

Research Article

Induced spawning and culture techniques for the invasive ascidian *Didemnum vexillum* (Kott, 2002)

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Editor's note:

This paper is a contribution to the proceedings of the 3rd International Invasive Sea Squirt Conference held in Woods Hole, Massachusetts, USA, on 26–28 April 2010. The conference provided a venue for the exchange of information on the biogeography, ecology, genetics, impacts, risk assessment and management of invasive tunicates worldwide.

Abstract

The colonial ascidian *Didemnum vexillum* has become relatively widespread in New Zealand, since its initial discovery in 2001. Despite the potential economic and ecological impacts of *D. vexillum*, there are still considerable knowledge gaps surrounding its key biological attributes. The ability to obtain larvae and culture colonies in the laboratory is crucial to research into larval longevity and dispersal potential, and the factors affecting colony survivorship and growth. Here we present methods for spawning and culture of *D. vexillum* under laboratory conditions. A 'light shocking without cycles' technique was used to stimulate larval release in adult colonies, with > 500 larvae being produced from ~ 100 g of tissue at the peak of the reproductive season. Following release, the larvae were allowed to metamorphose and the juveniles were cultured under controlled conditions for four weeks. Recruit survival during the four weeks of culture was > 85 % with the majority having formed small colonies of 4 to 6 zooids with a dense cover of white spicules throughout the tunic. The most effective laboratory spawning conditions are described with respect to light and temperature. The ability to obtain *D. vexillum* larvae on demand will enable increased research into several aspects of this species' reproductive biology and ecology.

Key words: tunicate, light shock, settlement, metamorphosis, tadpole larvae, non-indigenous species

Introduction

The ongoing human-mediated spread of non-indigenous ascidian species is causing growing concern globally (Lambert 2001; Carver et al. 2003; G. Lambert 2005; LeBlanc et al. 2007). In particular, ascidians pose a significant threat for aquaculture industries, as they are often strong spatial competitors that are able to reach a very high density or biomass in relatively short timeframes (Stachowicz et al. 2002; Blum et al. 2007; Arsenault et al. 2009). The potential for adverse effects on natural ecosystems has also been documented for some species, including the colonial ascidian *Didemnum vexillum* (Kott, 2002) (Valentine et al. 2007; Lengyel et al.

2009). Despite the potential impacts of *D. vexillum*, there are still considerable knowledge gaps surrounding its key biological attributes; information that underpins successful management. In particular, research into the factors affecting colony survivorship, growth and the potential spread of this species is still relatively scarce (but see Daniel and Therriault 2007). For other ascidian species, a range of abiotic and biotic factors that affect both the larval and post-settlement life-stages have been shown to dramatically influence subsequent adult populations (Grosberg 1981; Young and Chia 1984; Stoner 1990; Osman and Whitlatch 1995, 1996, 2004; reviewed by Bates 2005 and C.C. Lambert 2005). Hence, in order to better

understand the factors controlling the distribution and persistence of *D. vexillum*, increased research on the early life-stages (i.e., larvae and newly settled juveniles) is required. For example, research on larval longevity will aid our understanding of ascidian dispersal potential, as this is heavily dependent on the initial planktonic phase of the life history. Furthermore, knowledge of the environmental conditions that favour ascidian larvae and juveniles may be applied when predicting areas susceptible to new invasions or range expansions (Epelbaum et al. 2009a).

The reproductive biology of many colonial ascidians has been well-documented (Millar 1971; Harvell and Grosberg 1988; Svane and Young 1989; C.C. Lambert 2005; G. Lambert 2005). Colonies are made up of morphologically identical individuals, termed zooids, which are enclosed in a common tunic and share a circulatory system. Colonies undergo both asexual and sexual reproduction. Colonial ascidian embryos are brooded within the colony, and develop for one to several weeks until they are mature and tadpole larvae are released. Brooding in *D. vexillum* occurs within the tunic of the colony (Figure 1) and the embryos are believed to take several weeks to fully develop into free-swimming larvae about 1.4 mm in length (Lambert 2009). Previous research with other colonial ascidian species indicates that colonies release larvae in response to light stimulation, often spawning at dawn under natural conditions (Whittingham 1966; Lambert and Brandt 1967; Watanabe and Lambert 1973; West and Lambert 1976; Grosberg 1988; Svane and Havenhand 1993; Bingham 1997; Forward et al. 2000).

