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Research Article

Post-metamorphic attachment by solitary ascidian *Ciona intestinalis* (Linnaeus, 1767) juveniles from Newfoundland and Labrador, Canada

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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. We 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 we 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.

Key words: *Ciona intestinalis*, tadpole larvae, first ascidian stage, pre-attached metamorphosis, post-metamorphic attachment

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 and Herbinger 2009). Control and mitigation of invasive tunicate infestations and predicting potential dispersal rates is 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. 2016).

C. intestinalis reproduces sexually via external cross fertilization (Byrd and Lambert 2000; Cirino et al. 2002; Carver et al. 2006). Spawning and settling behaviors appear to be controlled by changes in light intensity (Lambert and Brandt 1967; Svane and 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 which 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 behavior 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 signaling 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 1 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 *C. intestinalis* is typically considered limited to the time between spawning and attachment to a substrate at the end of the swimming larval stage (Svane and 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 *C. 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* affect their ability to attach to a substrate post-metamorphosis.

Materials and methods

Sample collection and spawning

Sexually mature *C. 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 psu, 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. We exchanged 80–100% of seawater once a week, 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). We 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 6×10^6 cells/ml was allowed to drip for approximately 3 h per day at a rate of 1 drop per second; 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 our experiment, we 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, *C. 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, we 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.

Experiment 1: Larval pre-attachment metamorphosis

Swimming tadpole larvae were spotted by looking across the water surface at eye level and 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 *C. 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 a week using a disposable pipette to avoid disturbing larvae at the water surface. We 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 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.

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

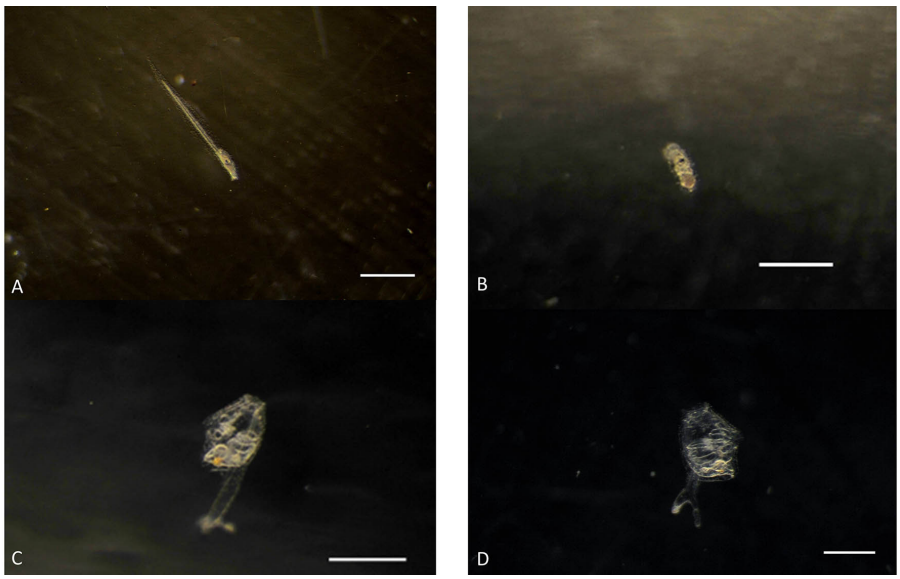

Figure 1. Photographs of the developmental stages of *Ciona intestinalis* tested for pre-attachment metamorphosis in Experiment 1 and for post-metamorphic attachment in Experiment 2. A) Tadpole larvae tested in Experiment 1; and for stages used in testing juvenile post-metamorphic substrate attachment B) Rotation Stage C) FAS I D) FAS II. Scale bar is 0.5 mm in each image.

metamorphosis). We 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 1). For each of these three developmental stages, we 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, we 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.

Experiment 2 Trial 2: Bottom, side, and top substrate attachment

In Trial 2, we 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 *C. 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 methanol 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, we 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.

