Intra and inter-specific variation in the reproductive strategies of two *Bolboschoenus* species from south-eastern Australia

by

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Summary

Bolboschoenus caldwellii and *Bolboschoenus medianus* (Cyperaceae) are two emergent macrophytes common in south-eastern Australian wetlands. This thesis investigates the sexual and asexual recruitment dynamics of both species within the Gippsland Lakes region of Victoria. Over the past 100-150 years the Gippsland Lakes region has undergone major landscape-scale changes, including the building of dams, levee banks and the permanent opening of Lakes Entrance to Bass Strait in 1889. One highly influential consequence of these changes has been the migration of salt throughout the Gippsland Lakes and its associated wetlands. The increase in salinity has altered the vegetation communities of much of the region, from dominance by freshwater macrophytes to dominance by salt-tolerant species. Although *B. caldwellii* and *B. medianus* have persisted within most of the wetlands, their ability to reproduce sexually under increased salt loads appears to be highly restricted, which has left them reliant on asexual recruitment mechanisms. This scenario is predicted to have a negative effect on the genetic diversity and fitness of populations.

Both *B. caldwellii* and *B. medianus* are keystone members of sedge wetland vegetation communities. Sedges are typically found in the ecotone region of ephemeral wetlands. Recruitment of sedge species occurs mainly by vegetative means, such as rhizomes, tubers and stolons. Sedge Wetland is one of several Ecological Vegetation Classes (EVC) within the Gippsland Lakes region that has undergone serious decline in the recent past. The Department of Sustainability and Environment (DSE) lists Sedge Wetland as Vulnerable, with around 1000 ha remaining, 90% of which is found in small patches and subject to continuing threatening processes. Sedge Wetland is critical within the region at providing a range of wetland services such as the prevention of shoreline erosion and pollution assimilation, as well as food, shelter and nesting sites for waterbirds. Many of these

birds are listed under various international migratory-bird agreements, thus the conservation of sedges is critical not only to the ongoing function of wetlands but also to waterbird welfare and international treaties. Determining the recruitment patterns of keystone sedge species may illuminate the specifics of their decline in the wetlands of the Gippsland Lakes and provide ways to improve the conservation of an important vegetation community.

There is a complete lack of information on the recruitment requirements of *Bolboschoenus* species in Australia, nor has any research been conducted on their genetics. This study has several objectives: 1) to investigate the sexual reproductive ecology and germination requirements of *B. caldwellii* and *B. medianus*, 2) to examine the asexual growth mechanisms and responses of *B. caldwellii* and *B. medianus* to increasing salinity, and 3) to assess the genetic diversity of *B. caldwellii* and *B. medianus* stands from three wetlands with contrasting environmental conditions in the Gippsland Lakes region.

Sexual reproductive ecology

Achene production and sediment seed (achene) bank formation was assessed by collecting entire flower heads and recovering achenes from soil cores across a number of populations and field sites. Achene production and sediment seed bank formation was low in both *Bolboschoenus* species and is likely a contributing factor to poor sexual recruitment in the field.

Achene viability was assessed for fresh and 1-year-old achenes, via direct-cut tests and also chemically using 2,3,5-triphenyl-tetrazolium-chloride (TTC). Both methods showed that achenes of *B. caldwellii* and *B. medianus* had consistently high viability (~80%), irrespective of age or sample location. The results indicated that poor viability was unlikely to be a strong contributing factor to the lack of sexual recruitment of these plant taxa in wetlands of the Gippsland Lakes.

The potential for achene dispersal of each species was assessed through buoyancy trials and by examining achene anatomy. Contrasting dispersal mechanisms were displayed by *B. medianus* and *B. caldwellii*. Achenes of *B.*

medianus contained little aeriferous tissue in their pericarp layer and sank almost immediately. Thus it is likely that they do not disperse far from parental populations. In contrast, *B. caldwellii* achenes contained substantial aeriferous tissue, which enabled them to remain afloat for up to 3 months. This ability offered *B. caldwellii* achenes a much higher probability of long-distance dispersal and allowed them to find recruitment windows in both time and space. While dispersal should not affect the ability of *B. caldwellii* achenes to find safe germination sites, achene dispersal may be a limiting factor for *B. medianus*.

Achene germination was tested under light and dark conditions and a range of temperature and salinity concentrations. Germination for both species was light-dependent and required wide diurnal temperature variations of at least 20-25°C. Salinity as low as 2 g L^{-1} significantly restricted germination compared to controls (especially for *B. medianus*), though achenes of both species were able recover from salinity as high as 32 g L^{-1} when transferred to freshwater. Sexual recruitment of both species is, therefore, restricted to late spring and summer on exposed, though not dry, substrata, with low salinity.

Achene pre-treatments of cold-wet stratification and scarification in weak acid, or the manual removal of the micropyle region by razor blade, were assessed for their capacity to improve germination. All scarification treatments significantly improved germination, as did cold-wet stratification of at least 4 weeks. The effects of stratification and scarification were especially significant at improving germination of *B. medianus* achenes. Most importantly, stratification and scarification trials widened the temperature range at which germination could take place in both species. Winter flooding of sediment seed banks is a near pre-requisite for the germination of *B. medianus* in spring, though stratification is less important for *B. caldwellii*. With sufficient stratification or disturbance of the seed coat, germination of *Bolboschoenus* achenes is not dependent on wide diurnal temperature range shifts, as achene pre-treatments encourage embryo activity to commence under normal climate averages.

The importance of hypocotyl hair formation to germination was assessed in *B. caldwellii* and *B. medianus* and a number of other species from the Cyperaceae

family. Hypocotyl hairs were found in all test species and were critical to germination and the establishment of seedlings. In all cases where hypocotyl hairs were absent, radicle formation was compromised and achenes failed to complete germination. Hypocotyl hairs have not been reported for Cyperaceae species in scientific literature to date, an overlooked factor for sedge germination and sexual recruitment.