Several studies have documented laboratory procedures to induce spawning and to culture ascidians (Berrill 1937; Grave 1937; Costello and Henley 1971; Cloney 1987); however, there has been no assessment of these methods for *D. vexillum*. Controlled research on the dispersal and growth of this species has been hampered by difficulties in obtaining sufficient larvae on demand. Previous work on colonial ascidians has shown that the time of larval release can be manipulated through maintaining the colonies in complete darkness for several hours prior (termed the dark adaptation period) (Costello and Henley 1971; Watanabe and Lambert 1973; Svane and Young 1989; Forward et al. 2000). It has also been suggested that increasing this dark adaptation period will result in a shorter duration

between light exposure and larval release (termed the latency period), as well as increasing the number of larvae that are released (Watanabe and Lambert 1973). The present study documents procedures we have recently developed to successfully induce spawning in *D. vexillum* colonies, as well as techniques for the successful settlement and metamorphosis of the larvae, and culture of the juvenile recruits. A range of methods were assessed over the course of this study, with those presented here found to be the most successful for this species.

Collection and maintenance of laboratory cultures

The *D. vexillum* colonies used during this study were collected from beneath a floating pontoon in Port Nelson, New Zealand, between December 2008 and June 2009. We found that small (< 20 cm² area, ~ 30 g) lobe-shaped colonies were most suitable for the trials as they generally had less detritus associated with them. Also, as the larvae are brooded within the tunic beneath the zooids in *Didemnum* species (Lambert and Lambert 2010), damage to these larvae is less likely to occur when using lobe or tendril-shaped specimens than from removing encrusting or two-dimensional colonies from the substratum. Following collection, the colonies were immediately transported to the nearby laboratory in 2 L plastic containers filled with natural seawater. Upon arrival at the laboratory, the colonies were rinsed in filtered seawater (0.35 µm) and any debris or associated epibionts removed. The colonies collected were used immediately for induced spawning trials, following the procedures outlined below. The initiation of spawning trials as soon as possible after field collection was vital, as our attempts to induce larval release in colonies collected from distant locations (several hours from the laboratory) consistently failed.

Induced spawning of adult colonies

Dark adaptation period

The *D. vexillum* larvae produced in this study were obtained using a 'light shocking' technique (Bullard and Whitlatch 2004) that stimulates and enables control of the time of larval release through the manipulation of a dark adaptation period. The colonies need to be maintained in

Figure 1. Cross section of a *Didemnum vexillum* colony. Numerous individual zooids are visible directly beneath the outer layer of the tunic, and several brooded embryos are visible in the central region between these layers. The location of one such embryo (e) is indicated. Scale bar 2 mm. Photograph by L. Fletcher.

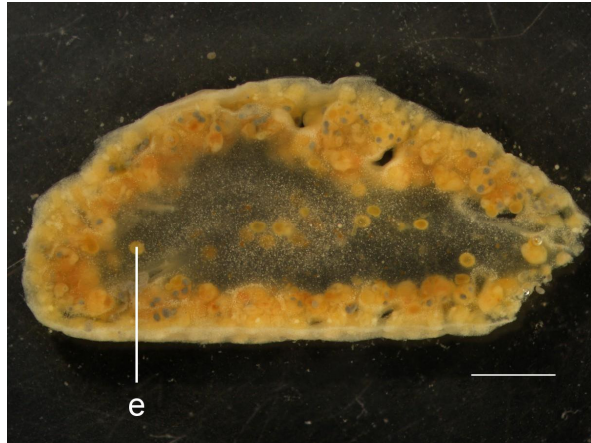
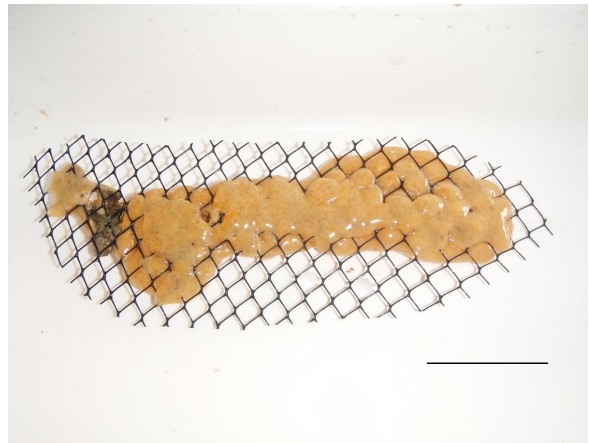


