Aspects of the growth and reproductive ecology of the introduced ascidian Didemnum perlucidum (Monniot, 1983) in Western Australia

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Abstract

In 2010, the colonial ascidian Didemnum perlucidum (Monniot, 1983) was first recorded in Western Australia and is now widespread across the state. This species is classified as a target biosecurity species by the Department of Fisheries, Government of Western Australia. However, limited information on the biology of this ascidian is available for Australian waters. The aim of this study was to gather baseline information on the temporal variation in growth, survival, and reproduction of D. perlucidum. The mean average colony area was significantly higher in summer (29.7 cm²) decreasing towards winter (3.3 cm²). Colonies produced larvae year round with maximum production (1109.0 larvae cm⁻²) and highest recruitment (0.90 settlers cm⁻²) in summer. Larva density was positively correlated to salinity (r = 0.965) and chlorophyll a concentration (r = 0.958) in the water column. From a management point of view, the decrease in colony size, larvae production, and recruitment of D. perlucidum during winter could offer the best opportunity for the control or eradication of this ascidian at specific locations in the state.

Key words: sea squirt, invasive, reproduction, marine pest, growth, temporal patterns

Introduction

Ascidians are a key ecological group because of their ability to thrive in eutrophic environments, high capacity to colonise and overgrow natural and artificial substrates (such as floating pontoons, pilings, buoys, and vessel hulls) in protected harbours, and high invasive potential (Shenkar and Swalla 2011). Introductions of non-native colonial ascidians to natural and artificial substrates in tropical and temperate environments are now commonplace (Minchin and Sides 2006; Valentine et al. 2007; Lambot 2009; Lengyel et al. 2009). Once introduced, ascidians have the potential to negatively affect the biodiversity and ecological function of the recipient location (Dijkstra et al. 2007; Lengyel et al. 2009; Morris and Carman 2012) and may cause negative economic impacts. For example, heavy ascidian fouling in aquaculture facilities has been linked to a reduction in growth and increased mortality of cultivated shellfish (Rocha et al. 2009) and finfish (Tan et al. 2002). Despite several recorded negative impacts, the costs associated with ascidian biofouling have yet to be fully quantified.

Ascidians are sessile marine filter feeders with a short-lived, non-feeding, motile larva (Berrill 1951; Millar 1971). Most ascidians are hermaphrodites, live as solitary individuals or form colonies, and often display complex life history cycles. These cycles include phases of sexual and asexual reproduction and growth phenomena such as fission, fusion, and re-juvenescence as well as periods of recession and senescence (Turon and Becerro 1992). Reproduction and growth are affected by environmental factors (e.g. temperature and food availability); for example, populations from temperate locations often show large variation
between summer and winter months (Ribes et al. 1998). Some colonial ascidians concentrate growth and reproductive activity to the winter/autumn seasons (Turon 1988; Turon and Becerro 1992; Dijkstra et al. 2007; Ritzmann et al. 2009) while others, such as Didemnum vexillum Kott, 2002 (Fletcher et al. 2013) and Didemnum perlucidum Monniot, 1983 (Kremer et al. 2010; Smale and Childs 2011), show highest levels of reproduction and growth during summer months. It remains unclear, however, whether these seasonal patterns are consistent among different geographic locations and species.

Didemnum perlucidum is a tropical colonial ascidian (Didemnidae) that has been introduced to many locations worldwide (Culbertson and Harper 2000; Coles et al. 2002; Golbuu et al. 2005; Kremer 2008; Kremer et al. 2010). In Australia, despite extensive surveys of ascidian fauna being undertaken around the country by Kott (1985, 1990 a, b, 1992 a, b, 1997, 2001, 2005) and by McDonald (2005 a, b), this species had not been reported in Australian waters. In 2010, D. perlucidum was recorded for the first time in Australia from the Swan River Estuary, Perth, Western Australia (WA) (Smale and Childs 2011). Later in 2011, an unidentified “sponge look alike” organism was recorded heavily fouling a mussel farm in the Cockburn Sound in WA and subsequently identified as D. perlucidum.