Asexual growth mechanisms

Individual *B. caldwellii* and *B. medianus* clones were grown in separate mesocosms to assess their asexual growth mechanisms under three different salinities. In contrast to almost all aspects of sexual reproduction, which were significantly inhibited at salinities > 4 g L^{-1}, asexual reproduction in both species continued largely unaffected at a salinity of 12 g L^{-1}. Clonal growth, therefore, is critical to population maintenance and dynamics for both species in salinised wetlands. The most prominent response of *B. medianus* to increasing salinity was to increase biomass allocation to tubers, whereas *B. caldwellii* allocated greater biomass to rhizomes under increasing salinity. Rhizome lengths did not differ between salinity treatments, indicating a lack of genet plasticity in the plagiotropic (horizontal) plane. In contrast, tuber sizes were highly variable within each clone and many tubers did not produce culms as salinity increased. This pattern suggested that plasticity in the orthotropic (vertical) plane was of great value to genet survival and spatial organisation.

The allocation of greater biomass into under-ground organs (rhizomes and tubers) is a trade-off mechanism used by sedge and rush species that allows resources to be stored over unfavourable growing periods. This mechanism increases the likelihood of future vegetative offspring, though is unlikely to contribute to genetic diversity. *Bolboschoenus* populations growing under brackish to highly saline conditions were therefore predicted to contain minimal genetic diversity, despite large population sizes and densities.

Genetic diversity

Amplified fragment length polymorphism (AFLP) analysis was used to construct DNA fingerprints of *B. caldwellii* and *B. medianus* populations in the

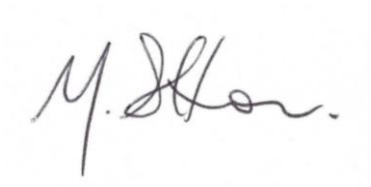
Gippsland Lakes region. The AFLP analysis confirmed predictions that the inherent genetic diversity of populations of *Bolboschoenus* can be highly cryptic. Despite the presence of many thousands of individual stems at each field site, three out of four sampled populations were genetically homogeneous. The findings suggested a strong relationship between site quality and clonal structure, as molecular fingerprints were polymorphic only from the population growing in freshwater conditions. It appears that salinity influences the genetic diversity of *B. caldwellii* and *B. medianus*. The AFLP analysis confirmed that asexual reproduction is responsible for the majority of biomass at each of the chosen field sites and indicated that caution must be exercised when population assessments of status and fitness (genetic diversity) are based purely on ramet numbers or morphological differences.

Implications

The change in many of the wetlands in the Gippsland Lakes from freshwater to brackish conditions over the past ~100-years has shifted the recruitment dynamics of *B. caldwellii* and *B. medianus* to an exclusive reliance on clonal reproduction. Prevailing salinity concentrations are now beyond the tolerance limits for achene germination in both species. Because of this salinity shift, local populations could become extinct without appropriate windows of opportunity for sexual recruitment from the sediment seed bank. The clonal growth habit enables *B. caldwellii* and *B. medianus* to cope with year-to-year changes in wetting and drying conditions but further increases to salinity throughout the Gippsland Lakes system may eventually prevent asexual or clonal growth mechanisms and thereby all forms of recruitment for these species.

Candidate's Declaration

This thesis is submitted for examination in accordance with the regulations for the degree of Doctor of Philosophy and contains no material that has been accepted for the award of any other degree or diploma in any other university or tertiary institution. The work in this thesis is entirely my own and contains no material previously published or written by another person, except where due reference is made in the text.



Matt Hatton

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Section I:

Introduction to the study

Chapter 1.
Introduction

1.1 The Gippsland Lakes region in south-eastern Australia

The Gippsland Lakes region in Victoria is the largest estuarine lagoon system in Australia, and consists of Lake Wellington (138 km^2), Lake Victoria (110 km^2) and Lake King (90 km^2) (Bird 1961). The lake system receives flows from seven major rivers: the Latrobe, Thomson, Avon, Macalister, Mitchell, Nicholson and Tambo Rivers and has a combined catchment area of about 2,000,000 ha (DSE 2003) (Fig. 1.1). Many wetlands fringe the Gippsland Lakes including: Dowd Morass, Clydebank Morass, Sale Common, Heart Morass, Red Morass and Backwater Morass. The first three of these wetlands were selected as field sites for this thesis and are discussed in greater detail in Chapter 2.

The wetlands surrounding the Gippsland Lakes are important within regional, State, Commonwealth and international contexts, as they contain 45 threatened fauna species and 11 threatened flora species (Parks Victoria 2008). In addition, the wetlands encompass four of Victoria's most threatened Ecological Vegetation Classes[1] (EVCs), eight sites of geological and geomorphological significance and have highly important cultural significance to the traditional owners of the region, the Tatungoloong people of the greater Gunai/Kurnai nation, due to their rich archaeological zones of: burial sites, scar trees, shell middens and artefact scatters (Parks Victoria 2008).

The Gippsland Lakes region is economically productive and as such has been a point of focus for large-scale enterprise since the time of European settlement.

[1] Over 300 EVCs are used in Victoria as an hierarchical system of classification for plant communities. EVCs are defined by a combination of floristics, life-form, position in the landscape and the environment" (www.dse.vic.gov.au).

Figure 1.1 shows the extent of landscape-scale alterations that have occurred over the past ~150 years in the region (e.g. coal mines, dams, large-scale irrigation and agriculture as well as the artificial opening of Lakes Entrance to Bass Strait). All these factors have irreversibly altered the historical hydrological and salinity regimes of the overall system, including its rivers and wetlands, and significantly influenced the ecology of the region (DSE 2003).

