# Recruitment patterns and post-metamorphic attachment by the solitary ascidian, *Ciona intestinalis* (Linnaeus, 1767) in an invading population from Placentia Bay Newfoundland and Labrador

By

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ABSTRACT

*Ciona intestinalis* (Linneaus, 1767) is a non-indigenous species discovered in Newfoundland (NL) in 2012. It is a bio-fouler with potential to cause environmental distress and economic strain for the aquaculture industry. Key in management of this species is site-specific knowledge of life history and ecology. This study defines the environmental tolerances, recruitment patterns, habitat preferences, and attachment behaviours of *C. intestinalis* in Newfoundland.

Over two years of field work, settlement plates and surveys were used to determine recruitment patterns, which were correlated with environmental data. The recruitment season extended from mid June to late November.

Laboratory experiments defined the growth rate and attachment behaviours of *Ciona intestinalis*. I found mean growth rates of 10.8% length·$d^{-1}$. The ability for *C. intestinalis* to undergo metamorphosis before substrate attachment, forming a feeding planktonic juvenile, thus increasing dispersal time was also found. These planktonic juveniles were then able to attach to available substrates post-metamorphosis.

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## CO-AUTHORSHIP STATEMENT

I am the principal author responsible for the contents of this thesis including literature review, formulation of questions, experimental design and execution, data collection and analysis as well as preparation of this thesis document. Chapter 3: Post-metamorphic attachment by solitary ascidian *Ciona intestinalis* (Linnaeus, 1767) juveniles from Newfoundland and Labrador, Canada has been published in Management of Biological Invasions (Reid et al 2016). Dr. Cynthia H. McKenzie, Cyr Couturier, and Dr. Gretchen Lambert assisted in the identification and design of the research. Chapter 2 – including any resulting publications unless requested otherwise – is co-authored by Dr. Cynthia H. McKenzie, Dr. Don Deibel, Cyr Couturier, Kyle Matheson and Terri Wells. Matheson and Wells assisted in data collection and Deibel and Matheson contributed to experimental design and statistical analysis of Chapter 2. Chapter 3 – including any resulting publications unless requested otherwise – is co-authored by Dr. Cynthia H. McKenzie, Kyle Matheson, Terri Wells and Cyr Couturier. Kyle Matheson contributed to experimental design and provided statistical advice.

# CHAPTER 1: INTRODUCTION AND OBJECTIVES

## 1.1 Introduction and Objectives:

Ascidian Aquatic Invasive Species (AIS) are a growing concern around the world both environmentally and economically. Newfoundland is currently dealing with three such species, tunicates (Ascidiacea, Tunicata) *Botryllus schlosseri* (Pallas, 1766), *Botrylloides violaceus* (Oka, 1927), and *Ciona intestinalis* (Linnaeus, 1767) previously known as Type B (Brunetti et al. 2015). They pose threats to native plants and animals through predation, space competition, alteration of habitats (Martin et al. 2011) and decreased species richness (Blum et al. 2007). These threats have resulted in moderate too severe ecological and economic impacts (Lambert & Lambert 2003). AIS are often introduced by boat traffic or floating debris, and artificial structures such as wharves, barges, and aquaculture gear are known to create novel niches for opportunistic colonizers (Dumont et al. 2011).

In Atlantic Canada, shellfish industries are severely affected by fouling tunicates due to increased processing costs and decreased product yields (Daigle & Herbinger, 2009). *Ciona intestinalis*, are filter-feeding, solitary ascidians belonging to the Order Phlebobranchia (Fox 2001, Carver et al. 2006). They were first discovered in Newfoundland in Placentia Bay in 2012 (Sargent et al. 2013). This report was of substantial concern as it was Newfoundland's first high-risk, ascidian invasion (McKenzie et al. 2016a).

The potential impacts of *Ciona intestinalis* are of concern, and the need for a research-based approach to controlling invasions is supported by industry and policy makers in Newfoundland and Labrador. Therefore, the purpose of this thesis is to carry out baseline studies to document the environmental parameters surrounding Newfoundland *Ciona*, and to understand its life history and population dynamics which often vary geographically. Secondly, I aim to

better understand the pattern of metamorphosis and attachment of *C. intestinalis* larvae to add to local knowledge of dispersal potential, and impacts on aquaculture.

With these objectives in mind, the purpose of Chapter 1 of this thesis is to discuss, according to the literature, a brief history of the distribution of *Ciona intestinalis,* with special attention on Atlantic Canadian populations. In addition, I will describe the biology and life history of *C. intestinalis* and discuss potential vectors and impacts of invasion, and methods of control. In Chapter 2, I document the habitat and environmental parameters in which *C. intestinalis* are found in Newfoundland, identify co-occurring organisms, and describe population recruitment dynamics in Little Bay, NL. In Chapter 3, I focus on the percent of larvae that undergo metamorphosis to feeding juveniles prior to settling and attachment, leading to increased time for dispersal by currents. I also determine the settlement success of these precocious, planktonic early life stages. Chapter 4 is a summary of the results from these studies and their significance to the aquaculture industry of Newfoundland and Labrador.

**1.2 Distribution and History**

*Ciona intestinalis* are known around the world as biofouling, opportunistic, nuisance species (Lambert & Lambert 1998, Carver et al. 2006, Sargent et al. 2013). Although considered to be cryptogenic, *C. intestinalis* is likely native to Northern Europe (Dybern 1965), which is presumably the origin of Canadian populations (Carver et al. 2006). While the first record of *C. intestinalis* in Canada was made in the early 1800s, it has only recently been recorded in high densities (Carver et al. 2006). The first occurrence in Eastern Canada was off Grand Manan Island, in the Bay of Fundy, in the mid-1800s (Carver et al. 2006). However, the first documented case of biofouling by *C. intestinalis* was reported in 1997 on a mussel farm in Lunenburg, Nova Scotia (Cayer et al. 1999, Clancey and MacLachlan 2004, Daigle & Herbinger,

2009). Prince Edward Island (PEI) is also dealing with significant infestations by *C. intestinalis*, which was introduced in 2004 (Carver et al. 2006, Locke et al. 2007). *C. intestinalis* is an aggressive competitor, often replacing other fouling ascidians, such as *Styela clava*, in PEI (Ramsay et al. 2009). A combination of increasing temperatures and the rapid growth and reproduction of *C. intestinalis*, indicate potentially serious implications of *C. intestinalis* spread in Atlantic provinces, including Newfoundland (Dybern 1965, Lambert & Lambert 2003, Sargent et al. 2013).

## 1.3 Biology and the Environment

### *1.3.1 Physical Description*

The body of *Ciona intestinalis* may be up to 15 cm in length, 3 cm in diameter, and is cylindrical in shape (Figure 1.3.1 a). Size may vary within a population as well as between populations, depending on the environmental conditions, such as temperature (Dybern 1965). The soft body tissues range in color from greenish/yellow to orange (Figure 1.3.2 b & d) due either to age, the presence of vanadium metals in the blood, or varying population morphs (Fox 2001, Sato et al. 2012). Located at the anterior end of *C. intestinalis* are two siphons, a smaller, more posterior atrial siphon, with six lobes, and a second more anterior buccal siphon with eight lobes (Figure 1.3.1b). Lobes of both siphons will have some variation of yellow margins and orange/red pigment spots (Figure 1.3.2 a & c) (Sato et al. 2012). At the posterior end are short, fleshy projections called villi, which function as holdfasts for substrate attachment (Figure 1.3.1a).

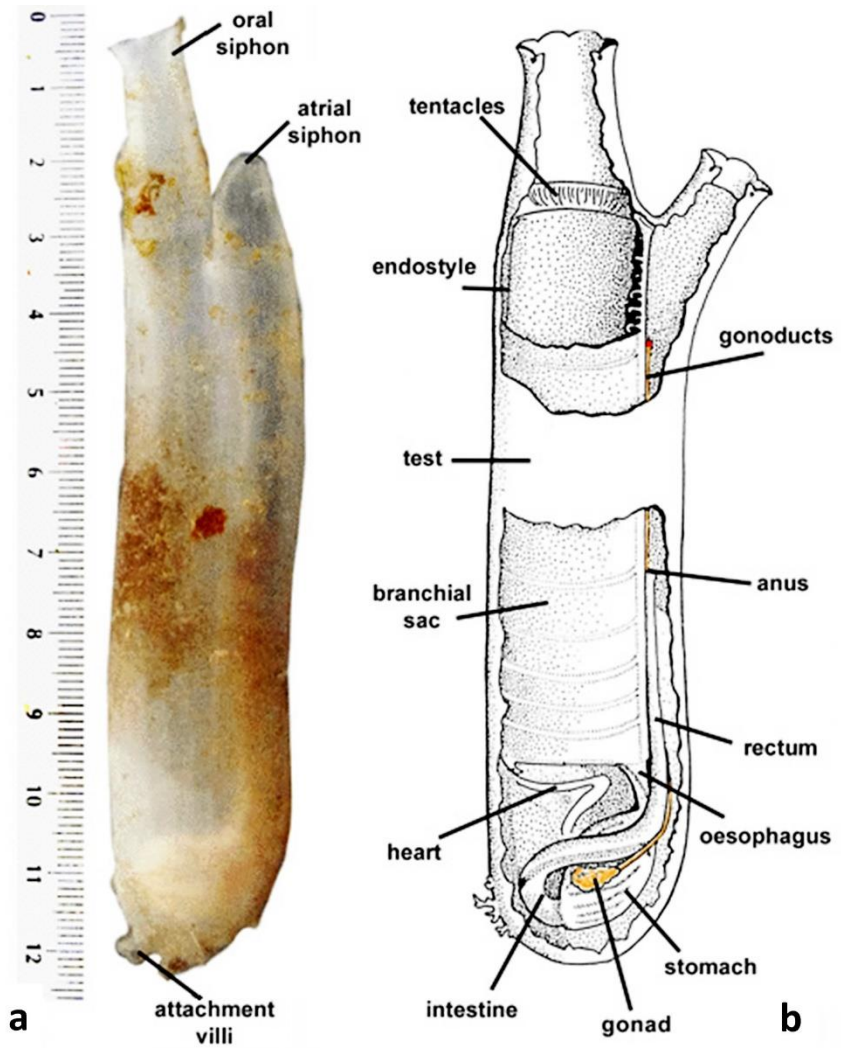



Figure 1.3.1: a) External anatomy of *Ciona intestinalis*; b) Internal anatomy of *C. intestinalis*. (from Cirino et al. 2002)

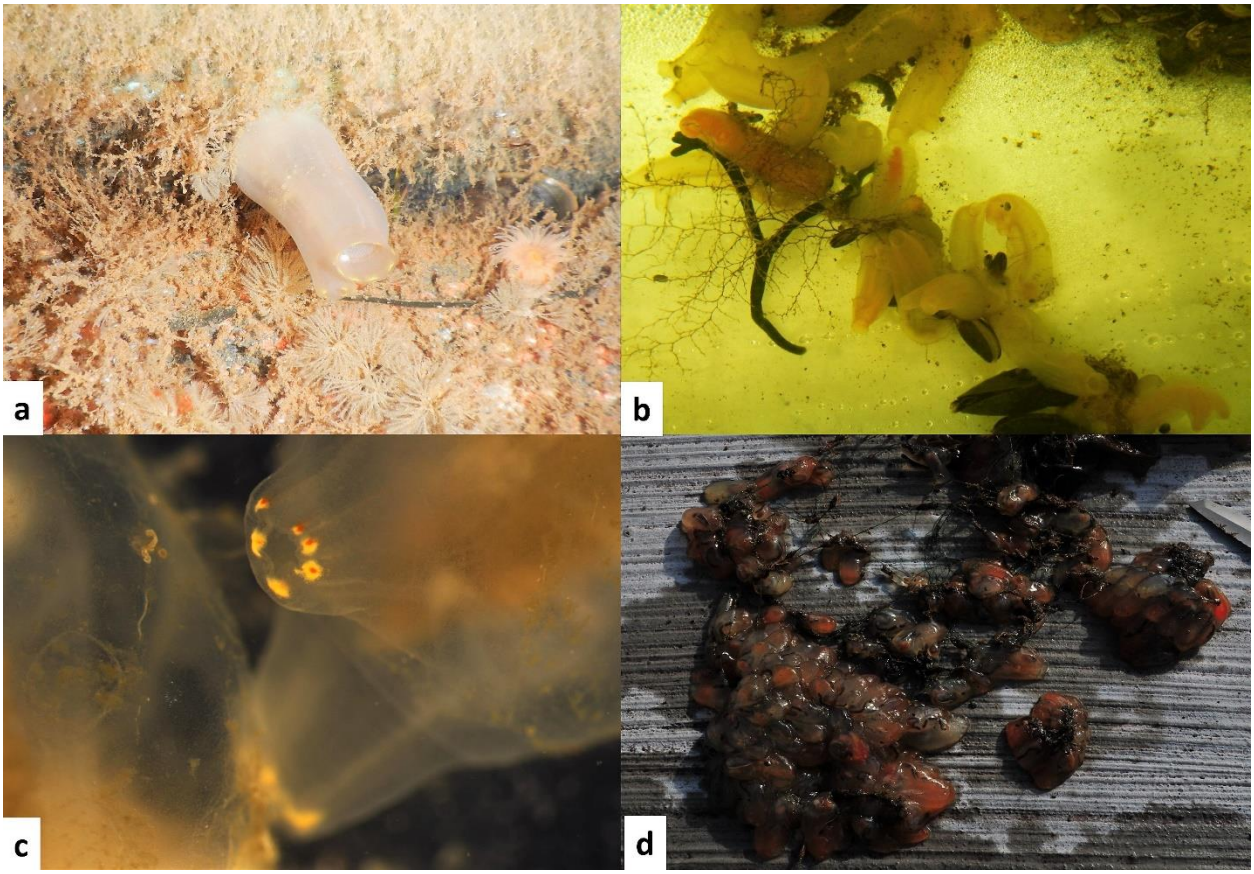



Figure 1.3.2: *Ciona intestinalis* physical appearance and color morphs: a) *C. intestinalis* attached to a boat hull in Burin, Newfoundland; b) samples collected from Burin indicating variation in color; c) yellow and red pigment at siphon lobes; d) *C. intestinalis* attached to an abandoned fishing net in Burin showing color variation.

Inside the outer protective tunic is a thin sac-like membrane composed of an external epithelium, connective tissue, muscles, and blood vessels which enclose the internal organs (Figure 1.3.1 b). The internal anatomy is visible through the translucent tunic, especially in younger individuals, as the tunic of adults is more opaque and may be covered by algal and bacterial fouling (Carver et al. 2006) (Figure 1.3.2 a-c). The longitudinal muscle bands are visible on each side of the body and may be pale yellow, white, or bright orange in color. These muscles retract the siphons when stimulated. The body is divided into two cavities, the atrial and the visceral cavities. The atrial cavity takes up the majority of the body and contains the

branchial sac, which takes on the water from the inhalant buccal siphon (Figure 1.3.1b). The visceral cavity is the smaller of the two and contains the intestine, stomach, testes and ovary, heart, and gonoducts and anus, which empty through the atrial siphon (Figure 1.3.1 b). For a more detailed description of morphological and taxonomic features, see Van Name (1945).

*1.3.2 Life History*

*Ciona intestinalis* is a self-sterile, protandric hermaphrodite (Morgan 1945). They are classified as oviparous, and fertilization is external after gametes are expelled through the atrial siphon. The point at which they reach sexual maturity depends on the individual's size rather than its age, and sizes-at-age depends on the temperature history of its environment (Dybern 1965, Yamaguchi 1975).

Spawning and settlement in *Ciona intestinalis* appear to be controlled by changes in ambient light, tending to occur at dawn (Lambert & Brandt 1967, Svane & Havenhand 1993). Svane & Havenhand (1993) suggested that both spawning and development time are synchronized so that larval settlement occurs when light conditions are optimal for finding highly shaded areas. Temperature also plays a significant role in controlling spawning. *C. intestinalis* are able to withstand wide ranges of environmental parameters (i.e., temperatures from -1 to 30 °C, and salinities between 12 and 40), but thrive within a much narrower range which may vary from one location to the next (Dybern 1965, Carver et al. 2003, 2006). While some populations spawn all year long (Yamaguchi 1975), *C. intestinalis* has a lower temperature limit for spawning of 8ºC in Scandinavian and Atlantic Canadian populations (Carver et al. 2003, Ramsay et al. 2008, 2009). Some suggest this temperature limit is set by degenerative changes in the gonads (Dybern 1965). However, one cannot ignore the potential impact of a decline in nutrient resources that commonly occurs at lower temperatures.

Once conditions are favorable, *Ciona intestinalis* will spawn continuously until conditions deteriorate. Individual egg production rate for populations in Scandinavian and Nova Scotian waters ranges from 150-500 eggs $d^{-1}$ (Petersen & Svane 1995, Carver et al. 2003). Yamaguchi (1975) however reported double these rates in Japanese populations. This variation in reproductive effort is likely due to differences in the range of temperatures and duration of favorable temperatures and associated nutrient levels. After spawning, gametes may be in the water column as individuals or many be clumped together in a mucus string. Successful fertilization is increased by sperm chemoattractants which effectively increase the detectable diameter of the egg to 2 mm (Jantzen et al. 2001) (Figure 1.3.3 a). In the absence of homospecific eggs, energy reserves of the sperm are conserved. If there are no homospecific eggs, the sperm will be viable for up to 16 h, but this decreases to 1.5 h with eggs present (Jantzen et al. 2001).

When fertilization is complete, a complex embryonic development occurs in six periods as outlined by Hotta et al. (2007). The stages are zygote, cleavage, gastrula, neurula, tailbud, and larva, and the duration of embryonic development will vary with temperature from 12 - 24 h (Cirino 2002, Carver et al. 2006, Liu et al. 2006). *Ciona intestinalis* larvae are 1.2 ± 0.04 mm long and are non-feeding (Bullard et al. 2004, Carver et al. 2006, Liu et al. 2006) (Figure 1.3.3 b). They consist of an elongated trunk with developed organ systems, and a slender, muscular tail surrounded by test cells (Katz 1983) (Figure 1.3.3 b). Noticeable sensory vesicles in the trunk of newly hatched larvae, known as the ocellus and statoliths (Millar 1971), initially cause upward swimming toward light, followed by downward swimming in search of shaded, attachment locations (Berrill 1947, Millar 1971). At the anterior end of the larvae are attachment organs which will become the initial stalk when metamorphosis begins (Berrill 1947, Bullard et al.

2004). The distance traveled and longevity of a larva before settlement vary depending on environmental conditions (Berrill 1947). Once larvae have settled, they begin metamorphosis, a process which transforms a non-feeding, planktonic larva into a filter feeding, benthic juvenile (Berrill 1947). Details of visible characteristics throughout metamorphosis are outlined by Cirino et al. (2002) and Bullard et al. (2004). The growth from juvenile to a sexually mature individual is dependent on environmental factors like temperature and available nutrients (Dybern 1965, Yamaguchi 1975, Carver et al. 2006).

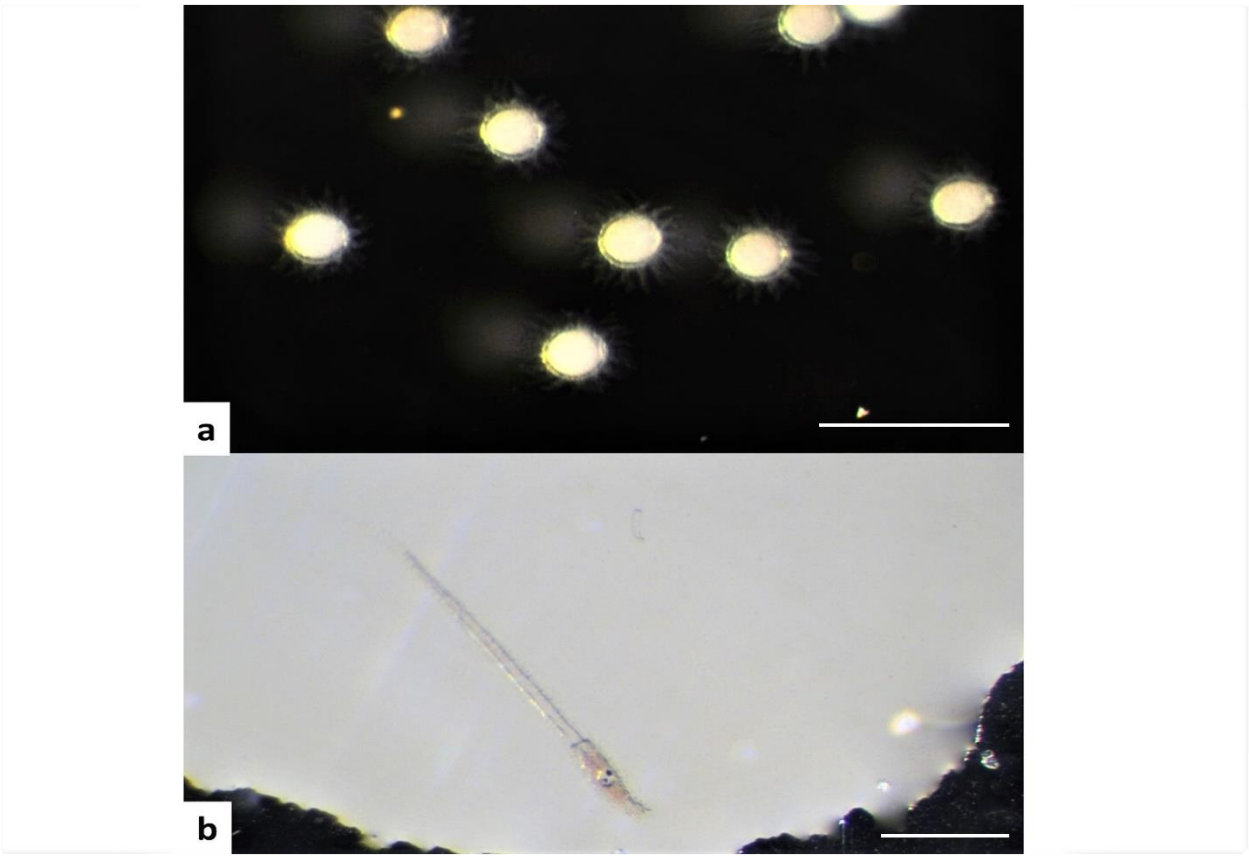



Figure 1.3.3: *Ciona intestinalis* a) eggs and b) larvae. Scale bar is 0.5mm in each image.