Figure 2. *Didemnum vexillum* colony overgrowing plastic mesh netting, following containment in the laboratory for 48 hours. Scale bar 3 cm. Photograph by L. Fletcher.



constant darkness for a duration that is long enough to induce sufficient larval release, but not so long as to lead to deterioration of colony health. Based on multiple observations of colony health and spawning success in relation to dark adaptation, a period of 48 hours is recommended for this species. To start this dark adaptation period, five colonies were placed into two 50 L black PVC bins (2–3 colonies per bin) with secure lids that excluded light. Each bin was filled with ambient temperature seawater (~16 °C, salinity ~35, filtered to 0.35 µm and UV treated). The colony fragments were supported by 60 mm² plastic mesh, suspended horizontally to raise them approximately 10 cm off the base of the bin and allow adequate water flow to all zooids within the colony. If placed directly on the base of the bin the underside of the colony did not receive adequate water flow and the health of the tissue deteriorated. The

colonies often grew around, and naturally attached to, the mesh during the time held in the bins (Figure 2), suggesting they were not overly stressed during this period. During the dark adaptation phase each bin contained a single air stone that provided a constant stream. The water temperature was maintained between 18 and 20 °C. Before the lids were sealed each bin had 50 ml of concentrated *Isochrysis* sp. algal solution added to the filtered seawater to provide a food source for the colonies (1.5 × 10⁷ cells.L⁻¹ algal concentrations in the bins).

Light exposure to induce larval release

Following the dark adaptation period the colonies were removed from the bins and suspended individually within five 1 L glass beakers of filtered (0.35 µm) seawater. If the colonies had attached themselves to the plastic

mesh, the mesh was cut adjacent to the tissue to prevent damage to the zooids (Figure 2). The beakers were placed in an area receiving natural sunlight and were also exposed to bright artificial light, to stimulate larval release (Cloney 1987). The artificial light was provided by two standard lamps, each containing a single Osram 18W warm-white fluorescent bulb (producing 1200 lumen), positioned 20 cm from the beakers. There was no need for aeration of the beakers. The water temperature increased due to the heat produced by the lights, although this was kept below 22 °C by placing the beakers in a cold water bath when necessary. The colonies are likely to become stressed at higher temperatures as growth of this species has been shown to be adversely affected by water temperatures above ~23 °C (McCarthy et al. 2007).

Patterns of larval release

Larvae appeared to be released from the common cloacal apertures that were spread across the surface of the tunic. The larvae were easily visible and often aggregated at the sides of the beakers nearest to the light source, as they are positively phototactic at this stage (Grave 1937). When the water was maintained between 20 and 22°C, the first larvae were consistently released within 3 to 4 hours following initial light exposure; this latency period was the shortest recorded in all trials. The frequency of larval release was initially slow but increased steadily over the following hour until release was at intervals of approximately 10–20 seconds. The colonies continued to release larvae for approximately 5 hours, although the frequency decreased considerably after the initial couple of hours.

Patterns of larval settlement

As the *D. vexillum* larvae were visible to the naked eye, they could be collected using a standard pipette, after which they could be examined directly or allowed to settle onto a hard substratum and metamorphose into juveniles. Pilot studies suggested less settlement occurred when larvae were exposed to well lit or completely dark environments, supporting the recommendation for shaded conditions to facilitate metamorphosis in ascidian larvae (Lambert and Lambert 2010). The duration of swimming activity and timing of larval