In a series of pioneering papers in the 1960s, Bird (1961; 1962; 1966) predicted that the Gippsland Lakes and its surrounding wetlands would become increasingly salinised as a result of the artificial opening of Lakes Entrance to Bass Strait in 1889. Bird (1966) predicted also that, as salinity levels increased throughout the lake system, vegetation communities would shift in response. For example, it was predicted that freshwater macrophytes such as *Phragmites australis* (Common Reed) would be replaced by more salt tolerant swamp scrub species such as *Melaleuca ericifolia* (Swamp Paperbark), which in turn would be succeeded by salt marsh species such as *Sarcocornia quinqueflora* (Beaded Glasswort) with continued salinity increases. The compositional changes to vegetation patterns proposed by Bird (1966) have recently been demonstrated in several research and development projects within the Gippsland Lakes region (Boon *et al.* 2007; Hatton *et al.* 2008; Boon *et al.* 2008; Saunders *et al.* 2008). For example, through the use of historical aerial photographs of Dowd Morass (see Fig. 1.1 and 2.1), percentage coverage of *P. australis* declined by ~30% over the past four decades, while *M. ericifolia* expanded its territory by ~73% (Boon *et al.* 2008). Furthermore, examination of both the floristic composition and distribution of species via ground surveys and belt transects at a neighbouring wetland site (Clydebank Morass – see Fig. 1.1 and 2.1), revealed that the floristic community was now entirely composed of moderate to highly salt tolerant species and that all aquatic species were clonal, with the exception of one annual species (Hatton *et al.* 2008).

Of primary interest to this thesis is the question of what effect landscape-scale changes to environmental conditions have had on the emergent component of wetland vegetation. While sedge species are relatively common in most wetlands throughout the Gippsland Lakes, EVC mapping of sedge wetlands (EVC #136) in this region has revealed that around 1000 ha remain (compared with pre-European estimates of

Source: Google Maps (photograph taken 2006)

Figure 1.1. Aerial photograph of the Latrobe Valley and Gippsland Lakes region, Victoria, south-eastern Australia. The image illustrates a number of landscape-scale alterations that have significantly influenced the ecology of the area (e.g. coal mines, dams, large-scale irrigation and agriculture as well as the artificial opening of Lakes Entrance to Bass Strait). The dotted red line indicates the main study area (*cf* Fig. 2.1).

around 1,000,000 ha) and that 90% of the total area is made up of small patches subject to continuing threatening processes (DSE Interactive maps website, accessed August 2006). Indeed, the conservation status of the majority of vegetation types around the Gippsland Lakes is now listed as either endangered or vulnerable (Fig. 1.2).

Many sedges are emergent species found in the ecotone region of ephemeral wetlands, such as *Baumea arthrophylla* and *Eleocharis sphacelata*. Recruitment of sedge species occurs via both sexual and vegetative means, such as rhizomes, tubers and stolons.

The distribution of sedges throughout the Gippsland Lakes is relatively wide, as they are found in wetlands with intermittent and near permanent water regimes, as well as fresh and saline sites (Figure 1.3). The ability of sedges to fill niches in most habitat types underwrites their consideration as keystone species and highlights their ability to persist under changing climates (e.g. *Carex curvula,* Steinger *et al.* 1996). One of the greatest uncertainties for sedge species, however, is the effect of altered environmental conditions (particularly increased salinity) to their sexual recruitment.

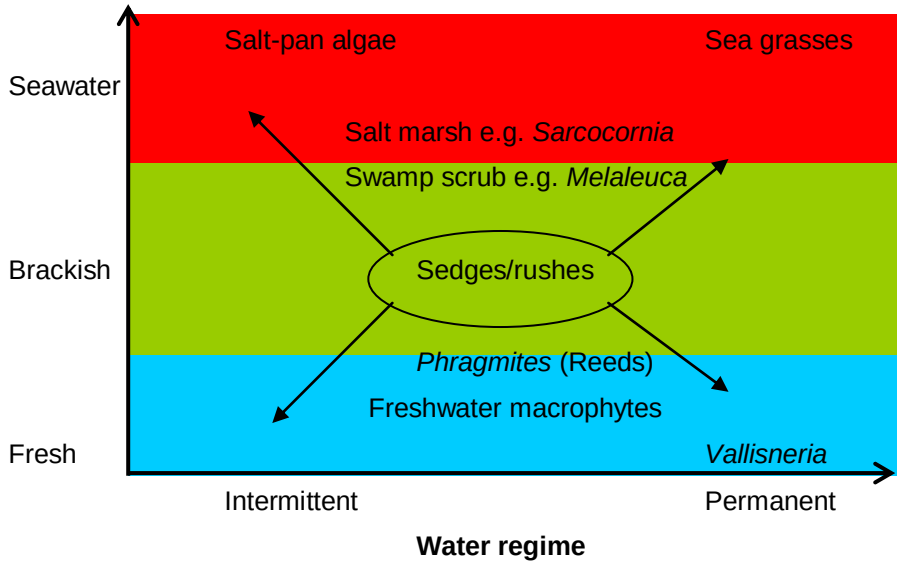
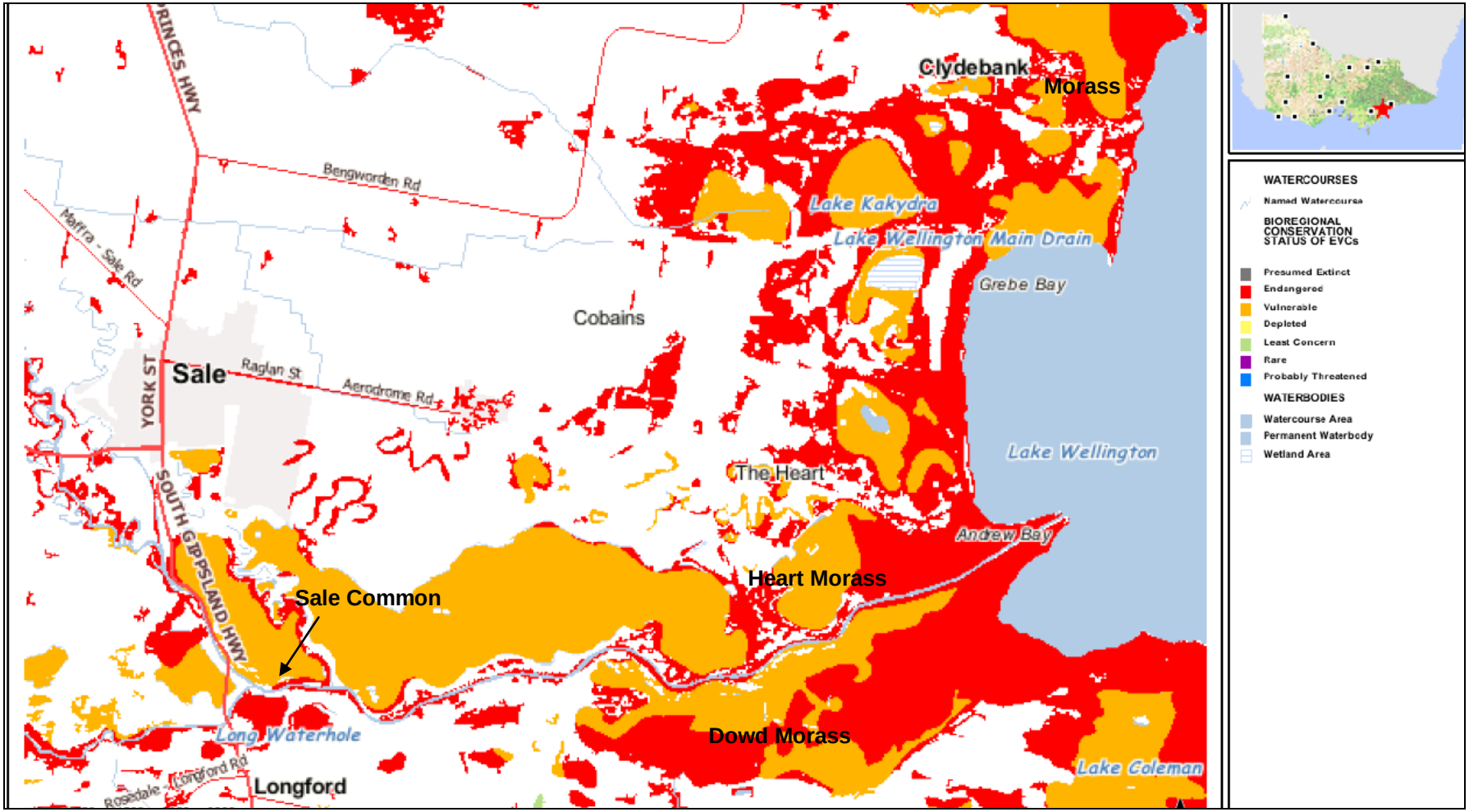


