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The role of true viviparity in *Amphibolis antarctica*: implications for the genetic diversity in the context of seagrass restoration

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Amphibolis antarctica (Labill.) Asch. produces large, well-developed viviparous seedlings, raising a fundamental question: Are these seedlings the result of sexual or asexual reproduction? This distinction carries significant implications for understanding dispersal, genetic diversity, and ecosystem resilience. Here, we present our observations on population structure, the development of embryos, morphological features of *A. antarctica* and testing genetic variability using new microsatellite markers, leading us to conclude that there is sexual reproduction in this species. While direct evidence of zygote fusion is lacking in the species, the application of new methods for detecting genetic diversity data suggest the presence of genetic variability allowing inference that there is no obligate asexual production of seedlings. We further evaluate the effectiveness of *Amphibolis* seedlings within the broader context of seagrass reproductive strategies. We argue that understanding whether reproduction is sexual or asexual is critical: asexual reproduction implies clonal dispersal, favoring spatial colonization, while sexual reproduction supports gene flow and long-distance dispersal. We review existing observational data, placing *Amphibolis* at the extreme end of the spectrum of mobile propagule dispersal and how this strategy may optimize recruitment success in marine environments. Our findings underscore the necessity of recognizing sexual reproduction and wide-scale genetic dispersal as pivotal components of effective seagrass conservation.

KEYWORDS
***Amphibolis antarctica*, hydrophilous pollination, pollen germination, reproduction, seagrass, restoration**

Introduction

The long-term survival of aquatic plants relies on an effective dispersal of seed or other propagules (McMahon et al., 2014). Seagrasses, along with a number of other aquatic plant species, possess unusually wide geographic ranges (Waycott and Les, 1996, Les et al., 2003). These wide-spread species distributions are ascribed to efficient dispersal mechanisms and effective long-distance dispersal (Les et al., 2003). Improvements to understanding the pattern and process of dispersal have come from investigating population connectivity using

indirect measures such as gene flow (Kinlan and Gaines, 2003). As connectivity plays a significant role in maintaining population size and biodiversity it is the final component in determining the overall effectiveness of sexual reproduction (Wang and Smith, 2002).

Migration and subsequent recruitment in aquatic environments, particularly marine environments, is reliant on hydrological processes that dictate the scale of connectivity such as has been described for seagrasses (Orth et al., 2006; Kendrick et al., 2012). Dispersal of both pollen and propagules is dependent on water movement and plant-flow interactions as well as the biological and physical characteristics of the propagule(s) itself (Ackerman, 1995; Verduin and Backhaus, 2000). Long distance dispersal will be principally derived from the movement of propagules (seeds, seedlings, vegetative fragments) where there are strategies for such movement through adaptive traits such as floating fruits (Kendrick et al., 2012; McMahon et al., 2014). Connectivity may be measured where there are measurable levels of genetic diversity among populations estimated from the proportion of shared traits (e.g. alleles) among populations (Lowe and Allendorf, 2010; McMahon et al., 2018).

Seagrasses have the ability to survive as clonal plants (e.g. Waycott and Les, 1996, Arnaud-Haond et al., 2012) and through sexual reproduction (Orth et al., 2006). Mature seagrass habitats, sometimes also known as seagrass meadows, are usually comprised of numerous individual plants that are both sexual and clonal in origin (Waycott, 1998; Reusch et al., 1999; Procaccini et al., 2001; Billingham et al., 2003; Olsen et al., 2004; Waycott et al., 2006). Meadow formation would begin with the establishment of seeds or seedlings then expand via rhizome extension to colonize larger areas (Marba and Duarte, 1998). The relative importance of long-distance dispersal is therefore most likely to be critical when new locations are being colonized. The maintenance of genetic diversity is important to the long-term evolutionary potential of species and confers advantages including improved survivorship under stressful conditions (Ehlers et al., 2008; Hughes and Stachowicz, 2004, 2011, Reynolds et al., 2012).

When sexually reproducing, seagrasses produce flowers and transfer pollen from the male flower to the ovary of the female flower setting seed (Ducker et al., 1978). This is a highly evolved trait as hydrophilous pollination is rare across flowering plants (Les et al., 1997). Most seagrass species are dioecious and produce flowers of a single sex on each individual plant, so there are separate male and female plants (Waycott et al., 2006). This means that for dioecious species, seed set in seagrasses means delivering pollen between separate plants (Waycott et al., 2006). Seagrass pollen is among the largest for all angiosperms and is modified for water pollination in its shape, buoyancy characteristics, and size (Ducker et al., 1978; Cox and Knox, 1988). Despite differences in size and shape of pollen, pollen is transported as aggregated filaments (Figure 1) rather than individual pollen grains in submerged pollinated species of seagrass such as *Thalassia* and *Halophila* (Ackerman, 1995), *Posidonia* (Smith and Walker, 2002) and *Amphibolis* (Verduin, 1996a).