The presence of D. perlucidum in WA is regarded as a significant threat to the shellfish aquaculture industry because of its ability to quickly smother mussels and oysters in aquaculture facilities as well as in natural habitats (Lambert 2009; Kremer 2010, 2011). Local anecdotal information suggests this ascidian can increase the mortality of farmed mussels and oysters possibly by outcompeting native fouling populations (Glenn Dibbin pers. comm.).

Despite multiple records of D. perlucidum introductions worldwide, most of the information gathered has related to its distribution, taxonomy, and morphological description (Monniot 1983; Monniot et al. 1991; Dias et al. 2008; Kremer 2010, 2011; Smale and Childs 2011; Bridgwood et al. 2014) while few studies have addressed its biology and ecology. In Western Australia, D. perlucidum is well established in marinas such as the Hillarys Boat Harbour, where it is often found colonising floating pontoons and pylons from the water line to a depth of 4 m. Preliminary observations have shown that in summer, colonies formed extensive mats covering areas of ~100 to 900 cm², contracting to a much smaller size in winter of ~1 to 25 cm² (Bridgwood et al. 2014). The aim of the present study was to gather baseline information on the temporal variation in colony growth, survival, and reproductive characteristics of Didemnum perlucidum in Western Australian waters.

Methodology

Study site

This research was carried out within the boundaries of the Hillarys Boat Harbour, Western Australia (31°49′30.70″S, 115°44′07.71″E). An area of 90 m² was selected and monitored over a period of one year from June 2012 to June 2013 (Figure 1).

Water quality

Water quality was measured monthly from June 2012 to June 2013 at a depth of 50 cm (n=39). Water temperature, salinity, pH, and dissolved oxygen were measured with a YSI® multiparameter sensor (YSI Pro Plus®, Perth Scientific, Perth, Australia) while water transparency was measured with a Secchi disc of 20 cm diameter. Chlorophyll a and pheophytin a analyses were performed on filtered seawater samples (5 L) and analysed with standard spectrophotometric methods (Strickland and Parsons 1972) at the Marine and Freshwater Research Laboratories (MAFRL), Murdoch University, Western Australia. Chlorophyll a and pheophytin a concentration was then used as an indirect measurement of food availability (plankton) in the water column.

Specimen collection, preservation and identification

Four different types of samples were used in this study: 1) marked colonies for determination of colony size, 2) samples collected for the reattachment experiment, 3) samples collected for reproductive analysis and colony thickness determination, and 4) new colonies settled on settlement plates. In all the cases, the identity of the samples suspected of being D. perlucidum was confirmed using molecular methods. This entailed collecting a subsample (~1 cm) preserved in absolute ethanol. All samples were barcoded by amplifying the cytochrome oxidase subunit I (COI) mitochondrial region using the COI primers following the methods developed by Stefaniak et al. (2009). The criteria for positive molecular identification was established when sequences had a 99–100% match with a sequence
Growth and reproductive ecology of *Didemnum perlucidum* in Western Australia

**Figure 1.** Map showing the relative location of Hillarys Boat Harbour, Western Australia. The study site within the harbour is indicated by a red rectangle, the location of settlement arrays is indicated with orange dots.

previously identified as *D. perlucidum* (Genbank accession JQ731735, Swan River, WA, identified by Gretchen Lambert, University of Washington).

**Colony area**

To evaluate changes in the area of *D. perlucidum*, colonies were permanently marked (n = 780) and observed over one year (June 2012–June 2013). Each marked colony was photographed on a monthly basis within a 10 cm × 10 cm quadrat. Selected photographs were standardised to a 700 × 700 pixel size, calibrated using a linear scale of 10 cm and analysed using the software ImageJ® (National Institute of Health, USA). The area of each *D. perlucidum* colony was calculated by using the freehand measuring tool to trace the perimeter of the colony. The total area occupied by *D. perlucidum* per quadrat was the sum of all colony areas measured in a photograph.