Figure 1.3. Diagram of likely vegetation succession and niche differentiation of broad vegetation types due to changing salinity and water regimes in the Gippsland Lakes region of Victoria.



Source: DSE interactive maps, accessed August 2008

Figure 1.2. Example of current EVC conservation status for wetlands within the Gippsland Lakes region. Red and Orange colours indicate Endangered and Vulnerable EVCs respectively.

1.2 The importance of diverse wetland sedge and rush vegetation

The building of dams, large-scale irrigation schemes and land conversion for agriculture, as well as the spread of urban and industrial development, have resulted in a significant decline in the area of sedge wetlands around the world (Kingsford 2000; Middleton 2002). Sedge-dominated wetlands provide a suite of environmental services such as nutrient and pollution assimilation, demobilisation of sediments and erosion control, though perhaps more importantly, members of the Cyperaceae family play a vital role in providing food and habitat for many different species of aquatic invertebrates and waterbirds (George 1963; O'Neill 1972; Landers *et al.* 1976; Corrick and Norman 1980; Prevost and Gresham 1981; Norman and Mumford 1982; Kantrud 1996; Espinar *et al.* 2006).

At least 185 bird species have been recorded within the Gippsland Lakes region, of which 87 regularly use its surrounding wetlands for feeding, resting and breeding (DSE 2003). Approximately 59,000 ha of wetlands in the Gippsland Lakes region have, therefore, been listed under the Ramsar convention (1971) as wetlands of international significance, as they may regularly support up to 40,000 waterbirds (NPS 1995; DSE 2003). Twenty-seven bird species that use the wetlands are also protected under bilateral migratory bird agreements between Australia and Japan (JAMBA 1974), China (CAMBA 1986) and more recently with the Republic of Korea (ROKAMBA 2007). Sustaining diverse sedge and rush communities will play an important role in maintaining these agreements. For example, Norman and Mumford (1982) noted that plant material derived from Cyperaceae species accounted for almost one quarter of the total food items found in the gizzards of Chestnut teal ducks (*Anas castanea*) over a 21-month period in the Gippsland Lakes region. Importantly, the research also highlighted the presence of Cyperaceae seeds in the gizzards of birds outside of known fruiting seasons, indicating that birds were eating fallen seeds lying within the sediment seed bank. Dormancy mechanisms allow seeds and tubers to persist for long periods (>2 yrs) under water, so dormant plant parts may still be used as a food source when all other above ground parts have ceased to persist (Norman and Mumford 1982). Similar findings have been recorded for Cyperaceae species such as *Scirpus* and waterbirds along the southern and mid-Atlantic coasts of

America (Landers *et al.* 1976; Prevost and Gresham 1981) and across the Mediterranean (Espinar *et al.* 2006).

As the Cyperaceae family consists of some 100 genera and ~5,000 species, field-site assessments of sedge populations may be limited through a lack of information on species-specific establishment requirements (Clevering 1995; Schütz 2000; Budelsky and Galatowitsch 2000; Budelsky and Galatowitsch 2004; Leck and Schütz 2005). This thesis examines the reproductive ecology of two commonly occurring wetland sedge species found within the Gippsland Lakes region: *Bolboschoenus caldwellii* and *Bolboschoenus medianus* (Cyperaceae).

