank the notochord in the tail of *M. oculata* and are critical for larval swimming. In the head is the otolith, a gravity-sensing organ that is important for larval settlement at metamorphosis. The embryo of *M. occulta* develops only 20 notochord cells, these cells do not converge and extend to form a tail during larval development, and no muscle cells develop because larval muscle genes have become pseudogenes. *M. occulta* also has no otolith in the larval stage. We show by transcriptome analyses that the ascidian metamorphosis program begins earlier in molgulid ascidians than in other ascidian species. A radical heterochronic shift has been documented in another tailless ascidian, *Molgula tectiformis*, and is now reported for both the tailed, *M. oculata* and tailless *M. occulta*. Further, gene networks involved in cell communication, and transport and catabolism are more highly expressed in the tailed *M. oculata* than the tailless *M. occulta* early in development. Additionally, the regulation of an autophagy gene network is more highly expressed in the tailless *M. occulta* than the tailed *M. oculata*, which suggests this network may be involved in the early degradation of cells that form the tail. These findings support the hypothesis that the pronounced metamorphosis gene network heterochrony is a preadaptation for evolution of tailless development in molgulid ascidians.

3.2 Introduction

Most of the approximately 3,000 described invertebrate chordate ascidians (Tunicata: Ascidiacea) (Shenkar and Swalla, 2011) develop as a tadpole larva, which swims for a period of several hours to days depending on the species, then reaches metamorphic competence, settles and metamorphoses into a sessile, filter-feeding adult (Davidson et al., 2003). Phylogenomic evidence suggests Tunicata is the sister-group to vertebrates (Delsuc et al., 2006; Bourlat et al., 2006). Ascidians in the Molgulidae are a monophyletic group of solitary ascidians (Ascidiacea: Stolidobranchia) that are sister to the families Styelidae and Pyuridae (Huber et al., 2000; Zeng and Swalla, 2005; Tsagkogeorga et al., 2009). Molgulidae contains species that have tailed, chordate larval development with notochord and muscle, and closely related species that have tailless larval development and lack the sensory otolith, and muscle and notochord in the tail (Swalla and Jeffery, 1990). The loss of a tailed larva has happened multiple times independently (Hadfield et al., 1995; Huber et al., 2000), has led to a decrease in evolutionary diversification in lineages that have tailless larvae (Maliska et al., 2013), and is a study-system that is tractable at the molecular level (Swalla and Jeffery, 1996; Swalla et al., 1999).

Studying the evolution of chordate tissues such as notochord, dorsal nerve cord, and pharyngeal slits can provide insights into how complex evolutionary traits evolve and are lost (Brown et al., 2008). Like the majority of ascidians, *Molgula oculata* undergoes tailed larval development and has 40 notochord cells that converge and extend (Swalla and Jeffery, 1990), similar to development in the widely studied ascidian species *Ciona intestinalis* (Di Gregorio et al., 2002; Jiang et al., 2005; Kugler et al., 2011). In contrast, its sister-species *M. occulta* undergoes a modified larval development in which the tail does not develop; *M. occulta* has only 20 notochord cells that do not converge or extend (Swalla and Jeffery, 1990). As a result of tailless larval development, *M. occulta* and other tailless ascidians lack chordate features like the otolith, a neural sensory organ, and a tail with both a notochord and striated muscle (Swalla and Jeffery, 1990; Swalla et al., 1999). *M. oculata* and *M. occulta* develop similarly until neurulation, when *M. oculata* undergoes convergence and extension of notochord cells and makes a tail (Swalla and Jeffery, 1990). After hatching, the tailed larva of *M. oculata* swims; the larva reaches metamorphic competence while swimming, settles to the substrate on the anterior of the head or trunk, then the tail is absorbed and metamorphosis into the adult body plan is completed, similar to metamorphosis in *Boltenia villosa* (Davidson et al., 2003). The tailless *M. occulta*, however, proceeds to metamorphosis directly after hatching (Swalla and Jeffery, 1990). Comparisons of *M. oculata* and *M. occulta* showed that *manx*, a zinc finger protein, and *p68/bobcat*, a DEAD-box RNA helicase, are critical for the formation of the tail (Swalla and Jeffery, 1996; Swalla et al., 1999).

The tailless larval phenotype in *M. occulta* is rescued in some embryos when hybrid embryos are produced with the tailed *M. oculata* (Swalla and Jeffery, 1990; Swalla et al., 1993; Swalla and Jeffery, 1996; Swalla et al., 1999). When sperm from the tailed *M. oculata* fertilizes an egg from the tailless *M. occulta*, some of the resulting hybrid larvae have an eyespot, an otolith, and a converged and extended tail half the size with half of the amount of notochord cells (Swalla et al., 1993).

The exact molecular trigger for ascidian metamorphosis is still not known, but inhibitory nitric oxide signaling and the expression of the *hsp90* chaperone are involved in pyurid ascidians (Bishop et al., 2001). Previous work on metamorphosis in ascidians has shown that a suite of genes – ‘metamorphosis genes – are expressed at metamorphic competence in the phlebobranch ascidian *Ciona intestinalis* (Nakayama et al., 2001), and in the pyurid ascidians *Boltenia villosa* (Davidson and Swalla, 2001, 2002; Davidson et al., 2003) and *Herdmania curvata* (Woods et al., 2004).

In *Ciona intestinalis*, *Ci-meta1*, *Ci-meta2* and *Ci-meta3* were discovered as novel genes expressed in metamorphically competent larvae or metamorphosed juveniles (Nakayama et al., 2001). Differentially expressed genes were identified at metamorphic competence, larval settlement and at the juvenile stage in *Boltenia villosa* (Davidson and Swalla, 2002).

Some of the ‘metamorphosis genes in *B. villosa* are involved in innate immunity (Davidson and Swalla, 2002), and inhibition of *mannose binding lectin associated serine protease* (*masp*), which is a critical component of the innate immunity machinery (Fujita, 2002), causes metamorphic arrest (Roberts et al., 2007). A large set of ‘metamorphosis genes was identified in the pyurid *Herdmania curvata*, and these genes included *meta1*, *meta2*, *meta3*, *masp* and other innate immunity genes first identified in *B. villosa*, and other genes involved in cell-cell communication and transport and catabolism (Woods et al., 2004).

Previous transcriptome data published on a tailless molgulid ascidian from Japan, *Molgula tectiformis*, showed expression in ‘metamorphosis genes identified from *C. intestinalis*, *B. villosa* and *H. curvata* as early as the gastrula stage (Gyoja et al., 2007). Gyoja et al. (2007) hypothesized tailless larval development in *M. tectiformis* was caused by the early expression of genes expressed at or shortly before metamorphosis in other ascidians. While Gyoja et al. (2007) only examined the tailless *M. tectiformis*, understanding the differences in expression has yet to be done between molgulid species with normal tadpole larval development and species with tailless larval development.

Here, we present transcriptome data showing there is an early heterochronic shift in the expression of ‘metamorphosis genes in molgulid species (Stolidobranchia: Molgulidae). Cell-cell communication and transport and catabolism pathways are more highly expressed in the tailed *M. oculata* than the tailless *M. occulta*. We also find that genes in the regulation of autophagy are more highly expressed in the tailless *M. occulta* than the tailed *M. oculata*. Additionally, hybrids from *M. occulta* eggs and *M. oculata* sperm show high expression of the maternal alleles, and significantly higher expression of ‘metamorphosis genes from the maternal alleles.

3.3 Methods

3.3.1 Embryo Collection

Molgula oculata and *M. occulta* were collected from sand flats near Station Biologique de Roscoff in France. The gonads were dissected to retrieve gametes from both species and gametes were fertilized with sperm from the same species. Hybrids were produced with *M. oculata* sperm and *M. occulta* eggs. RNA was collected from gastrula, neurula, and tailbud stage embryos of *M. oculata*, *M. occulta*, and hybrid embryos and further purified for transcriptome sequencing.

3.3.2 Transcriptome Assembly and Counts

Ten lanes were used to sequence the mRNA, one lane each for *M. oculata*, *M. occulta*, and the hybrid at the gastrula, neurula, and tailbud stages, and the *M. occulta* gastrula stage was repeated. We sequenced libraries on an Illumina Hi Seq 2000 at the Research Technology Support Facility at Michigan State University. We used 76 bp paired-end reads from a 250 bp insert size. Since the Molgulidae did not have genomic data available at the time, we assembled transcriptomes *de novo* using a novel pipeline. We quality-filtered reads, then filtered the reads using Digital Normalization, a process developed in the Brown lab (http://arxiv.org/abs/1203.4802). Reads were scanned to see if they contained new information to a coverage of 20, removing redundant and erroneous reads. The filtered reads were then assembled using Velvet v.1.7.07 (Zerbino and Birney, 2008) and Oases 0.2.08 (Schulz et al., 2012). The expression levels were calculated using the khmer package (https://github.com/ctb/khmer) developed by the Brown Lab. Reads were loaded into a counting Bloom filter at a k-mer–read length–of size 21. Once the reads are loaded, the median k-mer abundance is calculated for each transcript in the assembled transcriptomes for both *M. occulta* and *M. oculata.* Next the counts are normalized by the total kmer abundance per transcriptome. To compare *M. occulta* and *M. oculata* transcripts to the *Ciona* *intestinalis* gene models, we used a dataset of 55,017 predicted transcripts from 15,254 gene models for *C. intestinalis* (Satou et al., 2008) then used BLAST+ (Camacho et al., 2009) with an amino acid query (tblastn) and an acceptance e-value score of less than 10^{-6}.

3.3.3 'Metamorphosis Gene Identification

In order to identify orthologous genes in the *Molgula oculata* and *M. occulta* transcriptomes, we collated a list of 133 genes expressed in other ascidians at metamorphosis (Davidson and Swalla, 2002; Woods et al., 2004; Gyoja et al., 2007) (Table S1). We also included in this list, *manx* and *p68/bobcat*, two genes part of the same locus shown to be down-regulated in the tailless *M. occulta*, and required for the formation of the tail in *M. oculata* (Swalla and Jeffery, 1996). We used BLAST+ (Camacho et al., 2009) to examine the expression of these 'metamorphosis genes in the *M. occulta* and *M. oculata* transcriptomes, using an amino acid query (tblastn) and an acceptance e-value score of less than 10^{-6}. We then used BLAST+ (Camacho et al., 2009) to identify nucleotide mRNA transcripts using an amino acid query (tblastn) and an acceptance e-value score of less than 10^{-6}.

3.3.4 Test for Heterochrony of 'metamorphosis genes' in Molgulids

We examined the timing and expression of 'metamorphosis genes using the transcriptome data for *M. oculata* and *M. occulta* for the gastrula, neurula and tailbud stages, and previously published data for the tailless *Molgula tectiformis* (Gyoja et al., 2007) and the tailed phlebobranch, *C. intestinalis.* We examined expression of the 133 'metamorphosis genes in *M. tectiformis* in the gonads, and at the cleavage, gastrula and larval stages (Gyoja et al., 2007). We used the cleavage, gastrula, juvenile, and adult stages to compare the expression of metamorphosis genes in *C. intestinalis.* This unpublished data from Satou et al. (2004) was available on Genbank. To score the presence or absence of a gene at a stage we used blast+ (Camacho et al., 2009) with an acceptance e-value score of less than 10^{-6}; if an e-value score was less than 10^{-6}, then it was counted as present, if greater than 10^{-6}, then absent. In order to test if stages for each species had different sets of ‘metamorphosis genes present and absent than would be expected by chance, we compared presence/absence matrices using a two-tailed Fisher‘s Exact Test. All plots and statistical analyses were done using R (Team, 2012). Phylogenetic relationships were drawn from established relationships published previously (Huber et al., 2000; Zeng and Swalla, 2005; Tsagkogeorga et al., 2009).