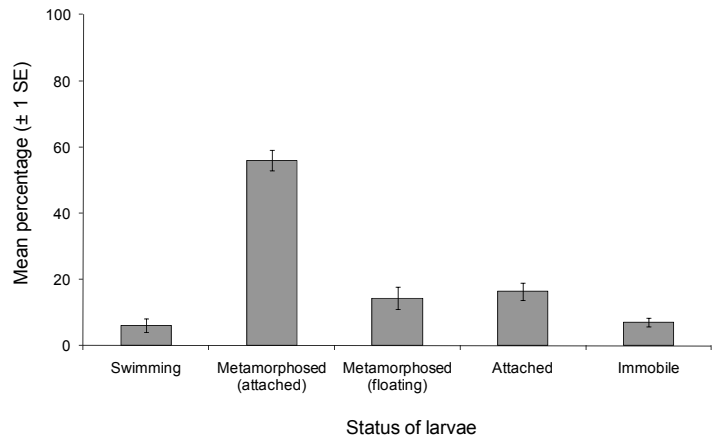
settlement varied considerably; some larvae only swam for a few minutes while others took several hours to initiate the metamorphosis process. In a study examining larval settlement rates of 193 larvae, 56.0 % (on average) had successfully settled onto the substratum and metamorphosed into juveniles 24 hours after release from the colony (Figure 3). On average, 6.1 % of the larvae examined were recorded to be actively swimming at this point. When assessed again a further 24 hours later, a small proportion (~18 %) of these larvae had subsequently metamorphosed into juveniles, although most had become immobile and unresponsive to light or tactile stimuli. Consequently, to ensure sufficient time for completion of all metamorphosis events, we found the larvae were best left undisturbed for at least 48 hours after release from the parent colony.

Even under ideal conditions, a consistent 10–15 % of the larvae metamorphosed at the water surface and were subsequently suspended, inverted, within this layer (e.g., 14.4 % in Figure 3). This phenomenon has been found in many ascidian species, and may be normal for a proportion of ascidian larvae (Millar 1971). These recruits, and others that have attached to non-target substrata, can be carefully removed and repositioned on more suitable substrata if desired. This method has been previously used to position newly settled juveniles of other colonial ascidian species in precise locations (Berrill 1937; Boyd et al. 1986; Epelbaum et al. 2009b).

Techniques to delay metamorphosis

Research into the competency period and energetic reserves of ascidian larvae require techniques to delay metamorphosis. We investigated a range of methods to achieve this delay, including the use of aeration, mechanical agitation, and varying light regimes. The most successful delay method was found to be a combination of exposure to a white surface (the larvae were in transparent containers and placed on the white surface) and continuous bright light. Once exposed to this regime the larvae became dormant and remained inactive for the period of light exposure, as also described for the colonial ascidian *Diplosoma listerianum* (Marshall et al. 2003; Bennett and Marshall 2005). *D. vexillum* larvae were able to successfully metamorphose into juveniles following artificial delays of up to 36 hours using this method (L. Fletcher, unpublished data).

Figure 3. Status of *Didemnum vexillum* larvae, 24 hours after release from the parent colony. Larvae were randomly allocated across 10 tissue culture plates (total larvae = 193, sourced from five separate colonies) and classified as either: swimming; attached to the substrate and metamorphosed; metamorphosed within the water column (floating); attached to the substrate but still a larva; or immobile.



Culture of juvenile recruits

Culturing methods

In a separate study, larvae that had successfully metamorphosed into recruits ($n = 174$) were cultured under laboratory conditions for four weeks, with colony survivorship and development documented. Recruits were situated within individual wells (6 ml) of sterile 12-well Falcon™ tissue culture plates and were maintained at 18 °C in a 12 : 12 hour light : dark regime. Ascidian recruits quickly develop atrial and branchial siphons, and begin feeding within one to two days (Takeuchi 1980; Epelbaum et al. 2009b). The recruits in our study were fed an algal solution of *Isochrysis* sp. diluted in filtered seawater (5×10^7 cells.L⁻¹) every second day. The containers were not aerated, as pilot studies had shown replacing the water every second day was sufficient to enable colony growth. Aeration of the culture containers has sometimes been shown to negatively affect feeding efficiency through the suspension of fecal pellets and detritus, leading to a reflex cessation of pumping (Milkman 1967). There was often considerable fecal pellet build up over the two days, so the area around the recruit was gently cleaned with a soft paintbrush to dislodge fecal matter before the water was changed as required.

Description of juvenile colonies

Following settlement and metamorphosis of the tadpole larva, the juvenile recruits were initially transparent, with pale yellow digestive organs

often visible within the posterior region. In addition, black ‘eyespot’ (the ocellus and statolith within the tadpole larva) were often visible near the anterior region of the recruit (Figure 4A). Paired yellow-brown coloured lateral organs of the thorax were generally visible two days following settlement, and these subsequently turned white following the production of calcium carbonate spicules from within these structures (as documented in Valentine et al. 2009). The spicules were generally visible throughout the surface of the tunic, four days post-settlement. The production of fecal matter by the recruits over this time indicated that they were successfully feeding (Valentine et al. 2009). Visible contractions in the recruits, occurring during water inhalation and exhalation, were witnessed after approximately one week.