## 1.4 Potential Vectors

*Ciona intestinalis* are sessile animals that have a relatively short planktonic stage before non-feeding larvae find an attachment location. Aside from natural dispersion of sperm, eggs, the developing embryo, and larvae, this species relies heavily on attachment to a substrate in order to populate new locations. Vectors are largely anthropogenic, such as boats, barges, and gear, but may also include floating debris (natural or manmade) (Bravo et al. 2011, Adams et al. 2014). According to Carver et al. (2006), *C. intestinalis* was repeatedly found in Nova Scotia rafting on pieces of *Codium fragile*. *C. intestinalis* can be relocated while attached to boat hulls and various types of gear, such as fishing, aquaculture, and diving equipment. Hull fouling of slow moving vessels, such as barges, small fishing and recreational boats, are likely responsible for regional dispersion within many coastal areas following an initial introduction (Lambert & Lambert 2003, Adams et al. 2014).

## 1.5 The Impact

### *1.5.1 Environmental*

The environmental impacts caused by *Ciona intestinalis* infestation can be extensive and increase with population size. They can alter habitat structure, nutrient and oxygen content of the surrounding water, decrease biodiversity, and contribute to benthic habitat degradation. Entire habitats can be altered by changing or blocking currents, obstructing light penetration, and occupying space for other settlers (Blum et al. 2007, Martin et al. 2011). Because of the high rate of growth and reproduction, *C. intestinalis* depletes dissolved oxygen and nutrients, and can outcompete native species, decrease species richness, and alter community structure (Blum et al.

2007). Lutz-Collins et al. (2009) found that invasive tunicates have particularly negative effects on sessile communities where they are in direct competition for space and nutrients (Colarusso et al. 2016). In cases where *C. intestinalis* is a dominant member of a biofouling community, they also contribute to the deposition of faecal matter to the ocean floor. This ongoing organic enrichment may eventually lead to development of anoxic sediments, the accumulation of hydrogen sulfide, and the degradation of the benthic community (Carver et al. 2006). *C. intestinalis* do well under low water flow and polluted conditions, and tend to become the dominant member of communities under these conditions (Mazzola and Riggio 1977, Carver et al. 2006).

Another significant environmental concern is the occurrence of tunicates on eelgrass, *Zostera marina* (Linnaeus, 1753). Eelgrass habitats have many important ecological functions (Barbier et al. 2011), including providing a nursery habitat for juvenile fish. The attachment of tunicates may inhibit eelgrass growth and cause shoot mortality, effectively depleting its effectiveness and coverage (Carman et al. 2016a).

Environmental damage caused by populations of *Ciona intestinalis* is of concern for the aquaculture industry, which relies heavily on healthy aquatic ecosystems. Because every aquaculture site is different, varying in stock, equipment, and environmental conditions, the impact of *C. intestinalis* populations will be site specific.

### *1.5.2 Economic*

The major threat of *Ciona intestinalis* in Newfoundland waters is to the farming of blue mussels, *Mytilus edulis*. Tunicate invasions can lead to fouled equipment and infrastructure, such as boats, barges, buoys, ropes, cages, and nets, as well as the product itself (Durr and Thomason

2009). Additional costs of cleaning and replacement of equipment, combined with decreased product yield, greatly affect profit. A tunicate infestation on mussel socks can be so dense that the socks become too heavy and break free of the lines (Daigle & Herbinger, 2009). With increased cost of operation, and loss or decreased product quality, *C. intestinalis* fouling can decrease product yields by 90% (Durr and Thomason 2009). Daigle & Herbinger (2009) showed that on farms in Nova Scotia, the denser the tunicate population the smaller the meat yield. In fact, the study showed that the number of marketable-sized mussels was three times higher in low tunicate densities than in high tunicate densities. *C. intestinalis* not only directly compete with mussels for space but compete for nutrients as well, leading to decreased resources and lower growth of the mussels. While mussels have three times higher individual filtration rates than *C. intestinalis* (Daigle & Herbinger, 2009), the tunicates are able to colonize mussel socks and rapidly become the dominate species in terms of abundance and biomass. This makes space and nutrient competition a major problem for mussel farmers. Fouled mussels may also have degraded shell quality, whereby the shells are weakened leading to damage and loss during processing, or a decrease in market value (Durr and Thomason 2009).

### 1.6 Control and Mitigation

There have been a variety of studies focused on an effective method to control or mitigate *Ciona intestinalis* populations. Three main types of control are physical, chemical, and biological. Many farms carry out physical control by scrubbing, scraping, or high pressure spraying of equipment or mussel lines themselves (Carman et al. 2010). However, well established populations quickly recolonize, so physical removal is needed repeatedly throughout the growing season (Clancey & Hinton 2003). Often physical controls are marginally effective. The ineffectiveness is likely due to lack of total removal of tunicates from the water or release of

gametes by rupture of the test. Cleaning of gear on dry land would increase the success of this method.

Carver et al. (2003) tested some chemical treatments, including sodium hypochlorite, salt brine, lime, acetic acid, and freshwater, at both 15ºC and 40ºC. While acetic acid and 40ºC-freshwater were shown to be effective, they also caused a certain amount of mortality in mussels and oysters. In this same study, biological control was tested using rock crab, *Cancer irroratus*, green crab, *Carcinus maenas*, and sea stars, *Asterias vulgaris*, all of which share a habitat with *C. intestinalis* populations in Newfoundland. While the crab species fed on the tunicates in nature, dense populations are normally safe from predation on wharves, pilings, and other suspended structures such as mussel socks (Carver et al. 2003). Carman et al. (2016b) found that freshwater baths are effective at killing tunicates attached to blue mussels without killing the mussels. Farmers in Nova Scotia lower mussel lines to the bottom allowing for predation by benthic crabs and sea stars (Clancey & Hinton 2003). However, the timing and duration of lowering lines must be carefully executed to avoid predation of the product and the extent of exposure to smothering seafloor sediments must be carefully controlled.

While these methods are reactive in dealing with established populations of *Ciona intestinalis*, there is also a proactive method known as cultural control which focuses on minimizing recruitment using management practices (Clancey & Hinton 2003, Ramsey et al. 2009). For instance, growers might clean gear more often and at scheduled times when tunicate populations are not reproductive, to avoid the unintended release of gametes. Davidson et al. (2016) found that beginning seawater spraying early in the season and repeating 3 - 4 times during the season, reduced tunicate number and size on mussel socks allowing for increased mussel growth and productivity. Also, before returning clean gear to the water, air drying to

insure no living tunicates are reintroduced is effective in preventing recolonization. Bakker et al. (2011) focused on preventing settlement of *C. intestinalis* larvae by applying food-grade oil to aquaculture gear. This method however proved ineffective.

## 1.7 Conclusion

Baseline information on environmental tolerances and recruitment patterns, as well as dispersal potential, is of the upmost importance in devising a plan to control invasive tunicate populations for nearly all methods of control. This thesis provides some of these vital details on this high risk invader to Newfoundland waters, *Ciona Intestinalis,* to assist in its control in local waters and aquaculture sites.

# CHAPTER 2: ECOLOGY AND RECRUITMENT OF *C. INTESTINALIS* IN COASTAL NEWFOUNDLAND WATERS.

**Abstract**

When the ascidian, *C. intestinalis* was found in Little Bay, NL, it was vital to gather site-specific information on its ecology and recruitment to develop a control plan. Mean growth rate in laboratory experiments at 15°C was 18 mm·mo$^{-1}$ or 10.8 % length·d$^{-1}$. Abundance estimated from quadrat photos of removed floating docks ranged from 0-88 individuals·m$^{-2}$. The recruitment season extended from mid-June to late November, with one major recruitment event. This study took place in the presence of mitigation experiments, resulting in a low maximum recruitment rate of 7.4 individuals·m$^{2}$·d$^{-1}$. Locations with higher recruitment rates were those with increased protection from currents and sunlight, more artificial substrates for attachment, and increased vessel traffic.

## 2.1 Introduction

The Aquatic Invasive Species (AIS) *Ciona intestinalis* is a marine solitary ascidian recognized worldwide as a fouling pest. In September 2012, small numbers of *C. intestinalis* were first detected during underwater surveys in southwestern Placentia Bay, Newfoundland (NL), Canada, at the public wharf in Burin (Sargent et al. 2013). A month later, further rapid assessment surveys revealed high abundances of *C. intestinalis* at a small nearby (~25 km) public wharf in Little Bay. This species was found fouling boat hulls, permanent and floating wharves, ropes and floats, and eelgrass, which is of high ecological importance (Barbier et al. 2011). Upon early detection of this high risk invader, a collaborative rapid response mitigation by Fisheries and Oceans Canada, NL Department of Fisheries and Aquaculture, Memorial University of Newfoundland and the Newfoundland Aquaculture Industry Association in Little Bay, with subsequent AIS removal and monitoring for effectiveness over a two-year period (2013 and 2014) (McKenzie et al. 2016a).

Generally, *Ciona intestinalis* can be found in shallow coastal waters often as a dominant member of the biofouling community on artificial substrates (Lambert & Lambert 1998, 2003). In its invasive range, *C. intestinalis* prefers to attach in habitats with low light and low current velocities (Howes et al. 2007). The species is a global invader and has had significant ecological and economic impacts, affecting local biodiversity and mussel aquaculture operations, respectively. To date, *C. intestinalis* has had the greatest impact on blue mussel (*Mytilus edulis*) aquaculture operations in southern regions of Atlantic Canada, which is a concern for the aquaculture industry in NL. Thus far, no invasive tunicates have been detected on aquaculture sites in NL (Sargent et al. 2013). Aquaculture operations provide clean surfaces for attachment of *C. intestinalis* (i.e., nets, cages, wharf structures, and shells of product) and a refuge from predation. The added costs of cleaning or replacing of equipment (Daigle and Herbinger 2009), along with possible decreases in product yield caused by nutrient competition, can be devastating for the industry (Durr and Thomason 2009).

By attaching to solid substrates *Ciona intestinalis* can alter the physical habitat, which can lead to lower light penetration, changes in current flow and nutrient availability, and greatly increase organic enrichment, creating anoxic sediments (Petersen and Riisgaard 1992, Stenton-Dozy et al. 2001). Established populations have the potential to out compete other species for resources, leading to decreases in biodiversity (Blum et al. 2007). Furthermore, Osman et al. (1989) reported that *C. intestinalis* can prey directly on oyster larvae. While often found attached to artificial structures, they have been observed in NL attached to eelgrass in small numbers (Sargent et al. 2013, Carman et al. 2016a). This is of concern because of the importance of eelgrass habitat as juvenile fish nurseries (Duarte 2002, Carman et al. 2016a). For example, in NL, eelgrass beds serve as nurseries for juvenile cod (both Greenland cod *Gadus ogac* and

Atlantic Cod *G. morhua*) which are economically valuable (Laurel et al. 2003, Bradbury et al. 2008).

*Ciona intestinalis* can survive a wide range of environmental conditions, including temperatures ranging from -1 to 30 °C (Dybern 1965, Carver et al. 2003, 2006) and salinities between 12 and 40 ppt. However, they thrive within more narrow ranges of environmental conditions (Carver et al. 2006). For example, the lower temperature threshold for occurrence of successful embryogenesis is 8 ºC (Dybern 1965), and when temperatures are above this threshold egg production and spawning (up to 500 eggs per day per individual in Atlantic Canada populations) is continuous, leading to rapid population expansion (Carver et al. 2003, 2006).

When an AIS is detected in a region, it is important to understand its ecology in the new environment, as this can vary among locations. Shifts in recruitment rates, for example, are likely due to environmental fluctuations, as growth and longevity vary in response to temperature and food levels (Millar 1952, Peterson et al. 1995). After reviewing the life history of various populations, Dybern (1965), concluded that growth rate, size at maturity, and longevity depend on cumulative temperature exposure. For example, in Maritime provinces and Scandinavian waters (annual temperatures ranging from -1.5 to 21 °C), *Ciona intestinalis* reach 150 mm in length, become sexually mature between 50-80 mm in length (at approximately 2.5 – 3 months of age), growing at a rate of 10-20 mm $mo^{-1}$, or 1-3 % length $d^{-1}$ (Dybern 1965, Petersen et al. 1995). The organisms live in these conditions for 12-18 months and only spawn when temperatures are above 8 °C, producing 2 generations $yr^{-1}$ (Dybern 1965, Carver et al. 2003, Ramsay et al. 2009). However, in coastal regions of Japan (where seawater temperatures range from 13.6-25.5 ºC), *C. intestinalis* reach a maximum length of 60 mm, live approximately six months, reach sexual maturity at 20 mm in length after only one month of summer growth, and

spawn all year (Yamaguchi 1975). In these conditions there may be as many as four generations per year, with two or three generations overlapping at a time.

Of particular interest in this study are the comparisons of *Ciona intestinalis* ecology in NL to populations found elsewhere in Atlantic Canada. Research has thoroughly examined populations of *C. intestinalis* in southern regions of Atlantic Canada, such as Nova Scotia (NS) and Prince Edward Island (PEI), including environmental and habitat preferences, recruitment patterns, and growth rates (Carver et al. 2003, Howes et al. 2007, Ramsay et al. 2008, 2009). *C. intestinalis* are exposed to temperatures ranging from -1°C in January/February to 18-20°C in September in Nova Scotia, and salinities ranging from 26.8 to about 31.2 (Howes et al. 2007, Vercaemer et al. 2011). Ramsay et al. (2009) indicated that populations of *C. intestinalis* in PEI experience temperatures from -1.5-21°C and salinities from 15-30.1. In Nova Scotia, *C. intestinalis* recruitment is spatially patchy, but can reach up to 140 individuals per petri dish collector, after a one-week deployment between early July and mid-November (Howes et al. 2007, Sephton et al. 2011). The first of two recruitment peaks occurs between early July and early August, and the second peak occurs between early September and mid-November. However, other research in Nova Scotia has demonstrated that timing of recruitment peaks of *C. intestinalis* can vary temporally and spatially due to variations in environmental conditions, such as temperature and food levels (Carver et al. 2003, 2006, Howes et al. 2007). Comparatively, the recruitment season of *C. intestinalis* in PEI spans from Early-June to the end of November, but has only one recruitment peak in mid-August, with an average of up to 429 individuals per collector plate (Ramsay et al. 2009). It should be noted here that Howes et al. (2007) used petri dish collectors while Ramsay et al. (2009) used PVC collector plates which may have contributed, to some degree, in the variation in settlement. In NS, Howes et al. (2007) found no

obvious relationship between temperature time series and the timing of recruitment peaks, except that recruitment only occurred above 8°C. While other studies carried out in both PEI and NS confirmed a lower temperature threshold for recruitment of 8ºC, they report a relationship between recruitment and temperature throughout the recruitment season (Ramsay et al. 2009; Vercaemer et al. 2011). These differences can influence population expansion, and an understanding of seasonal development and recruitment is essential in formulating management plans for these invaders.

## 2.2 The Objectives

The objectives of this study are to define the population dynamics and environmental parameters of *Ciona intestinalis* in Little Bay, NL, to assist in further control measures. Over the course of two years (2013 and 2014), the objectives of this study are 1) to determine the growth and development rate from larval settlement to sexual maturity in the laboratory; 2) to determine the pre-mitigation population density and the impact of mitigation on the target population, and 3) to describe temporal and spatial recruitment patterns, the relationship between recruitment and fluctuations in temperature, the number of generations present per year, and define the species composition of the biofouler community.

## 2.3 Materials and Methods

### *2.3.1: Growth and Development Rate*

Growth and development rate experiments were conducted under laboratory conditions. Sexually mature, adult *Ciona intestinalis* were collected by SCUBA divers from Burin, NL (47° 01’ 51” N, 55° 10’26” W), in November 2014. Tunicates were placed in coolers of sea water with Ziploc®bags filled with ice to keep them cool for transportation to the Northwest Atlantic

Fisheries Centre (Fisheries and Oceans Canada – DFO) in St. John's, NL. In the laboratory, they were held in 54 L, quarantined tanks filled with unfiltered sea water. Temperature was maintained at approximately 15 °C (the average spawning season temperature in Little Bay for 2013) using a tank chiller, and air supply tubes were operating 24 h a day. Lights in the lab were set to ambient cycles during the breeding season (7 h of darkness and 17 h of light). Tunicates were fed a mixture of *Chaetoceros muelleri, Isochrysis* sp.*, Pavlova* sp.*, and Thalassiosira pseudonana* in Shellfish Diet 1800® once daily using a slow release drip apparatus. A mixture of $6x10^6$ cells · $ml^{-1}$ was allowed to drip for approximately 3 h $d^{-1}$ at a rate of 1 drop $s^{-1}$.

Three days prior to the beginning of the experiment, tunicates were exposed to continuous light to prevent spawning and allow an accumulation of gametes. Following light exposure, 12 mature individuals were chosen based on the fullness of the bright orange oviduct and white sperm duct. Adults were split into two, small, 3L tanks of fresh, raw sea water with an air supply and 5 ml of the above food mixture. Both tanks were exposed to constant dark for a 24 h period. Spawning began within five minutes after dark exposure ended. Adults were allowed to spawn for two hours before being removed from the spawning tanks to avoid loss of gametes through filter feeding. Fertilization and embryogenesis occurred in the spawning tanks, and after 48 h, 10 swimming larvae were moved using disposable pipettes into one of 8, 9-cm petri dishes of 50 ml of raw seawater. Petri dishes were exposed to 15-17°C and ambient light cycles as mentioned above.

Over the course of 90 days, photographs were taken of individuals using a Nikon D300S. In this study, the length of an individual undergoing metamorphosis was measured from the outer edge of the tunic surrounding the internal organs to the anterior end of the buccal siphon, while the length of juveniles and fully developed individuals was measured from the base of the

attachment stalk to the tip of the buccal siphon. Individuals less than 1 cm in length were photographed using an AmScope CA-NIK-SLR microscope camera mount and measured using ImageJ to the nearest 0.01 mm. Larger individuals were photographed while submerged in sea water in a glass dish with a scale visible beneath them. Tunicates were given time to relax before photographing to ensure accurate length measurements. Beyond 57 days of age, tunicates were sensitive to manipulation and would often not relax after being moved into the glass dish for measuring. For this reason, a ruler was submerged into the tank and measurements were taken by eye without disturbance to the nearest 1 cm. Water in the petri dishes containing young tunicates was fully changed 5-6 times weekly, and feeding occurred daily using a dilution of Shellfish Diet 1800® algae (see above). At day 25, petri dishes were attached to plastic hangers by Velcro® and hung vertically inside a 54 L tank of raw seawater with 2 air supplies and a temperature control coil (15°C). From 80-100% of the tank volume was replaced once a week with unfiltered sea water. The tank was cleaned and 20-30% of the volume replaced twice weekly. Feeding was carried out 4-7 times weekly depending on demand (determined by monitoring intestine fullness) for the next two months, and length measurements and stages of development were recorded weekly.

Growth rates ($GR$) were calculated by,

$$GR = L_2 - L_1/t_2 - t_1$$

where $L_1$ was the length in mm of all individuals in the previous measurement period ($t_1$), in days and $L_2$ was the mean length of all individuals at time $t_2$.

The developmental stage and presence of full sperm and oviducts were recorded during each measurement. Developmental stages were rotation, first ascidian stage (FAS) I, II, III, IV

and V, and second ascidian stage (SAS) as illustrated in Chapter 3 of this thesis (which mirror stages 4 and 5 in (Chiba et al. 2004)).

Log transformed length was plotted against time (days after larval attachment) for use in estimating the age of *Ciona intestinalis.* The equation of this regression was used to estimate the age of field specimens (x, days) based on their length (y, mm).

$$x = \{\ln(y) + 0.594\}/0.0618 \text{ (}n=186, p=0.00, r^2=0.8963\text{)}$$

This enabled back-estimation of the day of recruitment of individuals of known size on settlement plates in the recruitment portion of this study.

*2.3.2: Mitigation*

The mitigation effort in Little Bay, NL (47° 09’ 50” N, 55° 06’ 45” W) began in early June 2013, when water temperatures were still below the documented spawning temperature threshold of 8 °C. Two floating docks, ropes, and other objects with *Ciona intestinalis* were removed from the water (and not returned for the duration of this study), and wharf pilings and boat hulls were scraped clean by SCUBA divers. The number of *C. intestinalis* was very low in early May of 2014 when mitigation efforts took place for the second year.

Pre-mitigation Population Density

Two of the floating docks removed during mitigation in 2013 were used for estimating the abundance of *Ciona intestinalis*. Once on land, the docks were inverted to expose the previously submerged areas. The bottoms of the floating docks consisted of large floats with wooden beams, creating 18 rectangular depressions inside which the quadrat photos were taken. Quadrat photos (0.25 $m^2$) were taken in all 18 depressions of each floating dock. Using ImageJ

software, all *C. intestinalis* were counted in the quadrats and a pre-mitigation abundance was calculated (individuals 0.25 $m^{-2}$).

Mitigation Impact - Monitoring

Following the cleaning of the wharf pilings in 2014, the perimeter of the permanent wharf was measured and monitoring quadrat locations were chosen by using the location of 10 randomly generated numbers representing measurements along the wharf perimeter. Permanent bolts with number labels were placed 1 m below the low tide mark on wharf pilings at these locations. Once a month, underwater quadrat photos were taken and visual inspection was carried out by SCUBA divers on the 0.34 $m^2$ piling area (Figure 2.3.2.1).

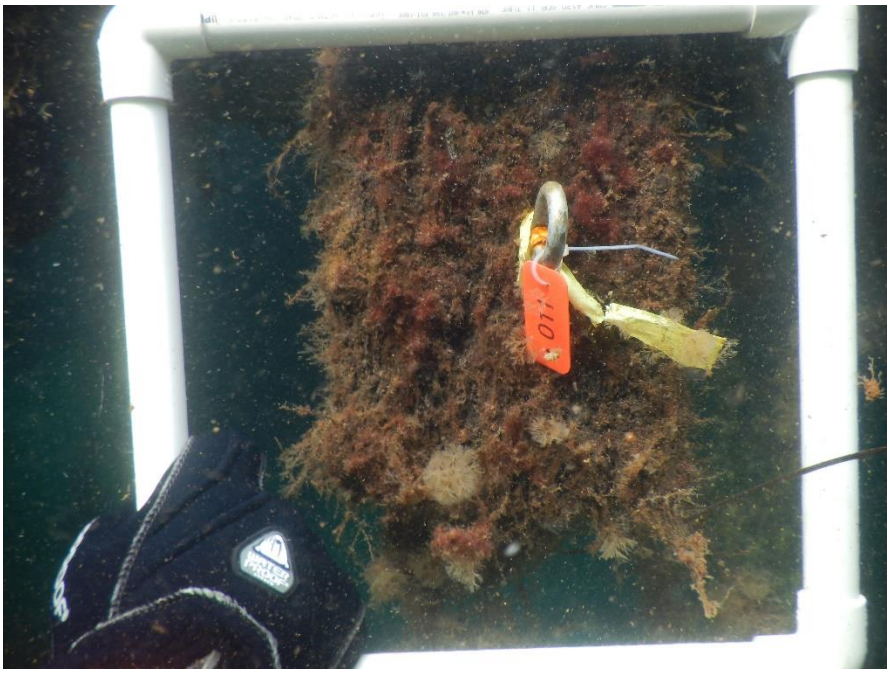


Figure 2.3.2.1: Underwater quadrat (0.034$m^2$) photograph sample from permanent wharf structure, used to monitor the reestablishment of *Ciona intestinalis* and the impact of mitigation.