3.3.5 Differential Expression

We examined differential expression between *M. occulta*, *M. oculata*, and the differential expression of the parent alleles in the hybrid. We log2-transformed the normalized counts (see above) from the transcripts blasted to the 133 ‘metamorphosis genes for *M. occulta* embryos, *M. oculata* embryos and the *M. occulta* and M oculata alleles in the hybrid embryos. We calculated the log2 fold change between counts by taking the difference between the log2-transformed counts of *M. oculata* and *M. occulta* or the *M. oculata* and *M. occulta* alleles in the hybrid. If the value is positive, there are more counts in *M. oculata*; if the value is negative there are more in *M. occulta.* We then calculated whether the mean expression of ‘metamorphosis genes found in 1) *B. villosa*, 2) *H. curvata*, 3) *M. tectiformis* or 4) all three species combined was significantly different from the mean expression of all the *C. intestinalis* transcripts, which were shared in *M. oculata* and *M. occulta* using a two-sample Mann-Whitney-Wilcoxon non-parametric test.

3.3.6 KEGG Pathways

We identified the role and potential function of genes from the ‘metamorphosis genes dataset to identify potential pathways that may be activated during metamorphosis in ascidians.

Based on examination of these 133 genes, we identified many genes to be involved in cell-cell communication, cell growth and death, immune system function and transport and catabolism. To further explore all the gene networks involved in these areas, we downloaded amino acid sequences for entire pathways from the Kyoto Encyclopedia of Genes and Genomes (Ogata et al., 1999). We downloaded all the gene models implicated to function in these three areas of gene function for *Ciona intestinalis* or in *Strongylocentrotus purpuratus* if a gene model for that specific gene was not found in *C. intestinalis*. We then calculated whether the mean expression of the genes in a pathway was significantly different from the mean expression of all the *C. intestinalis* transcripts, which were shared in *M. oculata* and *M. occulta* using a two-sample Mann-Whitney-Wilcoxon non-parametric test.

3.4 Results and Discussion

3.4.1 Transcriptome Analysis

We sequenced embryos of the tailed *Molgula oculata*, the tailless *M. occulta* and a hybrid – from sperm of the tailed *M. oculata* and eggs from the tailless *M. occulta* – at the gastrula, neurula and tailbud stages. Each library was sequenced on an individual lane of an Illumina Hi Seq 2000, and the *M. occulta* gastrula stage was sequenced twice. We obtained 75 million, 148 million (two lanes), and 73 million reads at the gastrula stage for *Molgula oculata*, *M. occulta* and the hybrid, respectively. For the neurula stage, we obtained 79 million, 82 million, and 73 million reads for *M. oculata*, *M. occulta* and the hybrid, respectively. For the tailbud stage, we generated 75 million, 85 million, and 84 million reads for *M. oculata*, *M. occulta* and the hybrid, respectively.

Using pooled reads of all stages, we assembled transcriptomes for *M. oculata*, *M. occulta* and hybrid. We recovered 40,175 transcripts for *M. oculata* and 87,163 transcripts for *M. occulta*. Transcriptome sequences are available from C. Titus Brown, by request.

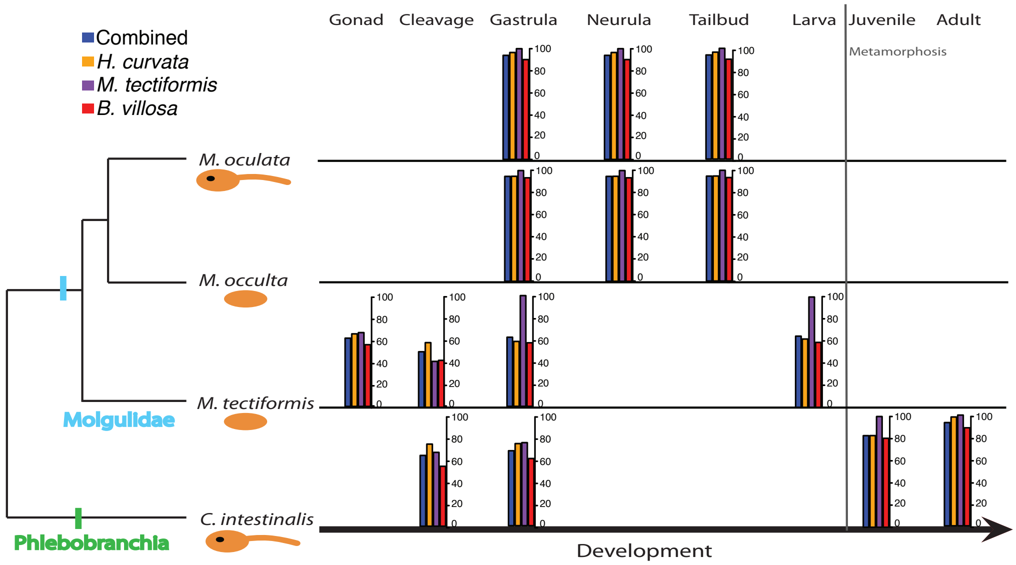

Figure 3.1: Diagram showing the percentage of ‘metamorphosis genes expressed through development in the tailed *Molgula oculata*, the tailless *M. occulta*, the tailless, *M. tectiformis* and the tailed *Ciona intestinalis*. Phylogenetic relationships shown are results from previous studies (Huber et al., 2000; Zeng and Swalla, 2005; Tsagkogeorga et al., 2009).

We assessed quality of coverage by comparing the Molgula transcripts to the *Ciona intestinalis* genome using an amino acid (*C. intestinalis*) to translated nucleotide (*Molgula*) query (tblastn) with an acceptance e-value score of less than 10^{-6}. We found 96/98 molgulid genes already in Genbank that were identified with the *M. oculata* and *M. occulta* transcriptomes. 43,866 of the 55,017 transcripts predicted in *Ciona intestinalis* map to *Molgula oculata* transcripts with high support (e-values less than 10^{-6}), and 43,672 of the *C. intestinalis* transcripts map to the *M. occulta* transcripts with high support. 8,928 of the 55,017 transcripts predicted in *Ciona intestinalis* map to unique *Molgula oculata* transcripts with high support, and 9,839 of the *C. intestinalis* transcripts map to the *M. occulta* transcripts with high support. If we consider the amount of unique transcripts that map uniquely from *M. oculata* and *M. occulta* to the *C. intestinalis* transcripts as distinct genes, then comparing these numbers to the 15,254 estimated genes in the *C. intestinalis* genome (Satou et al., 2008), *M. oculata* shares about 59% of its genes with high statistical support, *M. occulta* shares about 65% of its genes with high support values. Still, this relatively low percent of transcripts in common between molgulids and *C. intestinalis* is likely due to the evolutionary divergence of molgulids and *C. intestinalis*, which may be as long as over 400 million years ago (Erwin et al., 2011). We then used a combined dataset of 8,740 *C. intestinalis* gene models that were shared between the *M. occulta* and *M. oculata* with high support to compare gene expression of 'metamorphosis genes via heterochrony.

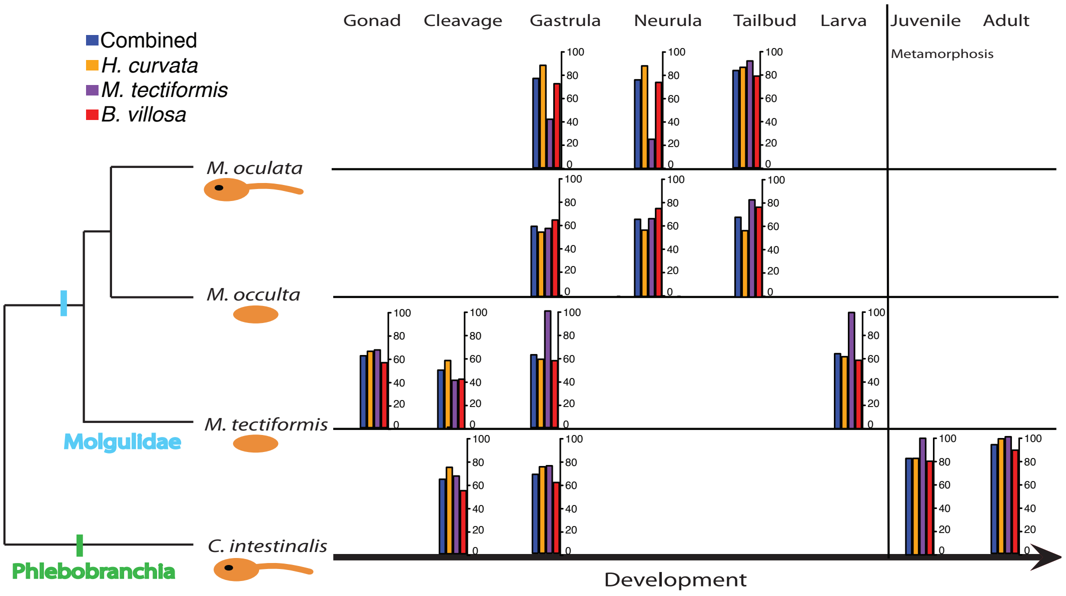

Figure 3.2: Diagram showing the percentage of 'metamorphosis genes expressed through development in the tailed *Molgula oculata*, the tailless *M. occulta*, the tailless, *M. tectiformis* and the tailed *Ciona intestinalis*. Phylogenetic relationships shown are results from previous studies (Huber et al., 2000; Zeng and Swalla, 2005; Tsagkogeorga et al., 2009). Since these transcriptomes were assembled based on pooled reads gene percentages for *M. oculata* and *M. occulta* 'metamorphosis genes are based on only genes found with an e-value of less than 10^{-6} with high support or genes that had a count of 0 at each stage.

3.4.2 Early Expression of 'Metamorphosis Genes

We compared timing of gene expression relative to morphogenesis for 133 'metamorphosis genes in three molgulid species, *Molgula oculata*, *Molgula occulta* and *Molgula tectiformis*, and one phlebobranch, *Ciona intestinalis*. The expression of these 'metamorphosis genes was compared for 133 genes, and for each species where the expression of this gene set was described previously (*Boltenia villosa*, *Herdmania curvata* and *Molgula tectiformis*) (Figure 3.1). We could not test for differences between the gastrula, neurula and tailbud stages of *M. occulta* and *M. oculata* because our assemblies were dependent on pooled data. We looked for statistical differences between the gastrula, neurula and tailbud transcriptomes for *M. oculata* and *M. occulta* by inferring genes with normalized count values of zero at a given stage as absent. Using the normalized count values of zero to also infer absence, we found a significant increase in the amount *M. tectiformis* 'metamorphosis genes expressed in the tailbud stage than the neurula stage in the tailed *M. oculata* ($p=0.0028$; Figures 3.4, 3.5).

In *Molgula tectiformis* transcriptomes there is a significant increase in the 'metamorphosis genes that were found to be expressed in *M. tectiformis* previously (Gyoja et al., 2007) from the cleavage to gastrula ($p=0.0046$; Figure 1) and the cleavage to larval stage ($p=0.0046$). Similarly, in the combined dataset of 'metamorphosis genes from all three species previously examined, there is a significant increase from the cleavage to larval stage ($p=0.025$) in *M. tectiformis.*

Based on our analysis of expression in 'metamorphosis genes in *Ciona intestinalis* through development, we found a significant increase in expression from the cleavage stage to the juvenile stage in the combined and *B. villosa* datasets ($p=8.1 \text{ X } 10^{-4}$, $p=0.0035$, respectively; Figure 3.1). There was a significant increase in genes expressed in the combined dataset of metamorphosis genes between the gastrula and juvenile stages in *C. intestinalis* ($p=0.0067$). We also found a significant increase in expression of genes from the combined, *H. curvata* and *B. villosa* datasets between the gastrula and adult stages of *C. intestinalis* ($p=1.4 \text{ X } 10^{-7}$, $p=2.1 \text{ X } 10^{-4}$, $p=7.1 \text{ X } 10^{-4}$, respectively).