Two weeks after settlement and metamorphosis, the majority of recruits had undergone asexual budding and divided into a two-zooid colony. Each zooid had its own inhalant oral siphon and a shared cloacal aperture out of which the filtered water was exhaled (Figure 4B). At the four week assessment, there was >85% recruit survival (L. Fletcher, unpublished data), with the majority of these recruits having formed small colonies of four to six zooids having a dense cover of white spicules throughout the tunic. The numbers of zooids observed in these colonies are similar to those found in other laboratory-raised colonial ascidian species (Milkman 1967; Boyd et al. 1986; Epelbaum et al. 2009b).

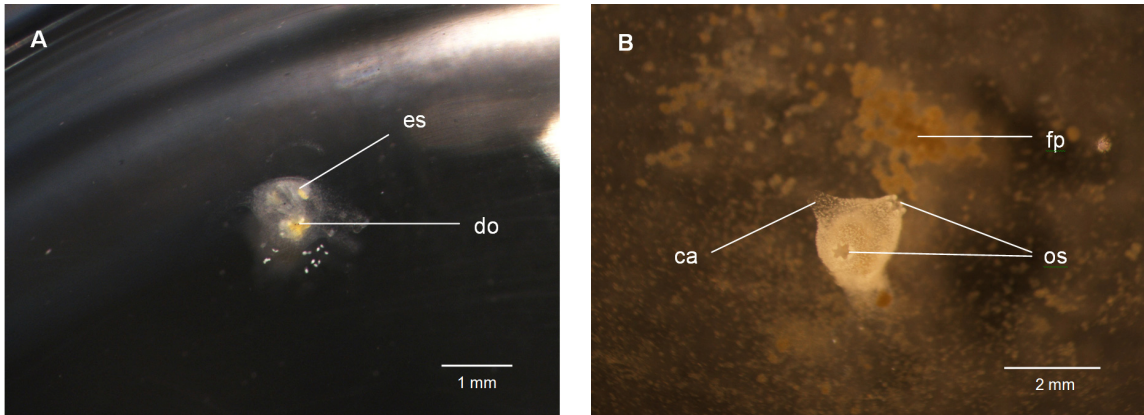


Figure 4. A. Newly settled *Didemnum vexillum* recruit. The location of the eyespot (es) in the anterior region and the digestive organs (do) in the posterior region of the zooid are indicated. Spicules have not yet been produced at this early stage. Scale bar 1 mm. B. Juvenile *D. vexillum* colony two weeks after initial settlement of the larva. The colony has undergone asexual reproduction and consists of two zooids (one has budded from the oozooid). The location of the two oral siphons (os), shared cloacal aperture (ca) and fecal pellets (fp) are indicated. White calcium carbonate spicules are distributed throughout the tunic. Scale bar 2 mm. Labels correspond to those used by Valentine et al. (2009). Photographs by L. Fletcher.

Discussion

The techniques described in this paper can be applied to research into several aspects of the reproductive biology and ecology of *D. vexillum*. The ability to obtain larvae on demand has already enabled research into the larval competency period of this species, which has indicated some larvae are capable of successful settlement and colony growth following 36 hours of delayed metamorphosis (L. Fletcher, unpublished data). This information is currently being used to predict the spread of *D. vexillum* within the Marlborough Sounds aquaculture region of New Zealand. These techniques can also be applied to determine factors such as the preferred settling time of *D. vexillum* larvae and the potential carry-over effects of delayed settlement of the recruits in terms of decreased fitness and growth rates. Similarly, the ability to culture juvenile colonies with precise control of environmental variables and adequate replication of trials enables greater isolation of various factors affecting colony survivorship, growth and reproduction. Using these methods, it is hoped to further investigate the growth rates of *D. vexillum* colonies in order to determine the length of time to reproductive maturity, and hence the number of larvae potentially released over a spawning season. Together with information on larval longevity, this knowledge

will enable predictions of the potential annual spread of this species by natural dispersal.