The thickness was measured to determine if this variable could be an indicator of growth for a colony. As colonies used for growth were permanently attached to the substrate, this variable was measured on colonies collected for reproductive analysis (Refer to Asexual and sexual reproduction section below). Colony thickness was measured by taking three replicate measurements from the centre of the colony with a digital calliper straight after collection and prior to preservation.

**Fragment reattachment**

A field experiment was undertaken to determine if *D. perlucidum* fragments could survive and reattach to another substratum after becoming detached from the parent colony. To test this, fragments (approx. 3 cm length) from ten colonies were placed into individual cages and
suspended vertically 20 cm above the seabed by a sub-surface float. The cages were constructed from plastic “gutter guard” mesh made of high density polyethylene plastic with a 5 mm mesh size. After ten days, the cages were retrieved and transferred to the laboratory. The total number of attached and unattached fragments was quantified by observing whether the colony fragments moved or floated from the cage when washed with seawater. If the fragment moved or floated it was considered unattached. This experiment was repeated on a seasonal basis (autumn, winter, spring, summer; n = 40).

Asexual and sexual reproduction

Samples of *D. perlucidum* colonies were collected seasonally and dissected to determine: 1) the abundance of eggs and larvae; 2) the presence of testes, ova and vegetative buds in the zooids; and 3) the maturity index of the colony. The latter was used as an indicative measure of reproductive output and recruitment as suggested by Fletcher et al. (2013).

Fifteen *D. perlucidum* colonies were collected each season for one year: winter (June 2012–August 2012), spring (September 2012–November 2012), summer (December 2012–February 2013) and autumn (March 2013–May 2013). After collection, a subsample of each colony was relaxed in seawater with menthol crystals then preserved in 4% formalin-seawater for morphological analysis. A 0.25 mm² section was removed from each colony sample and the total number of eggs and larvae counted. Additionally 10 zooids from each of the 15 colonies (150 zooids per season; n = 600) were dissected to quantify the presence or absence of visible ova and testes (sexual reproduction) and vegetative buds (asexual reproduction) in each zooid.

The maturity index (MI) of the colonies was calculated for each season using the equation of Fletcher et al. (2013):

\[
MI = \Sigma nF/N
\]

Where n is the number of colonies in stage F, F is the reproductive stage score (1 to 5), and N is the total number of colonies examined. Each colony was scored as follows: 1) absence of visible gonads; 2) presence of eggs in the abdomens; 3) presence of coiled testes; 4) presence of eggs in the matrix; and 5) presence of mature larvae (visible tail and ocelus) in matrix.

**Larval density and recruitment**

To determine whether larvae were settling and developing into new colonies, a settlement array was deployed at 1 m depth at two locations in the study area (Figure 1). Each array consisted of an aluminium frame with 4 arms each with 4 polyvinyl chloride plates (10 cm × 10 cm) resulting in a total of 16 plates per array. Each season (winter, spring, summer and autumn) the arrays were soaked for one month and then retrieved to collect the fouled plates. Collected plates were kept cool in seawater and transported to the laboratory where they were observed under a dissecting microscope to determine the presence of newly settled colonies suspected of being *D. perlucidum*. These colonies were removed and processed for molecular identification as previously described.

**Statistical analysis**

Data was tested for normality and homogeneity of variances to determine the most suitable analyses. SPSS V16 (IBM, USA) was used to perform the following analyses. Colony area was analysed using repeated measures ANOVA at 0.05 level to determine differences in average colony area across months. The Greenhouse-Geisser sphericity correction test was used to test for violations in sphericity in the data prior to running the repeated measures ANOVA. Difference in colony thickness between months was tested using a one way ANOVA. Tukey HSD *a posteriori* test was subsequently performed to determine specific differences in colony size and thickness between months.

The effect of seasons on larval densities in the colonies and on the number of new colonies on the settlement plates was determined using two independent ANOVA for each variable. Specific effects were tested using Tukey HDS *a posteriori* test at the 0.05 level.