While we have an incomplete dataset to compare expression of 'metamorphosis genes between the tailed *Molgula oculata*, tailless *M. occulta*, tailless *M. tectiformis* and tailed *Ciona intestinalis*, there are differences in presence and absence of these genes that suggest an early shift of 'metamorphosis genes in molgulids. There is a significantly higher number of the combined, *H. curvata* and *B. villosa* ‘metamorphosis genes datasets expressed in the gastrula stage in *M. oculata* and *M. occulta* than in *C. intestinalis* (p=4.2 X 10^{-8}, p=0.0011, p=2.6 X 10^{-3}, respectively comparing *M. oculata* and *C. intestinalis*; p=1.1 X 10^{-8}, p=0.0038, p=2.3 X 10^{-5}, respectively comparing *M. occulta* and *C. intestinalis*). The increase in the expression of ‘metamorphosis genes at the gastrula stage suggests a heterochronic shift in the expression of these genes in molgulid ascidian species with tailed and tailless larval development (Figure 3.2). This increase in number of genes found in molgulids early, however, may be due to our improved ability to recover transcripts with low expression in the transcriptomes we sequenced. If the genes with normalized expression values of zero are removed from the *M. oculata* and *M. occulta* transcriptomes we found no significant difference in metamorphosis gene expression at the gastrula stage when we compared our two molgulid species to *C. intestinalis* (Figure 3.2).

In all three molgulid species examined, we found 9 of 133 ‘metamorphosis genes in the gastrula stage that were not in the *Ciona intestinalis* gastrula stage transcriptomes, and we found 20 of 133 genes in the gastrula stage of *M. oculata* and *M. occulta* that were not in *C. intestinalis.* These genes include *mannose binding lectin* (*mbl*) and complement control protein 1/complement 1 (*c1*), which are both genes involved in innate immunity and found expressed at metamorphic competence in *B. villosa* (Davidson and Swalla, 2002). Inhibition of *mannose binding lectin associated serine protease* (*masp*), a gene shown to bind to *mbl*, and forms a protein complex that can recognize sugars or antigens on the surface of bacteria (Fujita, 2002), causes metamorphic arrest in *B. villosa* (Roberts et al., 2007). Additionally, *mbl* is expressed in the anterior or head of the trunk of *B. villosa*, which is the region in the larva that first contacts the substrate in settlement before going through metamorphosis (Roberts et al., 2007). This early activation of the innate immune system may imply that newly hatched tailed larvae of molgulids or even un-hatched larvae may already be metamorphically competent.

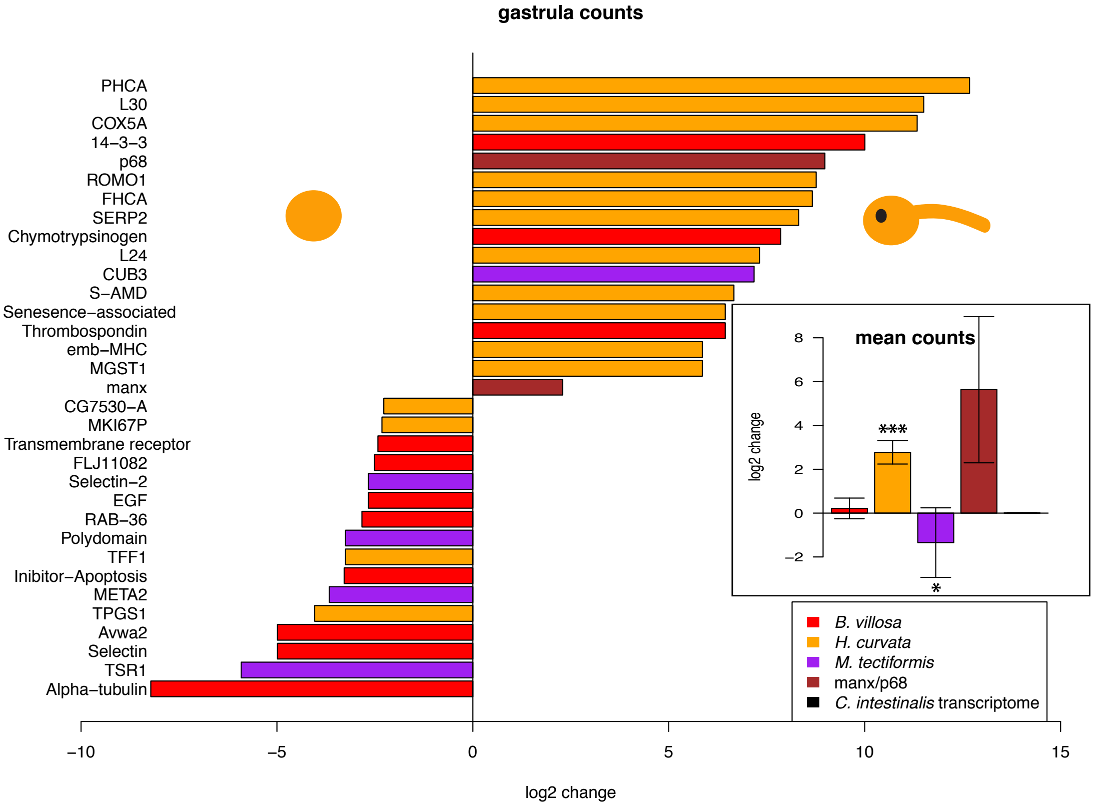

Figure 3.3: Counts for 8,740 gene models from *Ciona intestinalis* (in black) that map to both the *M. oculata* and *M. occulta* gastrula stage transcriptomes, and for 133 genes expressed during metamorphosis in other ascidians. 'metamorphosis genes for *Herdmania curvata*, *Boltenia villosa* and *Molgula tectiformis* are in orange red and purple, respectively. Counts were log2 transformed and counts to the right of the line show counts for genes expressed more highly in the tailed species and counts to the left show genes more expressed in the tailless species. Inset graph shows the mean of log2 fold differences in transcript counts between *M. oculata* and *M. occulta* for *H. curvata*, *B. villosa* and *M. tectiformis* metamorphosis genes. * denotes $p < 0.05$ and *** $p < 0.001$.

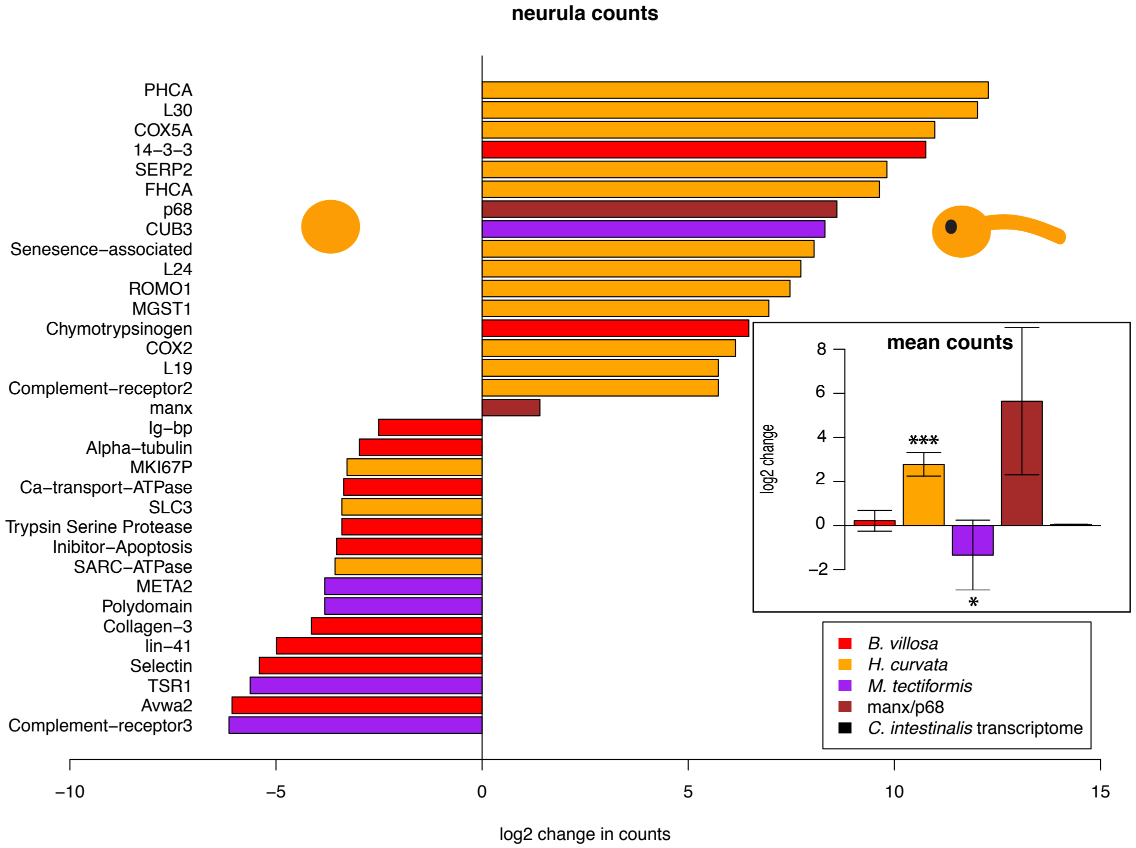

Figure 3.4: Counts for 8,740 gene models from *Ciona intestinalis* (in black) that map to both the *M. oculata* and *M. occulta* neurula stage transcriptomes, and for 133 genes expressed during metamorphosis in other ascidians. ‘metamorphosis genes for *Herdmania curvata*, *Boltenia villosa* and *Molgula tectiformis* are in orange red and purple, respectively. Counts were log2 transformed and counts to the right of the line show counts for genes expressed more highly in the tailed species and counts to the left show genes more expressed in the tailless species. Inset graph shows the mean of log2 fold differences in transcript counts between *M. oculata* and *M. occulta* for *H. curvata*, *B. villosa* and *M. tectiformis* metamorphosis genes. * denotes $p < 0.05$ and *** $p < 0.001$.

Additionally, *pentraxin*, which is found in *B. villosa* after metamorphosis (Davidson and Swalla, 2002), and part of a gene family that plays a role in innate immunity/complement signaling (Goodman et al., 1996) and activating phagocytosis in red blood cells (Rovere et al., 2000), is expressed in the gastrula stage of all three molgulids and not in *C. intestinalis*. A *rab-36* like protein found in *B. villosa* (Davidson et al. 2002), is part of a protein family largely involved in vesicular trafficking (Pereira-Leal and Seabra, 2001), and is found expressed at the gastrula stage of *M. oculata* and *M. occulta* and not in *C. intestinalis*. *sulfatase-like* protein, found expressed in *B. villosa* at settlement, is part of a protein family involved in hormone regulation, cellular degradation, and modulation of signaling pathways (Hanson et al., 2004), and expressed early in the three molgulids and not in *C. intestinalis*. *rab-36* and *sulfatase* are examples of genes that are expressed early in molgulids that are likely involved in some sort of cell-cell signaling pathway and transport and catabolism that are being activated early in development in molgulids.