*2.3.3: Ecology and Recruitment*

I studied recruitment dynamics of *Ciona intestinalis* at five sites in Little Bay, NL over two years (2013 and 2014). Three sites were located at the main public wharf (sites 1-3), the location of heaviest *C. intestinalis* infestation when the species was first detected in 2012, site 4 was a private dock across the bay (~ 240 m from the main wharf), and site 5 was in an adjacent eelgrass bed (~ 40 m from the main wharf) (Figure 2.3.3.1 a & b). Sites 1-4 are the main focus of this study, while site 5 was selected to monitor possible recruitment on eelgrass in an area with no immediately adjacent artificial structures for recruitment. The main wharf (Sites 1-3) creates a shaded habitat with low currents, which is ideal for *C. intestinalis*. The private dock (Site 4) was approximately ¼ the size of the main wharf and constructed primarily of debarked logs, providing less protection from sunlight and currents than the main wharf. We recorded sea water temperature using HOBO® Water Temp Pro V2 sensors from May 1 2013 to Nov 25 2014. Salinity was recorded both instantaneously using a Castaway ® CTD and continuously using a YSI 6600 V2 Multi-Parameter Water Quality SONDE. Mean daily salinities were calculated from June 17 to Oct 19 in both 2013 and 2014.

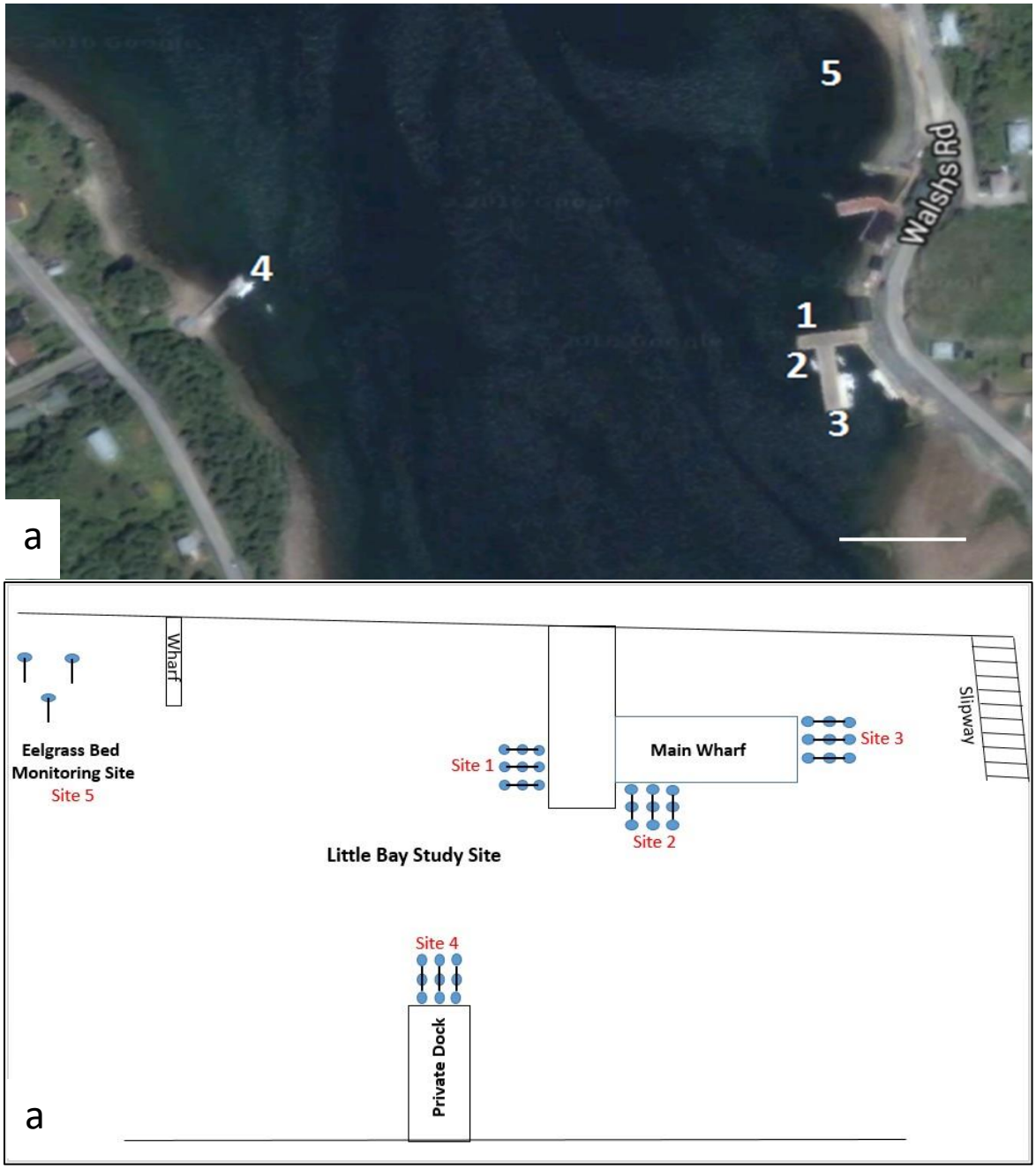



Figure 2.3.3.1: Study sites 1-5 in Little Bay, NL: a) Arial view showing proximity of sites to one another (Google Maps). Scale bar is 60 m; b) Illustration indicating collector plate placement (not to scale).

The settlement plate design was a variation of that described in Howes et al. (2007), and was deployed without prior biofilm conditioning. Each collector consisted of a line with three inverted flower pot bases (hereafter referred to as bases) spaced approximately 66 cm apart and suspended vertically in the water column (Figure 2.3.3.2 a). Each base had three petri dishes (measuring either 8, 8.5 or 9 cm in diameter), zip-tied to the inside, facing downwards (Figure 2.3.3.2 b). A total of three collectors were deployed at each site. A brick was tied to the bottom of the line to ensure it remained vertical in the water column, while the other end was secured to the wharf for easy access during collection. Each petri dish (hereafter referred to as plate) was considered one sampling unit and on each collection date three samples were taken from each collector (one plate per base), a total of nine samples per site. Each base had a rim that provided a shaded environment and likely reduced currents, which would further encourage settlement of *Ciona intestinalis* and reduce the likelihood of edge effects on plates (i.e., light or micro-hydrodynamics). At sites 1-4, I deployed three collectors spaced approximately 1 m apart. Collectors were deployed so the shallowest base was approximately 1.5-2 m below low tide. At Site 5 (i.e., eelgrass monitoring site), I deployed three collectors, but because of shallow depths (~ 5 m), and lack of protection from passing boats, each collector consisted of only one base instead of three. The collectors at Site 5 were suspended from a submerged float and anchored with a brick. The bases were attached approximately 3 m below low tide.

In the second year (2014), I used a larger base, with 7 petri dishes, at site 5 to increase settlement surface area. Howes et al. (2007) found that most recruitment occurred at depths of 4.5 m, which agreed with previous observations that this species settles at 4-6 m depth and not below 14 m. Therefore, given the shallow nature of the sites (with plates deployed in the range of 1.5-5 m below low tide), it was assumed there would be no difference in recruitment between

bases. Counts from the three plates collected were combined to give one count per collector per deployment period.

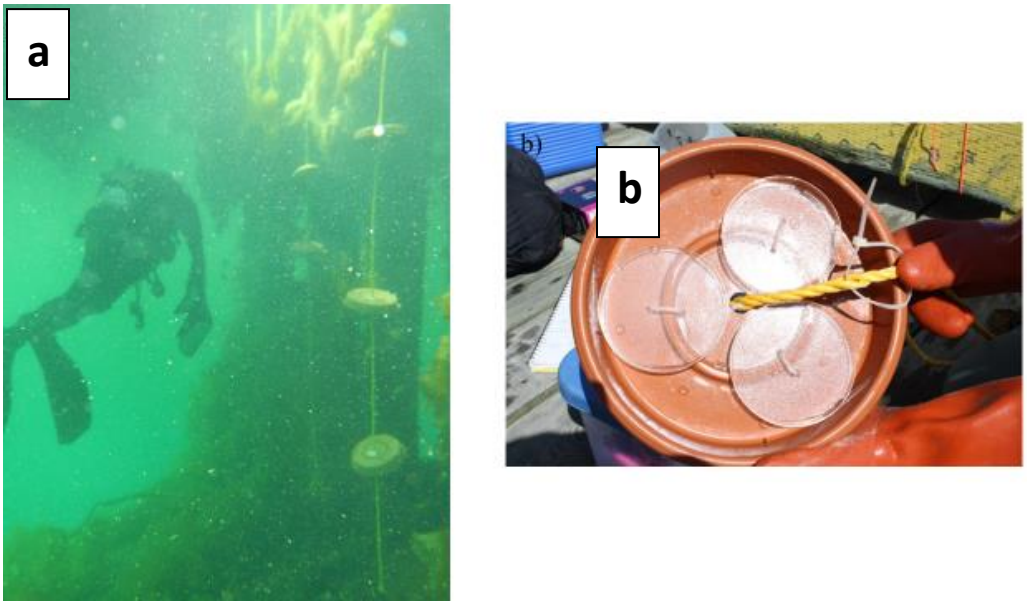



Figure 2.3.3.2: Collector design: a) Collectors suspended in the water column; b) plates zip-tied to bases on collectors.

On June 6, 2013, collectors were deployed at sites 1-5, one day after the start of mitigation in Little Bay. In 2013, there were three collection dates (Aug 22, Sept 30, and Oct 30). For each collection, we removed one plate from each base and replaced it with a new plate labeled by deployment date. All 2013 replacement plates remained in the water through the winter and were collected on the first sampling date the next spring, May 6, 2014. Deployment periods were named: June6Aug22, June6Sept30, June6Oct30, Aug22May6, Sept30May6, and Oct30May6. Collectors deployed in eelgrass remained in the water for the entire season (June 6 to Oct 30, 2013) and no replacement collectors were deployed at this site over the winter.

In 2014, collectors at all 5 sites were deployed May 6, 2014, and plates removed and replaced as in 2013. However, in 2014, plates were removed and replaced monthly at all 5 sites (June 16, July 16, Aug 14, Sept 16, Oct 8, and Nov 24). The resulting deployment periods were named: May6June16, May6July16, May6Aug14, June16Sept16, July16Oct8, Aug14Nov24, Sept16Nov24, Oct8Nov24 (Figure 2.3.3.3).

| Site | Plate | 6-Jun 2013 | 1-Jul 2013 | 22-Aug 2013 | 30-Sept 2013 | 30-Oct 2013 | Nov 2013 | Dec 2014 | Jan 2014 | Feb 2014 | Mar 2014 | Apr 2014 | 6-May 2014 | 16-Jun 2014 | 16-Jul 2014 | 14-Aug 2014 | 16-Sept 2014 | 8-Oct 2014 | 24-Nov 2014 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1-4 | 1 | | | | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | | | | |
| **5** | 1 | | | | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | | | | | | | |
| | 5 | | | | | | | | | | | | | | | | | | |
| | 6 | | | | | | | | | | | | | | | | | | |
| | 7 | | | | | | | | | | | | | | | | | | |

Figure 2.3.3.3: Deployment periods for 2013 and 2014 at sites 1-5. A change in color indicates a plate replacement and white indicates that no plate was deployed.

Once each plate was removed from the collector, it was labelled, placed in a nylon sleeve to protect the sample and allow for water exchange, and kept in a cooler of sea water along with Ziploc bags of ice. Plates were transported to the Northwest Atlantic Fisheries Centre in St. John's, NL, where they were kept at 5°C and photographed and analyzed within 48 h. Sea water was exchanged at least once to ensure specimens were alive when photographed for accurate length measurement. Each petri dish was photographed using a Nikon D300s with a macro lens and polarizing filter. Plates were then visually inspected with a dissecting microscope to count the number of *Ciona intestinalis* recruits.

Recruitment rates of *Ciona intestinalis* (individuals $m^{-2}d^{-1}$) were determined by dividing the number of recruits by the area of each plate and the time of deployment. To obtain a more accurate determination of the seasonal onset of recruitment, peaks in recruitment, and recruitment duration, the length of all recruits was measured using ImageJ software. Day-of-recruitment was then back-estimated for each individual using the regression equation between length and time-since-recruitment from the laboratory study. All other organisms on the plates were noted to the lowest taxonomic level possible.

When analyzing plates that overwintered after the 2013 deployment periods (Aug22May6, Sept30May6 and Oct30May6), I assumed that no growth occurred at temperatures below 6 ºC (typical in Little Bay from ~ Nov 30 – May 16), which is the lower temperature threshold for *Ciona intestinalis* larval development (Dybern 1965). Specimen age was calculated (to the nearest week) based on deployment dates and exposure to temperatures greater than 6 ºC.

Using the temporal recruitment rates of *Ciona intestinalis* and the rate of growth determined in the lab experiment, I was able to predict the number of generations present per season. For the purpose of this study, individuals < 10 mm long were considered a "new recruit", and those ≥ 50 mm long were considered "sexually mature".

*2.3.4 Statistical Analysis*

Due to low recruitment throughout this study, the data were zero inflated. Therefore, deployment periods with no recruitment were not included in statistical analyses (2013: Oct 30May6; 2014: May6June16 and May6July16). A value of 0.1 was added to each recruitment rate to eliminate remaining zeros. To compare seasonal recruitment rates spatially across sites and temporally across deployment periods, I conducted a two-way Analysis of Variance (ANOVA) with factors Deployment period (2013: June6Aug22, June6Sept30, June6Oct30, Aug22May6, Sept30May6; 2014: May6Aug14, June16Sept16, July16Oct8, Aug14Nov24, Sept16Nov24, Oct8Nov24) and Sites (1-4) for each year. Analyses were conducted separately for 2013 and 2014 because of uneven deployment periods (i.e., five in 2013 and six in 2014). Due to low recruitment in both years (low n value), transformation did not correct for non-normality and heteroscedasticity, however effects were minimized by a rank transformation of the data. In 2013, plates with the highest abundances of *Ciona intestinalis* were retrieved during the first collection date, and hence, not at random. Therefore, caution was taken when interpreting these results. Statistical analyses were only conducted on data from sites 1-4. Results from site 5, the eelgrass site, was solely for the purpose of observation and sampling was conducted differently. Regression analyses were used to examine the relationship between weekly temperature and the number of recruits per week (based on back-estimated recruitment data) in 2013 and 2014.

## 2.4 Results

### *2.4.1: Growth and Development Rate*

The length of *Ciona intestinalis* increased exponentially from larval attachment (day 0) to 90 d of age in the laboratory at 15° C and surplus food (Fig. 2.4.1.1). The mean growth rate, determined between pairs of time points, was 18 mm·mo$^{-2}$, or 10.8 % length·d$^{-1}$. The linear regression equation in Fig. 2.4.1.1 was used to estimate the age of *C. intestinalis* recruits from their size in the field study. The $R^2$ value of 0.90 indicates that this estimate of age was robust. However, age was less well estimated at the smallest and largest body sizes, due to typical growth lag at small sizes, and growth deceleration at large body sizes at the time of sexual maturity, oogenesis, and spawning.

Table 2.4.1.1: *Ciona intestinalis*. Mean length-at-age, (*n*), and range […] from the onset of metamorphosis to 12 weeks of age at 15° C and surplus food in the laboratory. (See Methods for details of food conditions). Changes in *n* over time were due to mortality and recruitment into the measurable size class at smaller body sizes, and to difficulty in obtaining relaxed specimens at the larger body sizes. Two animals of large body size died towards the end of the 90-day period.

| Age (d) | Mean size ± SD (mm) | Developmental Stage |
|---|---|---|
| 1 | 0.34 ± 0.1 (5) [0.23-0.6] | Rotation |
| 4 | 0.53 ± 0.1 (8) [0.32-0.7] | FAS I |
| 6 | 0.59 ± 0.07 (11) [0.48-0.7] | FAS I & II |
| 11 | 0.89 ± 0.14 (7) [0.63-1.1] | FAS I II & III |
| 16 | 1.2 ± 0.2 (7) [0.77-1.4] | FAS III & IV |
| 20 | 1.8 ± 0.3 (9) [1.3-2.2] | FAS V & SAS |
| 26 | 2.9 ± 0.4 (6) [2.4-3.4] | SAS & Fully Developed |
| 34 | 5.4 ± 1.5 (16) [3.3-8.3] | Fully Developed |
| 47 | 15 ± 5.9 (25) [4.3-27] | Fully Developed |
| 57 | 33 ± 12 (25) [5.0-50] | 1 sperm duct visible |
| 63 | 39 ± 17 (21) [5.0-60] | 5 sperm ducts visible |
| 72 | 53 ± 17 (20) [30-80] | Sperm ducts in all >50mm long |
| 90 | 73 ± 15 (22) [35-95] | Sperm ducts in all >50mm long |

FAS: First Ascidian Stage. SAS: Second Ascidian Stage. Fully Developed: Metamorphosis to adult morphology.

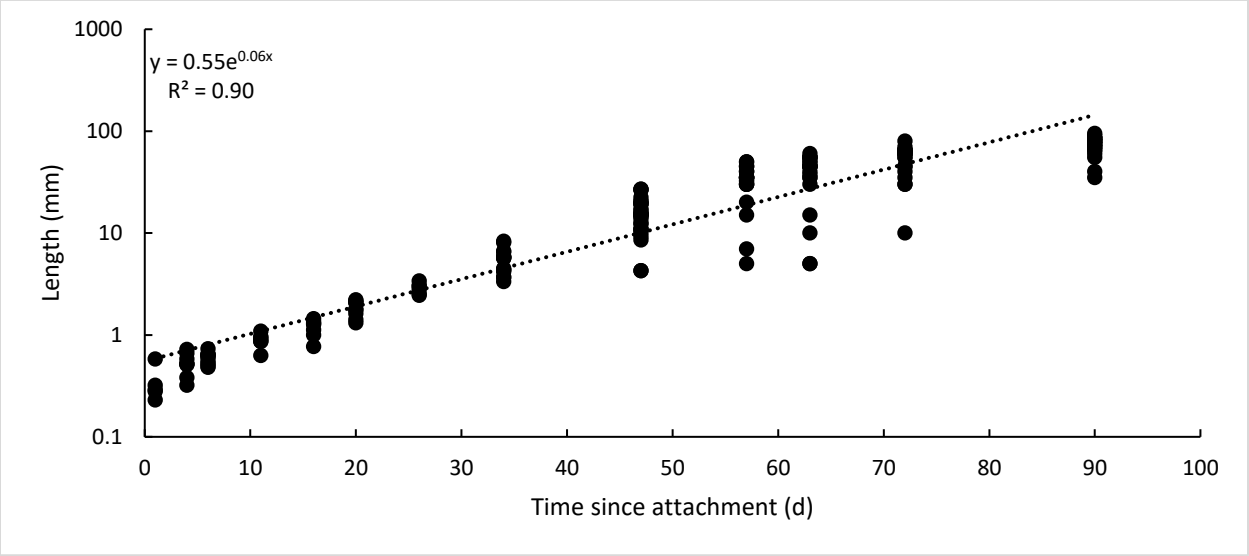



Figure 2.4.1.1: *Ciona intestinalis*. $Log_{10}$ of length (mm) *vs.* time since attachment (d) in the laboratory at 15° C. See Methods for details of food conditions. The exponential equation shown represents estimation of length (y) at age (x).

The first signs of sexually maturity (appearance of a white sperm duct) appeared at 57 days of age in one individual measuring 50 mm in length (Table 2.4.1.1). On day 63, 5 of the 6 individuals ≥ 50 mm long had developed white sperm ducts, and by day 90 all but 2 individuals ≥ 50 mm long were sexually mature. By the end of the experiment only two individuals were showing early signs of ripe eggs in the oviducts.

*2.4.2: Mitigation*

Pre-mitigation Population Estimates

According to observations by underwater video and SCUBA surveys, the most concentrated settlement was found on the underside of horizontal or quasi-horizontal surfaces, such as boat hulls, and the submerged portion of the floating docks. Lesser concentrations were found on vertical surfaces, such as pilings of the permanent wharf. Population densities on the two

floating docks varied from 0-88 individuals 0.25 $m^{-2}$, with a mean of 31 ± 27 individuals 0.25 $m^{-2}$ on Dock 1, and 36 ± 23 individuals 0.25 $m^{-2}$ on Dock 2 (Figure 2.4.2.1). Those areas with higher densities were columns 3 and 4 on Dock 1 (middle of the floating dock), and columns 1 to 4 on floating Dock 2, which were protected both by the permanent wharf and the floating dock itself. The ends of the floating docks (closest to columns 1 and 6) have the lowest recruitment except for the end closest to column 1 on dock 2. It should also be noted here that Dock 1 was in shallower water than Dock 2, and column 1 on Dock 1 was found to be covered in sediment.