To test the effect of season on the presence of eggs, testes, and vegetative buds a MANOVA (Pillai’s trace) analysis was conducted. Specific effects on each were tested using Tukey HDS *a posteriori* test at the 0.05 level. The actual P values obtained for all the tests are presented in the results section.

A Pearson’s correlation was used to determine if any correlation existed between water quality variables (temperature, salinity, pH, transparency, dissolved oxygen, chlorophyll *a* and pheophytin *a*) and colony size and larval density in the colony.
Growth and reproductive ecology of Didemnum perlucidum in Western Australia

Results

Water quality

The water quality in the study area varied across months (Figure 2). Mean water temperature ranged from 17 °C in August to 24.3 °C in January/February, salinity ranged from 33.4 in August to 36.4 in March, dissolved oxygen ranged from 4.5 mg L⁻¹ in February to 6.9 mg L⁻¹ in June while pH ranged from 7.2 to 8.18. Chlorophyll a (Chl a) ranged from 0.09 to 2.4 µg L⁻¹ while phaeophytin a ranged from 0.1 to 1.13 µg L⁻¹ and water transparency ranged from 2.9 to 5 m.

Colony area

The area of D. perlucidum colonies varied significantly (P = 0.001) between months (Figure 3). During colder months (Jul-Sep, ~17 °C), colonies underwent retraction and fragmentation and were significantly smaller than the colonies observed in the warmer months (Jan-Feb, ~24 °C). In September 2012, the area covered by the colonies was the smallest measured (3.2 cm²) increasing to an average area of 29.7 cm² by March 2013.

Colony thickness varied across months (Figure 3). Colonies were thinnest (1.2 mm) in November becoming significantly (P = 0.021) thicker towards the summer months in January (1.7 mm).

Fragment reattachment

D. perlucidum fragments were able to survive when detached from the parent colonies. In autumn, spring, and summer, 100% of the colonies were able to survive and attach to the plastic mesh of the cages while in winter 90% of the colonies survived and reattached.

Asexual and sexual reproduction

D. perlucidum reproduced sexually as indicated by the presence of ova and testes, and asexually via vegetative buds throughout the year. Results from the MANOVA analysis demonstrated a significant (P = 0.001) effect of season on these variables.

Dissected zooids contained ova, testes, and vegetative buds arising from the oesophageal region of the zooid. The density of ova, testes and buds varied seasonally. Zooids had a significantly greater number of testes in summer (7.5 testes zooid⁻¹) compared to winter (3.3 testes zooid⁻¹; P = 0.003) and the largest number of ova (5.2 ova zooid⁻¹; P = 0.002) and vegetative buds (2.5 buds zooid⁻¹; P = 0.011) in autumn (Figure 4).

The maturity index (MI) of the colonies observed in spring, summer and autumn was 1.0 with a slight decrease to 0.93 in winter.

Figure 2. Water quality recorded at the sampling site over a period of one year (June 2012 to June 2013, mean ± SE; n=39).
Figure 3. Mean colony size (n=780) and thickness (n=60) of *Didemnum perlucidum* over a period of one year (June 2012 to June 2013) at Hillarys Boat Harbour, Western Australia; bars indicate the SE of the mean.

Figure 4. Abundance of ova, testes and vegetative buds in *Didemnum perlucidum* zooids over a period of four seasons (2012–2013) (n=600; mean ± SE). Different letters indicate significant differences between seasons.

Figure 5. Larval development of *Didemnum perlucidum*. A. Cross section showing matrix with eggs and larvae; B. Morulla stage, C. Gastrula stage, D. Formation of tail with well-defined miomers. E-F. Fully developed larva. Photomicrographs by Department of Fisheries WA.
Growth and reproductive ecology of *Didemnum perlucidum* in Western Australia

**Table 1.** Temporal variation in larval density in *Didemnum perlucidum* colonies and density of settlers on settlement plates. Different letters indicate significant differences between seasons at 0.05 level.