3.4.3 Expression Differences in ‘metamorphosis genes

We examined expression differences of ‘metamorphosis genes – previously identified in *B. villosa*, *H. curvata* and *M. tectiformis* – between the tailed *Molgula oculata* and tailless *Molgula occulta* at the gastrula, neurula and tailbud stages (Figures 3.3, 3.4, 3.5). Additionally, we examined expression differences between *manx* and *p68/bobcat*, genes shown to be critical for formation of the tail in *M. oculata* (Swalla and Jeffery, 1996; Swalla et al., 1999). We then used a two-tailed Mann-Whitney-Wilcoxon non-parametric test to see if the mean expression of ‘metamorphosis genes was different than the mean expression of 8,740 transcripts that map to the *Ciona intestinalis* genome, and are found in both the *M. oculata* and *M. occulta* transcriptomes. The tailed *M. oculata* showed significantly higher expression of *H. curvata* ‘metamorphosis genes from the gastrula, neurula and tailbud stages ($p=2.47 \times 10^{-7}$, $p=2.79 \times 10^{-8}$, $p=7.46 \times 10^{-6}$, respectively; Figures 3.3, 3.4, 3.5).

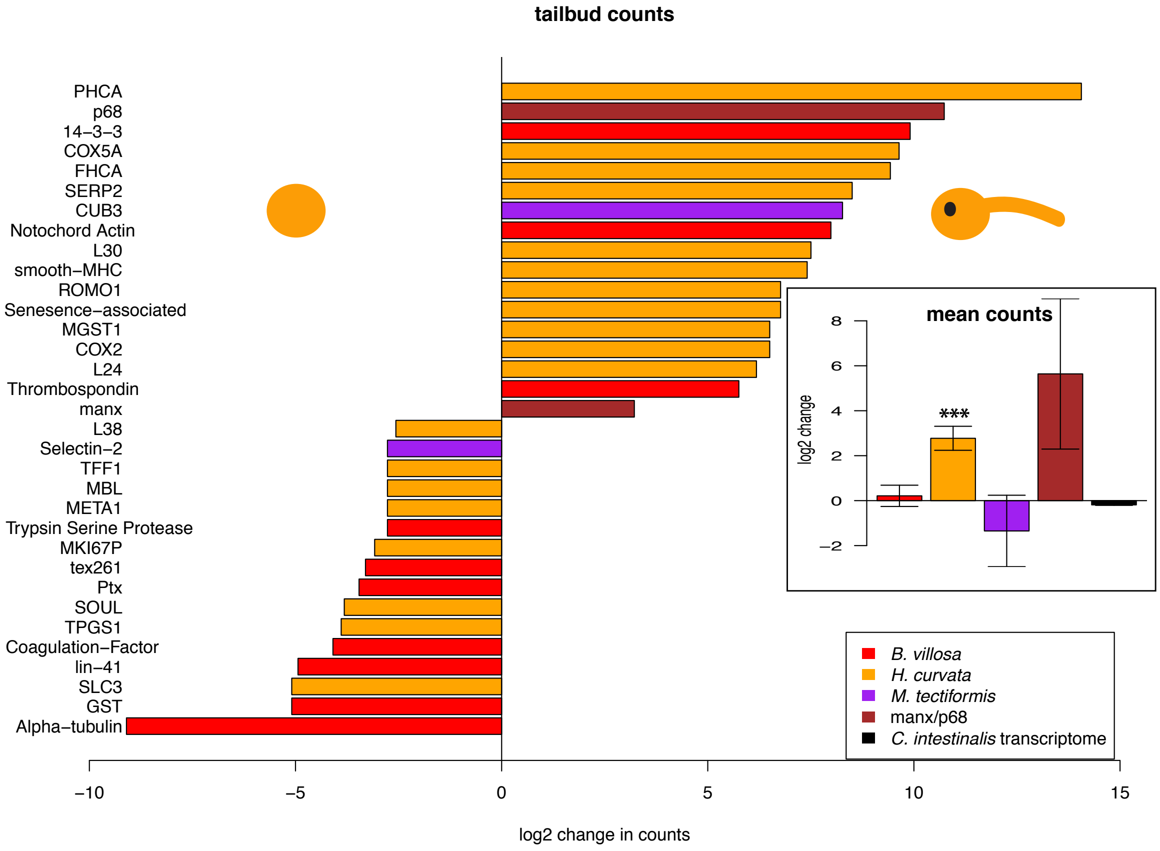

Figure 3.5: Counts for 8,740 gene models from *Ciona intestinalis* (in black) that map to both the *M. oculata* and *M. occulta* tailbud stage transcriptomes, and for 133 genes expressed during metamorphosis in other ascidians. ‘metamorphosis genes for *Herdmania curvata*, *Boltenia villosa* and *Molgula tectiformis* are in orange red and purple, respectively. Counts were log2 transformed and counts to the right of the line show counts for genes expressed more highly in the tailed species and counts to the left show genes more expressed in the tailless species. Inset graph shows the mean of log2-fold differences in transcript counts between *M. oculata* and *M. occulta* for *H. curvata*, *B. villosa* and *M. tectiformis* metamorphosis genes. * denotes $p < 0.05$ and *** $p < 0.001$.

Some of the most up-regulated genes in *M. oculata* from the *H. curvata* ‘metamorphosis genes are *plasmin heavy chain a* (*phca*), *stress-associated endoplasmic reticulum protein family member 2* (*serp2*) and *reactive oxygen species modulator 1* (*romo1*) (Figures 3.3, 3.4, 3.5). *phca* is a serine protease, cleaved component of *plasminogen*, plays a role in muscle cell migration in mice, and is involved in blood clot removal in mice (Lijnen et al., 1998). *serp2* is hypothesized to interact with proteins during the translocation of proteins into the lumen of the endoplasmic reticulum, and *serp1* stabilizes membrane proteins during stress in rats, so it may be possible *serp2* has a similar role in ascidians (Yamaguchi et al., 1999). *romo1*, is localized in the mitochondria and induces mitochondrial reactive oxygen species production through complex III of the mitochondrial electron transport chain, and also plays a role in upstream activation of *c-jun n terminal kinase* to promote apoptotic cell death (Kim et al., 2010). The upregulation of genes involved in cell migration, protein transport and metabolism and cell death in the tailed *M. oculata* early in development suggests that the tailless *M. occulta* may be suppressing basic cellular movement and function. While the higher expression of genes in *M. oculata* could show differences in expression based on more stress in embryos of *M. oculata* than *M. occulta* before they were fixed; since these embryos were fixed simultaneously, this seems unlikely. Additionally *manx* and *p68/bobcat*, genes shown to be critical for the formation of the tail in *M. oculata* (Swalla and Jeffery, 1996; Swalla et al., 1999), are upregulated in the tailed *M. oculata*, as expected.

The tailless *M. occulta* showed significantly higher expression of *M. tectiformis* ‘metamorphosis genes in the gastrula and neurula stages (p=0.041, p=0.028, respectively; Figures 3.3, 3.4), but not the tailbud stage (p=0.15; Figure 3.5). Some of these genes are involved in innate immunity like *complement receptor 3* (*c3*) (Fujita, 2002). *polydomain* or *p-selectin*, *selectin-2* and *tsr1* – a thrombospondin repeat protein – may play a role in innate immunity as well as in ascidian metamorphosis (Beutler, 2004). Interestingly though, *cub and sushi multiple domains 3 isoform 3* (*cub3*), which has similar domains to *masp*, an integral component of the innate immune system (Endo et al., 2003), is expressed more highly in the tailed *M. oculata*. While the *M. tectiformis* ‘metamorphosis genes are composed mostly of genes involved in innate immunity, a variant of *meta2* is also seen more highly expressed in the tailless *M. occulta*. While the expression of *M. tectiformis* ‘metamorphosis genes being expressed more highly in the gastrula and neurula stage (Figures 3.3, 3.4) may suggest earlier activation of the innate immune system in the tailless *M. occulta*. Therefore, further examination of the full pathway involved in innate immunity is needed (see below).

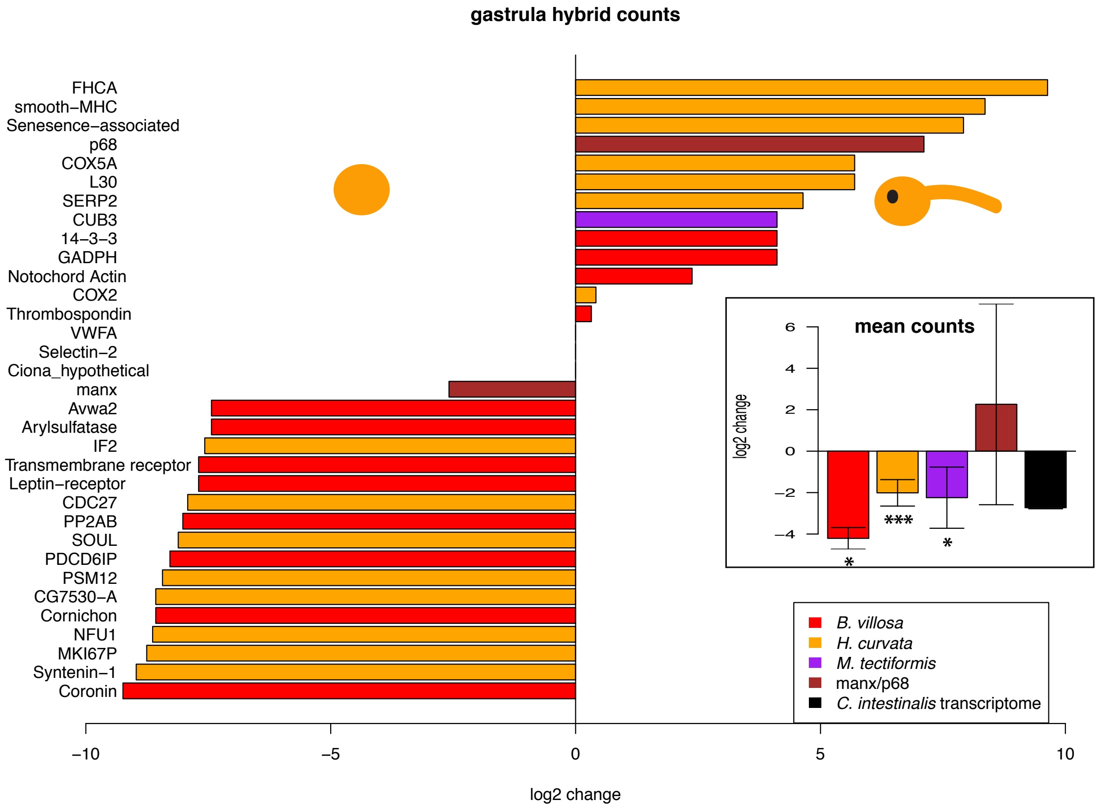

Figure 3.6: Counts for 8,740 gene models from *Ciona intestinalis* (in black) that map to both the *M. oculata* allele and *M. occulta* allele in hybrid gastrula stage transcriptomes, and for 133 genes expressed during metamorphosis in other ascidians. ‘metamorphosis genes for *Herdmania curvata*, *Boltenia villosa* and *Molgula tectiformis* are in orange red and purple, respectively. Counts were log2 transformed and counts to the right of the line show counts for genes expressed more highly in the tailed allele and counts to the left show genes more expressed in the tailless allele. Inset graph shows the mean of log2-fold differences in transcript counts between *M. oculata* and *M. occulta* for *H. curvata*, *B. villosa* and *M. tectiformis* metamorphosis genes. * denotes $p < 0.05$ and *** $p < 0.001$.