In many seagrasses, pollination appears to occur predominantly at a local scale within meadows as most pollen is trapped within the canopy when released (Verduin et al., 2000; Verduin and Backhaus, 2000; Verduin et al., 2002; Backhaus and Verduin, 2008). Given that clone sizes can be extensive, the capture of pollen within the meadow is unlikely to be restrictive, as individual clones are capable of interacting with a broad range of others. In species that release pollen above the canopy, the potential for long-distance dispersal is enhanced but limited by the viability of the pollen. The few studies on the viability of seagrass pollen report viable pollen lasting from several hours for *Zostera* (Cox et al., 1992) to 50% reduction in viability after 18h for *Posidonia australis* and 36h for *Posidonia sinuosa* (Smith and Walker, 2002) and 24h for *Amphibolis antarctica* (Verduin, 1996a). The spatial scale of pollen dispersal processes will remain relatively small when compared to the distribution of species ranges, thus the major contribution to connectivity will be propagule movement. Interestingly genetic analyses have revealed high outcrossing rates in a number of seagrasses, indicating the effectiveness of pollination in natural populations (Waycott et al., 2006; McMahon et al., 2018).

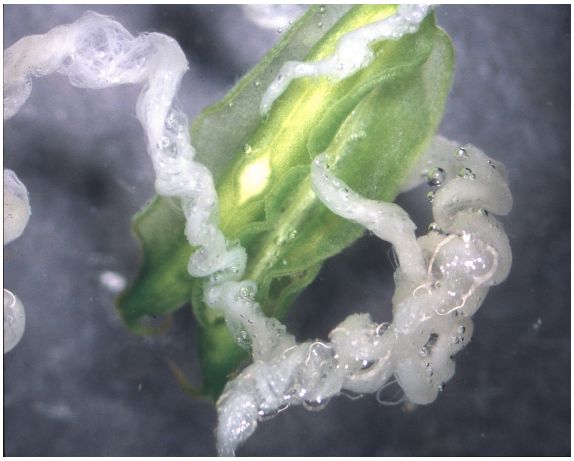

FIGURE 1
Amphibolis antarctica pollen and pollen sack; 1 cm = 1 mm.

Seagrasses invest significant effort in sexual reproduction producing seeds and seedlings such as *Amphibolis antarctica* (Labill.) Asch. (commonly known as wireweed or sea nymph) (Kendrick et al., 2012). *Amphibolis* seedlings (Figure 2) have a high capacity for long-distance dispersal that enables them to potentially colonize distant new locations. Although the frequency, extent, influences and consequences of long-distance dispersal are still poorly understood for many seagrass species, recent assessments of range-wide population genetic variation in widespread seagrass species have revealed extensive dispersal at scales much larger than was previously thought possible (McMahon et al., 2014; van Dijk et al., 2018). *Amphibolis antarctica* is a dominant seagrass across the more exposed reef environments of southern and south-western Australia (Carruthers et al., 2007; Kilminster et al., 2018) and is an ecologically significant Australian endemic seagrass. The viviparous seedlings of *Amphibolis* remain attached to the mother plant for a significant period (ca. seven months to a year (Ducker et al., 1977; Kuo and Kirkman, 1990; Verduin, 1996a). This large investment in the development of "offspring" indicates these seedlings are important to the reproductive strategy of this plant, and indeed the genus as the sister species *A. griffithii* also produces viviparous seedlings similar in structure (Den Hartog, 1970).

Despite many studies on the physiology, inflorescence development and pollen-stigma interaction of *A. antarctica* (Ducker et al., 1978; Pettitt et al., 1981, 1983; Pettitt, 1984; Cox and Knox, 1988; Cox, 1991; Ackerman, 2002; 2006), the status of sexual reproduction and overall reproductive strategy of this species remains obscure. Den Hartog (1970) described *A. antarctica* as having fertilization but gave no evidence. Cox and Knox (1988) observed surface pollination in intertidal populations of *A. antarctica*, but found neutrally buoyant as well as floating pollen, noting for populations at greater depths: "pollination, if it occurs at all, must of necessity be submarine". *Amphibolis antarctica* is known to grow and flower at considerable depths (Ducker et al., 1978; Cambridge, 1979; Walker, pers. obs. 1982, 1995; Verduin et al., 1996; Verduin, 1996b) and we propose the occurrence of three-dimensional submarine fertilization is therefore more likely. However, preliminary studies using allozymes to establish genetic variability in *A. antarctica* (Waycott et al., 1996) showed no variability and although these markers are known to be limited in their ability to detect individual level genetic variability, they provide no additional evidence to confirm seedlings are sexually derived. Recent microsatellite studies reveal significant genetic diversity both within and between *A. antarctica* populations (van Dijk et al., 2018).