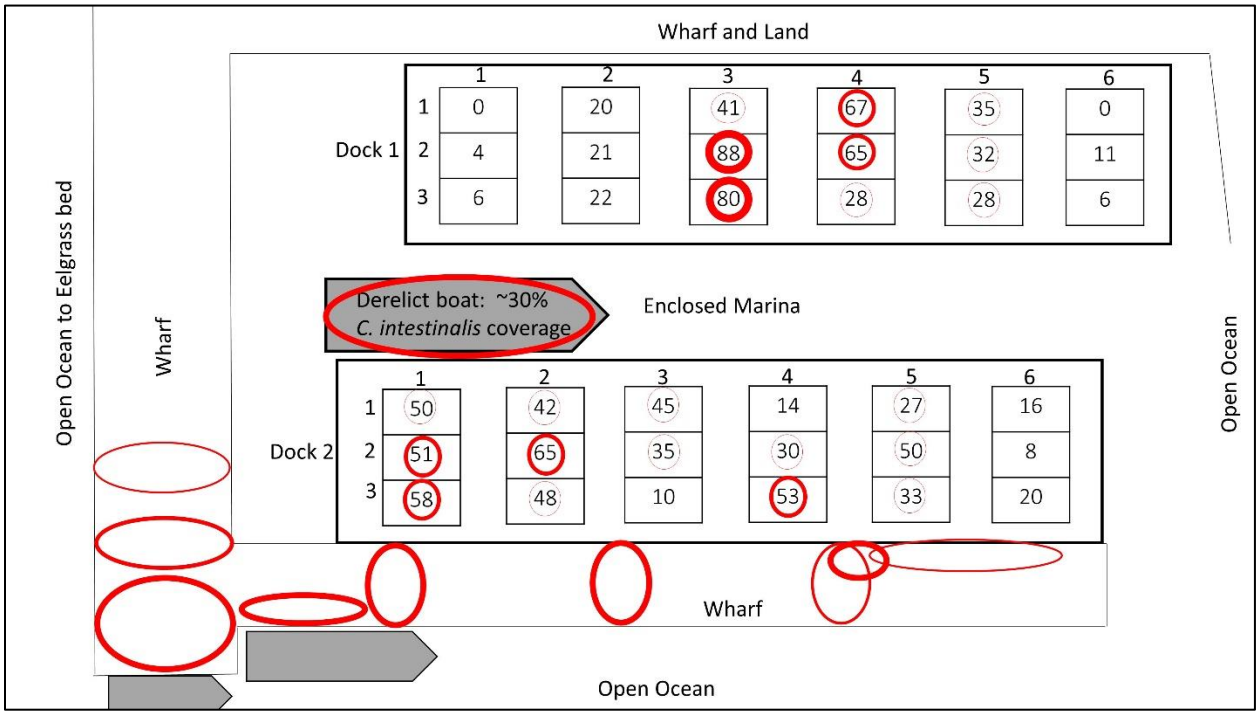



Figure 2.4.2.1: *Ciona intestinalis*. Pre-mitigation density (individuals 0.25 $m^{-2}$) on the Little Bay, Newfoundland, public wharf and nearby floating docks. Thickness of the red lines is proportional to density estimates (by SCUBA divers on the wharf and quadrat population analysis on floating docks) and numbers on floating docks are counts from quadrat population analysis. Known location of boats is indicated by grey polygons, while those moved during floating dock removal is unknown. Schematic is not to scale.

Impact of Mitigation - Monitoring

Follow up monitoring of recruitment, including underwater quadrat pictures of the permanent wharf pilings and SCUBA surveys, detected no *Ciona intestinalis* recruits on the structure for the duration of the monitoring study. Collection plates used in the recruitment portion of this study however did detect low recruitment in the area.

*2.4.3: Ecology and Recruitment*

Temporal Pattern of Recruitment

Temporal recruitment patterns, including the duration of the recruitment season and peaks in recruitment, were determined using a combination of observed recruitment rates, derived from counts of individuals on the collection plates, and back-estimated day-of-recruitment, derived from length measurements of individuals on the plates and the length-at-age regression equation found in laboratory experiments (Fig. 2.4.1.1). In 2013, the start of the recruitment season was not clear based on recruitment rates alone, as the initial plate retrieval occurred late in the season (August 22$^{nd}$) when recruitment was well underway (Fig. 2.4.3.1a). The end of the season occurred between 30$^{th}$ September and Oct 30$^{th}$ 2013, as plates for deployment period Sept30May6 had low recruitment, and those for Oct30May6 had zero recruitment. There was a significant difference in mean recruitment rate among deployment periods (i.e., time), in 2013 ($p < 0.001$) (Table 2.4.3.1). June6Aug22 and June6Sept30 were each significantly different from all other deployment periods, with mean recruitment rates of 7.31 ± 3.8, and 4.6 ± 3.7 individuals $m^{-2}\ d^{-1}$, respectively ($p < 0.05$) (Fig. 2.4.3.2a). June6Oct30, Aug22May6, and Sept30May6 were not significantly different from one another, with

recruitment rates of 1.70 ± 2.0, 0.95 ± 1.7, and 0.75 ± 1.2 individuals $m^{-2}d^{-1}$, respectively (> 0.1). In 2013, there was only one peak in recruitment according to the recruitment rates alone, occurring in June6Aug22 deployment period.

In 2014, the first recruits of *Ciona intestinalis* were observed on May6Aug14 plates (Fig. 2.4.3.1b). Since May6June16 and May6July16 had no settlers, recruitment began sometime between July 16 and August 14. Recruitment was observed during all subsequent collections, including Oct8Nov24. As in 2013, we found a significant difference in mean recruitment rate among deployment periods (i.e., time) in 2014 ($p < 0.001$) (Table 2.4.3.2). May6Aug14 was significantly lower than all other deployment periods, with a mean rate of 0.32 ± 0.51 individuals $m^{-2}\ d^{-1}$ ($p < 0.01$), except for Aug14Nov24, with a mean recruitment rate of 1.4 ± 1.6 individuals $m^{-2}\ d^{-1}$ ($p > 0.573$) (Fig. 2.4.3.2b). Aug14Nov24 was only significantly lower than the highest mean recruitment rate found in Sept16Nov24, of 7.0 ± 5.3 individuals $m^{-2}d^{-1}$) ($p < 0.002$). While Sept16Nov24 had a higher mean recruitment rate than all other recruitment periods, it was only significantly higher than both of the lowest rates for deployment periods May6Aug14 and Aug14Nov24 ($p < 0.001$). Thus, in 2014 there were two pulses in recruitment at sites 1-3, with the larger peak occurring in the Sept16Nov24 deployment period at sites 1 and 3, and during the July16Oct8 deployment period at site 2 (Fig. 2.4.3.1b).

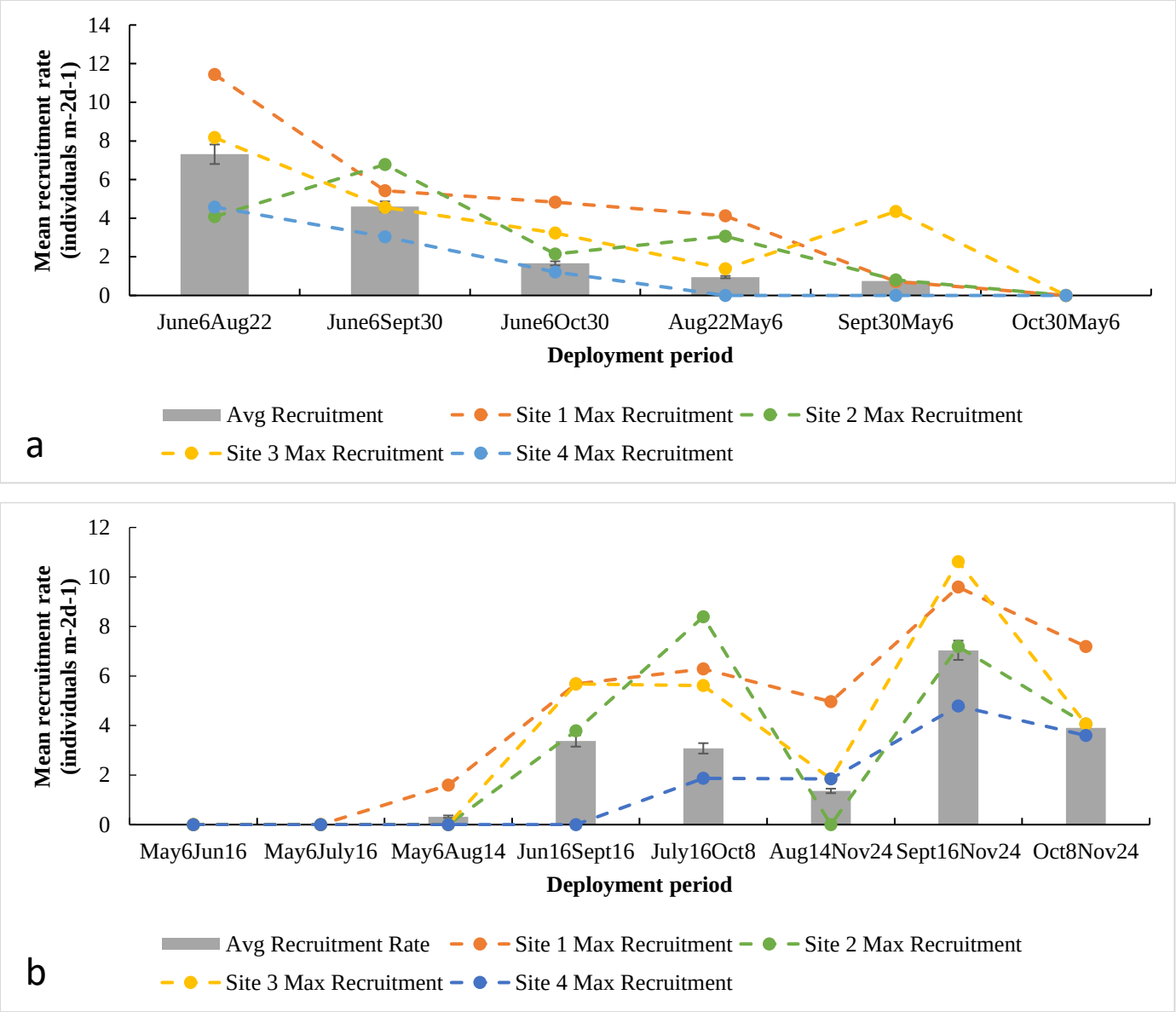


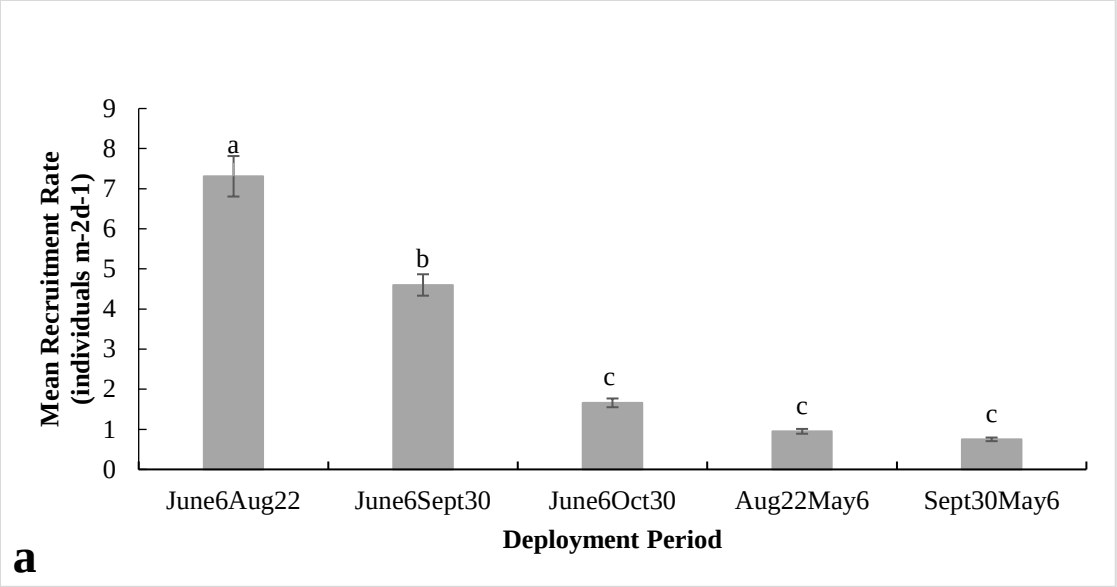

Figure 2.4.3.1: *Ciona intestinalis*. Mean recruitment rate (± SE) (individuals $m^{-2}d^{-1}$)for each deployment period in a) 2013 and b) 2014. Bars represent the mean recruitment rate per deployment period across all sites. Dashed lines represent the maximum recruitment rate at each site for each deployment period. The mean rate in June6Aug22 may be biased high due to non-random selection of plates during that retrieval (see Methods).

Table 2.4.3.1: *Ciona intestinalis*. Summary of two-way ANOVA (applied to ranked data) showing the effect of deployment period and site on recruitment rate for 2013. Note that the interaction term is not statistically significant.

| Source | Type III Sum of Squares | df | Mean Square | F-Value | P-Value |
|---|---|---|---|---|---|
| Deployment | 9931.125 | 4 | 2482.78 | 25.99 | 0.00 |
| Site | 1450.50 | 3 | 483.50 | 5.06 | 0.005 |
| Deployment *Site | 2105.38 | 12 | 175.45 | 1.84 | 0.075 |
| Error | 3821.00 | 40 | 95.53 | | |
| Total | 73123.00 | 60 | | | |

Table 2.4.3.2: *Ciona intestinalis*. Summary of two-way ANOVA (applied to ranked data) showing the effect of deployment period and site on the recruitment rate for 2014. Note that the interaction term is not statistically significant.

| Source | Type III Sum of Squares | df | Mean Square | F-Value | P-Value |
|---|---|---|---|---|---|
| Deployment | 10272.50 | 5 | 2054.50 | 7.91 | 0.00 |
| Site | 2942.33 | 3 | 980.78 | 3.78 | 0.016 |
| Deployment*Site | 3735.83 | 15 | 249.06 | 0.96 | 0.51 |
| Error | 12465.83 | 48 | 259.71 | | |
| Total | 125338.50 | 72 | | | |



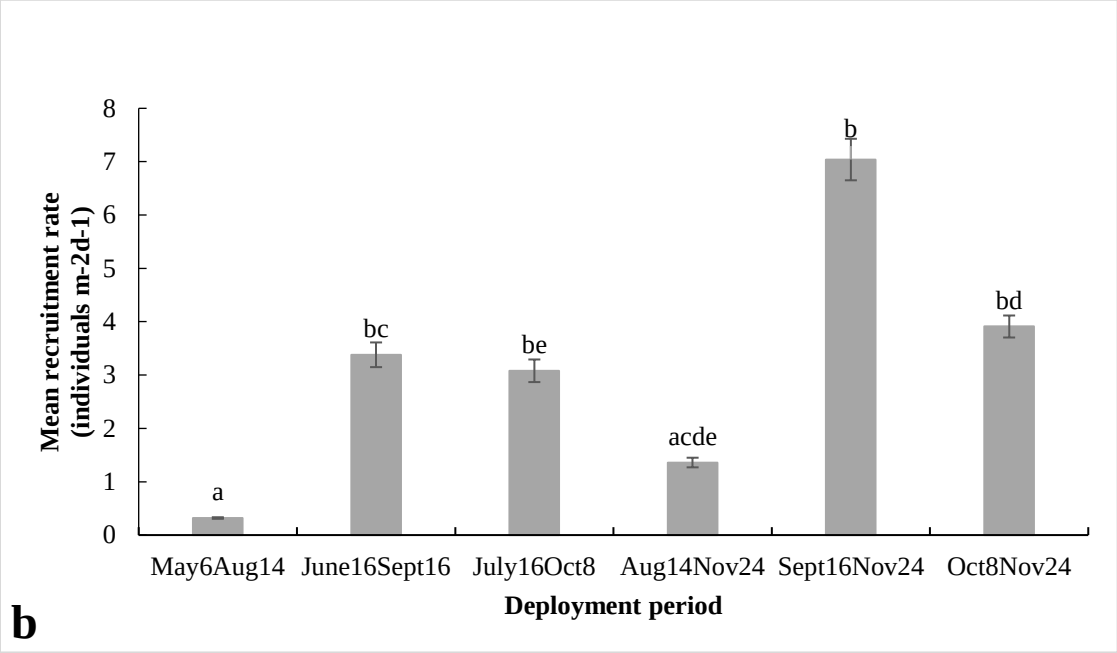



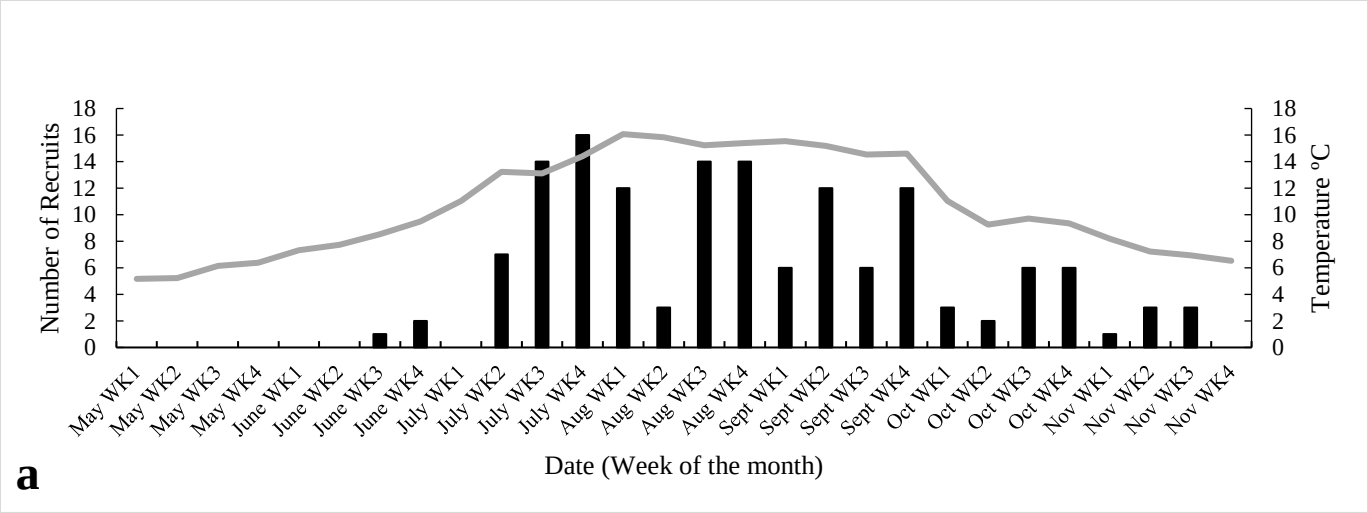

Figure 2.4.3.2: *Ciona intestinalis*. Mean recruitment rate ± SE (individuals $m^{-2}d^{-1}$) for each deployment period for a) 2013 and b) 2014. Different lower case letters indicate statistically significant differences in mean recruitment rate (LS means, $p < 0.05$, $n = 6$ for each mean). The mean rate of June6Aug22 (panel a) may have been over-estimated due to non-random selection of plates during that retrieval.

The back-estimated day-of-recruitment for 2013 indicated that recruitment began in the third week of June and continued until the third week of November (Figure 2.4.3.3.a). There was

a single recruitment peak the last week of July, with 16 recruits across all sites. There was a gradual decrease in back-estimated day-of-recruitment following this peak, interrupted by occasional weeks ($n$ = 6) with low recruitment. Recruitment began when water temperature was > 8° C, and ceased when temperature was < 7° C.

In 2014, back-casted day-of-recruitment indicated that recruitment began during the second week of July, and lasted until the third week of November (Figure 2.4.3.3 b). There were two recruitment pulses, the largest during the last week of October with 19 recruits across all depths and sites, and a secondary peak the last week of July, with 12 recruits. Low recruitment occurred the second week of August, and again during the end of September and early October.

The temperature profile in 2014 was different than in 2013, with a sustained decrease of ca. 8° C lasting 5 weeks, from the end of August to the end of September (Fig. 2.4.3.3b). Over the same time period in 2013, temperatures remained > 14° C. Recruitment began at ca. 10°C in 2014, and ended at temperatures < 6° C. Very low recruitment the last two weeks of September and first week of October 2014, coincided with the end of the five-week period of declining temperatures.



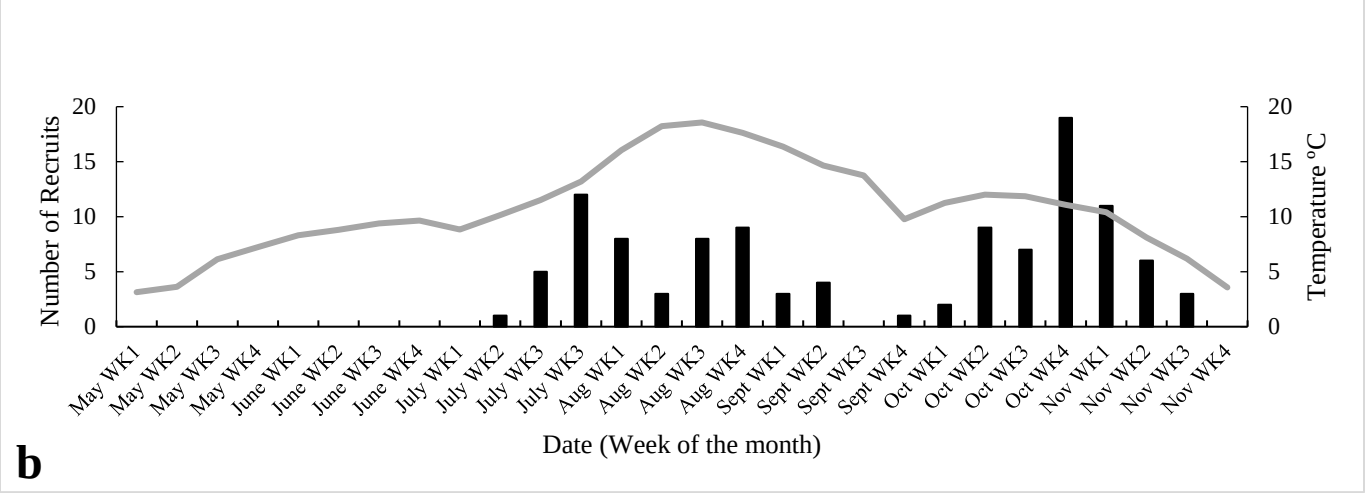
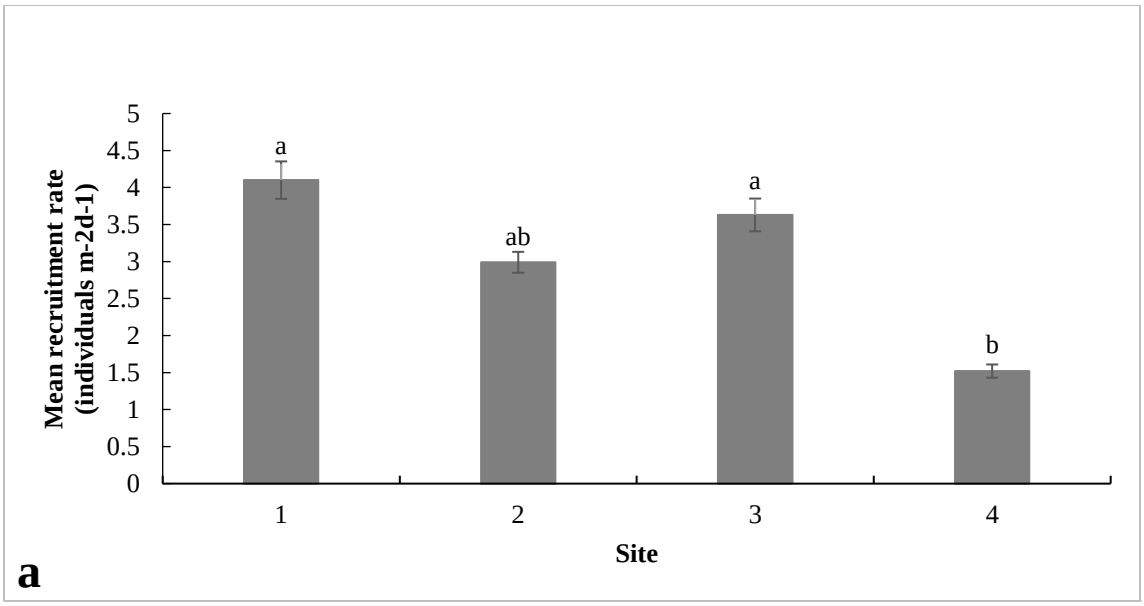




Figure 2.4.3.3: *Ciona intestinalis*. Back-estimated, weekly recruitment in Little Bay, Newfoundland, summed over all depths and sites (black bars), and weekly mean temperature (grey lines) *vs* week-of-the-month for a) 2013 and b) 2014.