3.4.4 Expression of Maternal Alleles in Hybrids

Hybrids from the sperm of the tailed *Molgula oculata* and the egg of the tailless *M. occulta* show higher expression largely in the maternal alleles for the metamorphosis genes examined (Figures 3.6, 3.7, 3.8). In the gastrula stage of the hybrid the *B. villosa* and *M. tectiformis* ‘metamorphosis genes are more highly expressed in the tailless, maternal alleles (p=0.038,

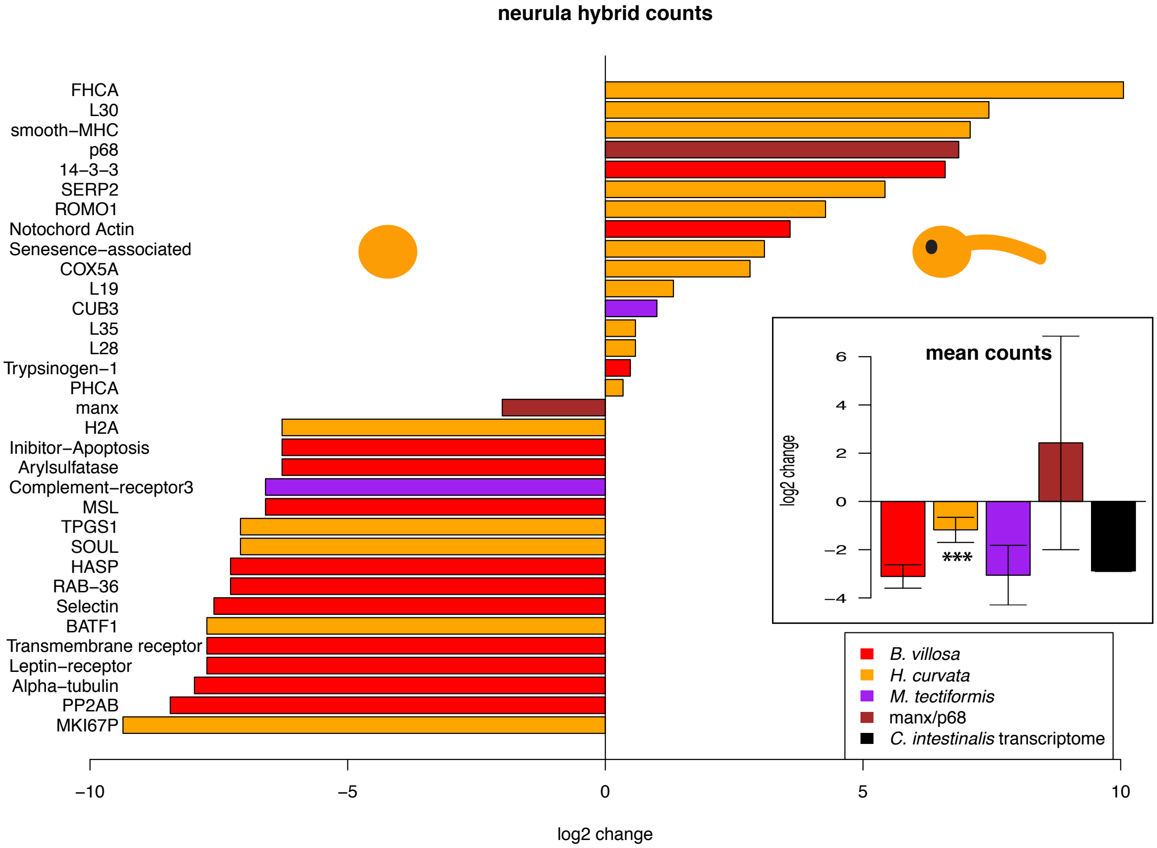

Figure 3.7: Counts for 8,740 gene models from *Ciona intestinalis* (in black) that map to both the *M. oculata* allele and *M. occulta* allele in hybrid neurula stage transcriptomes, and for 133 genes expressed during metamorphosis in other ascidians. ‘metamorphosis genes for *Herdmania curvata*, *Boltenia villosa* and *Molgula tectiformis* are in orange red and purple, respectively. Counts were log2 transformed and counts to the right of the line show counts for genes expressed more highly in the tailed allele and counts to the left show genes more expressed in the tailless allele. Inset graph shows the mean of log2 fold differences in transcript counts between *M. oculata* and *M. occulta* for *H. curvata*, *B. villosa* and *M. tectiformis* metamorphosis genes. *** denotes $p < 0.001$.

p=0.022; Figure 3.6). Interestingly, in the tailbud stage the *M. tectiformis* ‘metamorphosis genes are more highly expressed in the paternal alleles (p=0.014; Figure 3.7). Expression of the tailed paternal alleles is higher for *H. curvata* ‘metamorphosis genes from gastrula to tailbud stages in the hybrid ($p=5.77 \times 10^{-7}$, $p=8.15 \times 10^{-9}$, $p=3.5 \times 10^{-10}$, respectively; Figures 3.6, 3.7, 3.8). While it appears that a large amount of the expression of genes in the hybrids comes from the maternal alleles, it is interesting that many genes seen highly expressed in the paternal alleles are genes found to be critical for formation of the tail like *p68/bobcat* (Swalla and Jeffery, 1996) or genes found to expressed in the notochord like notochord actin, which is a gene expressed in the notochord of cephalochordates (Suzuki and Satoh, 2000). Further work examining expression of transcripts in the eggs and zygotes will be needed to differentiate if these transcripts are present before fertilization.

3.4.5 KEGG Pathway Expression

We examined the expression for pathways recognized in the Kyoto Encyclopedia of Genes and Genomes (Ogata et al., 1999) to be involved in Cell communication (CC), Cell growth and death (CGD), Immune system function (IS) and Transport and catabolism (TC) (Figure 3.9). The tailed *M. oculata* showed differential expression of the complement/innate immune system in the gastrula stage (p=0.0049, Figure 3.9a). However, this *M. oculata*-specific expression was largely due to the very high differential expression of *plasminogen*, and when this gene was removed, there was no significant difference in mean expression of the complement pathway from the mean expression of the 8,740 *C. intestinalis* gene models that map with high support to the *M. oculata* and *M. occulta* transcriptomes.

In the tailbud stage, there is heightened expression in the tailed *M. oculata* of KEGG pathways involved in cell communication: adherens junction and focal adhesion (p=0.0027, $p=3.8 \times 10^{-5}$, respectively; Figure 3.9C). Adherens junctions are specialized forms of cadherin-based adhesive contacts important for tissue organization in developing and adult organ-

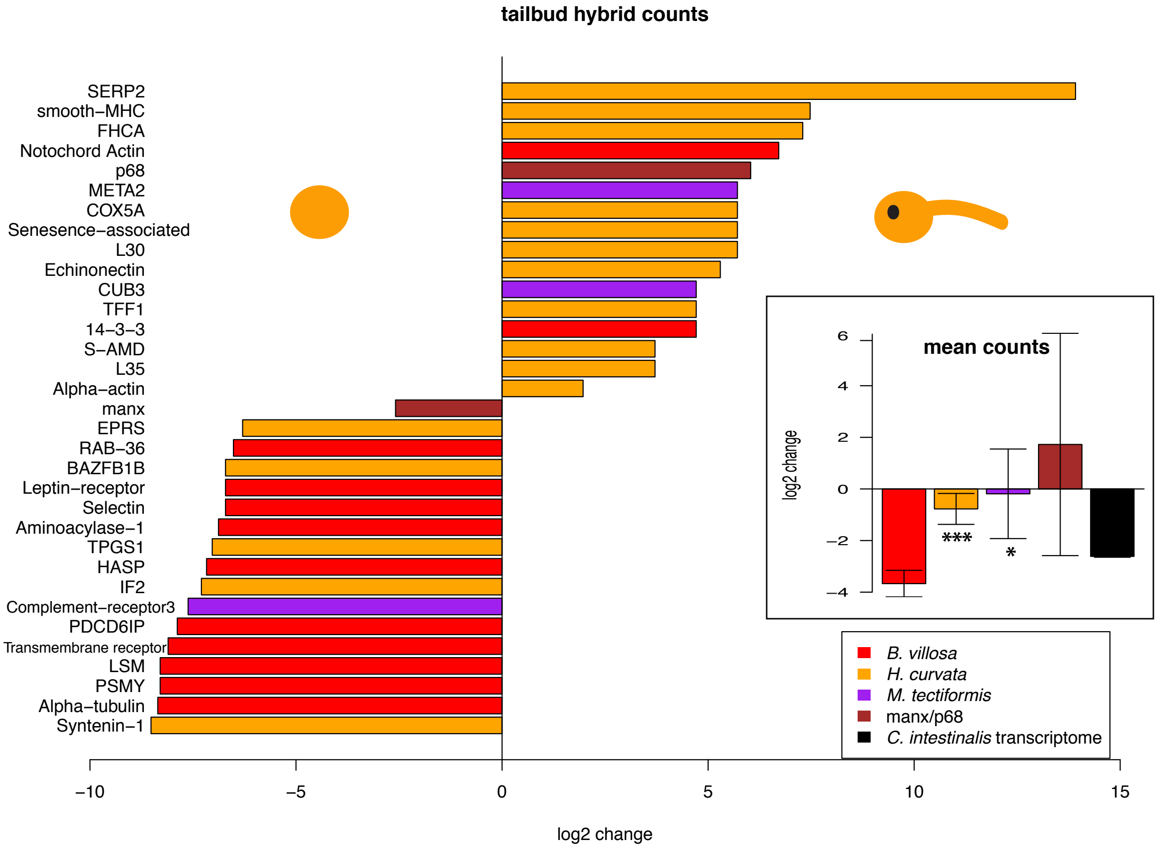

Figure 3.8: Counts for 8,740 gene models from *Ciona intestinalis* (in black) that map to both the *M. oculata* allele and *M. occulta* allele in hybrid neurula stage transcriptomes, and for 133 genes expressed during metamorphosis in other ascidians. ‘metamorphosis genes for *Herdmania curvata*, *Boltenia villosa* and *Molgula tectiformis* are in orange red and purple, respectively. Counts were log2 transformed and counts to the right of the line show counts for genes expressed more highly in the tailed allele and counts to the left show genes more expressed in the tailless allele. Inset graph shows the mean of log2-fold differences in transcript counts between *M. oculata* and *M. occulta* for *H. curvata*, *B. villosa* and *M. tectiformis* metamorphosis genes. * denotes $p < 0.05$ and *** $p < 0.001$.

isms (Yap et al., 1997). The focal adhesion pathway involves focal adhesion kinase, which is a crucial signalling component that is activated by numerous stimuli and functions as a biosensor or integrator to control cell motility (Mitra et al., 2005)), and downstream genes involved in cell motility. There is also heightened expression in the tailed *M. oculata* at the tailbud stage for the transport and catabolism pathway, which regulates the phagosome or phagocytosis (p=0.016; Figure 3.9C), and is a central mechanism in the tissue remodeling, inflammation, and defense against infectious agents (Aderem and Underhill, 1999).

The upregulation of adherens junctions and focal adhesion pathways at the tailbud stage in *M. oculata* is concordant with previous studies identifying specific genes that are activated downstream of *brachyury* and expressed in the notochord of *C. intestinalis* at the tailbud stage, but some genes are expressed at the gastrula and neurula stages (Hotta et al., 2008). The tailed *M. oculata* shows upregulation of *brachyury* in the notochord cells as early as the gastrula stage and into the tailbud stage, but there is a downregulation of *brachyury* expression in the tailless *M. occulta* and *M. tectiformis* (Takada et al., 2002). The upregulation of KEGG pathways involved in cell-cell communication at the tailbud stage in *M. oculata* could be due to *brachyury* downstream genes in the notochord being more highly expressed in the tailed *M. oculata* than the tailless *M. occulta*, and not an early heterochronic shift in expression of metamorphosis genes. However, many of the metamorphosis genes involved in innate immunity and transport and catabolism have not been identified as playing a role in notochord formation (Aderem and Underhill, 1999). Therefore, the significantly higher expression in the tailed *M. oculata* than the tailless *M. occulta* of the KEGG phagosome pathway, responsible for tissue remodeling, is likely an early activation of metamorphosis genes.