Improved methods in combining empirical data and modelling can facilitate predictions of recruitment through connectivity resulting from propagule dispersal (e.g. Grech et al., 2016). Such models rely on detailed understanding of the biological and hydrological interactions of seagrass dispersal unit, and the efficacy of predictions are dependent on (Grech et al., 2016). Such detailed modelling can significantly improve our understanding of the complex relationship between potential population recovery in some seagrass species where dispersal distances and the interactions with complex coastal topography and propagule dispersal properties have been able to be aligned (Grech et al., 2016; Evans et al., 2021).

We describe the plant morphology and flowering biology of two *A. antarctica* populations in a southwest coastal area of Western Australia. Observations of *in situ* submarine pollination and resulting germination of pollen are presented, as well as subsequent embryonic developmental stages, leading to successful sexual reproduction in *A. antarctica*. This study was conducted to determine whether *in situ* pollen capture resulted in fertilization or whether *A. antarctica* forms viviparous seedlings via apomixes. We provide evidence that seedlings are in fact sexually derived and discuss the implications of this to the reproductive strategies of this important seagrass.

Methods

Shoots of *A. antarctica* were collected using SCUBA from two populations off the coast of south–west Western Australia located at 32° 16' S, 115° 41' E and 32° 19' S, 115° 43' E. To characterize shoot and canopy structure, haphazard selections of 100 male and 100 female *A. antarctica* shoots were collected using SCUBA weekly during October and November (Australian spring) when pollination events in *A. antarctica* occurred. Morphology of shoots was recorded as total length, number and position of leaf clusters and number and position of male and female flowers per shoot. Position of flowers and leaf clusters were measured as numbers per 5 cm interval along the length of the shoot. Sex ratios were expressed as the percentage of male and female shoots (plants) from a total of n=400 shoots for each site based on the collections.

The flowering biology of *A. antarctica* was studied over a period of two flowering seasons. One hundred male and 100 female shoots were collected weekly during October and November with further monthly collections, of female shoots only, until May. Timing and duration of pollen release, pollination and onset of viviparous seedling formation were established from these collections and *in situ* observations. Mature female flowers were collected during and after the pollination events in November and December. These mature female inflorescences were preserved in 2.5% glutaraldehyde, 0.05 M PO4 buffer pH 7 (n=1200).

To test whether female plants would set seed in the absence of male plants, ten immature (initiating) female shoots were collected at 32° 16' S, 115° 41' E in September. These shoots were transplanted to an aerated seawater tank and their root system kept buried under sand. Temperature was increased in three stages from 18 to 20 °C over a period of two months to resemble field conditions. Natural light conditions (ca. 200 μmol quanta m-2 s-1) were mimicked by 12-hour light-dark cycles provided by fluorescent lights. A further tank containing both male and female shoots (collected in October) was kept under the same conditions.

Fresh inflorescences from the study sites were examined for the presence of pollen on stigmas with an Environmental Scanning Electron Microscope (ESEM) at 15 kV. Initially, pollinated inflorescences were prepared for freeze-microtome sectioning. Specimens were allowed to harden in a glycerol-alcohol preservative, fitted onto a freeze-microtome (Mectron [Frigistor] Ltd.) with Hamilton's freezing microtome solution. Sections, ca. 25 μm thick, were cut with razor blades and stained with Toluidine Blue for light microscopy examination (Feder and O'Brien, 1968 cited in: O'Brien and McCully, 1981). Further inflorescences were prepared for infiltration and embedding in Glycol Methacrylate [GMA] (Feder and O'Brien, 1968 in: O'Brien and McCully, 1981). GMA sections, 3-5 μm thick, were obtained by glass knife microtomy (Porter-Blum ultra-microtome, JB-4).

Stigmas, preserved in 2.5% glutaraldehyde, were examined for presence of pollen tubes. The stigmas were softened by heating in an 8 N sodium hydroxide (NaOH) solution at 60 °C for one hour (Martin, 1959). Prior to softening, longitudinal incisions were made in the style to allow penetration of NaOH. Stigmas were squashed on microscope slides and stained according to standard fluorescence microscopy techniques (Martin, 1959). Specimen sections were examined and photographed using a Zeiss Axiophot transmitted light and incident light fluorescence photomicroscope.

Fresh male inflorescences (n = 40) were measured including the forked appendages of the anthers. The appendages, averaging a length of 2.1 ± 1.0 SE mm, have not been considered for the calculation of the number of pollen within the anthers.