<table>
<thead>
<tr>
<th></th>
<th>Winter</th>
<th>Spring</th>
<th>Summer</th>
<th>Autumn</th>
<th>df</th>
<th>MSS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larvae in colony</td>
<td>300 a</td>
<td>460 a</td>
<td>1109.0 c</td>
<td>953.3 d</td>
<td>3,52</td>
<td>201.0</td>
<td>3.11</td>
<td>0.034</td>
</tr>
<tr>
<td>Settlers on plates</td>
<td>0.00 a</td>
<td>0.00 a</td>
<td>0.90 b</td>
<td>0.10 c</td>
<td>3,45</td>
<td>184.3</td>
<td>65.2</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Table 2.** Pearson’s correlation coefficient (r) between water quality parameters, colony size and larval density, * denotes a correlation significant at 0.05 level.

<table>
<thead>
<tr>
<th></th>
<th>Larval density</th>
<th>Colony size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0.938</td>
<td>0.889</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.965*</td>
<td>0.978*</td>
</tr>
<tr>
<td>pH</td>
<td>-0.052</td>
<td>0.114</td>
</tr>
<tr>
<td>Dissolved Oxygen</td>
<td>-0.222</td>
<td>-0.104</td>
</tr>
<tr>
<td>Transparency</td>
<td>-0.842</td>
<td>-0.724</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>0.958*</td>
<td>0.892</td>
</tr>
<tr>
<td>Pheophytin a</td>
<td>0.766</td>
<td>0.689</td>
</tr>
</tbody>
</table>

**Larval density and recruitment**

The dissection of the colonies revealed the presence of different stages of eggs and larvae in the same colony, from eggs at morulla and gastrula stages to unhatched and fully developed larvae (Figure 5).

There was a significant (*P* = 0.03) difference in density of larvae in the colonies between seasons, increasing from winter (300 larvae cm⁻²) to a maximum density in summer (1109 larvae cm⁻²) (Table 1). Larval density was positively correlated to salinity and Chl *a* concentration in the seawater (Table 2). Colony size was also positively correlated to salinity (Table 2).

Recruitment was observed in summer and autumn when larvae settled on the arrays and developed into new colonies (settlers). In summer, up to 0.90 colonies cm⁻² were observed after only a one month soaking period. However, no recruitment was observed in winter and spring (Table 1). Initial weekly inspections on the plates revealed that many colonies were present on the settlement plates a few days after deployment but one month later, when the settlement arrays were retrieved from the water, the density of new settlers had decreased.

**Discussion**

*Didemnum perlucidum* colonies from Hillarys Boat Harbour, WA, reached peak colony area, larval density, and recruitment in summer and autumn and then decreased towards winter. This pattern is typical of many ascidians (Becerro and Turon 1992) including *D. perlucidum* from South America (Kremer et al. 2010). While this study reports on the temporal variation of *D. perlucidum* at a single location, these results likely are representative observations of other temperate populations in the state.

Water temperature is one of the main factors correlated to growth and reproduction of ascidians (Hirose et al. 2007). We observed a peak in growth and reproduction in the warmer seasons of summer and autumn. It was also during this time that we measured the highest concentrations of Chl *a*, which was positively correlated to higher larval densities. In temperate environments, food availability is highest during the summer due to an increase in phytoplankton growth, increased temperature and higher irradiance (Coma et al. 2000; Petersen 2006). The sex allocation theory suggests that sexual reproduction is associated with periods of higher food availability and water temperature (Newlon et al. 2003). For example, it has been observed that feeding levels affect the reproductive efforts of the colonial ascidian *Botryllus schlosseri* (Grosberg 1988). Lambert (2005) also found that the reproductive season in ascidians usually coincides with the period when phytoplankton productivity is highest. Similarly, in our study, *D. perlucidum*’s sexual and asexual reproduction peaked in summer when water temperature and Chl *a* concentration were highest. The higher larval density and colony size also coincided with the highest salinity at the study suite, which occurred in summer.