Molgula occulta shows significantly higher expression of the KEGG pathway involved in the regulation of autophagy in the gastrula and tailbud stages (p=0.029, p=0.040, respectively; Figure 3.9A,C). The regulation of autophagy pathway activates the autophagosome, which then helps to transport proteins in the cytosol or entire organelles to the lysosome or vacuole where protein breakdown and recycling occurs (Klionsky and Emr, 2000). The regulation of autophagy pathway can be activated from cell starvation, and can lead to cell death that occurs independent of the apoptosis pathway (Lum et al., 2005). The increased activation of the regulation of autophagy pathway in the tailless *M. occulta* as early as the gastrula stage, but the non-significant increase of the apoptosis pathway suggests that the packaging and shipping of proteins to the lysosome and vacuole by the autophagosome may be occurring without significant activation of caspase-dependent apoptosis. However, previous work has shown that apoptosis is being activated in cells that would normally form the tail in stages as early as the gastrula in tailless molgulid species (Jeffery, 2002). Therefore, functional studies will be needed to disentangle the roles of caspase-dependent apoptosis and autophagy in the inability to form a tail during larval development in molgulids.

In the hybrid, from the gastrula to tailbud stages all of the KEGG pathways involved in cell-cell communication, cell growth and death, immune system function and transport and catabolism show significantly higher expression of the tailless allele except for the complement pathway during the gastrula stage and the phagosome pathway during the neurula stage, which both showed no difference in expression (Figure 3.9D-F). These results suggest that the mRNAs for these KEGG pathways that are upregulated in the tailless *M. occulta* allele may be passed to the embryo in the cytosol of the egg. However, we need further transcriptomic data from eggs and after fertilization in the hybrid to know that these transcripts are being produced in the egg. Additionally, there may also be some maternal or paternal specific methylation of certain genes, which can deactivate certain transcripts from the paternal and maternal genome copies as has been shown to occur in rice hybrids (Chodavarapu et al., 2012).

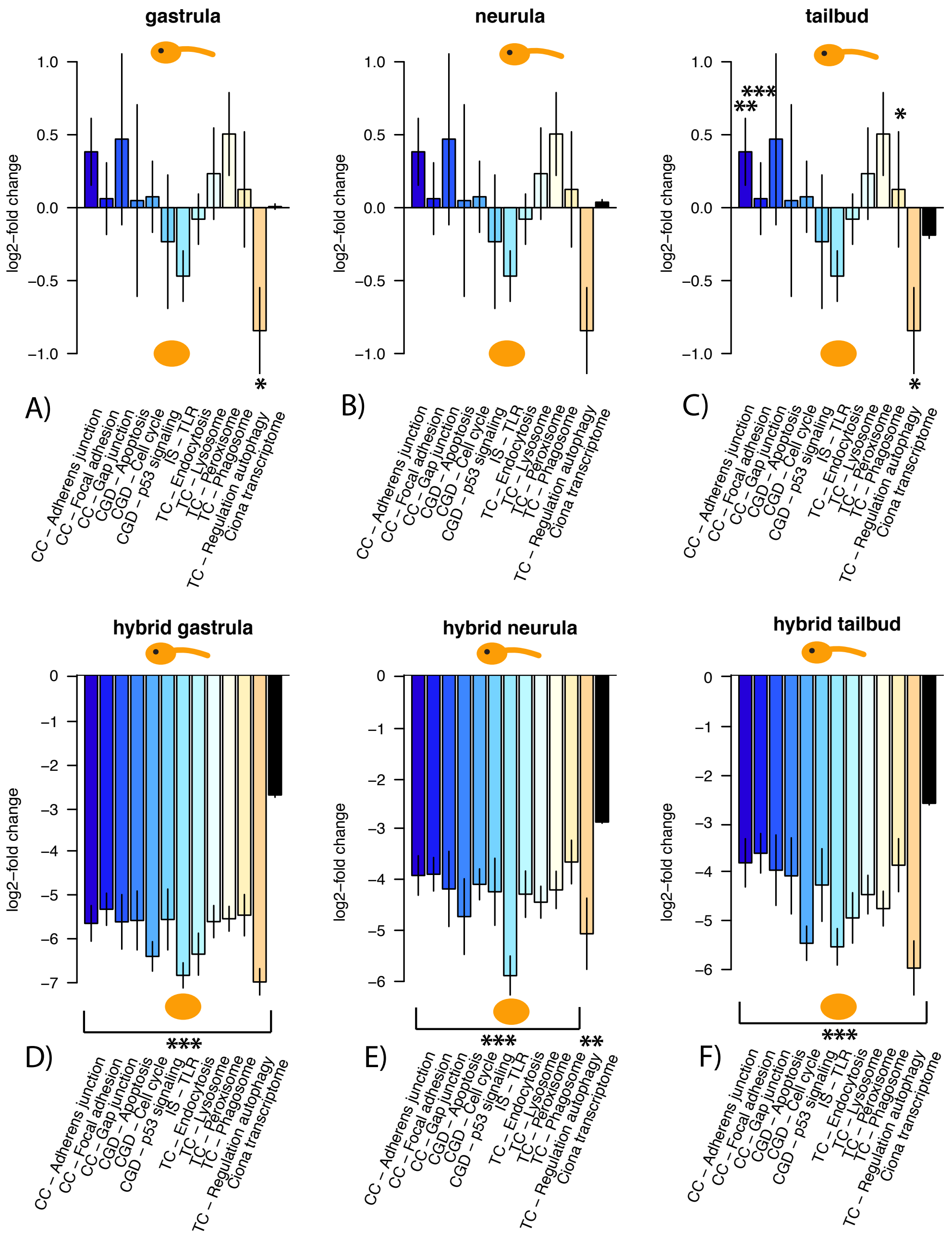

Figure 3.9: Mean log2 fold difference in counts for KEGG pathways involved in cell-cell communication (CC), cell growth and death (CGD), immune system function (IS) and transport and catabolism (TC). Mean log2 fold difference counts for 8,740 gene models from *Ciona intestinalis* are in black. A-C show mean counts for the gastrula, neurula and tailbud stages for *Molgula oculata* and *M. occulta*, respectively. D-F show mean counts for the gastrula, neurula and tailbud stages for the paternal (tailed *M. oculata*) and maternal (tailless *M. occulta*) alleles in the hybrids. Mean counts above the horizontal line show expressed more highly in the tailed allele of the hybrid and counts to the left show genes more expressed in the tailless species. * denotes $p < 0.05$, ** denotes $p < 0.01$, and *** denotes $p < 0.001$.

3.4.6 Molgulids – ancestral or derived?

Molgulids share several uncommon features that most other groups of ascidians do not share. Molgulids are only one of two groups (with the Styelidae) of ascidians that have tailless larvae (Maliska and Swalla, 2010). In the molgulids there are two kinds of species with tailless larval development: 1) species that hatch out of the chorion before going through metamorphosis, and live primarily in sandy or muddy substrate as adults, and 2) species that do not hatch out of the chorion before going through metamorphosis into the adult form, and live attached to hard substrate (Maliska and Swalla, 2010; Tagawa et al., 1997). Most of the molgulids that have been examined have some of the shortest developmental periods found in ascidians (Berrill, 1935). The derived tailless species of molgulids that hatch and do not hatch have lower speciation rates over evolutionary time than species that have the ancestral condition of hatching and having tadpole larva (Maliska et al., 2013).

Molgulids are a sister group to stolidobranch ascidians, yet show this early heterochronic shift of metamorphosis genes, and also do not contain any colonial species (Zeng and Swalla, 2005). In studies using *18S rRNA* on a large set tunicate and ascidian species, the molgulids are a sister group to the other stolidobranchs (Swalla et al., 2000; Zeng and Swalla, 2005; Tsagkogeorga et al., 2009). In a phylogenomic study that examined higher-level relationships in the deuterostomes, from three ascidian species, the molgulids were also found to be sister to other stolidobranchs (Delsuc et al., 2006). More data will be needed from more ascidian species at independent loci to determine whether molgulids are highly derived or basal ascidians.

3.4.7 Heterochronies in larval development

We propose that this heterochronic shift for tailed and tailless molgulids of genes expressed at metamorphosis in other ascidians is a preadaptation that has allowed for the early degradation of the tail in *M. occulta* and at least 14 other molgulid species, including *M. tecti-*

formis (Gyoja et al., 2007) and *M. pugetiensis* (Maliska and Swalla, 2010). Heterochronies in larval development have been shown in several groups of marine invertebrates, including echinoderms (Wray and McClay, 1989) and polychaetes (Pernet and McHugh, 2010). An early heterochronic shift in larval development has been shown to be involved in the evolution of direct development in sea urchins (Raff, 1987) and sea stars (Byrne and Barker, 1991). Repeated phenotypic changes in closely related species has been investigated in wing pigments in Drosophila, and these studies showed the importance of transcription factors and their binding sites, including the affinity of binding for these transcription factors in promoting different phenotypes (Wittkopp et al., 2002; Gompel et al., 2005).

While we have identified critical gene networks that may be involved in the early degradation of the tail like the ‘metamorphosis genes found to be expressed in *Boltenia villosa* (Davidson and Swalla, 2002), *Herdmania curvata* (Woods et al., 2004) *Molgula tectiformis* (Gyoja et al., 2007), and the KEGG regulation of autophagy pathway (Ogata et al., 1999), functional studies will be needed to examine the effect of inhibition of certain pathways and the resultant phenotypes. Additionally, until further genomic data of multiple tailed and tailless species is available, the exact molecular, genetic, and developmental underpinnings of tailless evolution and development will remain a mystery.

BIBLIOGRAPHY

Aderem, A. and Underhill, D. M. 1999: Mechanisms of Phagocytosis in Macrophages. Annual Review of Immunology 17(1):593–623.

Akaike, H. 1974: A new look at the statistical model identification. IEEE Transactions on Automatic Control 19(6):716– 723.

Bates, W. R. 1993: Evolutionary modifications of morphogenetic mechanisms and alternate life history strategies in ascidians. Microscopy Research and Technique 26(4):285–300.

———. 1995: Direct Development in the Ascidian Molgula retortiformis (Verrill, 1871). Biological Bulletin 188(1):16–22.

Bates, W. R. and Mallett, J. E. 1991: Anural development of the ascidian Molgula pacifica (Huntsman). Canadian Journal of Zoology 69(3):618–627.

Berrill, N. J. 1931: Studies in Tunicate Development. Part II. Abbreviation of Development in the Molgulidae. Philosophical Transactions of the Royal Society of London. Series B, Containing Papers of a Biological Character 219:281–346.

———. 1935: Studies in Tunicate Development. Part III. Differential Retardation and Acceleration. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 225(525):255–326.

———. 1948: The Gonads, Larvae, and Budding of the Polystyelid Ascidians Stolonica and Distomus. Journal of the Marine Biological Association of the United Kingdom 27(03):633–650.

Beutler, B. 2004: Innate immunity: an overview. Molecular Immunology 40(12):845–859.

Birand, A.; Vose, A.; and Gavrilets, S. 2012: Patterns of Species Ranges, Speciation, and Extinction. The American Naturalist 179(1):1–21.

Bishop, C. D.; Bates, W. R.; and Brandhorst, B. P. 2001: Regulation of metamorphosis in ascidians involves NO/cGMP signaling and HSP90. Journal of Experimental Zoology 289(6):374–384.