The number of pollen grains per male flower was estimated by dividing the volume of a hemispherical cylinder [the assumed geometric structure of the male anthers divided by the theoretical

volume of an individual filiform pollen grain, 3000 μm long and 20 μm in diameter (Ducker et al., 1977; McConchie and Knox, 1989a)]. The theoretical radius was taken as 10 μm.

Data were analyzed for the effects of sex (male, female), year site and position of flowers on length of shoots, number of flowers and ratio of number of flowers to clusters. Cramer-von Mises and Watson test statistics were used to show normal distribution of the data. Analyses of variance were undertaken using regression analysis for an unbalanced design for all variables tested. A generalized linear model of GENSTAT 5 (Developed by: Payne and Rothamsted Experimental Station, 1994) was adopted to analyze data on the position of mean number of flowers as these data were highly skewed. The data were log- transformed and normal distribution was achieved accordingly. Ratio of flowers to leaf clusters was expressed as a percentage of the number of flowers per leaf cluster.

Results

Female inflorescences of *Amphibolis antarctica* consisted of two ovaries located on a common stalk. Each carpel included a single ovule, and it continued as a short style, which extended into three stigmas. Generally, only one ovule formed a seedling from each pair. Only in 1.25% of inflorescences studied (n = 1200) were both ovules observed to begin the initial stages of seedling formation. Female plants kept in isolation under controlled conditions, although exhibiting healthy somatic growth over the two-month period did not show any evidence of formation of seedlings. These female plants were kept under controlled conditions, which may have affected the growth rate of flowers. However, seedling formation was observed under controlled conditions where both male and female plants were present. However, these plants were collected at a more advanced stage of floral development than those in the male exclusion experiment and so the comparison is not identical.

Fluorescence microscopy examination of mature female inflorescences (Figure 3A) collected during November and December [post-pollination event] revealed several pollen tubes in the style just before entry into the ovary (Figure 3B). A single ovule with a pollen tube within the stigma (Figure 3C) shows the fluorescent lining of the embryo sac, possibly hypostase material. Along the outside of the ovary, a further area of fluorescence represents phloem and possibly some pollen tubes. Callose of pollen tubes can be seen as thickened parts of fluorescent stained pollen tubes in the style (Figures 3B, C). The callose of pollen tubes in *A. antarctica* was confined to localized, but closely spaced, plugs.

Light microscopy of mature inflorescences revealed several stages of globular embryos within the embryo sac (Figures 3D–F). The earliest stage of post-fertilization found in the studied specimens consisted of what appeared to be collapsed synergids at the micropylar end of the embryo sac (Figure 3D). Within the middle of the embryo sac two nuclei, possibly polar nuclei, can be seen (Figure 3D). The nucellus showed invaginations within the surrounding ovule. Remnants of a micropyle, stained dark purple with Toluidine blue, were present between the inner and outer integument formations Figure 3E.

Shoot length varied significantly among sites and years but did not differ between sexes (Table 1). Unbalanced ANOVA showed no main effect of sex on shoot length (p = 0.249), whereas significant differences were detected between sites (p = 0.027) and between years (p = 0.019). A significant sex × site interaction (p = 0.021) indicated that differences in shoot length between male and female plants were site dependent, while the sex × year interaction was not significant (p = 0.825).

The number of male flowers per shoot was higher than the number of female flowers per shoot (Table 2 (p= <0.001). Male to female flower ratios per shoot varied from 1.5:1 to 2:1. The populations at both collection sites were male biased with an overall ratio of 3.7:1 (male to female plants).

Highest concentrations of male and female flowers occurred between 30 and 45 cm from the base of the shoot. The mean number of female flowers was less than the mean number of male flowers, but a similar pattern of distribution was apparent. Most male flowers occurred between 25 and 40 cm from the base of the shoot. The location of highest number of flowers coincided with the leaf area distribution along shoots of *A. antarctica*. The data for the position of number of flowers along the shoot were highly skewed. The position term was significant with a substantial difference in the number of male and female flowers for each of the 14 defined positions on a shoot.

The ratio of flowers to leaf clusters differed significantly between sexes and between sites (Table 3). The male flower to leaf cluster ratio varied from 17.9 to 35.7%, whereas the female flower to leaf cluster varied from 32 to 34.8% between sites. These differences were significant between male and female plants (p = 0.03), between sites (p = 0.002) and the site x sex interaction was significant (Table 3, (p = 0.005)).

FIGURE 2
Amphibolis antarctica seedling and anchoring "comb".