1.3 The genus *Bolboschoenus* (Cyperaceae)

1.3.1 Taxonomy

Formerly incorporated within the genus *Scirpus* L., the genus *Bolboschoenus* (Asch.) Palla (Cyperaceae) has undergone a major taxonomic revision in recent times. The genus is now recognised to contain 15 species world-wide (Edgar 1970; Oteng-Yeboah 1974; Wilson 1981; Browning *et al*. 1997; Hroudová *et al*. 2005). The new classification is based mostly on morphological differences in achene and embryo characteristics (Browning and Gordon-Gray 1992; 1993; 1995; Browning *et al*. 1996; Browning *et al*. 1997; Browning *et al*. 1999; Hroudová *et al.* 1999; Pignotti and Mariotti 2004; Hroudová *et al.* 2005). Australasian *Bolboschoenus* species were once all described as variants of *Scirpus maritimus*, though three species are now recognised: *B. caldwellii, B. medianus* and *B. fluviatilis* (formerly also *B. perviridis*) (Edgar 1970; Wilson 1981; Browning *et al*. 1997). The smallest species is *B. caldwellii* (up to 1 m in height) and is distinguished by having achenes that are consistently obovate and biconcave in shape and light brown in colour. In contrast, achenes of *B. medianus* may be either trigonous or biconcave shaped and black to dark brown in colour, while the achenes of *B. fluviatilis* are consistently trigonous, elliptical and grey in colour (Browning *et al*. 1997). Both *B. medianus* and *B. fluviatilis* grow to similar heights (1.5-2 m) (Fig. 1.4).

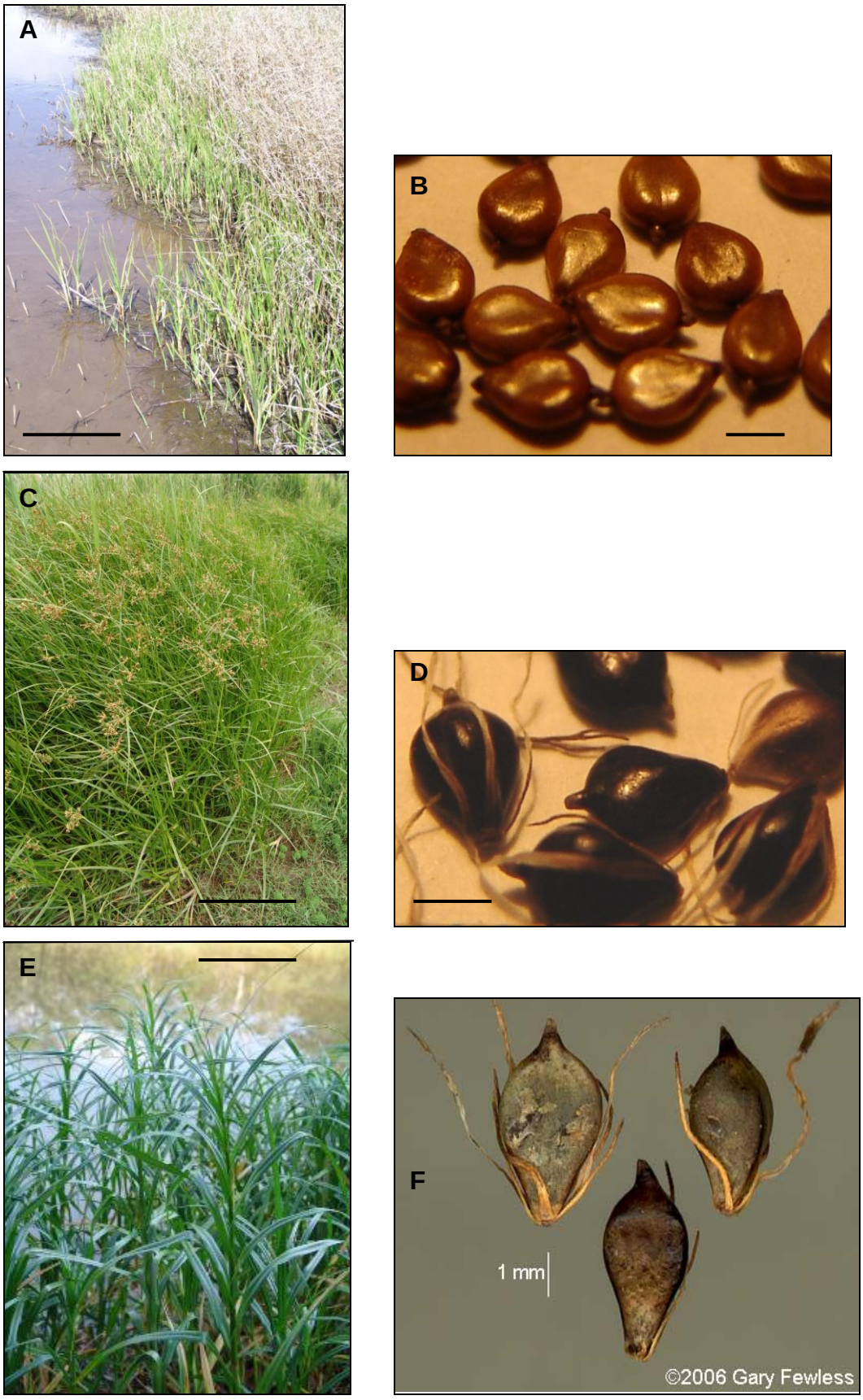


Figure 1.4. Typical growth habits and achenes of Australasian species of *Bolboschoenus*: A & B) *B. caldwellii*, C & D) *B. medianus* and E & F) *B. fluviatilis*. Scale bars = 50 cm for whole plants, whereas scale bars = 2 mm for achenes.

1.3.2 Distribution

The distributions of the three Australasian *Bolboschoenus* species differ across continental Australia. While *B. caldwellii* (Sea clubrush) is found in all Australian States and Territories, *B. medianus* (Marsh clubrush) is restricted to the south-eastern States (Australian Virtual Herbarium – August 2006) (Fig. 1.5.A & 1.5.B). River clubrush (*B. fluviatilis*), in contrast, is rare in Victoria but extends northward into Queensland and into Asia (Romanowski 1998; Australian Virtual Herbarium – August 2006). Two patches of ~500-1000 stems on the margins of the Yarra River (Warrandyte) are reputedly the largest population of *B. fluviatilis* in Victoria (Margaret Bourke, *pers. comm.*). For reasons of both its isolation and small population size in Victoria, *B. fluviatilis* was not examined further in this thesis.