Bock, D. G.; MacIsaac, H. J.; and Cristescu, M. E. 2012: Multilocus genetic analyses differentiate between widespread and spatially restricted cryptic species in a model ascidian. Proceedings of the Royal Society B: Biological Sciences 279(1737):2377–2385.

Bourlat, S. J.; Juliusdottir, T.; Lowe, C. J.; Freeman, R.; Aronowicz, J.; Kirschner, M.; Lander, E. S.; Thorndyke, M.; Nakano, H.; Kohn, A. B.; Heyland, A.; Moroz, L. L.; Copley, R. R.; and Telford, M. J. 2006: Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. Nature 444(7115):85–88.

Brown, F. D.; Prendergast, A.; and Swalla, B. J. 2008: Man is but a worm: Chordate origins. genesis 46(11):605–613.

Brown, F. D. and Swalla, B. J. 2007: Vasa expression in a colonial ascidian, Botrylloides violaceus. Evolution & Development 9(2):165177.

Byrne, M. and Barker, M. F. 1991: Embryogenesis and Larval Development of the Asteroid Patiriella regularis Viewed by Light and Scanning Electron Microscopy. The Biological Bulletin 180(3):332–345.

Camacho, C.; Coulouris, G.; Avagyan, V.; Ma, N.; Papadopoulos, J.; Bealer, K.; and Madden, T. L. 2009: BLAST+: architecture and applications. BMC Bioinformatics 10(1):421.

Chodavarapu, R. K.; Feng, S.; Ding, B.; Simon, S. A.; Lopez, D.; Jia, Y.; Wang, G.-L.; Meyers, B. C.; Jacobsen, S. E.; and Pellegrini, M. 2012: Transcriptome and methylome interactions in rice hybrids. Proceedings of the National Academy of Sciences 109(30):12040–12045.

Davidson, B.; Smith Wallace, S. E.; Howsmon, R. A.; and Swalla, B. J. 2003: A morphological and genetic characterization of metamorphosis in the ascidian Boltenia villosa. Development Genes and Evolution 213(12):601–611.

Davidson, B. and Swalla, B. J. 2001: Isolation of genes involved in ascidian metamorphosis: epidermal growth factor signaling and metamorphic competence. Development Genes and Evolution 211(4):190–194.

———. 2002: A molecular analysis of ascidian metamorphosis reveals activation of an innate immune response. Development 129(20):4739 –4751.

Davis, M. P.; Midford, P. E.; and Maddison, W. 2013: Exploring power and parameter estimation of the BiSSE method for analyzing species diversification. BMC Evolutionary Biology 13(1):38.

Delsuc, F.; Brinkmann, H.; Chourrout, D.; and Philippe, H. 2006: Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature 439(7079):965–968.

Di Gregorio, A.; Harland, R. M.; Levine, M.; and Casey, E. S. 2002: Tail Morphogenesis in the Ascidian, Ciona intestinalis, Requires Cooperation between Notochord and Muscle. Developmental Biology 244(2):385–395.

Drummond, A. and Rambaut, A. 2007: BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evolutionary Biology 7(1):214.

Duda, T. F. and Palumbi, S. R. 1999: Developmental shifts and species selection in gastropods. Proceedings of the National Academy of Sciences of the United States of America 96(18):10272–10277.

Emlet, R. B. 1995: Developmental Mode and Species Geographic Range in Regular Sea Urchins (Echinodermata: Echinoidea). Evolution 49(3):476–489.

Endo, Y.; Nonaka, M.; Saiga, H.; Kakinuma, Y.; Matsushita, A.; Takahashi, M.; Matsushita, M.; and Fujita, T. 2003: Origin of Mannose-Binding Lectin-Associated Serine Protease (MASP)-1 and MASP-3 Involved in the Lectin Complement Pathway Traced Back to the Invertebrate, Amphioxus. The Journal of Immunology 170(9):4701–4707.

Erwin, D. H.; Laflamme, M.; Tweedt, S. M.; Sperling, E. A.; Pisani, D.; and Peterson, K. J. 2011: The Cambrian Conundrum: Early Divergence and Later Ecological Success in the Early History of Animals. Science 334(6059):1091–1097.

Fedonkin, M. A.; Vickers-Rich, P.; Swalla, B. J.; Trusler, P.; and Hall, M. 2012: A new metazoan from the Vendian of the White Sea, Russia, with possible affinities to the ascidians. Paleontological Journal 46(1):1–11.

FitzJohn, R. G. 2012: Diversitree: comparative phylogenetic analyses of diversification in R. Methods in Ecology and Evolution page nono.

FitzJohn, R. G.; Maddison, W. P.; and Otto, S. P. 2009: Estimating Trait-Dependent Speciation and Extinction Rates from Incompletely Resolved Phylogenies. Systematic Biology 58(6):595 –611.

Fujita, T. 2002: Evolution of the lectin–complement pathway and its role in innate immunity. Nature Reviews Immunology 2(5):346.

Gaston, K. J. 1996: Species-range-size distributions: patterns, mechanisms and implications. Trends in Ecology & Evolution 11(5):197–201.

Gavrilets, S. and Vose, A. 2005: Dynamic patterns of adaptive radiation. Proceedings of the National Academy of Sciences of the United States of America 102(50):18040–18045.

Goldberg, E. E.; Kohn, J. R.; Lande, R.; Robertson, K. A.; Smith, S. A.; and Igi, B. 2010: Species Selection Maintains Self-Incompatibility. Science 330(6003):493 –495.

Gompel, N.; Prud'homme, B.; Wittkopp, P. J.; Kassner, V. A.; and Carroll, S. B. 2005: Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. Nature 433(7025):481–487.

Goodman, A. R.; Cardozo, T.; Abagyan, R.; Altmeyer, A.; Wisniewski, H.-G.; and Vilek, J. 1996: Long pentraxins: an emerging group of proteins with diverse functions. Cytokine & Growth Factor Reviews 7(2):191–202.

Gyoja, F.; Satou, Y.; Shin-i, T.; Kohara, Y.; Swalla, B. J.; and Satoh, N. 2007: Analysis of large scale expression sequenced tags (ESTs) from the anural ascidian, Molgula tectiformis. Developmental Biology 307(2):460–482.

Hadfield, J. D. and Nakagawa, S. 2010: General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multitrait models for continuous and categorical characters. Journal of Evolutionary Biology 23(3):494–508.

Hadfield, K. A.; Swalla, B. J.; and Jeffery, W. R. 1995: Multiple origins of anural development in ascidians inferred from rDNA sequences. Journal of Molecular Evolution 40(4):413–427.

Hansen, T. A. 1978: Larval Dispersal and Species Longevity in Lower Tertiary Gastropods. Science 199(4331):885–887.

———. 1983: Modes of larval development and rates of speciation in early tertiary neogastropods. Science (New York, N.Y.) 220(4596):501–502.

Hanson, S. R.; Best, M. D.; and Wong, C.-H. 2004: Sulfatases: Structure, Mechanism, Biological Activity, Inhibition, and Synthetic Utility. Angewandte Chemie International Edition 43(43):5736–5763.

Haydar, D.; Hoarau, G.; Olsen, J. L.; Stam, W. T.; and Wolff, W. J. 2011: Introduced or glacial relict? Phylogeography of the cryptogenic tunicate Molgula manhattensis (Ascidiacea, Pleurogona). Diversity and Distributions 17(1):68–80.

Hellberg, M. E. 2009: Gene Flow and Isolation among Populations of Marine Animals. Annual Review of Ecology, Evolution, and Systematics 40(1):291–310.

Herdman, W. 1898: Descriptions of simple ascidians collected in Puget Sound, Pacific coast. Proceedings and Transanctions of the Liverpool Biological Society 12.

Hijmans, R. J.; Williams, E.; and Vennes, C. 2012: geosphere: Spherical Trigonometry. R package version 1.2-28. http://CRAN.R-project.org/package=geosphere .

Hotta, K.; Takahashi, H.; Satoh, N.; and Gojobori, T. 2008: Brachyury-downstream gene sets in a chordate, Ciona intestinalis: integrating notochord specification, morphogenesis and chordate evolution. Evolution & Development 10(1):37–51.

Huber, J. L.; da Silva, K. B.; Bates, W. R.; and Swalla, B. J. 2000: The evolution of anural larvae in molgulid ascidians. Seminars in Cell & Developmental Biology 11(6):419–426.

Jablonski, D. 1986: Larval ecology and macroevolution in marine invertebrates. Bulletin of Marine Science 39:565–587.

Jeffery, C. H.; Emlet, R. B.; and Littlewood, D. T. J. 2003: Phylogeny and evolution of developmental mode in temnopleurid echinoids. Molecular Phylogenetics and Evolution 28(1):99–118.

Jeffery, W. R. 2002: Programmed cell death in the ascidian embryo: modulation by FoxA5 and Manx and roles in the evolution of larval development. Mechanisms of Development 118(1-2):111–124.

Jeffery, W. R.; Swalla, B. J.; Ewing, N.; and Kusakabe, T. 1999: Evolution of the ascidian anural larva: evidence from embryos and molecules. Molecular Biology and Evolution 16(5):646–654.

Jiang, D.; Munro, E. M.; and Smith, W. C. 2005: Ascidian prickle Regulates Both Mediolateral and Anterior-Posterior Cell Polarity of Notochord Cells. Current Biology 15(1):79–85.

Katoh, K. and Toh, H. 2008: Recent developments in the MAFFT multiple sequence alignment program. Briefings in Bioinformatics 9(4):286 –298.

Kim, J. J.; Lee, S. B.; Park, J. K.; and Yoo, Y. D. 2010: TNF--induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-XL. Cell Death & Differentiation 17(9):1420–1434.

Klionsky, D. J. and Emr, S. D. 2000: Autophagy as a Regulated Pathway of Cellular Degradation. Science 290(5497):1717–1721.

Kohn, A. J. 2012: Egg Size, Life History, and Tropical Marine Gastropod Biogeography*. American Malacological Bulletin 30(1):163–174.

Kott, P. 1985: The Australian Ascidiacea, Part 1. Phlebobranchia and Stolidobranchia. Memoirs of the Queensland Museum 23:1–440.

Kugler, J. E.; Kerner, P.; Bouquet, J.-M.; Jiang, D.; and Di Gregorio, A. 2011: Evolutionary changes in the notochord genetic toolkit: a comparative analysis of notochord genes in the ascidian Ciona and the larvacean Oikopleura. BMC Evolutionary Biology 11(1):21.

Lacaze-Duthiers, F. J. H. 1877: Histoire des ascidies simples des cotes de France II. Etudes des speces. Arch. Zool. Exp. Gen. 6.

Lambert, G.; Shenkar, N.; and Swalla, B. 2010: First Pacific record of the north Atlantic ascidian Molgula citrina bioinvasion or circumpolar distribution? Aquatic Invasions 5(4):369–378.

Lewin-Koh, R., N. J. Bivand. 2012: maptools: Tools for reading and handling spatial objects. R package version 0.8-20. http://CRAN.R-project.org/package=maptools .

Lijnen, H. R.; Hoef, B. V.; Lupu, F.; Moons, L.; Carmeliet, P.; and Collen, D. 1998: Function of the Plasminogen/Plasmin and Matrix Metalloproteinase Systems After Vascular Injury in Mice With Targeted Inactivation of Fibrinolytic System Genes. Arteriosclerosis, Thrombosis, and Vascular Biology 18(7):1035–1045.

Liu, L.; Yu, L.; Kubatko, L.; Pearl, D. K.; and Edwards, S. V. 2009: Coalescent methods for estimating phylogenetic trees. Molecular Phylogenetics and Evolution 53(1):320–328.