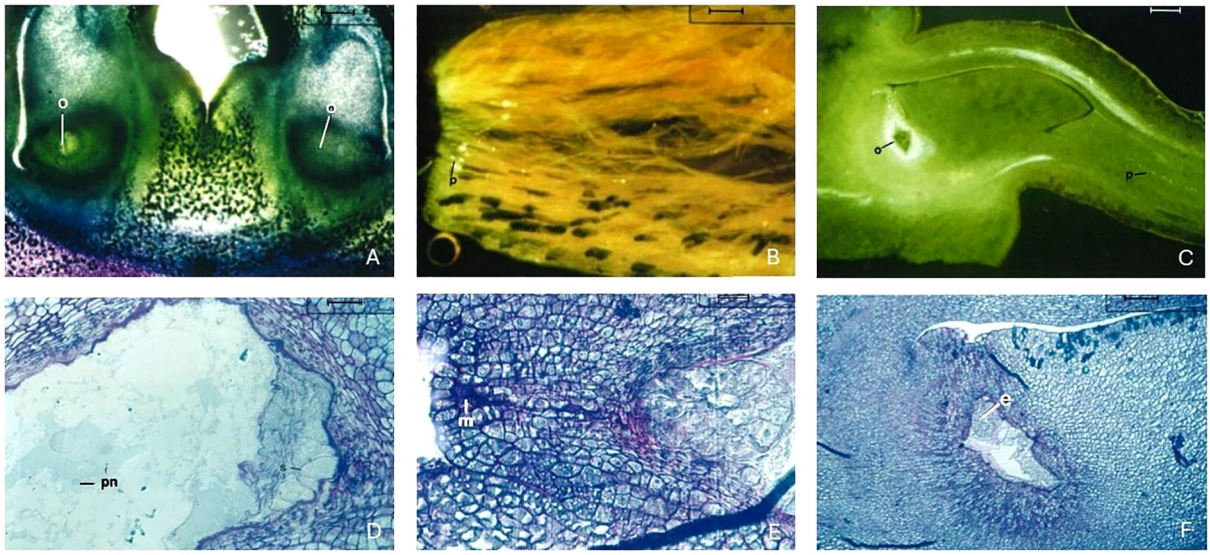

FIGURE 3
(A) Longitudinal section of a female Amphibolis antarctica inflorescence, stained with Toluidine Blue; ovules (o). Scale bar = 25 μm. **(B)** Longitudinal section of an *Amphibolis antarctica* stigma showing pollen tubes (p). Section stained with Aniline Blue. Scale bar = 10 μm. **(C)** Longitudinal section of a female *Amphibolis antarctica* carpel showing pollen tube (p), ovule (o). Section stained with Aniline Blue. Scale bar = 25 μm. **(D)** Longitudinal section of an *Amphibolis antarctica* ovary showing synergids (s) comprising the egg apparatus, and polar nuclei (pn), stained with Toluidine Blue. Scale bar = 25 μm. **(E)** Longitudinal section of an *Amphibolis antarctica* ovary showing remnants of a micropyle (m), stained dark purple with Toluidine Blue. Scale bar = 25 μm. **(F)** Longitudinal section of developing embryo (e) in *Amphibolis antarctica.* Section stained with Toluidine Blue. Scale bar = 100 μm.

Pollen release events and subsequent monitoring of seedling formation showed that all male flowers had released their pollen within 60 days of initial pollen release. Male shoots collected had scars where the male flowers had been attached to the pedicel, allowing male shoots to be identified as post dehiscence providing an indication of the rate of anther dehiscence. From 50 days after pollination event initial stages of seedling formation were observed. Seedling success rate was expressed as a percentage of the mean number of seedlings per shoot versus mean number of female flowers. Seventy percent of the flowers resulted in seedling formation.

The anthers of *A. antarctica* were 9.1 ± 1.1 SE mm in length, 2.3 ± 0.9 SE mm in diameter, with a radius of 1.14 ± 0.5 SE mm and a volume of 27.1 ± 7.7 SE mm^3, leading to a number of pollen grains of 27,128. Resulting pollen to ovule ratio was 13,564:1. Male to female flower ratio as well as male to female sho243ot (sex) ratio gave a pollen to ovule ratio of 75,000 to >100,000:1.

Discussion

An important component of sexual reproductive success is the reliability of pollination followed by fertilization resulting in seed or, as in the case of the viviparous *Amphibolis antarctica*, a seedling. This study demonstrates that *A. antarctica* produces sexually derived viviparous seedlings, rather than clonal propagules, with important consequences for population connectivity and restoration outcomes. A first indication that *A. antarctica* may reproduce sexually is the large number of pollen grains it produces. Evidence

TABLE 1 ANOVA table for analysis of an unbalanced design using GENSTAT 5 regression to compare length depending on sex, site and year.