Bolboschoenus caldwellii and *B. medianus* are often sympatric species and are both common components of wetland and riparian vegetation, particularly in shallow or semi-permanent water bodies along the coast of south-eastern Australia. While occasionally found on the margins of freshwater bodies (the theoretical niche), both species are generally out-competed by glycophytes in such conditions, especially the smaller *B. caldwellii*, and instead find that their realised niche is largely restricted to the littoral margins of brackish water wetlands (Walsh and Entwisle 1994; *pers. obs.*). As they are emergent species, both *B. caldwellii* and *B. medianus* are commonly found growing at the waterline, though when growing together the distributions of the two species differentiate by adopting different gradient elevations. For example, in the lower reaches and wetlands of the River Murray, *B. medianus* is frequent in regularly flooded and exposed sites (lower gradient elevations), whereas *B. caldwellii* typically favours infrequently flooded areas (higher gradient positions) (Walker *et al.* 1994; Blanch *et al.* 1999a, 1999b; Siebentritt and Ganf 2000).

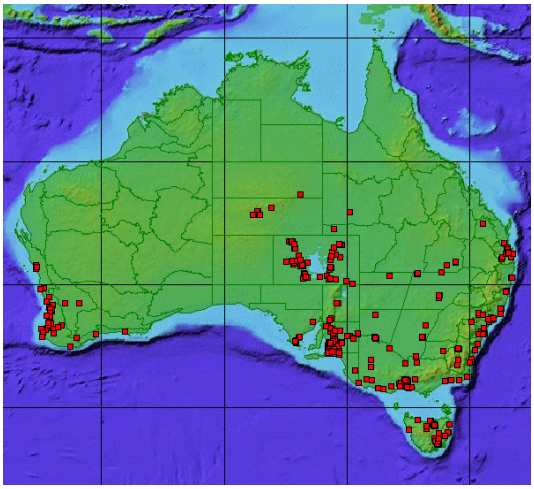


Figure 1.5.A. Australian distribution of *Bolboschoenus caldwellii* (Australian Virtual Herbarium).

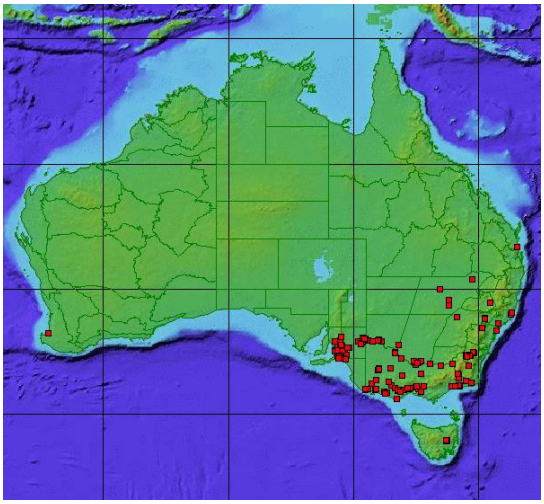


Figure 1.5.B. Australian distribution of *Bolboschoenus medianus* (Australian Virtual Herbarium).

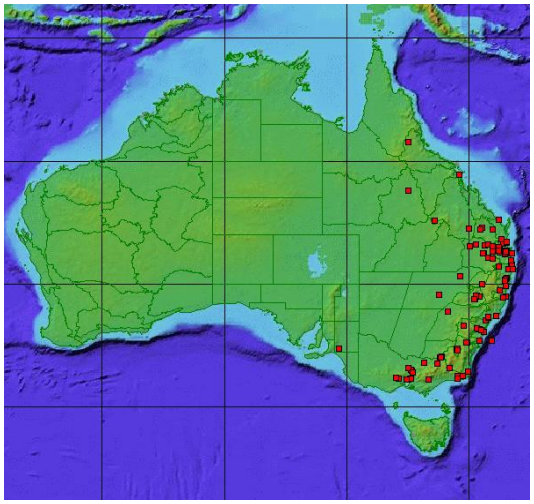


Figure 1.5.C. Australian distribution of *Bolboschoenus fluviatilis* (Australian Virtual Herbarium).

1.3.3 Ecology

Bolboschoenus species are monocotyledons. A single cotyledon/plumule is produced during germination, followed by several sheath leaves that form a pseudo-stem prior to the initiation of the triangular stem/culm that characterise their adult morphology (Fig. 1.6). Culms grow rapidly in *B. caldwellii* and may flower within the first season under favourable conditions. In contrast, *B. medianus* culms generally do not flower in the first season of growth (Walsh and Entwisle 1994; *pers. obs.*).

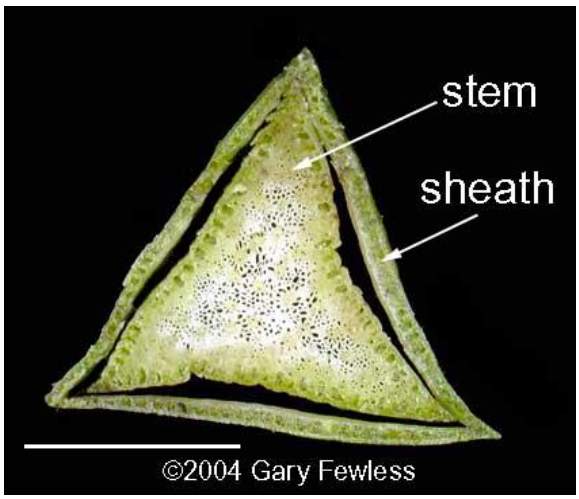


Figure 1.6. Cross-section of a mature *B. medianus* stem showing characteristic triangular morphology, surrounded by a leaf sheath. Scale bar = 5 mm.