Lum, J. J.; Bauer, D. E.; Kong, M.; Harris, M. H.; Li, C.; Lindsten, T.; and Thompson, C. B. 2005: Growth Factor Regulation of Autophagy and Cell Survival in the Absence of Apoptosis. Cell 120(2):237–248.

Maddison, W. P.; Midford, P. E.; and Otto, S. P. 2007: Estimating a Binary Character's Effect on Speciation and Extinction. Systematic Biology 56(5):701 –710.

Maliska, M. E.; Pennell, M. W.; and Swalla, B. J. 2013: Developmental mode influences diversification in ascidians. Biology Letters 9(3):20130068.

Maliska, M. E. and Swalla, B. J. 2010: Molgula pugetiensis is a Pacific Tailless Ascidian Within the Roscovita Clade of Molgulids. Biol Bull 219(3):277–282.

Millar, R. H. 1954: The breeding and development of the ascidian Pelonaia corrugata Forbes and Goodsir. Journal of the Marine Biological Association of the U.K. 33:681–687.

Millar, R. H. 1962: The Breeding and Development of the Ascidian Polycarpa Tinctor. Quarterly Journal of Microscopical Science s3-103(63):399–403.

Mitra, S. K.; Hanson, D. A.; and Schlaepfer, D. D. 2005: Focal adhesion kinase: in command and control of cell motility. Nature Reviews Molecular Cell Biology 6(1):56–68.

Monniot, F. and Monniot, C. 1997: Ascidians Collected in Tanzania. Journal of East African Natural History 86(1):1–35.

Nakayama, A.; Satou, Y.; and Satoh, N. 2001: Isolation and characterization of genes that are expressed during Ciona intestinalis metamorphosis. Development Genes and Evolution 211(4):184–189.

Nylander, J. A. A. 2004: MrModeltest v2. Program distributed by author. Evolutionary Biology Center, Uppsala University .

Ogata, H.; Goto, S.; Sato, K.; Fujibuchi, W.; Bono, H.; and Kanehisa, M. 1999: KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Research 27(1):29–34.

Pereira-Leal, J. B. and Seabra, M. C. 2001: Evolution of the rab family of small GTP-binding proteins. Journal of Molecular Biology 313(4):889–901.

Pernet, B. and McHugh, D. 2010: Evolutionary changes in the timing of gut morphogenesis in larvae of the marine annelid Streblospio benedicti. Evolution & Development 12(6):618–627.

Pineda, M. C.; Lpez-Legentil, S.; and Turon, X. 2011: The Whereabouts of an Ancient Wanderer: Global Phylogeography of the Solitary Ascidian Styela plicata. PLoS ONE 6(9):e25495.

Rabosky, D. L. 2010: Extinction rates should not be estimated from molecular phylogenies. Evolution 64(6):1816–1824.

Raff, R. A. 1987: Constraint, flexibility, and phylogenetic history in the evolution of direct development in sea urchins. Developmental Biology 119(1):6–19.

Ritter, W. E. 1913: The simple ascidians from the northeastern Pacific in the collection of the United States national museum. Nabu Press.

Roberts, B.; Davidson, B.; MacMaster, G.; Lockhart, V.; Ma, E.; Wallace, S. S.; and Swalla, B. J. 2007: A complement response may activate metamorphosis in the ascidian Boltenia villosa. Development Genes and Evolution 217(6):449–458.

Rocha, R. M. d. 2002: Bostricobranchus digonas Abbott (Ascidiacea, Molgulidae) in Paranaqu Bay, Paran, Brazil: a case of recent invasion? Revista Brasileira de Zoologia 19:157–161.

Rovere, P.; Peri, G.; Fazzini, F.; Bottazzi, B.; Doni, A.; Bondanza, A.; Zimmermann, V. S.; Garlanda, C.; Fascio, U.; Sabbadini, M. G.; Rugarli, C.; Mantovani, A.; and

Manfredi, A. A. 2000: The long pentraxin PTX3 binds to apoptotic cells and regulates their clearance by antigen-presenting dendritic cells. Blood 96(13):4300–4306.

Satou, Y.; Mineta, K.; Ogasawara, M.; Sasakura, Y.; Shoguchi, E.; Ueno, K.; Yamada, L.; Matsumoto, J.; Wasserscheid, J.; Dewar, K.; Wiley, G. B.; Macmil, S. L.; Roe, B. A.; Zeller, R. W.; Hastings, K. E.; Lemaire, P.; Lindquist, E.; Endo, T.; Hotta, K.; and Inaba, K. 2008: Improved genome assembly and evidence-based global gene model set for the chordate Ciona intestinalis: new insight into intron and operon populations. Genome Biology 9(10):R152.

Schulz, M. H.; Zerbino, D. R.; Vingron, M.; and Birney, E. 2012: Oases: robust de novo RNA-seq assembly across the dynamic range of expression levels. Bioinformatics 28(8):1086–1092.

Shenkar, N.; Gittenberger, A.; Lambert, G.; Rius, M.; Da Rocha, R. M.; Swalla, B. J.; and Turon, X. 2013: World Ascidiacea Database. http://www.marinespecies.org/ascidiacea. Accessed: 01/29/13.

Shenkar, N. and Swalla, B. J. 2010: Molecular data confirm synonymy of Roscovite molgulid ascidians. Cahiers de biologie marine 51(1):85–87.

———. 2011: Global Diversity of Ascidiacea. PLoS ONE 6(6):e20657.

Stanley, S. M. 1979: Macroevolution, pattern and process. W.H. Freeman, San Francisco.

———. 1986: Population Size, Extinction, and Speciation: The Fission Effect in Neogene Bivalvia. Paleobiology 12(1):89–110. ArticleType: research-article / Full publication date: Winter, 1986 / Copyright 1986 Paleontological Society.

Strathmann, R. R. 2007: Three functionally distinct kinds of pelagic development. Bulletin of Marine Science 81(2):167–179.

Sugimoto, K. and Nakauchi, M. 1974: Budding, Sexual Reproduction, and Degeneration in the Colonial Ascidian, Symplegma Reptans. The Biological Bulletin 147(1):213–226.

Suzuki, M. M. and Satoh, N. 2000: Genes Expressed in the Amphioxus Notochord Revealed by EST Analysis. Developmental Biology 224(2):168–177.

Swalla, B.; Just, M.; Pederson, E.; and Jeffery, W. 1999: A multigene locus containing the Manx and bobcat genes is required for development of chordate features in the ascidian tadpole larva. Development 126(8):1643 –1653.

Swalla, B.; Makabe, K.; Satoh, N.; and Jeffery, W. 1993: Novel genes expressed differentially in ascidians with alternate modes of development. Development 119(2):307 –318.

Swalla, B. J.; Cameron, C. B.; Corley, L. S.; and Garey, J. R. 2000: Urochordates Are Monophyletic Within the Deuterostomes. Systematic Biology 49(1):52 –64.

Swalla, B. J. and Jeffery, W. R. 1990: Interspecific hybridization between an anural and urodele ascidian: Differential expression of urodele features suggests multiple mechanisms control anural development. Developmental Biology 142(2):319–334.

———. 1992: Vestigial Brain Melanocyte Development During Embryogenesis of an Anural Ascidian. Development, Growth & Differentiation 34(1):1725.

———. 1996: Requirement of the Manx Gene for Expression of Chordate Features in a Tailless Ascidian Larva. Science 274(5290):1205 –1208.

Tagawa, K.; Jeffery, W. R.; and Satoh, N. 1997: The Recently-Described Ascidian Species Molgula tectiformis Is a Direct Developer. Zoological Science 14(2):297–303.

Takada, N.; York, J.; Davis, J. M.; Schumpert, B.; Yasuo, H.; Satoh, N.; and Swalla, B. J. 2002: Brachyury expression in tailless Molgulid ascidian embryos. Evolution & Development 4(3):205–211.

Team, R. D. C. 2012: R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/ .

Thiyagarajan, V. and Qian, P.-Y. 2003: Effect of temperature, salinity and delayed attachment on development of the solitary ascidian Styela plicata (Lesueur). Journal of Experimental Marine Biology and Ecology 290(1):133–146.

Thorson, G. 1950: Reproductive and larval ecology of marine bottom invertebrates. Biological Reviews 25(1):1–45.

Tsagkogeorga, G.; Turon, X.; Hopcroft, R.; Tilak, M.-K.; Feldstein, T.; Shenkar, N.; Loya, Y.; Huchon, D.; Douzery, E.; and Delsuc, F. 2009: An updated 18S rRNA phylogeny of tunicates based on mixture and secondary structure models. BMC Evolutionary Biology 9(1):187.

Van Name, W. G. 1945: The North and South American ascidians. Bulletin of the AMNH 84:1–476.

Vazquez, E. and Young, C. 1996: Responses of compound ascidian larvae to haloclines. Marine ecology progress series. Oldendorf 133(1):179–190.

Watanabe, H. and Lambert, C. C. 1973: Larva Release in Response to Light by the Compound Ascidians Distaplia occidentalis and Metandrocarpa taylori. Biological Bulletin 144(3):556–566.

Whittaker, J. R. 1979: Development of Vestigial Tail Muscle Acetylcholinesterase in Embryos of an Anural Ascidian Species. The Biological Bulletin 156(3):393–407.

Wittkopp, P. J.; Vaccaro, K.; and Carroll, S. B. 2002: Evolution of yellow Gene Regulation and Pigmentation in Drosophila. Current Biology 12(18):1547–1556.

Woods, R. G.; Roper, K. E.; Gauthier, M.; Bebell, L. M.; Sung, K.; Degnan, B. M.; and Lavin, M. F. 2004: Gene expression during early ascidian metamorphosis requires signalling by Hemps, an EGF-like protein. Development 131(12):2921 –2933.

Wray, G. A. and McClay, D. R. 1989: Molecular Heterochronies and Heterotopies in Early Echinoid Development. Evolution 43(4):803–813.

Yamaguchi, A.; Hori, O.; Stern, D. M.; Hartmann, E.; Ogawa, S.; and Tohyama, M. 1999: Stress-Associated Endoplasmic Reticulum Protein 1 (Serp1)/Ribosome-Associated Membrane Protein 4 (Ramp4) Stabilizes Membrane Proteins during Stress and Facilitates Subsequent Glycosylation. The Journal of Cell Biology 147(6):1195–1204.

Yap, A. S.; Brieher, W. M.; and Gumbiner, B. M. 1997: Molecular and Functional Analysis of Cadherin-Based Adherens Junctions. Annual Review of Cell and Developmental Biology 13(1):119–146. PMID: 9442870.

Young, C. M. and Braithwaite, L. F. 1980: Orientation and Current-Induced Flow in the Stalked Ascidian Styela montereyensis. Biological Bulletin 159(2):428–440.

Young, C. M. and Chia, F.-S. 1985: An experimental test of shadow response function in ascidian tadpoles. Journal of Experimental Marine Biology and Ecology 85(2):165–175.

Young, C. M.; Gowan, R. F.; Dalby, J.; Pennachetti, C. A.; and Gagliardi, D. 1988: Distributional Consequences of Adhesive Eggs and Anural Development in the Ascidian Molgula pacifica (Huntsman, 1912). The Biological Bulletin 174(1):39–46.

Zeng, L.; Jacobs, M. W.; and Swalla, B. J. 2006: Coloniality has evolved once in Stolidobranch Ascidians. Integrative and Comparative Biology 46(3):255 –268.

Zeng, L. and Swalla, B. J. 2005: Molecular phylogeny of the protochordates: chordate evolution. Canadian Journal of Zoology 83:24–33.

Zerbino, D. R. and Birney, E. 2008: Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Research 18(5):821–829.