Although laboratory culture of juvenile colonies can have several advantages, an awareness of differences between laboratory and natural environments is required. Life-history traits exhibited under laboratory conditions may not accurately reflect those occurring in nature where considerably more factors influence natural populations (e.g., predation and physical disturbance). For example, previous work on the larval duration of ascidians is mainly based on laboratory studies; however, as laboratory culture conditions are inherently different from nature, it has been suggested that larval swimming time may be over-estimated by experiments conducted in closed systems (Shanks 2009). This phenomenon is also illustrated by our research with reference to the morphology of the early one and two-zooid *D. vexillum* colonies that were quite different from those observed growing on settlement plates in the field. The laboratory-raised colonies were very erect in appearance, while those observed in the field are generally quite flat and dome-shaped (see Valentine et al. 2009 for a description). We propose that the laboratory-raised colonies exhibit this morphology as they are growing in static water with no forces to prevent upright growth. Although results of experiments using laboratory-raised colonies

should perhaps be interpreted with caution, cultured colonies still provide a valuable means for investigating aspects of a species' biology under controlled and replicated conditions.

Conclusions and future directions

The methods for induced spawning and culture of *D. vexillum* colonies presented in this work were adequate for our research purposes, which required the controlled release of moderate numbers of *D. vexillum* larvae in order to determine their competency period and make estimates of the dispersal potential of this species. However, some applications of this method, such as large-scale studies into the interactions between *D. vexillum* early life-stages and other species within fouling communities, may call for more larvae than we were able to produce. Hence, continued efforts to improve and refine methods to induce larval release in this species would be desirable. Similarly, additional research on techniques for maintenance of adult laboratory cultures is essential, as these individuals can be used as brood stock cultures, which will enable long-term experiments year round.

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References

Arsenault G, Davidson J, Ramsay A (2009) Temporal and spatial development of an infestation of *Styela clava* on mussel farms in Malpeque Bay, Prince Edward Island, Canada. *Aquatic Invasions* 4: 189–194, <http://dx.doi.org/10.3391/ai.2009.4.1.19>

Bates WR (2005) Environmental factors affecting reproduction and development in ascidians and other prochordates. *Canadian Journal of Zoology* 83: 51–61, <http://dx.doi.org/10.1139/z04-164>

Bennett CE, Marshall DJ (2005) The relative energetic costs of the larval period, larval swimming and metamorphosis for the ascidian *Diplosoma listerianum*. *Marine and Freshwater Behaviour and Physiology* 38: 21–29, <http://dx.doi.org/10.1080/1023624040029333>

Berrill NJ (1937) Culture methods for ascidians. In: Galstoff PS, Lutz FE, Welch PS, Needham JG (eds) Culture methods for invertebrate animals. Dover Publications, New York, USA, pp 564–571

Bingham BL (1997) Light cycles and gametogenesis in three temperate ascidian species. *Invertebrate Biology* 116: 61–70, <http://dx.doi.org/10.2307/3226925>

Boyd HC, Brown SK, Harp JA, Weissman IL (1986) Growth and sexual maturation of laboratory-cultured Monterey *Botryllus schlosseri*. *Biological Bulletin* 170: 91–109, <http://dx.doi.org/10.2307/1541383>

Blum JC, Chang AL, Lijsthröm M, Schenk ME, Steinberg MK, Ruiz GM (2007) The non-native solitary ascidian *Ciona intestinalis* (L.) depresses species richness. *Journal of Experimental Marine Biology and Ecology* 342: 5–14, <http://dx.doi.org/10.1016/j.jembe.2006.10.010>

Bullard SG, Whitlatch RB (2004) A guide to the larval and juvenile stages of common Long Island Sound ascidians and bryozoans. Connecticut Sea Grant College Program, CTSG-04-07

Carver CE, Chisholm A, Mallet AL (2003) Strategies to mitigate the impact of *Ciona intestinalis* (L.) biofouling on shellfish production. *Journal of Shellfish Research* 22: 621–631

Cloney RA (1987) Phylum Urochordata, Class Ascidiacea. In: Strathmann MF (ed) Reproduction and development of marine invertebrates of the Northern Pacific coast. University of Washington Press, Seattle, pp 607–646

Costello DP, Henley C (1971) Methods for obtaining and handling marine eggs and embryos. Marine Biological Laboratory, Woods Hole, MA, pp 202–223, <http://dx.doi.org/10.1575/1912/295>

Daniel KS, Therriault TW (2007) Biological synopsis of the invasive tunicate *Didemnum* sp. Canadian Manuscript Report of Fisheries and Aquatic Sciences 2788: vi + 53 p