An individual ramet consists of a single culm and tuber with roots and on average three rhizomes originating from the tuber (though possibly 5-6 rhizomes). Initial rhizomes are produced approximately one month after germination under favourable conditions. Each rhizome may then give rise to a new tuber and thereby form new daughter ramets (Fig. 1.7). *Bolboschoenus* species are helophytic, as their above-ground parts senesce during late autumn to early winter, leaving interconnected networks of underground (dormant) rhizomes and tubers to over-winter and re-sprout the following spring (Hroudová and Zákravský 1995). *Bolboschoenus* growth dynamics, therefore, incorporate a trade-off between above and belowground biomass and the mechanism of dormancy, which defines them as true helophytic species. The perennation of tubers is a mechanism specifically adapted to allow vegetative storage organs (totipotent tissue) to maintain respiration over winter or during unfavourable

growth periods (Sculthorpe 1967; Silvertown *et al.* 2001). In this way populations may recover via asexual growth and are not dependent on sexual recruitment. In unpredictable environments such as wetlands, the ability to form perennating organs is a critical factor for the recruitment dynamics of aquatic vegetation (Kautsky 1990).

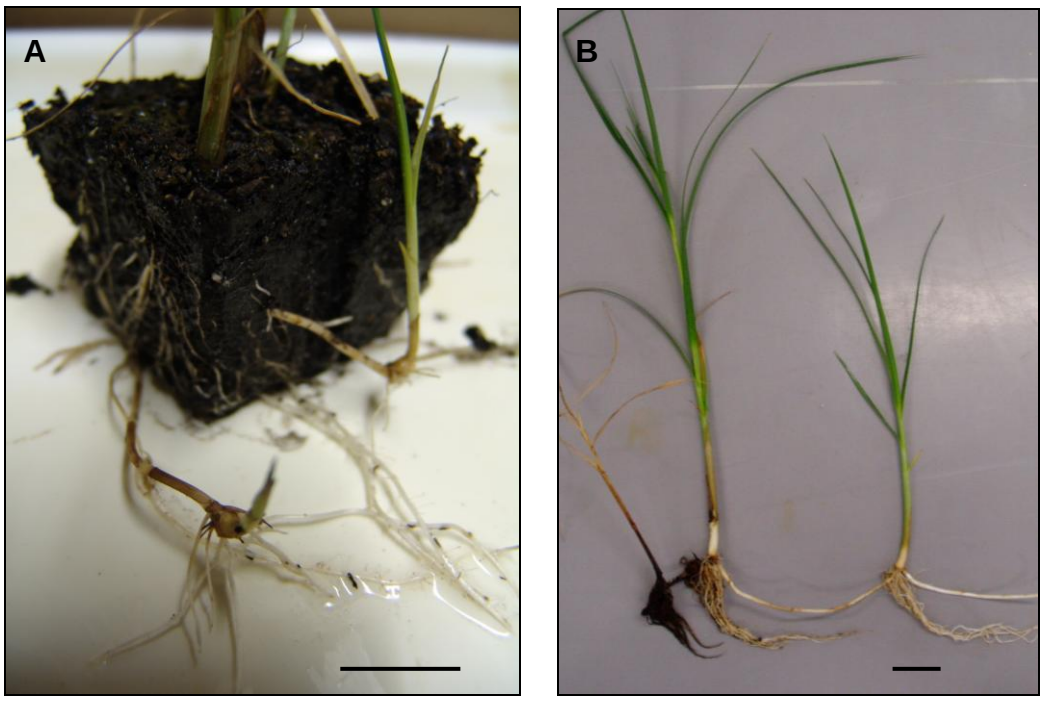


Figure 1.7. Ramet production in A) *B. caldwellii* and B) *B. medianus*. Scale bars = 50 mm.

While extensive taxonomic research has clarified phylogenetic and speciation issues within the confusing *Bolboschoenus* genus, little work has been conducted on the reproductive and dormancy requirements of the various species. This knowledge gap is of concern as *Bolboschoenus* species are commonly reported to fail to recruit sexually, especially into existing populations (Clevering 1995; Kantrud 1996; Clevering and Hundscheid 1998; Moravcová *et al.* 2002).

Seed dormancy is a common feature of the Cyperaceae family and is particularly strong within its wetland or aquatic species (Leck and Schütz 2005). For example, seed dormancy mechanisms in wetland *Carex* species were reported as initially stronger and harder to break than the dormancy capacity of their terrestrial relatives (Schütz 2000). Seed dormancy is endogenous in most Cyperaceae species,

meaning that germination is inhibited by a physiological mechanism of the embryo (Baskin and Baskin 2001). Dormancy breakage involves a balance between growth inhibitors and promoters, such as abscisic acid and gibberellic acid (Taiz and Zeiger 2002). Growth-inhibiting chemicals can be found in the endosperm, cotyledons, or coverings of seeds, where they must be leached out or reduced in concentration by water prior to germination. In some species, growth inhibitors reside in the embryonic axis of seeds and temperature and light changes are required to shift the chemical balance to growth promotion (Baskin and Baskin 2001).

Members of the *Bolboschoenus*/*Scirpus* complex all produce seeds that are encased within an achene. An achene is defined as a simple, dry, one seeded fruit in which the seed coat is not adherent to the pericarp, whereas a typical seed consists only of an embryo, endosperm and seed coat (Raven *et al.* 1987). Achenes have thick, multi-layered pericarp anatomy surrounding the seed coat and a waxy exterior surface, which makes them relatively waterproof and highly dormant (see Chapter 4). The structure of achenes suggests that their dormancy is regulated by the permeability of the seed coat rather than through an embryonic mechanism (Clevering 1995; Lacroix and Mosher 1995). Previous studies on European and North American species belonging to the *Bolboschoenus* / *Scirpus* complex have shown that achenes respond to cold-wet stratification treatment (which mimics a natural over-wintering period in flood-waters) as well as light and wide diurnal temperature regimes, though conditions for germination may differ substantially between closely related species (Harris and Marshall 1960; Dietert and Shontz 1978; Prevost and Gresham 1981; Clevering 1995; Moravcová *et al*. 2002). For example, although *Scirpus lacustris* and *Scirpus maritimus* (now *Bolboschoenus maritimus*) were able to germinate on exposed substrata (Clevering 1995), germination of *Scirpus juncoides* seeds was inhibited by oxygen (Pons and Schröder 1986).