Source of variation	*df*	*ss*	*ms*	*F pr*
Sex	1	126.42	126.42	0.249 *ns*
Site	1	469.42	469.42	0.027 *
sex × site	1	508.42	508.42	0.021 *
Year	1	528.87	528.87	0.019 *
sex × year	1	4.66	4.66	0.825 *
Residual	326	30925.12	94.86	
Total	331	32562.92	98.38	

Where *df* is degrees or freedom, *ss* is sum of squares, *ms* is mean squares, *Fpr* is probability. * indicates significance at 5%. ns, not significant.

TABLE 2 ANOVA table for analysis of an unbalanced design using GENSTAT 5 regression to compare number of flowers (log transformed) depending on sex, site and year.

Source of variation	*df*	*ss*	*ms*	*F pr*
Sex	1	3.59854	3.59854	<.001 ***
Site	1	0.31310	0.31310	0.065 *
sex × site	1	0.00881	0.00881	0.756 *ns*
Year	1	1.49142	1.49142	<.001 ***
sex × year	1	0.09791	0.09791	0.301 *ns*
Residual	326	29.68780	0.09107	
Total	311	35.19758	0.10634	

*, *** indicate significance at 5% and 0.1% respectively. ns, not significant.

TABLE 3 ANOVA table for analysis of an unbalanced design using GENSTAT 5 regression to compare ratio flowers to clusters depending on sex and site.

Source of variation	*df*	*ss*	*ms*	*F pr*
Sex	1	3043.9	3043.9	0.002 **
Site	1	1500.6	1500.6	0.028 *
sex × site	1	2483.6	2483.6	0.005 **
Residual	244	75361.0	308.9	
Total	247	82389.1	333.6	

*, ** indicate significance at 5% and 1% respectively.

of pollination and pollen tube formation under controlled conditions in *A. antarctica* emerged from studies on pollen-stigma interactions by Ducker et al. (1978); Pettitt et al. (1983) and McConchie and Knox (1989a, 1989b). The general sequence of pollen capture, germination and tube penetration for *A. antarctica* is typical of normal pollination in terrestrial plants (Heslop-Harrison, 1972).

The populations studied had male biased sex ratio. Generally, in apomictic dioecious plant species, very few male plants are present (Asker and Jerling, 1992; Waycott et al., 1996). The *Amphibolis* populations studied exhibited high pollen production in relation to number of female flowers, a phenomenon assumed to overcome the loss associated with three-dimensional dispersal (Faegri and van der Pijl, 1979). In terrestrial, wind pollinated plants a mere 2% of the total number of released pollen grains may result in successful pollination (Faegri and van der Pijl, 1979).

Pollinated female inflorescences generally resulted in formation of one seedling per inflorescence, despite the presence of two ovules. In certain terrestrial plants the abortion of one of the ovules expands the time window in which one of the ovules may be fertilised (Richards, 1986). These attributes of *A. antarctica* are all indicators of the likelihood of sexual reproduction and suggest that these plants are sexual.

The phasing of the pollen release can be attributed to the variability of female flower maturation over time. On any one female plant, both mature and immature flowers occur. On examination of female plants after the final pollen release, i.e. when 95% of pollen had been released, 98% of all female flowers were mature (Verduin et al., 1996). This, in turn, would maximize the probability of the success of pollen, and therefore of pollination. This possibility is supported by the production of many fruits on a single plant.

Examination of post-fertilization stages in *A. antarctica* in this study showed evidence of basal cells at the micropylar end of a mature embryo sac and may represent the initial stages of formation of a footing for the embryo. Later stages of embryonic development showed that embryos occurred in the basal part of the embryo sac. Observation of possible synergids, comprising the egg apparatus, and two polar nuclei, indicated that the embryo may have been derived from the egg apparatus. Pollination is necessary for the formation of endosperm, which functions as nutritive tissue (Asker and Jerling, 1992). Position of globular embryos and remnants of a pollen tube entering the embryo sac would suggest that embryos, as seen in later stages of development, were sexually derived. Haustoria were observed which serve as a pathway through the hypostase.

The pollen to ovule ratio for *A. antarctica* was estimated at 13,500:1, an estimate close to values for *Zostera marina* (10,000:1; based on a staminate flower to carpellate flower comparison) quoted by Ackerman (1993). *Zostera marina* is a well-studied seagrass species with respect to pollen dispersal and pollination efficiency (Ackerman, 2002). However, as *A. antarctica* and *Z. marina* differ in morphology, life history and habitat, comparison allows the reproductive traits of *A. antarctica* to be placed within a broader seagrass context. Multiplying the male flower to female flower ratio of 1.65:1 (M:F) with the male to female sex ratio of 3.7:1, this can be translated into an approximate pollen to ovule ratio of > 80,000:1. Estimated values of pollen to ovule ratio provide population and pollination efficiency (Richards, 1986; Faegri and van der Pijl, 1979). Les (1988) suggested that the three-dimensionality of hydrophily leads to high pollen wastage, although a higher efficiency may be attained in shallow water where pollen loads may concentrate.