Epelbaum A, Herborg LM, Therriault TW, Pearce CM (2009a) Temperature and salinity effects on growth, survival, reproduction, and potential distribution of two non-indigenous botryllid ascidians in British Columbia. *Journal of Experimental Marine Biology and Ecology* 369: 43–52, <http://dx.doi.org/10.1016/j.jembe.2008.10.028>

Epelbaum A, Therriault TW, Paulson A, Pearce CM (2009b) Botryllid tunicates: Culture techniques and experimental procedures. *Aquatic Invasions* 4: 111–120, <http://dx.doi.org/10.3391/ai.2009.4.1.12>

Forward Jr. RB, Welch JM, Young CM (2000) Light induced larval release of a colonial ascidian. *Journal of Experimental Marine Biology and Ecology* 248: 225–238, [http://dx.doi.org/10.1016/S0022-0981\(00\)00168-4](http://dx.doi.org/10.1016/S0022-0981(00)00168-4)

Grave C (1937) Notes on the culture of eight species of ascidians. In: Galstoff PS, Lutz FE, Welch PS, Needham JG (eds) Culture methods for invertebrate animals. Dover Publications, New York, USA, pp 560–564

Grosberg RK (1981) Competitive ability influences habitat choice in marine invertebrates. *Nature* 290: 700–702, <http://dx.doi.org/10.1038/290700a0>

Grosberg RK (1988) Life-history variation within a population of the colonial ascidian *Botryllus schlosseri*. I. The genetic and environmental control of seasonal variation. *Evolution* 42: 900–920, <http://dx.doi.org/10.2307/2408907>

Harvell CD, Grosberg RK (1988) The timing of sexual maturity in clonal organisms. *Ecology* 69 (6): 1855–1864, <http://dx.doi.org/10.2307/1941162>

Kott P (2002) A complex didemnid ascidian from Whangamata, New Zealand. *Journal of the Marine Biological Association of the United Kingdom* 82: 625–628, <http://dx.doi.org/10.1017/S0025315402005970>