Colonies decreased in size and fragmented during the cool winter season, which also coincided with the period of lowest Chl *a* concentration; suggesting a reduction in food availability. Colony fragmentation caused by zooid regression and the subsequent decrease in colony size is common in ascidians, especially in didemnids (Ritzmann et al. 2009; Page et al. 2011). This is thought to be a rejuvenation process that extends the lifespan of the zooids, and the survival of the colony (Turon and Becerro 1992). For *D. perlucidum*...
colonies in WA, winter may represent a resting season for growth and reproduction when resources are scarce and energy budgets allow for only colony maintenance until the next active-growth season. Salinity was also at lowest in winter and colonial ascidians are often negatively affected by low salinity (Lambert 2005; Fletcher et al. 2013). Salinity variation may affect reproduction in ascidians, such as recruitment (Fletcher et al. 2013) and metamorphosis (Vázquez and Young 2000), and may also influence the reproductive patterns of \( D. \) perlucidum. In our study, new settlers were not observed on settlement plates in winter, consistent the low larval density in the colonies. It is possible that larval production or survival during this period was so low that the presence of new settlers was not detected on our plates. Fletcher et al. (2013) found similar results for Didemnum vexillum, where low recruitment was explained as lack of detection capability rather than absence of new settlers.

As was observed for \( D. \) perlucidum in Brazil (Kremer et al. 2010), larvae in different developmental stages were observed year round. The presence of zooids with ova and testes year round would explain the continuous presence of fully developed larvae in the colonies (indicated by the high MI). Continuous asexual reproduction also was observed. While larvae production favours the formation of new colonies, vegetative budding provides the means for colony growth and expansion even when resources are being allocated to sexual reproduction. However, the \( D. \) perlucidum life cycle and the environmental factors affecting it remain poorly understood require further investigation.

Our study showed that there were considerably more zooids containing testes than ova. Similar results were observed by Dias et al. (2008) who found that \( D. \) perlucidum zooids had fewer ova when under the effect of competition. In hermaphroditic organisms, an emphasis on male reproduction is commonly observed in nutrient depleted situations and when organisms are found in high densities; both leading to greater competition for space and resources. This can be explained in terms of the greater energy investment required for production of female gametes than is required for the production of male gametes (Newlon et al. 2003; Dias et al. 2008). Western Australian waters are known to be oligotrophic in nature which can lead to competition for food (Smale and Childs 2011). While competition for food may be affecting \( D. \) perlucidum colonies and favouring male gamete production, this question needs further work to confirm the mechanism.

\( D. \) perlucidum fragments were shown to survive and capable of quickly reattaching and growing on a new artificial substrate. It is well known that fragments of colonial ascidians are capable of reattachment and further that enlargement of a colony is hastened by asexual budding (Stoner 1989; Fletcher and Forrest 2011). This suggests that \( D. \) perlucidum colony growth through vegetative budding may be an important process in the colonization of new substrates, and explains the presence of vegetative buds year round. The capacity for survival and reattachment may also be influenced by environmental factors such as water temperature. In our study we observed that reattachment of \( D. \) perlucidum is slightly decreased in winter (90% reattachment). Carman et al. (2014) found similar results for \( D. \) vexillum, where fragment reattachment success declined with during periods of low water temperature.

Knowledge of the temporal and spatial patterns of growth and reproduction of marine fouling organisms is of particular importance in formulating effective management strategies. This paper provides baseline information on the biology and ecology of \( D. \) perlucidum in Western Australia. From a management perspective it is possible that this species may be overlooked if sampling occurs only in winter when colonies undergo a significant reduction in colony size and fragmentation. The reduction in colony size and recruitment during the colder months also indicates that winter could be the most appropriate period to control this species if management is being considered. However, additional information on the biology of this species and other management strategies such as control of source population, larval dispersal, and ongoing human mediated introduction need to be considered to avoid reinfection events.

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273