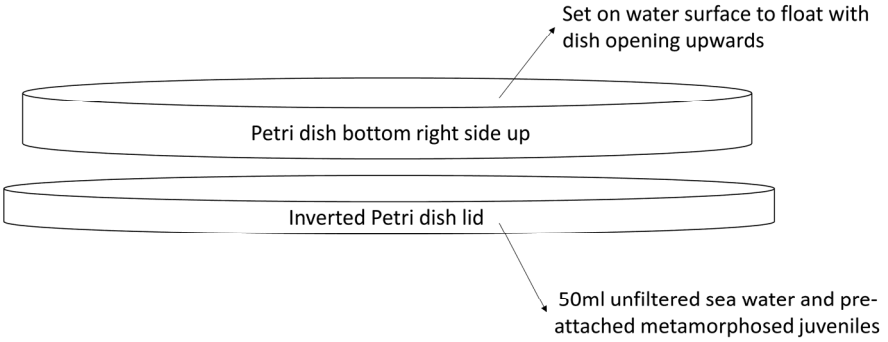

Figure 2. Increased surface area settlement plates used in Experiment 2 Trial 2.

This increased the substrate available for attachment for *C. intestinalis* juveniles (Figure 2). After 24 h, we 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.

Statistical analyses

In Experiment 1 (Larval Pre-attachment Metamorphosis), the number of unattached juveniles that underwent metamorphosis was converted into a percentage. We 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, we 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, we 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 we used Tukey HSD multiple comparison tests. All analyses were conducted using Minitab 17.0 using a significance threshold of 0.05 for all tests.

Results

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 our 3 trials (Figure 3). Statistical analyses indicated significant variation between trials in the percent of larvae that underwent pre-attachment metamorphosis after 24 h (p =0.01, Table 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), 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). 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.

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

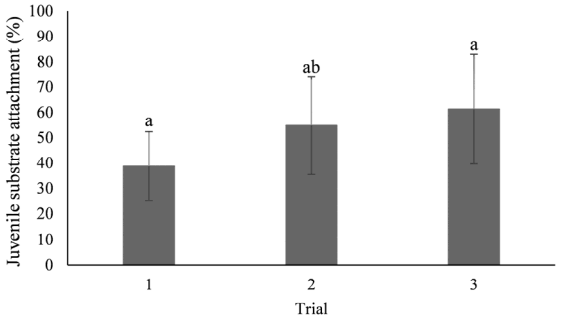

Figure 3. Pre-attachment metamorphosis in Trials 1, 2, and 3 in Experiment 1. Bars represent mean +/- SE; Different letters indicate differences in treatment means (LS means, p<0.05, n=8).

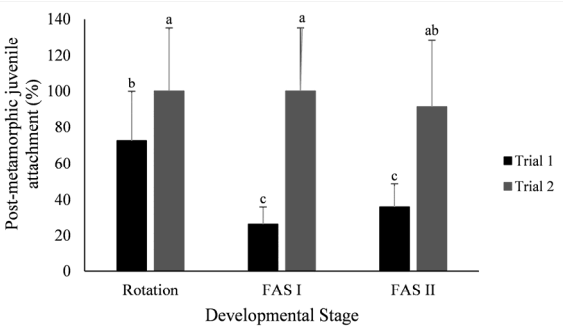

Figure 4. Percent of post-metamorphic juvenile attachment between Trials 1 and 2 and stage of development (Rotation, FAS I, and FAS II, respectively). Bars represent mean +/- SE. Bars with different letters are significantly different (LS means, P<0.05, n=6 to 8).

substrate attachment ranged widely from 0–90.9% of individuals and averaged 44.9 ± 24%. In Trial 2, we 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 4), but in Trial 2, there was no difference in the attachment rate between developmental stages (p >0.10, LS means, Figure 4).

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 *C. 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.

Discussion

According to previous descriptions of the life cycle of *C. 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 and 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, we 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%). We 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. We 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 and 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). Our findings indicate that metamorphosis of *C. intestinalis* tadpole larvae occurs with or without a substrate. Therefore, we hypothesize that just as other larvae postpone settlement in the absence of favorable environmental conditions (Svane and 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 our 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 we did observe *C. intestinalis* larvae that underwent pre-attached metamorphosis and proceeded to develop into the Second Ascidian Stage while remaining unattached. We 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 *C. 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 *S. 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 our 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, *C. 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, we presumed this result was partly due to lack of available substrate. Our assumption was validated when we 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 our 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, we 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) we 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, we 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 we continued the study for a longer time period to include more stages of development, we may have observed a decline in attachment ability. While our findings indicate that *C. 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. We also observed reattachment in our 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, we 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 we observed that *C. 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 *C. 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.

We conclude that pre-attached metamorphosis is known to occur in ascidians and we observed this process in up to 61 % of *C. intestinalis* in a laboratory setting. Post-metamorphic attachment of juvenile *C. intestinalis* has not previously been studied and we 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. Our 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 our 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.

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