- Lambert CC (2005) Historical introduction, overview, and reproductive biology of the protochordates. *Canadian Journal of Zoology* 83: 1–7, <http://dx.doi.org/10.1139/z04-160>
- Lambert G (2001) A global overview of ascidian introductions and their possible impact on the endemic fauna. In: Sawanda H, Yokosawa H, Lambert CC (eds) *The biology of ascidians*. Springer-Verlag, Tokyo, pp 249–257
- Lambert G (2005) Invasive sea squirts: a growing global problem. *Journal of Experimental Marine Biology and Ecology* 342:3–4, <http://dx.doi.org/10.1016/j.jembe.2006.10.009>
- Lambert G (2009) Adventures of a sea squirt sleuth: unravelling the identity of *Didemnum vexillum*, a global ascidian invader. *Aquatic Invasions* 4: 5–28, <http://dx.doi.org/10.3391/ai.2009.4.1.2>
- Lambert CC, Brandt CL (1967) The effect of light on the spawning of *Ciona intestinalis*. *Biological Bulletin* 132 (2): 222–228, <http://dx.doi.org/10.2307/1539890>
- Lambert G, Lambert CC (2010) *Ascidian taxonomy workshop*. International Invasive Sea Squirt Conference III, Woods Hole, MA, April 26, 2010, 4 pp
- LeBlanc N, Davidson J, Tremblay R, McNiven M, Landry T (2007) The effect of anti-fouling treatments for the clubbed tunicate on the blue mussel, *Mytilus edulis*. *Aquaculture* 264: 205–213, <http://dx.doi.org/10.1016/j.aquaculture.2006.12.027>
- Lengyel NL, Collie JS, Valentine PC (2009) The invasive colonial ascidian *Didemnum vexillum* on Georges Bank – Ecological effects and genetic identification. *Aquatic Invasions* 4: 143–152, <http://dx.doi.org/10.3391/ai.2009.4.1.15>
- Marshall DJ, Pechenik JA, Keough MJ (2003) Larval activity levels and delayed metamorphosis affect post-larval performance in the colonial ascidian *Diplosoma listerianum*. *Marine Ecology Progress Series* 246: 153–162, <http://dx.doi.org/10.3354/meps246153>
- McCarthy A, Osman RW, Whitlatch RB (2007) Effects of temperature on growth rates of colonial ascidians: A comparison of *Didemnum* sp. to *Botryllus schlosseri* and *Botrylloides violaceus*. *Journal of Experimental Marine Biology and Ecology* 342: 172–174, <http://dx.doi.org/10.1016/j.jembe.2006.10.036>
- Milkman R (1967) Genetic and developmental studies on *Botryllus schlosseri*. *Biological Bulletin* 132: 229–243, <http://dx.doi.org/10.2307/1539891>
- Millar RH (1971) The biology of ascidians. *Advances in Marine Biology* 9: 1–100, [http://dx.doi.org/10.1016/S0065-2881\(08\)60341-7](http://dx.doi.org/10.1016/S0065-2881(08)60341-7)
- Osman RW, Whitlatch RB (1995) Predation on early ontogenetic life-stages and its effect on recruitment into a marine community. *Marine Ecology Progress Series* 117: 111–126, <http://dx.doi.org/10.3354/meps117111>
- Osman RW, Whitlatch RB (1996) Processes affecting newly-settled juveniles and the consequences to subsequent community development. *Invertebrate Reproduction and Development* 30: 217–225, <http://dx.doi.org/10.1080/07924259.1996.9672548>
- Osman RW, Whitlatch RB (2004) The control of the development of a marine benthic community by predation on recruits. *Journal of Experimental Marine Biology and Ecology* 311: 117–145, <http://dx.doi.org/10.1016/j.jembe.2004.05.001>
- Shanks AL (2009) Pelagic larval duration and dispersal distance revisited. *Biological Bulletin* 216: 373–385
- Stachowicz JJ, Fried H, Osman RW, Whitlatch RB (2002) Biodiversity, invasion resistance, and marine ecosystem function: reconciling patterns and processes. *Ecology* 83: 2575–2590, [http://dx.doi.org/10.1890/0012-9658\(2002\)083\[2575:BIRAME\]2.0.CO;2](http://dx.doi.org/10.1890/0012-9658(2002)083[2575:BIRAME]2.0.CO;2)
- Stoner DS (1990) Recruitment of a tropical colonial ascidian: relative importance of pre-settlement vs. post-settlement processes. *Ecology* 71: 1682–1690, <http://dx.doi.org/10.2307/1937577>
- Svane I, Havenhand JN (1993) Spawning and dispersal in *Ciona intestinalis* (L.). *Marine Ecology* 14: 53–66, <http://dx.doi.org/10.1111/j.1439-0485.1993.tb00364.x>
- Svane I, Young CM (1989) The ecology and behaviour of ascidian larvae. *Oceanography and Marine Biology Annual Review* 27: 45–90
- Takeuchi K (1980) Oozoid formation in the ascidian *Botrylloides violaceus*. *Publications of the Seto Marine Biological Laboratory* 15 (1/4): 1–5
- Valentine PC, Collie JS, Reid RN, Asch RG, Guida VG, Blackwood DS (2007) The occurrence of the colonial ascidian *Didemnum* sp. on Georges Bank gravel habitat – Ecological observations and potential effects on groundfish and scallop fisheries. *Journal of Experimental Marine Biology and Ecology* 342: 179–181, <http://dx.doi.org/10.1016/j.jembe.2006.10.038>
- Valentine PC, Carman MR, Dijkstra J, Blackwood DS (2009) Larval recruitment of the invasive colonial ascidian *Didemnum vexillum*, seasonal water temperatures in New England coastal and offshore waters, and implications for spread of the species. *Aquatic Invasions* 4: 153–168, <http://dx.doi.org/10.3391/ai.2009.4.1.16>
- Watanabe H, Lambert CC (1973) Larva release in response to light by the compound ascidians *Dipstaplia occidentalis* and *Metandrocarpa taylora*. *Biological Bulletin* 144: 556–566, <http://dx.doi.org/10.2307/1540308>
- West AB, Lambert CC (1976) Control of spawning in the tunicate *Styela plicata* by variations in a natural light regime. *Journal of Experimental Zoology* 195: 263–270, <http://dx.doi.org/10.1002/jez.1401950211>
- Whittingham DG (1966) Light induction of shedding of gametes in *Ciona intestinalis* and *Molgula manhattensis*. *Biological Bulletin* 132: 292–296, <http://dx.doi.org/10.2307/1539896>
- Young CM, Chia FS (1984) Microhabitat-associated variability in survival and growth of subtidal solitary ascidians during the first 21 days after settlement. *Marine Biology* 81: 61–68, <http://dx.doi.org/10.1007/BF00397626>