Spatial Pattern of Recruitment

Site (1-4) was found to be a significant factor in mean recruitment rate in Little Bay in both 2013 and 2014 (Tables 2.4.3.1 & 2.4.3.3). According to the pairwise comparisons, there was a significant difference in mean recruitment rate across sites in both years ($p < 0.02$). In 2013, the sites were in the order 1 > 3 > 2 > 4, with mean recruitment rates of 4.1, 3. 6, 3.0, and 1.5 $m^{-2}\ d^{-1}$, respectively (Fig 2.4.3.4 a). While site 1 had the highest mean recruitment rate it was only significantly higher than site 4, and not significantly higher than sites 2 and 3. Although site 4 had the lowest mean recruitment rate, it was only significantly lower than sites 1 and 3. Mean recruitment rates at sites 1-4 for 2014 had an identical pattern (1 > 3 > 2 > 4) with mean recruitment rates of 4.9, 3.5,2.6 and 1.8 $m^{-2}\ d^{-1}$, respectively (Fig 2.4.3.4 b). Site 1 was significantly higher than site 4, but not significantly higher than sites 2 and 3, which were not significantly higher than site 4.

In Little Bay, *Ciona intestinalis* were concentrated on artificial structures (i.e., wharves, floating docks, ropes, and boat hulls), with highest densities in the most protected locations with shade and low currents. Before mitigation, the highest concentrations of *C. intestinalis* were found in the middle of floating docks, with greatest shelter from light and currents. There were also higher densities on centre and inside pilings of the permanent wharf, or on those pilings protected by moored vessels. Attachment on the collectors showed similar preferences for low current and shade, in that all attachment occurred on the underside of the collectors, and also in the small spaces between the petri dishes and the bases where currents would be extremely low. However, those recruits outside the collection plates were not quantified in this study and this was solely observation.



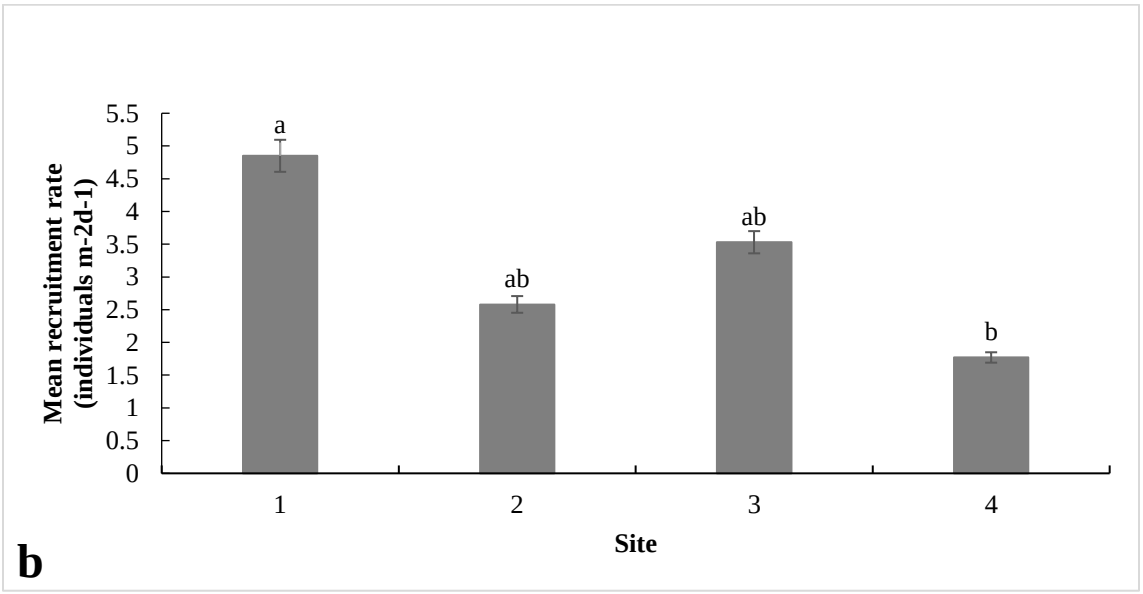




Figure 2.4.3.4: *Ciona intestinalis*. Mean recruitment rate ± SE (individuals $m^{-2}$ $d^{-1}$) *vs* site for a) 2013 and b) 2014. Different lower case letter indicates a statistically significant difference in mean recruitment rate between sites (LS means, < 0.05, n=9).

Recruitment and Temperature Relationship

Temperatures in Little Bay ranged from -1.2 ºC to 18.9 ºC (Fig 2.4.3.5). Maximum temperatures were ca. 16.6 ºC on August 3rd of 2013, and 18.9ºC on August 20th of 2014. Temperatures were above the spawning threshold of 8°C between mid-June and early November

in 2013, and between early June and mid-November in 2014 (Figure 2.4.3.5 a). The salinity in Little Bay ranged from 27.5 to 33.7, with an average of 32.0. According to the back-estimated day-of-recruitment, larval settlement began at a temperature of ca. 8° C in 2013, and 10° C in 2014, while recruitment ended at ca. 7° C in 2013 and 6° C in 2014 (Figure 2.4.3.3). Pulses in recruitment were significantly positively correlated with temperature in both years ($r$ = 0.80 for 2013 $r$ = 0.45 for 2014 [$n$ = 28]).

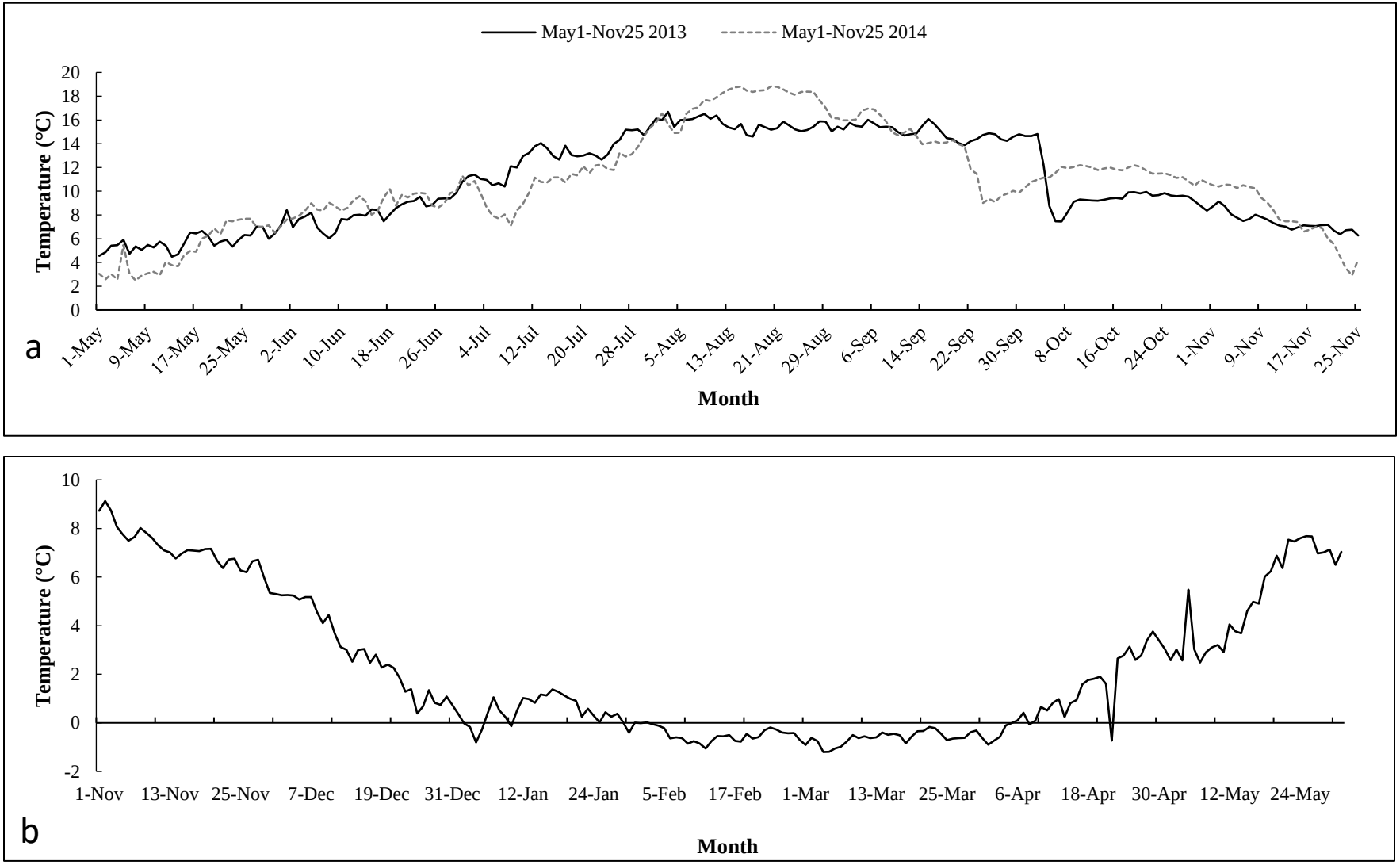
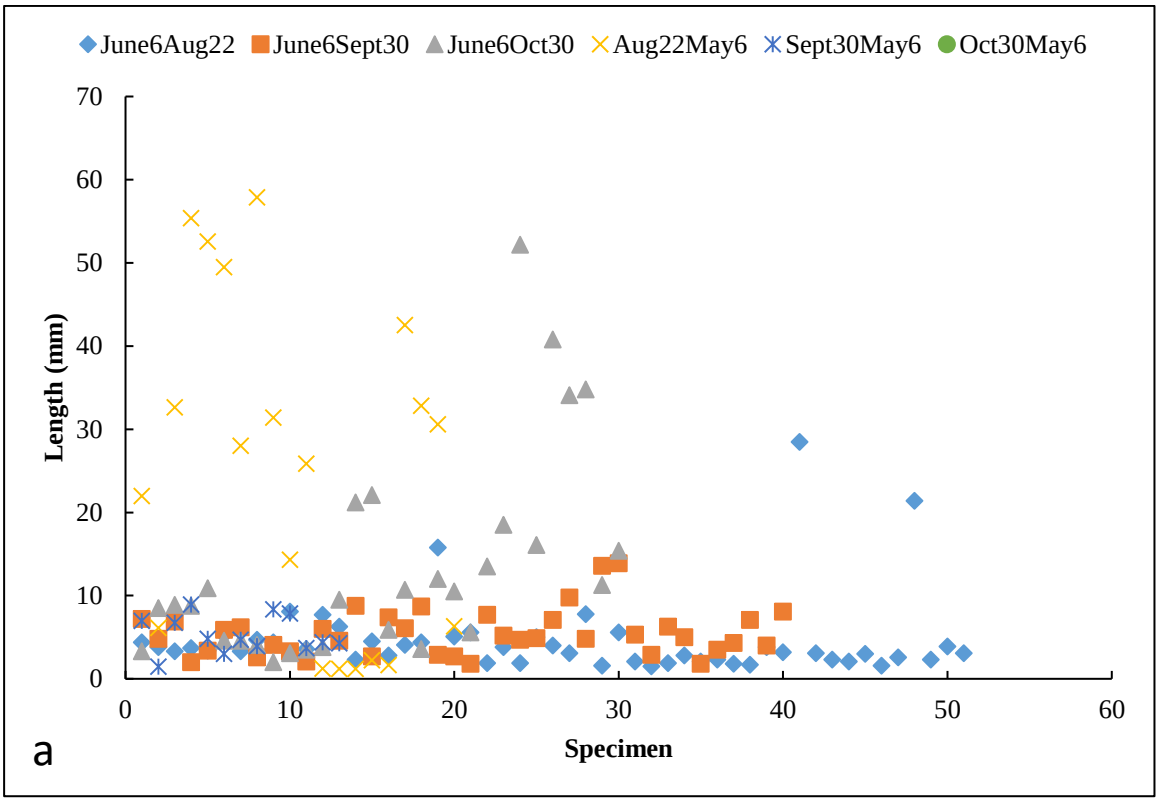



Figure 2.4.3.5: *Ciona intestinalis*. Panel a) Mean weekly temperature in Little Bay, Newfoundland, during the growing seasons in 2013 (black) and 2014 (grey). Panel b) Mean weekly temperature at the same location during the winter of 2013-2014.

Generations per Year

I have shown from my field study that *Ciona intestinalis* recruitment begins in Little Bay around 1 July (giving rise to Generation I which have been generated by the previous season's overwintering individuals), and from my laboratory study that these early recruits will be sexually mature after approximately 90 d, around mid-September to mid-October. These now sexually mature individuals then give rise to the second generation for that season. Since *C. intestinalis* recruits continuously after early July, a wide range of sizes should be present during the July-October period, including from new recruits (< 10 mm) to adults > 50 mm long. In 2013, the June6Aug22 and June6Sept30 deployment periods had mostly new recruits with the largest approximately 30 mm long, consistent with the 2013 Generation I (2013GI). In June6Oct30 and Aug22May6 however, I found individuals ranging in size from < 10 mm to nearly 60 mm, consistent with large, sexually mature individuals from 2013GI, producing new recruits, or 2013 Generation II (2013GII) (Fig 2.4.3.6a). Therefore, there were two generations coexisting after late August in 2013.

In 2014, deployment periods May6Aug14 and June16Sept16 had only small, new recruits, indicating 2014 Generation I (2014GI) (Figure 2.4.3.6b). July16Oct8 and Aug14Nov24 deployments had both large, mature individuals from 2014GI, and new recruits, 2014 Generation II (2014GII). Therefore, there were two generations coexisting in Little Bay after mid-September 2014. The Sept16Nov24 and Oct8Nov24 deployments had only small, new recruits, due to the short duration of deployment.



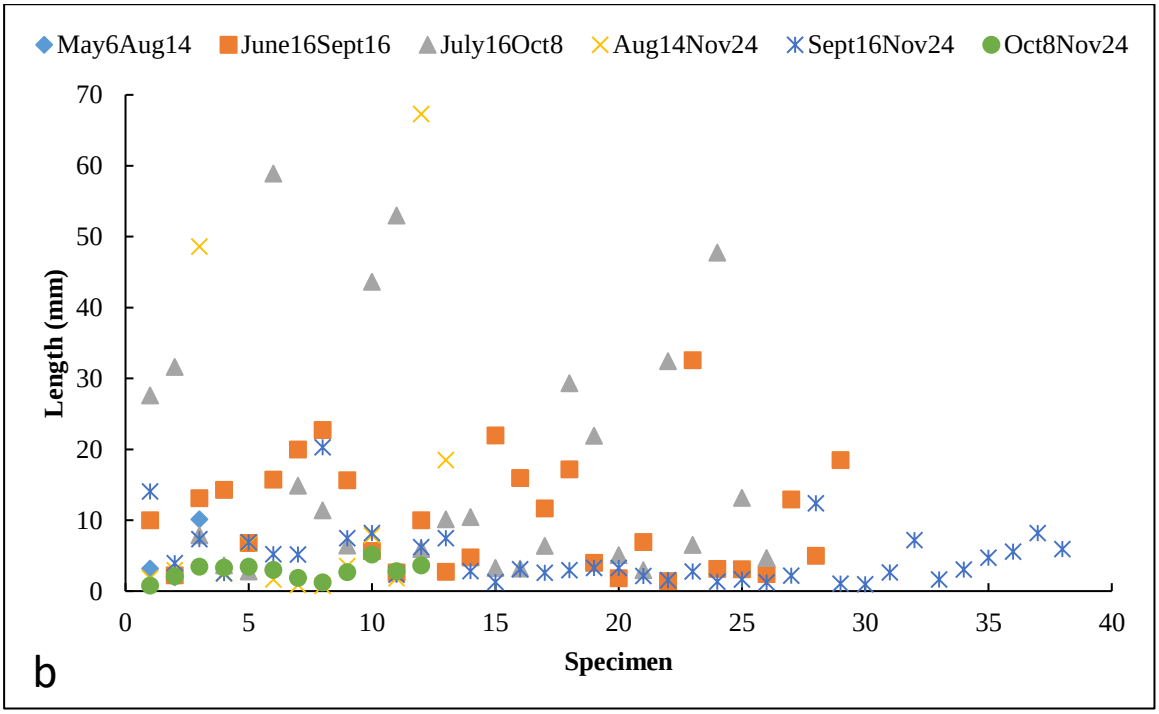




Figure 2.4.3.6: *Ciona intestinalis*. Length of each specimen on the settlement plates colour coded by deployment period for a) 2013 and b) 2014.

Species Composition of Biofouler Community on the Plates

A total of 31 taxa were found attached to collection plates during this study (Table 2.4.3.3). Twenty-one of the taxa were identified to the species level. Taxa included possible predators of *Ciona intestinalis,* such as Atlantic rock crab (*Cancer irroratus* (Say, 1917)) and the Northern sea star (*Asterias vulgaris* (Verrill, 1866)). Other taxa included grazers such as the common periwinkle (*Littorina littorea* (Linnaeus, 1758)), and red-finger, aeolis nudibranch (*Flabellina verrucosa* (M. Sars, 1829)).

Table 2.4.3.3: Little Bay, Newfoundland. Taxa found on collection plates in 2013 and 2014.

| **Phylum** | **Class** | **Order** | **Family** | ***Genus*** | ***Species*** |
|---|---|---|---|---|---|
| Porifera | Demospongiae | Halichondrida | Halichondrida | *Halichondriidae* | *H. panicea* |
| Cnidaria | Scyphozoa | Semaeostomeae | Ulmaridae | *Aurelia* | *A. aurita* |
| Cnidaria | Anthozoa | Actiniaria | Metridiidae | *Metridium* | *M. senile* |
| Annelida | Polychaeta | Canalipalpata | Serpulidae | *Spirorbis* | *S. spirorbis* |
| Annelida | Polychaeta | Phyllodocidae | Polynoinae | *Harmothoe* | *H. imbricata* |
| Annelida | Polychaeta | Phyllodocidae | Glyceridae | *Glycera* | *G. dibranchiata* |
| Nematoda | _ | _ | _ | _ | _ |
| Bryozoa | Gymnolaemata | Cheilostomatida | Bugulidae | *Bugula* | _ |
| Bryozoa | Gymnolaemata | Cheilostomatida | Electridae | *Electra* | |
| Bryozoa | Gymnolaemata | Cheilostomatida | Membraniporidae | *Membraniporidae* | *M. mebranacea* |
| Bryozoa | Gymnolaemata | Cheilostomatida | Cryptosulidae | *Cryptosula* | _ |
| Bryozoa | Stenolaemata | Cyclostomatida | Crisiidae | *Crisia* | _ |
| Bryozoa | Stenolaemata | Cyclostomatida | Lichenoporidae | *Disporella* | _ |
| Mollusca | Bivalvia | Mytiloida | Mytilidae | *Mytilus* | *M. edulis* |
| Mollusca | Bivalvia | Euheterodonta | Hiatellidae | *Hiatella* | _ |
| Mollusca | Bivalvia | Pectinoida | Anomiidae | | _ |
| Mollusca | Bivalvia | Ostreoida | Pectinidae | *Argopecten* | *A. irradians* |
| Mollusca | Gastropoda | Littorinimorpha | Littorinidae | *Littorina* | *L. littorea* |
| Mollusca | Gastropoda | Nudibranchia | Flabellinoidae | *Flabelina* | *F. verrucosa* |
| Mollusca | Gastropoda | _ | Littiidae | *Testudinalia* | *T. testudinalis* |
| Arthropoda | Malacostraca | Decapoda | Cancridae | *Cancer* | *C. irroratus* |
| Arthropoda | Malacostraca | Amphipoda | Maeridae | *Maera* | *M. danae* |
| Arthropoda | Malacostraca | Amphipoda | Gammaridae | *Gammarus* | *G. oceanicus* |
| Arthropoda | Arachnida | Pantopoda | _ | _ | _ |
| Echinodermata | Stelleroidae | Forcipulatida | Asteriidae | *Asterias* | *A. vulgaris* |
| Echinodermata | Ophiuroidae | Ophiurina | Ophiactidae | *Ophiopholis* | *O. aculeata* |
| Chordata | Ascidiacea | Stolidobranchia | Styelidae | *Botryllus* | *B. schlosseri* |
| Chordata | Ascidiacea | Stolidobranchia | Molgulidae | _ | _ |
| Chordata | Ascidiacea | Aplousobranchia | Polyclinidae | *Aplidium* | *A. glabrum* |
| Chordata | Ascidiacea | Aplousobranchia | Didemnidae | *Didemnum* | *D. albidum* |
| Chordata | Ascidiacea | Phlebobranchia | Ascidiidae | *Ascidia* | _ |
| Chordata | Ascidiacea | Phleobranchia | Cionadae | *Ciona* | *C. intestinalis* |

*2.4.4: Eelgrass Monitoring*

Recruitment of *Ciona intestinalis* occurred in the eelgrass site in both 2013 and 2014. In 2013, the mean recruitment rate for the only deployment period, June6Oct30, was approximately 1 individual $m^{-2}$ $d^{-1}$ with a maximum of 4.3 $m^{-2}$ $d^{-1}$ (data not shown). In 2014, the maximum recruitment rate was 5.7 individuals $m^{-2}$ $d^{-1}$ during the July16Oct8 deployment period, which also had the highest mean recruitment rate during 2014, of 1.9 individuals $m^{-2}$ $d^{-1}$ (data not shown).