The estimated pollen to ovule ratio for *A. antarctica* is consistent with estimates in wind pollinated species [>100 000: 1] (e.g. Pohl, 1937; Richards, 1986). Female flowers of *A. antarctica* began to develop into viviparous seedlings soon after fertilization. The number of seedlings per shoot stabilized around mid-January. This suggests that seedlings formed by then may have reached a size sufficient for the full term of their maternal attachment phase.

Since fertilization may be defined as the fundamental part of sexual reproduction involving the fusion of haploid male and female gametes to form a diploid zygote, this study has not conclusively established that *A. antarctica* is a sexual species as such a fusion of gametes has not been observed. Nevertheless, the observations presented here on population structure, the initial evidence from developing embryos, as well as the morphological features of *A. antarctica*, do indicate sexual reproduction in this species. Furthermore, a study of hydrodynamic interactions, predicted probabilities of capture (Verduin and Backhaus, 2000) suggest that submarine pollination of *A. antarctica* is functionally and biologically significant. Sexual reproduction enables gene flow among meadows, which is fundamental for maintaining genetic diversity at both local and regional scales.

Plant-flow interactions may have more general implications regarding the biology of seagrasses, particularly *A. antarctica*. For example, does the observed pollination ecology of *A. antarctica* arise from an adaptation (in the environmental sense) to its hydrodynamic habitat or has it evolved in response to selection for outcrossing as a dioecious species? The outcrossing model (Charlesworth and Charlesworth, 1978) would infer that, if *A. antarctica* evolved as a response to selection for outcrossing, genetic variability would be high as *A. antarctica* is a dioecious species. A study on genetic variability of *A. antarctica* showed that this species appears to be genetically uniform (Waycott et al., 1996). Recent studies using microsatellites have revealed significant genetic diversity within and among *A. antarctica* meadows (van Dijk et al., 2018). Among the relatively small sample set analyzed by van Dijk et al. (2018) clonality varied among sites and some populations had high genotypic diversity. This supports the inference that

Amphibolis antarctica, likely, exhibits sexual reproduction, i.e. seedlings are derived sexually. Why might this species be genetically invariable using allozyme markers? Waycott et al. (1996) suggest that the methods used did not allow for identification of genotypic differences or that inbreeding may have caused *A. antarctica* to lose diversity. While pollen dispersal in *A. antarctica* is largely constrained to within-meadow scales (Verduin et al., 2002) due to hydrodynamic trapping and limited pollen viability, this study highlights that long-distance connectivity is primarily achieved through dispersal of viviparous seedlings. This might be inferred from mating system studies in *Posidonia australis* where Waycott and Sampson (1997) identified most pollen genotypes within the local meadow, although occasional migrants occur in a similar hydrodynamic environment.

The filiform shape of *A. antarctica* pollen would appear to provide a significant mechanical advantage in a highly dynamic habitat as the pollen was not only sticky, but it also wrapped around the stigmatic area presented by female flowers during anthesis. This observation was confirmed by studies on the efficiency of filiform pollen of submarine pollinated seagrass species (Cox et al., 1991; Ackerman, 1995). In *Z. marina*, it has been estimated that 95% of pollen falls within 15 m of its source, and successful pollination is directly related to flower density; in *Posidonia*, mean pollen dispersal distances of around 30 m have been inferred; and in *Thalassia*, pollen dispersal is less than 1 m (Ruckelshaus, 1996; Reusch, 2003; Van Tussenbroek et al., 2012; Sinclair et al., 2014).

Dye studies in *A. antarctica* meadows indicate pollination is likely to occur within the meadow (Verduin et al., 1996, 2002). Capture probability is a function of plant-flow interactions (Verduin and Backhaus, 2000; Verduin et al., 2002; Backhaus and Verduin, 2008), including the spatial location and orientation of the reproductive organs, which is critical in submarine pollination (Ackerman, 2006). Flowers of *A. antarctica* in the highly dynamic swell dominated study area occur at 30–40 cm above the seabed coexistent with the highest occurrence of leaves, thus increasing the capture probability by creating a larger trapping area (Verduin and Backhaus, 2000). Backhaus and Verduin (2008) found the highest capture probability within the meadow associated with the local hydrodynamic current direction. This, and the proximity of male to female plants in a male biased way would substantiate that *Amphibolis* has invested in pollination to be successful as a population continuation persistence mechanism.