**2.5 Discussion**

*2.5.1: Growth and Development*

Experimental growth rates found here were constant at 10.8% length $d^{-1}$ from attachment to 90 days, with drop offs in rate found in the smallest and largest animals. During metamorphosis, growth rate in terms of length was low as indicated by the drop off of measurements below the regression line in Fig 2.4.1.1. This is likely due to a combination of recruitment of new individuals early on in the experiment and the allocation of energy resources to the process of metamorphosis. Similarly, growth rates approaching the onset of maturity (day 57 at lengths around 50 mm) begin to level off, falling below the regression line (Fig 2.4.1.1) indicating energy allocation towards gamete production (Carver et al. 2003). The onset of sexual maturity at 57 days of age is within the lower end of the expected range for populations in similar climates (2.5-3 months) (Dybern 1965). While full sperm ducts were noted, full maturity was not achieved within the 90-d scope of this experiment, and eggs were not produced. The production of sperm first was expected, as *Ciona intestinalis* is a protandric hermaphrodite (Carver et al. 2006). The reason for the lack of egg production in

this experiment is unknown. However, likely causes include food quantity or quality. Since food was provided at surplus, perhaps some essential fatty acid(s) required for oogenesis was missing from the diet. However, the primary goal of this experiment was to measure the growth rate to sexual maturity, and the production of sperm cells marks this event.

The 10.8 % $d^{-1}$ growth rate found in this study was much higher than the 1-3% found in field observations (Dybern 1965, Petersen et al. 1995). This is likely due to the environment provided in the lab for this study. The tunicates experienced little physical stress and therefore no interruption in feeding, whereas wild populations would experience currents and other physical stimuli. Tunicates in this study were also given a highly organic diet in surplus. The natural environment provides a mixture of inorganic and organic particles for ascidians, which is of lower nutritional value per unit volume than my lab diet. Some published field studies also did not include all stages of development when calculating growth rates. For instance, Petersen et al. (1995), who observed 2.3-2.8 % increase in length $d^{-1}$, carried out their study for a short period of time (7-8 d).

The overall growth rate found for 15ºC was slightly lower at 18 mm·$mo^{-1}$, than the 20 mm·$mo^{-1}$ found by Carver et al. (2003) in Nova Scotian populations. This difference is likely due at least in part to the fact that growth rates in this study are calculated under controlled laboratory conditions at a constant 15ºC while Carver et al. (2003) calculated growth rates based samples taken from a population under ambient conditions, in a temperature range of 10-20ºC, and used an estimated length (based on the relationship between body diameter when contracted and length while fully extended). Differences in growing conditions in both

experiments may include, food quantity and quality, current or other physical stimuli causing interruptions in feeding, or water quality.

*2.5.2: Mitigation*

Pre-Mitigation Population Estimates

*Ciona intestinalis* are often found attached to the underside of artificial surfaces, in low light and current speeds, where larvae prefer to settle (Millar 1971, Tursi 1980, Lambert & Lambert 1998, Carver et al. 2006). *C. intestinalis* also preferred low light and low current, protected microhabitats on the floating docks in Little Bay. Protected areas were the middle of the floating docks, such as columns 3 and 4 on Dock 1, and those parts of the docks with added protection from the permanent wharf, such as columns 1 and 2 on Dock 2. Boats moored to the floating docks likely provided additional protection, and perhaps sources of larvae. On Dock 1, we can see a cone shaped pattern of attachment densities radiating from column 3, rows 2 and 3. It is possible that this was the location of a fouled boat hull containing spawning adults. Similarly, on Dock 2 we can see that the highest population density is found in columns 1 and 2 and on the side of the dock attached to the permanent wharf structure where the highest concentration of individuals was found (Figure 2.4.2.1).

The lack of settlement on Dock 1 column 1 coincided with high amounts of sediment. This side of the dock would have been in less than 1 m of water at low tide, when sediments could be resuspended by waves. Further indication of this was the high sediment content within the quadrats at this location. Studies have found a decrease in larval settlement and in juvenile and adult survival of *Ciona intestinalis* in turbid environments (Lowen et al. 2016).

Monitoring

The lack of recruitment of *Ciona intestinalis* on permanent wharf structures during the two years of mitigation efforts in Little Bay, as indicated in the quadrat photos and SCUBA surveys of juveniles and adults, indicate that mitigation was a success. This result was likely due, in part, to the rapid response following the discovery of the invader in the fall of 2012. *Ciona intestinalis* may be re-introduced in the future.

*2.5.3: Ecology and Recruitment*

Temporal Pattern of Recruitment

Recruitment of *Ciona intestinalis* occurred in Little Bay, Newfoundland, from mid-June until late November. The recruitment time series for 2013 indicate a single peak in late July with only a slight pulse in late October. This single major peak in recruitment is similar to that seen in PEI populations, though it is unclear whether this would have occurred in the absence of mitigation. It is possible that mitigation resulted in the release of gametes contributing to the early peak in recruitment in 2013. Mitigation in 2013 took place at temperatures between 6-7º C and while inspection of a portion of the population indicated full sperm ducts but not full oviducts, it is possible that some ripe eggs were present at these temperatures (Carver et al. 2003), which could have been released during mitigation. Based on this rationale, I suggest that future mitigation be carried out in late April and early May at temperatures < 4°C, well before gametes begin to develop (Carver et al. 2003).

Unlike 2013, in 2014 there were two peaks in recruitment at 3 of the 4 sites. The first peak occurred in the last week of July, mirroring 2013, and the second peak in the last week

of October. Populations of *Ciona intestinalis* in NS also have two recruitment peaks, with the larger peak in the autumn (Carver et al. 2003). However, in Little Bay, the decrease in recruitment in August and September was likely caused by construction on the permanent wharf, resulting in high sedimentation at sites 1 to 3, but not site 4. High turbidity has the potential to affect fertilization success, larval settlement, and survival of juvenile *C. intestinalis* (McLaughlin et al. 2013). Lowen et al. (2016) found that settlement at ≥ 25 Nephelometric Turbidity Units (NTU) was only 10% that of the control group, and continuous exposure to suspended particles at this turbidity level resulted in 100% mortality of young individuals. The turbidity level at sites 1-4 is unknown during this time, as a turbidity sensor was not deployed. More research is needed to determine the impacts of turbidity in this area.

According to the temperature time series for 2014, there was a coincident decrease in temperature at the time of wharf construction, which may have further affected recruitment levels. For these reasons, it is unclear whether the two recruitment events would have occurred in the absence of wharf construction.

Carver et al. (2003) followed two adjacent populations and found them to have different recruitment patterns, one population with one main recruitment event, and the other population with two. While she suggests the possibility of delayed spawning in the second population creating two recruitment events, the condition index used was based on somatic dry weight, which may not be an accurate indicator of gonad fullness.

Howes et al. (2007) found interannual variability in the timing of recruitment peaks in populations in Nova Scotia. These variations in the timing of recruitment events within and

among adjacent populations were likely driven by environmental factors such as hydrodynamics and temperature, as well as variations in the distribution of spawning adults. More investigation is required, in the absence of mitigation and high sedimentation caused by wharf construction, to more clearly define the number of annual recruitment events in *Ciona intestinalis* populations in Newfoundland.

The end of the recruitment season in both years occurred below temperatures of 8 °C. In fact, recruitment continued until temperatures dropped to 6-7 °C. According to Dybern (1965), the end of the reproductive season is a more gradual process than is the onset of recruitment in the spring, where degenerative changes occur under the influence of decreasing temperatures. While 8 °C is the lower limit for embryonic development, once hatched, larvae can continue to develop in temperatures as low as 6 °C (Dybern 1965). Gulliksen (1972) also concluded that the lowest temperature for the production of cionid larvae in Norwegian population was 6-8 °C. Therefore, recruitment may still occur at temperatures lower than 8 °C at the end of the season.

Spatial Pattern of Recruitment

Although recruitment rates of *Ciona intestinalis* in Little Bay varied significantly among sites 1-4 in both years, the ordinal pattern was the same in 2013 and 2014, i.e., site 1 > 3 > 2 > 4. Lower recruitment rates at site 4 were expected, as the main wharf (sites 1 to 3) was the site of the original invasion in Little Bay, experienced greater boat traffic, was larger, and more sheltered. During the initial survey of this area, only two *C. intestinalis* were found at site 4 (which were removed), and the only vessel to use this dock was free of all invasive species. This meant that recruits found at site 4 during the course of this study were likely

carried there by currents from sites 1-3, ca. 242 m away. At the main wharf, mean recruitment rates at sites 1 to 3 were not significantly different from one another in either year. This was expected, given that the wharf is < 40 m long and sites 1-3 were relatively close together.

Recruitment and Temperature Relationship

Recruitment of *Ciona intestinalis* in Little Bay, Newfoundland, took place at temperatures near published thresholds, and increased with increasing temperature. The temperature range in Little Bay, -1.2 to 18.9º C, is similar to that of other populations in Atlantic Canada, such as those found in NS and PEI (-1 to 20º C, & -1.5 to 21º C, respectively). The mean salinity in Little Bay (32), is similar to that of NS (ca. 31), but less variable than that of PEI (15-31). Recruitment began soon after the published threshold temperature of 8 °C in 2013. However, in 2014, recruitment was not observed until 6 weeks of temperatures > 8°C. This was likely due to a combination of low recruitment in 2013 due to mitigation, overwinter mortality, and further mitigation in 2014 before water temperatures reached 4°C. Not only is the recruitment season limited by temperature, but mean weekly recruitment rate and temperature were positively correlated in both years.

Generations Per Year

In both years, recruitment begins from late June to early July, and continues until late November. However, the peaks in recruitment are not clear as both years yield different recruitment patterns. Taking into consideration the number of generations present each year may aid in clarification. Cold-temperate populations in Scotland, Scandinavia, and other Atlantic provinces of Canada experience temperatures ranging from -1.5 to 15-20° C, with values > 8°C from April or May until November. Animals have a life span of 12-18 months

and produce two generations $yr^{-1}$ (Millar 1952, Dybern 1965, Carver et al. 2003). I also found two generations $yr^{-1}$ in Little Bay, Newfoundland., Thus, we should see maximum recruitment rates as GI individuals become sexually mature and begin contributing to the population. According to the rate of growth determined in the laboratory, maximum recruitment rates should occur ca. 2.5-3 months after the onset of recruitment, or late September in 2013, and mid to late October in 2014. While this timing of peak recruitment was not the case in 2013, the largest recruitment event in 2014 season is during this time, late October. I do not know why the predicted peak did not occur in 2013. Clearly, more investigation is necessary to be certain of the causes of peaks in recruitment rate.

The Biofouling Community

Upon arrival to a new habitat, invasive species must overcome biotic resistance from competitors and predators in order to become established (Dumont et al. 2011). Of the other species observed along with *Ciona intestinalis* in Little Bay, predatory species are of particular interest due to their potential to help control invasive populations. Perhaps the most significant predatory species found is rock crab, which have two feeding strategies for *C. intestinalis* depending on prey size. Smaller individuals (15-35 mm) are eaten whole (though the tunic is rejected after extraction of body tissues), and larger individuals are torn open and the body tissue is extracted, leaving an empty tunic attached to the substrate (Carver et al. 2003). While empty tunics were observed infrequently on the collection plates in this study, in the spring of 2014, during an initial SCUBA survey and follow-up mitigation, a large number of *C. intestinalis* were torn and dead, and many empty tunics were observed. As suggested by Carver et al. (2003), rock crab may play a role in population control over winter months in

Little Bay, when the rate of feeding may impact the non-expanding population. Though Carver et al. (2003) reported no predation on *Ciona intestinalis* by sea stars in Nova Scotia, in Norway, *Asterias rubens* were shown to prey on *C. intestinalis*, and evidence suggests that they compete with various fish species for tunicate prey (Gulliksen & Skjaeveland 1973). Therefore, predation of *C. intestinalis* by the northern sea star in Little Bay is possible, though the effectiveness of these predator-prey relationships was not the focus of this study and would need further investigation.

Another hypothesis is that newly settled *Ciona intestinalis* may be vulnerable to dislodgement by surface grazers (Enright et al. 1983), such as the common periwinkle and nudibranchs found in this study. For example, the addition of periwinkles to lantern nets resulted in significant reduction in biofouling at aquaculture sites (Enright et al. 1983). However, Petersen & Svane (1995) found extremely low rates of dislodgement by the gastropods *Hydrobia ventrose* and *Littorina saxatilis.* Although adult moon jellyfish (*Aurelia aurita*) and stickleback fishes (Unknown species) were not listed in Table 2.4.3.2 as species found on the collection plates, they were present in the area and may also prey on *C. intestinalis*. Petersen & Svane (1995) found both eggs and larvae of *C. intestinalis* in the stomachs of *Aurelia aurita*, and the stickleback *Gasterosteus aculeatus* are also listed as another potential predator.

*2.5.4 Eelgrass Monitoring*

The preferred attachment location for *Ciona intestinalis* in Little Bay is in sheltered, shaded locations suspended above the sea floor. While recruits were recorded on artificial structures in greater numbers, recruitment on natural substrates was also observed, in

particular on eelgrass blades, bryozoan colonies, and the shells of molluscs. This tells us two things. First, there is a preference for attachment to the underside of artificial structures as is common for this and other invasive ascidian species (Tursi 1980, Carver et al. 2003, Bulleri & Chapman 2010). Secondly, these preferred locations likely aid in protecting the tunicates from predation. Dumont et al. (2011) showed that while recruitment of *C. intestinalis* occurred on both natural and artificial substrates, of those settled on natural substrates, only those inside a predator-exclusion cage survived to adulthood. It is conceivable that predation in Little Bay reduced the number of *C. intestinalis* found on natural substrates. Low numbers of *C. intestinalis* were found in the eelgrass itself, while recruits were observed on the collector plates, the only artificial substrate at this site. However, settlement on natural substrates may have been higher if the population were not under mitigation.

Eelgrass beds may be susceptible to colonization by *C. intestinalis* in north-temperate and boreal regions, such as those in this study (Carman et al. 2016a). This is not surprising, given that this species is primarily a benthic ascidian in its native range in northern Europe (Dybern 1965, Gulliksen & Skjaeveland 1973, Petersen & Svane 1995, Dumont et al. 2011). Eelgrass beds provide a wide range of ecosystem services including food, erosion control, maintenance of fisheries, water purification, carbon sequestration, and nursery habitat for many species (Heck et al. 2003, Barbier et al. 2011). Invasive tunicates can be detrimental to eelgrass beds. By attaching to the grass blades, tunicates effectively block sunlight slowing photosynthesis, and weigh down the blades damaging the plant and lowering the overall bed canopy (Wong & Vercaemer 2012). This eventually leads to reduced growth or death of the

plant. Thus, settlement of *C. intestinalis* recruits on eelgrass in NL should be of great concern and the focus of further investigation.

## 2.6 Conclusion

Following the arrival of a new invasive species, it is paramount that initial baseline information be gathered, to gain insight with regard to possible vectors, population densities, preferred habitats, ecology, and recruitment dynamics. The arrival of *Ciona intestinalis* in NL is of concern both environmentally, due to its ability to out compete local species for space and nutrients, and the negative impact on valuable eelgrass beds, and economically, to local tourism, fisheries, and in particular, the aquaculture industry. The objective of this study was to determine growth and development rates at locally relevant temperatures, to determine the effectiveness of trial mitigation efforts, and to define recruitment dynamics of *C. intestinalis* in Little Bay, NL.

*Ciona intestinalis* grew at a constant, exponential rate from larval attachment to 90 d of age. There was a moderate lag in growth for the first 20 d after settlement, and following sexual maturation. The specific growth rate was ca. 10.8 % $d^{-1}$ in the laboratory at 15° C and surplus food, equivalent to doubling in length ca. every 10 d. Sexual maturation began (males) at ca. day 60. This information enables the prediction of seasonal peaks in population growth, which will assist in designing control measures in the future.

In Little Bay, NL, *Ciona intestinalis* prefer to settle in areas of low current and sunlight. A primary vector is likely to be fouled boats moored at the main wharf and floating docks. Two successive years of mitigation, by physical removal of fouled objects and cleaning fixed objects, resulted in drastic reductions of *C. intestinalis* in Little Bay.

*Ciona intestinalis* is exposed to temperatures ranging from -1.2 to 18.9°C in Little Bay, and an average salinity of 32. Spawning begins in mid-June and terminates in late November. The temperature threshold for the initiation of spawning is 8-10° C, and for termination 6-7° C. There was a single peak in recruitment in 2013 (late July), and two peaks in 2014 (late July and late October). However, the October peak in 2014 may have been the indirect result of wharf construction in August and September. There was a clear preference for recruitment in sheltered habitats on artificial structures suspended above the sea. While recruitment was rare on eelgrass blades in Little Bay, in the absence of mitigation recruitment there may have been higher. Further investigation is needed to know the impact of *C. intestinalis* on this valuable habitat in NL coastal waters. This information on the temporal and spatial dynamics of recruitment of *C. intestinalis* in Little Bay will enable tailored, site-specific design of future mitigation protocols.

# CHAPTER 3: POST-METAMORPHIC ATTACHMENT BY SOLITARY ASCIDIAN *CIONA INTESTINALIS* (LINNAEUS, 1767) JUVENILES FROM NEWFOUNDLAND AND LABRADOR, CANADA

Citation:

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**Abstract**

*Ciona intestinalis* is an invasive marine biofouling organism first detected in coastal waters of Newfoundland and Labrador in 2012. As a sessile animal, it is essential that larvae locate a suitable substrate for attachment in an adequate environment, but the timing of this critical event may not be as important as once believed. I demonstrate that while swimming larvae may have limited time to locate and attach to a substrate, development into juvenile stages and prolonged survival is possible without substrate attachment. In laboratory experiments I demonstrate that between 38 and 61% of tadpole larvae undergo pre-attached metamorphosis at the water surface or free floating. These are the first experiments to confirm the ability of *C. intestinalis* juveniles to initiate post-metamorphic attachment when substrate is available. In the early stages of juvenile development (i.e., Rotation, FAS I, and FAS II) there are no differences in post-metamorphic attachment ability. Postponing attachment until after the onset of metamorphosis allows *C. intestinalis* larvae and juveniles to effectively prolong the planktonic stage and increase their dispersal potential. This information is of particular concern to aquaculture industries, but also may have implications for management efforts in regions where *C. intestinalis* has successfully invaded.

## 3.1 Introduction

The vase tunicate (*Ciona intestinalis* (Linnaeus, 1767)) is a solitary invasive ascidian with a global distribution that includes Atlantic Canada (Carver et al. 2006). It was first found in south-western Placentia Bay, Newfoundland in September 2012 (Sargent et al. 2013), and has since been confirmed in 3 neighbouring locations (Little Bay, Marystown, and Burin). *C. intestinalis* can threaten biodiversity through predation, competition for space, and alteration of habitats by limiting water flow, light penetration, or nutrient distribution (Blum et al. 2007; Martin et al. 2011), which ultimately can lead to severe ecological and economic impacts.

Although *C. intestinalis* has not yet invaded Newfoundland mussel aquaculture operations, that industry is concerned given the ability of *C. intestinalis* to foul equipment, infrastructure, and product (Carver et al. 2006, Ramsay et al. 2008, 2009). Elsewhere in Atlantic Canada, such fouling has been shown to decrease mussel meat yield due to nutrient competition and increase processing costs associated with cleaning of equipment and product, and gear replacement following damage due to the additional weight of tunicates (Daigle & Herbinger 2009). Control and mitigation of invasive tunicate infestations and predicting potential dispersal rates are critical to manage impacts. Since 2013, the Department of Fisheries and Oceans Canada (DFO) has collaborated with the provincial Department of Fisheries and Aquaculture (DFA) and the Newfoundland Aquaculture Industry Association (NAIA) to reduce populations of *C. intestinalis* in Little Bay to prevent its spread throughout the province, including nearby mussel aquaculture sites (McKenzie et al. 2016a).

*Ciona intestinalis* reproduces sexually via external cross fertilization (Byrd & Lambert 2000, Cirino et al. 2002, Carver et al. 2006). Spawning and settling behaviours appear to be controlled by changes in light intensity (Lambert & Brandt 1967, Svane & Havenhand 1993); in natural populations individuals typically spawn at dawn (Berrill 1947). Following fertilization is embryogenesis, which requires a minimum of 8ºC (Dybern 1965) and is a complex development process occurring in 6 stages, as outlined by Hotta et al. (2007). Ascidians complete this process as non-feeding (i.e., lecithotrophic), swimming tadpole larvae (Cloney 1978, Bullard et al. 2004, Carver et al. 2006, Liu et al. 2006). These larvae consist of an elongated trunk that contains the sensory vesicle composed of gravity sensing statoliths and a simple eye, the ocellus (Berrill 1947). The sensory vesicle initially causes positive

phototropism and negative geotropism, such that the larvae swim to the water surface (Berrill 1947, Millar 1971). Ultimately, this behaviour assists in their distribution via currents (Carver et al. 2006). As larvae begin to settle, they experience negative phototropism and positive geotropism and tend to seek out dark locations as they begin attachment (Berrill 1947, Liu et al. 2006). Larvae often have an air bubble at their anterior end, which aids in bringing them to the water surface and may explain why they often attach to underside of floats, wharves, and boats (Willey 1893, Berrill 1947). Typically, when larvae attach to a substrate they secrete adhesives from the anterior papillae (Cloney 1978), and metamorphosis begins, transforming non-feeding, mobile tadpole larvae into filter-feeding sessile juveniles. Larval acquisition of metamorphic competence and subsequent adhesion are the initiating point of metamorphosis (Karaiskou et al. 2014). Karaiskou et al. (2014) indicated that the adhesive papillae are a hot zone of signalling pathways that lead to the first event of metamorphosis, tail regression. Following tail regression, the juvenile stages of attachment are initiated by the ampullae at the posterior end of the stalk (Cloney 1978). Next, a series of rapid morphogenic changes leads to the opening of one anterior and two lateral siphons and the first pair of functioning stigmata which marks the beginning of the First Ascidian Stage (FAS). At this point feeding begins, the number of stigmata increases, and the 2 lateral siphons fuse into a single atrial siphon, which characterizes the beginning of the Second Ascidian Stage (SAS). After the definitive stigmata number is reached in the branchial sac and the only atrial siphon is formed, metamorphosis is complete (Berrill 1947, Cloney 1978, 1982, Cirino et al. 2002, Bullard et al. 2004).

The opportunity for non-anthropogenic distribution of *Ciona intestinalis* is typically considered limited to the time between spawning and attachment to a substrate at the end of the swimming larval stage (Svane & Young 1989). Sperm and eggs can remain viable for approximately 30 h (Carver et al. 2006) and then larvae can actively swim for up to 12 h (Berrill 1947), presumably to find suitable substrate rather than for dispersal (Olson 1985). Thus, there are approximately 42 h for *C. intestinalis* to distribute, although the actual dispersal zone will be highly dependent on environmental and physical factors such as temperature and currents. Interestingly, settlement is not always essential for metamorphosis in ascidians (Millar 1971). The ability of larvae to metamorphose into free-floating juveniles with siphons and initiate feeding (Carlisle 1961) suggests that substrate attachment is not prompted by depletion of larval energy reserves. The ability to metamorphose into a feeding, free-floating juvenile can effectively increase the time for potential dispersal. According to Feng et al. (2010), *Styela canopus* (Savigny, 1816), another solitary ascidian, is capable of temperature dependent pre-attached metamorphosis. This phenomenon becomes increasingly common with increases in water temperature from 12 to 30°C, and there are clear implications for dispersal via currents if the duration of the planktonic phase increases. Observations suggest that *C. intestinalis* has the ability to undergo pre-attached metamorphosis (Willey 1893, Berrill 1947), either at the water surface or free floating, however it has yet to be determined experimentally how often this occurs.

While pre-attached metamorphosis has been studied in ascidians (Feng et al. 2010), the ability to attach to a substrate after the onset of metamorphosis has not been examined, particularly in *Ciona intestinalis* juveniles. Feng et al. (2010) suggested that post-

metamorphic attachment may be possible and necessary in *S. canopus*, and Carlisle (1961) stated that attachment of *C. intestinalis* juveniles is at the posterior end by epidermal ampullae. In this study, laboratory experiments are used to 1) estimate the percent of *C. intestinalis* tadpole larvae that exhibit pre-attachment metamorphosis, while at the water surface or free floating, 2) determine the percent of *C. intestinalis* juveniles that undergo post-metamorphic attachment to a substrate, and 3) determine if the stage of development (i.e., Rotation, and two First Ascidian Stages, FAS I and FAS II) of juvenile *C. intestinalis* affects their ability to attach to a substrate post-metamorphosis.

## 3.2 Materials and Methods

### *3.2.1 Sample Collection and Spawning*

Sexually mature *Ciona intestinalis* were collected using SCUBA from two locations, Little Bay (47°09′50″N, 55°06′45″W) and Burin (47°01′51″N, 55°10′26″W), Newfoundland and Labrador (NL) between August and November, 2014. Tunicates were placed in coolers of sea water with Ziploc®bags filled with ice to keep animals cool and transported to the Northwest Atlantic Fisheries Centre in St. John's, NL. In the laboratory, tunicates were held in 54-L quarantined and self-contained tanks filled with unfiltered sea water. Salinity of raw sea water was 32, temperature was maintained at 15°C using a tank chiller and air was supplied continually. The chosen temperature was the average water temperature during the 2013 spawning season (i.e., July-September) in Little Bay, NL. I exchanged 80–100% of seawater once a week, and conducted smaller exchanges of 20–30% twice weekly, and cleaned the tank bottom during each water exchange. Lights in the laboratory were on timers and set to ambient cycles during the breeding season (7 h darkness: 17 h light). I fed the tunicates a

mixture of *Chaetoceros muelleri, Isochrysis* sp., *Pavlova* sp. and *Thalassiosira pseudonana* (Shellfish Diet 1800®) once daily using a slow-release drip apparatus. A mixture of $6\times10^6$ cells/ml was allowed to drip for approximately 3 h $d^{-1}$ at a rate of 1 drop $s^{-1}$; this was sufficient to maintain full intestines in up to 60 adult *C. intestinalis*.

Adults were defined as mature by the presence of a bright orange oviduct and white sperm duct visible through the transparent tunic. To collect gametes for my experiment, I moved 8–12 mature adults into a separate 3-L tank (hereafter referred to as the spawning tank) filled with unfiltered sea water, and used light level manipulation to induce spawning. This involved exposing animals to 3 days of continuous light, which deterred spawning (Georges 1971) and allowed gametes to accumulate, followed by exposure to 12–24 h of darkness. Following this dark period, *Ciona intestinalis* were again exposed to light and spawning was induced within minutes. After 2 hours of spawning, adults were removed from the spawning tank to avoid the loss of gametes due to filter feeding. Subsequently, I added an air supply to the spawning tank and allowed fertilization and embryogenesis to occur. This protocol was used for all experiments excluding Experiment 2 Trial 2.

### *3.2.2 Experiment 1: Larval Pre-Attachment Metamorphosis*

Swimming tadpole larvae were spotted by looking across the water surface at eye level, removed from the spawning tank using a disposable pipette, and placed in 95-mm petri dishes containing 50 mL of unfiltered sea water. Each dish was stocked with 20 larvae and three trials were conducted, each with eight replicates (i.e., 8 dishes of 20 larvae) to determine the percent of *Ciona intestinalis* tadpole larvae that undergo pre-attached metamorphosis. For this experiment, larval attachment is defined by substrate attachment at the most anterior

point, the attachment papillae, followed by notable metamorphosis (starting with tail regression and no larval movement). Pre-attached metamorphosis is defined by tail regression while not attached to a substrate (i.e., the petri dish) and remaining at the surface of the water with the tail under regression pointing downward and no larval movement. Water exchanges of 50% in the petri dishes occurred 5–6 times weekly using a disposable pipette to avoid disturbing larvae at the water surface. I counted the number of live individuals attached to the petri dish, at the water surface, and free floating every second day and recorded their stage of development for 2 weeks. The notable stages were Tadpole Larvae, Tail Regression, Rotation, and First Ascidian Stages I and II (FAS I & FAS II mirror stages 4 and 5 in Chiba et al. 2004) (Figure 3.2.2.1). Only larvae at the water surface or free floating and undergoing stages of metamorphosis were considered to be undergoing pre-attached metamorphosis. After the first 24 h, only live larvae/juveniles were used to estimate the percent of tunicates undergoing pre-attached metamorphosis. Larval development and survival were monitored for the following two weeks.

### *3.2.3 Experiment 2: Juvenile Post-Metamorphic Substrate Attachment*

Experiment 2 Trial 1: Bottom and Side Substrate Attachment

In Experiment 2, when swimming larvae were observed approximately 24 h after spawning, the air supply was removed from the spawning tank to reduce water movement and encourage metamorphosis at the water surface (i.e., pre-attached metamorphosis). I took samples of unattached juveniles from the water surface using a disposable pipette at each stage of development: Rotation Stage, and two early stages of the First Ascidian Stage, FAS I and FAS II (Figure 3.2.2.1). For each of these three developmental stages, I placed 10

juveniles in 95-mm petri dishes containing 50 mL of unfiltered seawater (8 replicates total). Each petri dish was left for 24 h to encourage settlement on available substrate, which included the sides and bottom of each dish. For juveniles in this experiment the term attached refers to attachment from the base of the attachment stalk at the extreme posterior end, the epidermal ampullae, to a substrate with the anterior portion pointing towards open water. The term post-metamorphic attachment is attachment occurring after metamorphosis has begun. Note that due to larval and juvenile shortages, plates contained between 6 and 10 larvae. After 24 h, I recorded the number of living juveniles attached to the dish, free floating, or still at the water surface and calculated a percentage value based on the total number of live individuals after 24 h.

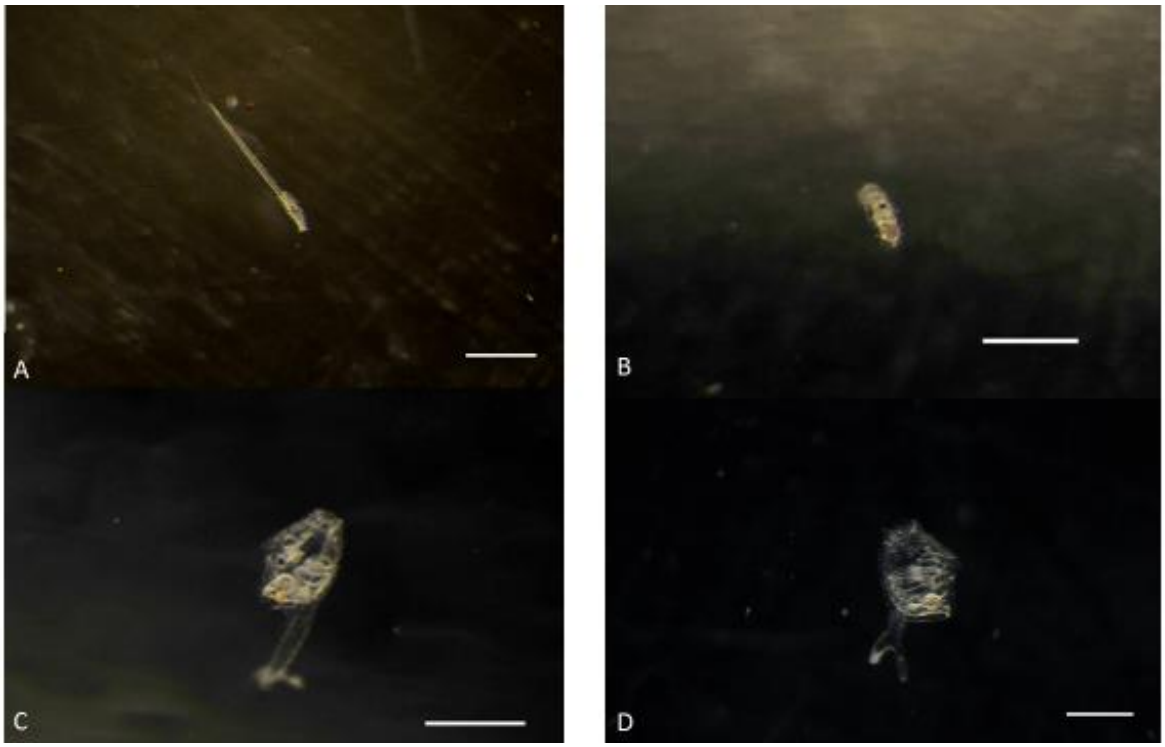


Figure 3.2.2.1: Photographs of the developmental stages of *Ciona intestinalis* tested for pre-attachment metamorphosis in Experiment 1 and for post-metamorphic attachment in Experiment 2. a) Tadpole larvae tested in Experiment 1; and for stages used in testing juvenile post-metamorphic substrate attachment b) Rotation Stage; c) First Ascidian Stage (FAS) I; d) FAS II. Scale bar is 0.5 mm in each image.

Experiment 2 Trial 2: Bottom, Side, and Top Substrate Attachment

In Trial 2, I provided more substrate surface area to increase the opportunity for juvenile tunicates to come into contact with a substrate. Adults were dissected to ensure the collection of the maximum number of gametes, as there were fewer gametes in the oviducts at the time of this experiment. Following a 3-day period of continuous light exposure to delay spawning and maximize the number of gametes available, eight mature *Ciona intestinalis* were selected by inspecting the oviduct and sperm duct for gametes. The animals were relaxed before dissection by leaving them for 3 h in a mixture of filtered sea water and

menthol crystals. To ensure animals were relaxed before dissection, tweezers were inserted into the buccal siphon to check their reflexes. When they no longer reacted to this test they were considered ready for dissection. Gametes were collected and mixed according to steps outlined by Cirino et al. (2002). Embryogenesis and development of swimming larval stages took place in small 150-mL glass dishes instead of spawning tanks to facilitate the collection of unattached juveniles by placing dishes under a dissecting scope. When larvae were observed, dishes were placed in the dark to encourage metamorphosis. Larvae actively seek dark locations for settlement and metamorphosis and yet are often found near the water surface due to their associated air bubble and initial upward swimming motion. Larvae development was checked daily and individuals from the same 3 stages as Trial 1 (Rotation, FAS I, and FASII) were transferred into 8 petri dishes using disposable a pipette (10 individuals/petri dish). However, in contrast to Trial 1, I inverted the petri dish lid making it the portion of the dish containing water. The dishes contained 50 ml of unfiltered sea water and the bottom portion of the dish was set to float on the water surface.

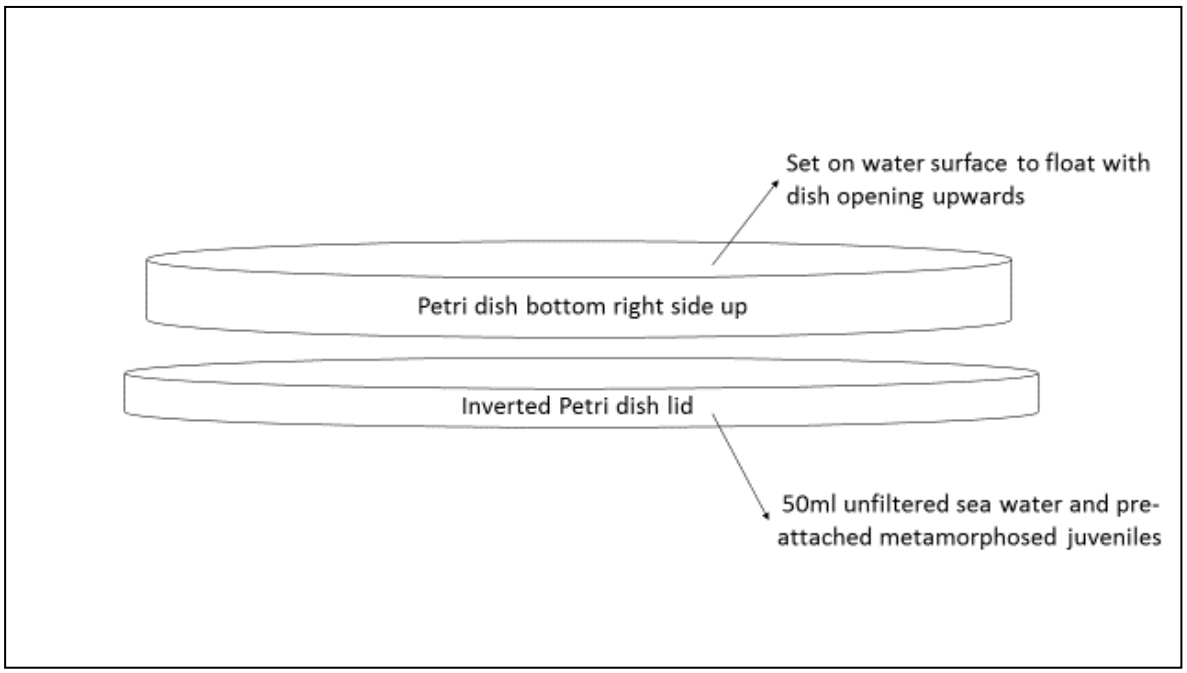


Figure 3.2.3.1: Plates of increased surface area used in Experiment 2, Trial 2.

This increased the substrate available for attachment for *C. intestinalis* juveniles (Figure 3.2.3.1). After 24 h, I counted the number of juveniles attached to any of the substrates (i.e., the bottom, side, or top floating plate surfaces) versus those that remained unattached.

### *3.2.4 Statistical Analyses*

In Experiment 1 (Larval Pre-attachment Metamorphosis), the number of unattached juveniles that underwent metamorphosis was converted into a percentage. I used a one-way analysis of variance (ANOVA) to test the effect of Trial on the percentage of larvae that underwent pre-attached metamorphosis. Analyses were applied to the raw data because they met the assumptions of normality and homoscedasticity. In Experiment 2, I conducted a two-way ANOVA to test the effect of Trial (1 and 2) and Stage of development (Rotation, FAS I, and FAS II) on the percentage of juveniles that attached to a substrate after beginning

metamorphosis (i.e., post-metamorphic settlement). Data transformation did not correct for lack of normality, and therefore this analysis was conducted on rank transformed data and results were compared to results from raw data (Conover and Iman 1981). In all analyses, I verified normality using the Anderson- Darling statistic and homogeneity of variance using Levene tests and examining the graphical distribution of the residuals. To detect differences among levels within a factor I used Tukey HSD multiple comparison tests. All analyses were conducted using Minitab 17.0 using a significance threshold of 0.05 for all tests.

## 3.3 Results

### *3.3.1 Experiment 1: Larval Pre-Attachment Metamorphosis*

The percent of *C. intestinalis* larvae that underwent pre-attached metamorphosis ranged from 21.1 to 80.0 % across petri dishes in my 3 trials (Figure 3.3.1.1). Statistical analyses indicated significant variation between trials in the percent of larvae that underwent pre-attachment metamorphosis after 24 h ($p = 0.01$, Table 3.3.1.1). For example, 61.3 ± 14 % of larvae underwent pre-attachment metamorphosis in Trial 3, which was significantly greater than Trial 1 (38.7 ± 17 %, $p < 0.01$, LS means, Figure 3.3.1.1), while the results of Trial 2 (54.8 ± 8 %) were not significantly different from either Trial 1 or Trial 3 ($p > 0.068$, LS means, Figure 3.3.1.1). After 1 week, the majority of individuals developed into the FAS I stage and after 2 weeks most juveniles reached FAS II. After the 2-week trial, the number of unattached juveniles decreased, because of a 15.8 % death rate and post-metamorphic attachment to the substrate, which was not recorded.

*3.3.2 Experiment 2: Juvenile Post-Metamorphic Substrate Attachment*

In Experiment 2, there was a significant interaction between the factors Trial and Stage of development ($p < 0.01$, Table 3.3.2.1), which demonstrates that the percent of juveniles which attached to a substrate differed depending on surface area availability (which differed between Trials) and their stage of development. In Trial 1, where only the bottom and sides of the petri dish were available for settling tunicates, post- metamorphic

Table 3.3.1.1: Summary of one-way ANOVA (applied to raw data) showing effect of Trial on the percent of larvae undergoing pre-attached metamorphosis. (Experiment 1: Pre-attached metamorphosis by *Ciona intestinalis* tadpole larvae).

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Trial | 2 | 2175 | 1087.5 | 5.8 | 0.01 |
| Error | 21 | 3914 | 186.4 | | |
| Total | 23 | 6089 | | | |

Table 3.3.2.1: Summary of two-way ANOVA (applied to ranked data) showing the effect of Trial and Stage of Development (Rotation, First Ascidian Stage (FAS) I, II, respectively) on the percent of post-metamorphic juvenile attachment. (Experiment 2: Post-metamorphic attachment of *Ciona intestinalis* juveniles).

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Trial | 1 | 4640.6 | 4640.6 | 197.7 | <0.01 |
| Stage | 2 | 479.7 | 239.8 | 10.2 | <0.01 |
| Trial × Stage | 2 | 256.4 | 128.2 | 5.46 | <0.01 |
| Error | 38 | 892.2 | 23.5 | | |
| Total | 43 | 6523.5 | | | |

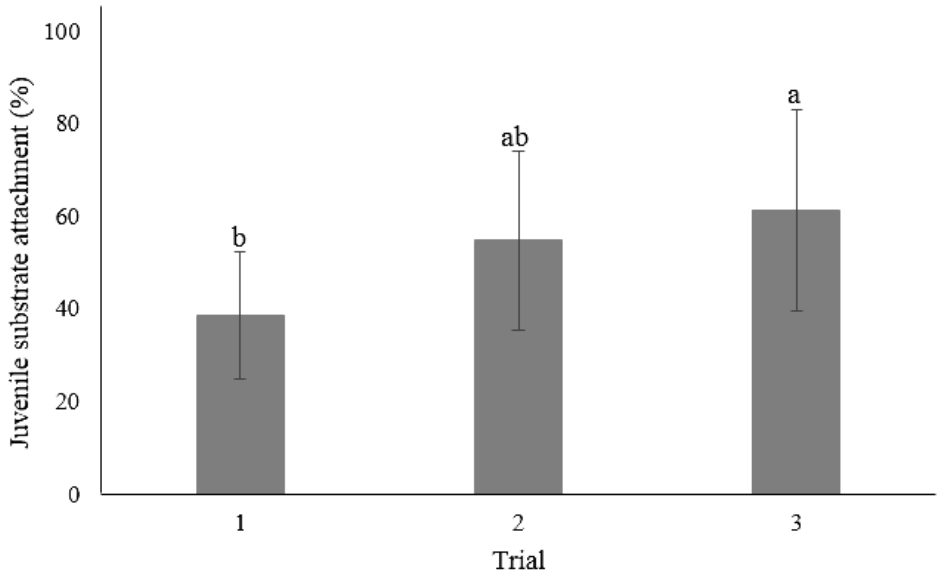


Figure 3.3.1.1: Pre-attachment metamorphosis in Trials 1, 2, and 3 in Experiment 1. Bars represent mean +/- SE; Different letters indicate differences in treatment means (LS means, $p < 0.05$, n=8).

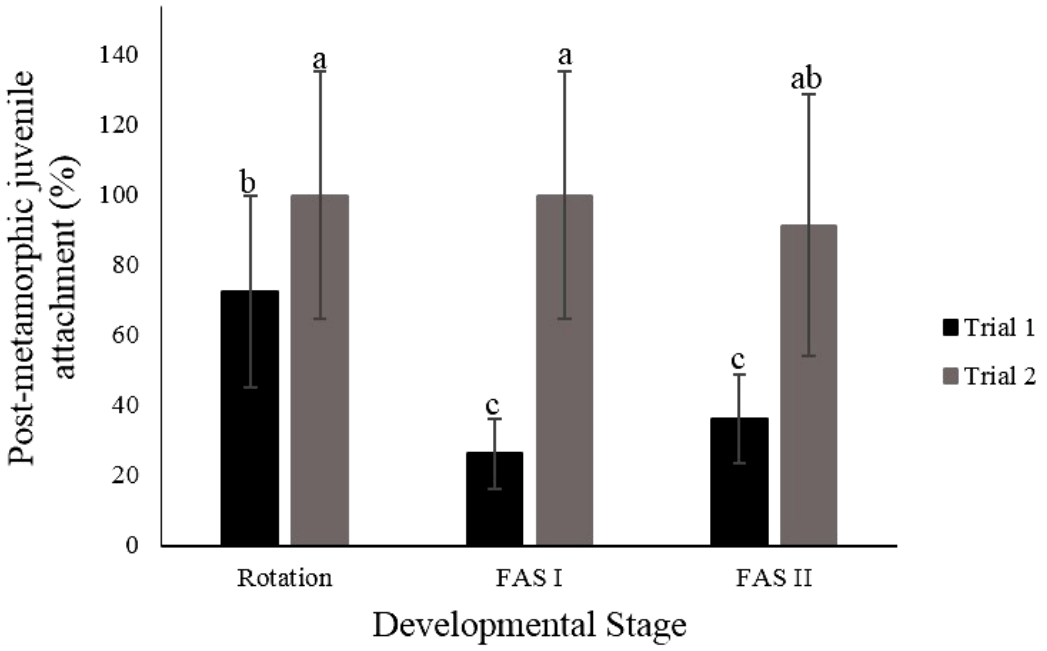


Figure 3.3.2.1: Percent of post-metamorphic juvenile attachment between Trials 1 and 2, and stage of development (Rotation, First Ascidian Stage (FAS) I, and FAS II, respectively). Bars represent mean ± SE. Bars with different letters are significantly different (LS means, $P < 0.05$, n = 6 to 8).

substrate attachment ranged widely from 0 – 90.9 % of individuals and averaged 44.9 ± 24 %. In Trial 2, I increased the substrate area to include a top substrate (i.e., allowing contact for floating juveniles) and the level of juvenile post-metamorphic substrate attachment ranged from 75 – 100 % and averaged 97.1 ± 5 %. Furthermore, in Trial 1 the percent of post-metamorphic attachment by juveniles at each of the 3 developmental stages, Rotation, FAS I, and FAS II was 72.5, 26.2, and 36.1 %, respectively. In comparison, the frequency of post-metamorphic attachment was significantly higher in Trial 2 during the Rotation and FAS I stages (100 and 91.4%, respectively; $p < 0.01$). Statistical analysis for Trial 1 showed that post-metamorphic substrate attachment in tunicates from the Rotation Stage (72.5 ± 20 %) was significantly higher than both FAS I and FAS II (26.2 ± 29 % and 36.1 ± 18 %; $p < 0.013$ LS means, Figure 3.3.2.1), but in Trial 2, there was no difference in the attachment rate between developmental stages ($p > 0.10$, LS means, Figure 3.3.2.1).