Once pollen gets dispersed out of the canopy, its further dispersal would be governed by turbulent diffusion and advection due to Stokes Drift. In this case the seagrass plants will no longer play an active role in dispersal. This comprises the far field dispersal of pollen which may eventually carry pollen away from a meadow. From hydrodynamic and model studies we know that a slow onshore drift superimposed on periodic wave motions are present at the study sites. Magnitudes of velocities were approximately 1 to 2cms^{-1} (Verduin and Backhaus, 2000; Backhaus and Verduin, 2008). This flow or 'Stokes Drift' (Pond and Pickard, 1983; Denny, 1988) is caused by non-linearities of wave dynamics, for instance by shoaling or breaking of waves. This flow is characterized by much longer time scales than the wave period and, in contrast to linear wave dynamics, accounts for a net transport of particles (Kundu, 1990), thus allowing a smaller proportion of viable pollen to be transported further away.

A net flow of 2cms^{-1}, as experienced along the coast off Western Australia (Verduin, 1996a) may carry particles over a distance of about 70 m within one hour. Depending on the viability of pollen, which ranges from several hours for *Zostera* (Cox et al., 1992) to 36 hours for *Posidonia* (Smith and Walker, 2002) pollen can potentially be transported by this Stokes Drift and fertilize populations further afield ranging from several hundred meters to several kilometers. Preliminary studies of the viability of *A. antarctica* pollen indicated survival from a few hours (Ducker et al., 1978) to at least 24 hours (Verduin, 1996a). However, pollen movement is small due to physiological limitations of the pollen itself.

The success and survival of *A. antarctica* is dependent not only on pollination success consequently, based on pollen viability findings and distance involved, the securing of continued genetic variability over larger distances would have to depend on the success of long-distance seedling dispersal. The path of the viviparous seedlings (~6 cm when first released) is dictated not only by hydrodynamic factors but also by its characteristic capability to attach itself to many things it encounters. Bristles of the pericarp form four lobed combs which act as a 'grappling apparatus' to anchor the seedling, presumably to withstand wave and tidal movement (McConchie et al., 1982; Cox and Knox, 1988; Kuo and Kirkman, 1990; Rivers et al., 2011). The interesting feature that suggests success of long-term survival of older seedlings is the fact that the grappling comb may remain on the young plant for one or more years (Kuo and Kirkman, 1990). Healthy *Amphibolis* seedlings of more than 25cm in length have been found with the grappling comb still intact (Waycott, pers. obs, 2014).

Conclusion

The confirmation of sexual reproduction in this study provides a mechanistic explanation for the genetic diversity recently detected within and among *A. antarctica* meadows using microsatellite markers (van Dijk et al., 2018). This genetic diversity is critical for population resilience ensuring adaptive capacity such as resistance to disturbance, and long-term persistence which are key objectives of restoration efforts. Restoration strategies that rely solely on near-field transplantation and subsequent clonal expansion, risk reinforcing genetic homogeneity, potentially limiting ecosystem resilience and inbreeding. *In situ* submarine pollination events in submerged meadows of *A. antarctica* have been observed and described by Verduin (1996a, 1996b); Verduin et al. (1996); Verduin et al. (2002) and Waycott and Les (1996). Pollen of fully submerged *A. antarctica* populations were retained mainly within the meadows and did not float to the surface (Verduin, 1996a, 1996b; Verduin et al., 2002). Examination of pollination and embryo development indicates that *A. antarctica* exhibits normal sexual reproduction. Further *In situ* observations showed that ca. 70% of female flowers developed seedlings as result of sexual reproduction, rather than forming as apomictic propagules.

In many seagrasses, pollination appears to occur predominantly at a local scale, within meadows because most pollen is trapped within the canopy when released (Verduin et al., 2002). In species that release pollen above the canopy, the potential for long-distance dispersal is enhanced but limited by the viability of the pollen. The spatial scale of pollen dispersal processes will remain relatively small (McMahon et al., 2014) when compared to the distribution of species ranges, thus the major contribution to connectivity will be propagule movement.

In the context of seagrass restoration, sexually produced seedlings offer clear advantages to the long-term success of restoration efforts. Seedlings can be collected and redistributed without damaging donor meadows, and their dispersal across broader spatial scales can promote genetic connectivity rather than reinforce local genetic homogeneity. Effective restoration of *Amphibolis antarctica* should therefore entail not only successful establishment but also the maintenance and enhancement of wide-scale genetic dispersal and gene flow, thereby supporting the long-term resilience and ecological functionality of restored meadows.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JV: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. MW: Data curation, Validation, Visualization, Writing – review & editing.

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