Three further observations worth noting include 1) the majority of post-metamorphic juvenile attachment in Trial 2 occurred within seconds of being introduced to the increased substrate area setup; 2) when *Ciona intestinalis* juveniles "stuck" to a substrate in an incorrect orientation, they righted themselves and attached to the substrate by the base of the attachment stalk (i.e., epidermal ampullae) within 24 h; and 3) air bubbles were found at the posterior ampullae (where attachment occurs) in unattached post-metamorphic juvenile stages similar to those often found at the anterior attachment papillae of tadpole larval stages (Willey 1893; Berrill 1947). In these juveniles, the bubble was always observed at the extreme posterior end of the attachment stalk where attachment eventually occurred.

## 3.4 Discussion

According to previous descriptions of the life cycle of *Ciona intestinalis*, there is a short period of time between spawning and attachment leading to metamorphosis, during which dispersal by non-anthropogenic means can occur (Svane & Havenhand 1993). During the larval stage, dispersal is estimated to be between 100 and 1000 m (Jackson 2008) or up to 6 km per generation (Jackson 2008, Kanary et al. 2011), although these values depend on many environmental and physiological factors. In this study, however, I demonstrate that attachment is not required for metamorphosis to occur in *C. intestinalis*; larvae may undergo metamorphosis in the absence of a substrate and later attach as a juvenile. In Experiment 1, at least 20 % of larvae underwent pre-attachment metamorphosis but this value varied greatly among trials (maximum 80 %). I used relatively few larvae in this experiment; larger numbers of larvae per dish may have reduced this variation. Overall, pre-attachment metamorphosis was observed in over 50 % of the larvae used in this study. I also observed larvae to have an affinity for undergoing pre-attached metamorphosis at the water surface (over 90 % of occurrences), which was also noted by Willey (1893). This is likely because tadpole larvae are commonly found with a gas bubble near the attachment papillae at the anterior end of the trunk, which encourages movement towards the water surface.

Some studies hypothesize that the phenomenon of metamorphosis before attachment is due to the depletion of larval energy reserves (Toonen & Pawlik 2001). This behaviour, known as the "desperate larva hypothesis" suggests that when lecithotrophic larvae only have enough energy to metamorphose into a juvenile and can no longer engage in expensive tail movements for swimming, they become "desperate". In this event, larvae will settle and undergo metamorphosis in less than ideal locations, such as an undesired substrate, or as noted here at the

water surface, or even free floating (Carlisle 1961). My findings indicate that metamorphosis of *Ciona intestinalis* tadpole larvae occurs with or without a substrate. Therefore, I hypothesize that just as other larvae postpone settlement in the absence of favourable environmental conditions (Svane & Young 1989), they may also postpone settlement and undergo pre-attached metamorphosis due to lack of available substrate. When compared to the alternative of depleted energy reserves and death before metamorphosis, undergoing pre-attachment metamorphosis provides more time to find a suitable substrate, increasing chances of survival. If 20 – 80 % of larvae can undergo pre-attachment metamorphosis, as my study suggests, filter-feeding juveniles can have more time to drift and disperse in the water column. It was beyond the scope of this study to determine how much dispersal time is extended, however I did observe *C. intestinalis* larvae that underwent pre-attached metamorphosis and proceeded to develop into the Second Ascidian Stage while remaining unattached. I also kept unattached juveniles alive and feeding for over a month. Although other environmental factors must be considered, the ability of larvae and juveniles to survive unattached for weeks rather than merely hours, could greatly increase dispersal.

Temperature plays a significant role in both the rate of embryonic development and the occurrence of pre-attached metamorphosis. This is significant as the *Ciona intestinalis* in Newfoundland is the northernmost confirmed population of this species in North America, according to the distribution indicated by Carver et al. (2006), and hence are exposed to cooler temperatures. Embryonic development occurs at a slower rate at lower temperatures (Berrill 1935), and therefore Newfoundland populations may benefit from a longer dispersal period during the embryonic development stages compared to more southern populations. Feng et al. (2010) found that *Styela canopus* swimming larvae exhibited greater levels of pre-attached

metamorphosis with increasing temperature due to increased tail movements which are energetically expensive. If *C. intestinalis* larvae respond similarly to temperature, they may follow the same trend as *S. canopus*. Feng et al. (2010) also suggests that pre-attached metamorphosis may be adaptive; increasing sea water temperatures globally may lead to higher numbers of larvae undergoing metamorphosis before settling. The scope of this research did not include experimenting with the effects of temperature; further research is required to examine whether this factor influences the timing of metamorphosis in *C. intestinalis*.

In this study, *Ciona intestinalis* juveniles exhibited a strong ability to attach to a substrate after metamorphosis has begun, a characteristic that would be essential for the survival of drifting juveniles. While a relatively low percent (26 %) of juveniles attached in Trial 1 of Experiment 2, I presume this result was partly due to lack of available substrate. My assumption was validated when I increased the available substrate in Trial 2 and observed nearly 100 % attachment after the onset of metamorphosis. The stages of *C. intestinalis* juvenile development tested in this study (i.e., Rotation, FAS I, and FAS II), are no longer motile like tadpole larva, and unlike in nature where currents move juveniles, the water in the petri dishes remains still making them less likely to find a substrate on their own. However, it may be argued that in nature there is less substrate per unit volume of water than in a petri dish and more predators, thus reducing the probability of successfully locating a substrate. Each of the 3 stages of juvenile development tested in this study demonstrated the ability to attach to a substrate. Therefore, attachment may be possible in nature, where moving water can transport free-floating juveniles to available small niches such as underwater structures, cracks and spaces in wharves, spaces between strands of rope, cracked paint on boat hulls, or spaces on and between living organisms. Pre-attached metamorphosed *C. intestinalis* juveniles have been found in nature in a planktonic

state (Jacobs et al. 2006) further supporting my findings that pre-attached metamorphosis can occur under natural conditions.

Until this study, there was no experimental evidence that planktonic juveniles can attach to a substrate after undergoing pre-attached metamorphosis. The percentage of juveniles settling post-metamorphosis in nature is unknown and likely varies depending on factors such as currents, established population size, temperature, and the amount of substrate available. For example, too much wave action may cause siphons to remain closed, which would limit feeding and reduce subsequent juvenile survival, or cause physical damage to juveniles. Conversely, if juveniles are not exposed to currents, the chances of reaching a suitable substrate may be low. However, we know that unattached juveniles can survive long periods of time. Willey (1893) kept unattached juveniles for weeks, and while not shown in this study, I kept juveniles alive at the water surface in a tank for up to three weeks without feeding and greater than one month when fed daily. Post-metamorphic attachment was not tested at these later stages of development and therefore, further work is needed to determine how late in the life cycle attachment to a substrate is still possible.

When comparing rates of post-metamorphic attachment between stages of development (i.e., Rotation, FAS I and FAS II), in Trial 1 (Experiment 2) I observed that significantly more juveniles attached during the Rotation stage than either stages FAS I or FAS II, respectively. At first one might infer that this was due to the difference in the developmental stage of the juveniles as this was the only difference between the experiments in this trial. However, in Trial 2 (i.e., increased substrate surface area) there was no difference in post-metamorphic attachment in juveniles across these stages of development. This observation can be interpreted in 2 ways. First, in this study, I have shown that if juveniles come into contact with a substrate, they can

attach to it. Therefore, considering the results from both trials in Experiment 2, settlement when exposed to ideal conditions may be independent of the stage of juvenile development. Second, it is possible that if I continued the study for a longer time period to include more stages of development, I may have observed a decline in attachment ability. While my findings indicate that *Ciona intestinalis* are capable of post-metamorphic attachment during the early stages of juvenile development (i.e., Rotation, FAS I, and FAS II), it has also been shown that adult solitary ascidians are capable of movement and reattachment. Adults "crawl" by tearing or dissolving old attachments and forming new ones (Carlisle 1961). Attachment at the adult stage is by epidermal ampullae at the posterior end, which was also observed for juvenile stages in this study. I also observed reattachment in the tanks of adult *C. intestinalis*. If attachment to a substrate is possible for both post-metamorphic juveniles (as shown here), and adult stages, it is unlikely that the ability to attach is lost between these 2 stages of development. Therefore, attachment or reattachment may occur at any stage of development and growth.

Throughout this study, I observed juvenile tunicate behavior and what appear to be adaptations to enhance post-metamorphic attachment. First, post-metamorphic attachment occurred almost instantaneously when contact with a substrate was made, which increases the chance of attachment of a free floating juvenile to a substrate in nature. Second, when juveniles were initially placed into petri dishes, they either attached instantly as mentioned, or they became "stuck" to the substrate, but in the wrong orientation. This sticky body was observed on *S. canopus* by Feng et al. (2010) who suggested that it was a post-metamorphic settlement mechanism. After 24 h I observed that *Ciona intestinalis* became properly orientated and firmly attached at the epidermal ampullae, which has not been noted in previous studies. It is possible that the epidermal ampullae extend towards the substrate (Carlisle 1961) allowing attachment at

the base of the stalk after becoming stuck to a substrate in the wrong orientation. Third, the repeated observation of an air bubble at the extreme posterior end of the attachment stalk in juveniles brings them to the water surface, attachment structures first, in the same way the air bubble at the anterior attachment papillae of the swimming larvae assisted them to the surface. Juveniles near the water surface in Trial 2 (Experiment 2) were almost always associated with an air bubble when placed into petri dishes. Therefore, when the top substrate was added, they were already at the surface with their epidermal ampullae ready for attachment. When checked 24 h later, the juveniles were attached to the top substrate and the bubble was gone. While further work is needed to know what exactly makes juveniles of *C. intestinalis* "sticky", have air bubbles present at the epidermal ampullae, and reorient their body after improper attachment, these observations suggest that *C. intestinalis* may be well adapted to post-metamorphic attachment.

This work suggests that the distribution time of juvenile *Ciona intestinalis* may increase from hours to weeks and even months, and that attachment to substrates and establishing populations at further distances, without intermediate steps, is conceivable. The ability to undergo metamorphosis in a planktonic state can influence potential dispersal of invasive tunicates by anthropogenic vectors such as ballast water (Carver et al. 2006). The ability to develop into a feeding juvenile while remaining in the water column may further support the hypothesis that introductions can result from larval survival in ship ballast water (Lambert 2001) in addition to the risk of transfer through hull biofouling.

### 3.5 Conclusion

I conclude that pre-attached metamorphosis is known to occur in ascidians and I observed this process in up to 61 % of *Ciona intestinalis* in a laboratory setting. Post-metamorphic

attachment of juvenile *C. intestinalis* has not previously been studied and I show that at least in the early stages of development (Rotation, FAS I and FAS II), up to 100% of juveniles may attach when a substrate is available. However, further work is needed to determine the ability of juveniles to undergo post-metamorphic attachment during subsequent stages of development. In order to manage spread of invasive tunicates, such as *C. intestinalis*, and keep shellfish aquaculture sites, wharves, boats, and other potential habitats clear, it is crucial to understand their life history. My findings increase our knowledge of the ability of *C. intestinalis* to metamorphose and attach to substrates, which may have further implications for their dispersal. During my laboratory study, the ability of juveniles to undergo metamorphosis before attachment, and to attach almost instantly when in contact with a substrate suggests that adequate environmental conditions (i.e., sufficient nutrients and appropriate water quality parameters) may play a larger role than substrate availability in the survival of *C. intestinalis* juveniles. This information can help in the development of future mitigation and antifouling strategies to manage populations and avoid spread to new locations.

# CHAPTER 4: CONCLUSION

## 4.1 Summary

Due to its fairly recent discovery, *Ciona intestinalis* invasions in Newfoundland (NL) waters have not been studied in depth with regards to population dynamics and metamorphosis in the NL environment. The focus instead has been on surveying potential hotspots, monitoring, early detection, and mitigation to control and prevent further spread. This is the focus of the Department of Fisheries and Oceans (DFO) Aquatic Invasive Species (AIS) program, in collaboration with the Department of Oceans Sciences at Memorial University of Newfoundland (MUN) (McKenzie et al. 2016b).

The research described in this thesis will provide site-specific information vital in assisting the mitigation and control. In particular, I have addressed the following questions regarding *Ciona intestinalis* in NL: (1) What are the growth and development rates at a mean growing season temperature of15° C? (2) What was the pre-mitigation population density and how effective was mitigation in Little Bay in 2013 and 2014? (3) What are the temporal and spatial patterns of recruitment patterns? (4) What are the metamorphosis and attachment patterns of early life stages?

In Chapter 2, I estimate the pre-mitigation population density of *Ciona intestinalis* in Little Bay, NL. Using quadrat pictures taken on two floating docks upon removal from the water, I found that population density ranged from 0-88 individuals $\cdot$ 0.25 $m^{-2}$, with the highest densities in areas of low currents and light. The spatial pattern of density indicated the possibility that a moored vessel may have seeded Little Bay with *Ciona intestinalis*.

I then carried out a laboratory experiment to estimate the growth and development rate of *Ciona intestinalis,* which was 18 $mm \cdot mo^{-1}$ or 10.8 % length $\cdot d^{-1}$. Sexual maturity was reached in some as early as 57 days after larval attachment. I then used the length *vs* age relationship to back-cast the day-of-recruitment of recruits found on collection plates. Collection plates were deployed at 5 sites in total, 4 of which were used in the analysis of recruitment patterns in Little Bay, while the $5^{th}$ was at an eelgrass bed and used purely for observation of *C. intestinalis* recruits. While temperatures in the area range from -1.2 °C to 18.9 °C, recruitment was constrained to temperatures above 6-8 °C in both years, which occurs from about the third week in June to the third week in November. There was one recruitment peak in 2013 and two in 2014. However, I conclude that a biased collection of plates at the start of 2013, and the possibility of stress-induced spawning caused by mitigation, may have shifted the peak to earlier in the season. For this reason, I accept the major recruitment peak in the autumn of 2014 to be a more accurate representation of the true recruitment dynamics of *C. intestinalis* in Little Bay.

Having estimated a growth rate to sexual maturity, I was also able to predict the time at which the season's early recruits would become sexually mature and begin to contribute to a second generation. A 2.5 to 3 month maturation period meant that the year's early recruits, or Generation I, (appearing in late June and early July) would potentially be spawning and contributing to the second generation at 3 months of age (in October). I show that this was the case given the peak in new recruits occurring in October of 2014, and therefore conclude that there are two generations of *C. intestinalis* occurring at the same time, each year, in Little Bay, NL.

Included in the list of 31 other taxa sharing the fouling habitat with *Ciona intestinalis* in Little Bay, I noted the presence of possible predator species Atlantic rock crab and Northern sea

star, which may have played a role in population control during cold winter temperatures when spawning did not occur. Other notable species were the common periwinkle and red-finger aeolis nudibranch, which have been shown in published studies to dislodge new recruits. However, the effectiveness of these co-habitating species in controlling *C. intestinalis* populations was not the focus of this study and needs further investigation.

In Chapter 3, a laboratory experiment was carried out to examine the settlement behaviour of *Ciona intestinalis* larvae at 15 °C. I found that between 38 and 61% of tadpole larvae will undergo metamorphosis in the water column, without immediate settlement. 100% of these precocious juveniles (Rotation, FAS I, and FAS II stages) later successfully attached to a substrate, indicating that they were behaviourally and energetically viable while in the plankton. Precocious metamorphosis and planktonic feeding results in increased dispersal time, and therefore distance.

**4.2 Discussion**

According to the Department of Fisheries and Aquaculture (2016), aquaculture in NL industry had a total production value of $161 million and is expected to grow in the future. In this Province the blue mussel is the main commercial shellfish, aquaculture species, with approximately 51 commercial site licences covering a combined area of 4,090 hectares (Figure 4.2.1) (DFA 2016). Mussel aquaculture production in the Province in 2015 was valued at $12.8 million, and contributes to rural employment with many farms being small, family-run businesses.

Control and effective mitigation of a new, high risk AIS, such as *Ciona intestinalis*, is paramount in avoiding the spread of this species to aquaculture sites in NL. Of particular

concern, due to close proximity to the known locations of *C. intestinalis*, are sites within Placentia Bay, including the first commercial oyster sites in the province. With documented cases worldwide of *C. intestinalis* fouling on shellfish aquaculture farms, causing reduction in product growth and eventual loss of crop, rapid response in combination with research-based information is vital for the local aquaculture industry.

The information provided in this thesis will assist in building a plan to control *Ciona intestinalis.* In Chapter 2, I illustrate that spawning occurs above 6-8 °C. Therefore, mitigation should occur early in the spring, at temperatures < 4 - 6° C, before the production of viable gametes to avoid stress-induced spawning. I also show that there is a clear preference for sheltered attachment locations, which will further assist in rapid assessment of local marinas and harbours increasing the likelihood of early detection. Growth rates calculated in this project help predict the timing of peaks in recruitment, which is important information for choosing effective timing of control measures. For example, increased effort may be needed during the time of increased recruitment.

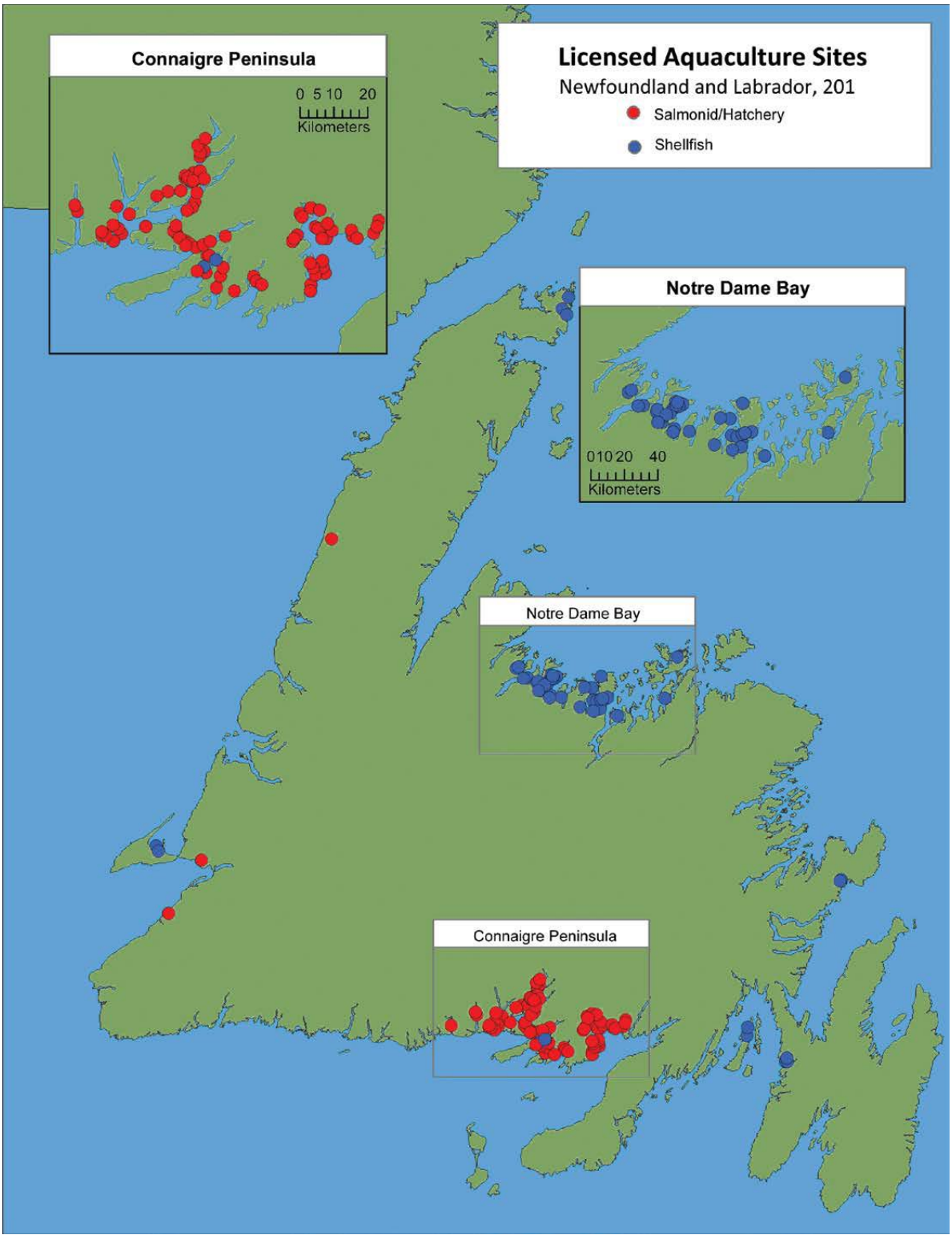


Figure 4.2.1: Insular Newfoundland: Licenced aquaculture sites as of 2015 (taken from Department of Fisheries and Aquaculture 2016).

In Chapter 3, I illustrate the occurrence of metamorphosis from a non-feeding larval stage into a feeding juvenile, before attachment to a substrate, up to 80 % of the time. This indicates a potentially significant increase in the time of life in the plankton, and thus greater dispersal capacity by means of natural currents. I also illustrate, for the first time, that while precocious, planktonic juveniles do not have the swimming ability of larvae, they do have the ability to attach to a substrate when it becomes available. From these findings I conclude that while it is important to consider the substrates available for attachment of *Ciona intestinalis*, it is also important to consider planktonic stages of development, which may populate new locations or be viable in the water column after mitigation.

### 4.3 Conclusion

In Newfoundland, the DFO-AIS Program, in collaboration with the Department of Ocean Science, NL Department of Fisheries and Aquaculture, industry, and policy makers have been focused on a proactive approach of rapid assessments and continued monitoring for early detection of AIS. Upon early detection of an AIS, rapid response and mitigation is of first priority. Research-based information, like basic biology, ecology, and recruitment dynamics of these species, as I have provided here, will optimize efforts to combat aquatic invaders.

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