Alternative methods that disrupt the integrity of seed coats, such as physical and chemical scarification treatments have also been successfully used to break dormancy in several *Scirpus* species (Clevering 1995). Bleach or acid scarification treatments are often used in germination trials as a form of pseudo endozoochory (seed passage through vertebrate digestive tracts) as the disruptive effects of acid on seed coats can significantly increase germination percentages and rates (Griffiths and

Lawes 2006). The cosmopolitan distribution of *Bolboschoenus* species and the resilience of their achenes suggests that achene morphology in *Bolboschoenus* species may have been, in part, selectively driven by dispersal through waterbirds. For example, De Vlaming and Proctor (1968) recorded higher germination percentages of *Scirpus paludosus* achenes following passage through the digestive tracts of Killdeer and Mallard ducks, compared with control treatments.

Members of the *Bolboschoenus/Scirpus* complex are frequently reported as the most systematically consumed helophytes by waterfowl (O'Neill 1972; Landers *et al*. 1976; George and Young 1977; Prevost and Gresham 1981; Norman and Mumford 1982; Green *et al*. 2002; Espinar *et al*. 2004). In parallel with stratification treatments, the effectiveness of endozoochory to germination success is dependent on retention time; hence seed responses differ in relation to the host (bird) species (Traveset *et al*. 2001b). A wide variety of waterbirds and other frugivores utilising *Scirpus / Bolboschoenus* achenes, equates to greater variation in scarification effects between individual achenes, effectively allowing a broader germination response from the collective sediment seed bank. Endozoochory or scarification, therefore, is thought to have both a spatial and temporal, risk spreading effect by enabling asynchronous germination (Izhaki and Safriel 1990; Traveset *et al*. 2001b; Espinar *et al*. 2006).

Improved permeability of the pericarp layer through stratification and scarification treatments increases the efficiency of water imbibition, which in turn raises the metabolic capacity of seed embryos and thereby arrests dormancy, priming seeds for rapid germination given the right temperature cues (Baskin and Baskin 2001). Storage in an imbibed state (i.e. cold-wet stratification) also offers benefits for seed viability. Water imbibition has been shown to suppress seed deterioration processes as it allows enough enzyme activity for internal maintenance and damage repair, which would otherwise cause viability loss (Berjak and Villiers 1972; Villiers 1974). The storage of seed in cold wet conditions to maintain viability is, therefore, recommended for members of the Cyperaceae family (Budelsky and Galatowitsch 1999). Stratification and scarification techniques have a further advantage in that they may also widen the parameters at which germination can occur for many species (Clevering 1995; Shütz and Rave 1999; Moravcová *et al*. 2002).

As far as I am aware, no research on the germination requirements of Australasian species of *Bolboschoenus* has been conducted in the past. Few studies have addressed the role of achene architectural differences within and among *Bolboschoenus* species and whether the polymorphisms have an adaptive role in recruitment processes (e.g. Hroudová *et al.* 1997). Nor is there any mention of specialised germination adaptations, such as hypocotyl hairs, in scientific literature for members of the Cyperaceae. Given that sedge wetlands are listed as vulnerable within the Gippsland Lakes, it is highly important to determine the germination requirements of their species. As emergent species that inhabit the ecotone between aquatic and terrestrial zones, *Bolboschoenus* species provide a suite of environmental services and much other biota depends on their integrity. Threatening processes to the abundance of *B. caldwellii*, *B. medianus* and other components of sedge wetlands are likely to have serious flow-on effects to many other species.

The bulk of this thesis focuses on the sexual reproductive ecology of *B. caldwellii* and *B. medianus*, as sexual reproduction is assumed to be of prime importance for plant biology, as it ensures genetic recombination and creates a diverse and resilient gene pool (Eriksson 1997). The fitness of a plant species is typically measured by estimating its potential seed output over a lifetime (Harper 1977). Estimating the expected seed or offspring output of clonal plants, however, is very difficult, as clonal species may trade-off resource expenditure between sexual and asexual reproduction (Wikberg 1995). In order to understand the dynamics of clonal plants it is important, therefore, to consider both sexual and asexual reproduction and how the two pathways operate together within the life-history strategy of a species. As highlighted by Eckert (2002), the relative importance of sexual versus asexual recruitment varies widely between species and populations and remains largely unknown for most organisms.

1.4 Reproduction in wetland plants

Reproduction in wetland plants occurs in two ways: sexually through the production of seeds, forming genetically distinct individuals, or asexually via vegetative extension, fragmentation or asexual propagules such as turions, resulting in genetically identical clones (Sculthorpe 1967; Grace 1993; Philbrick and Les 1996).

With exception of algae and aquatic ferns, all wetland plant species display an ability to flower, indicating that they have evolved from terrestrial angiosperm species that have reinvaded aquatic environments; a pathway which is said to have independently occurred among aquatic plant genera on at least 50-100 occasions (Cook 1990). The reproductive methods of aquatic species are essentially the same as those in terrestrial plants, as the majority of species maintain their flowering structures above the water line for fertilisation (Grace 1993). Because fluctuating water levels may inhibit flower production as well as seed germination, the majority of aquatic species have maintained or developed one or often multiple forms of asexual reproduction (Sculthorpe 1967; Hutchinson 1975; Grace 1993). Examination of botanical reference texts and recent surveys of the floristic compositions of wetlands and the life-history strategies of the plants they contain have demonstrated that ~65-70% of all aquatic and semi-aquatic species incorporate a clonal growth habit (Aston 1977; Klimeš *et al.* 1997; Song and Dong 2002; Sainty and Jacobs 2003; Hatton *et al.* 2008) (e.g. Fig. 1.8).

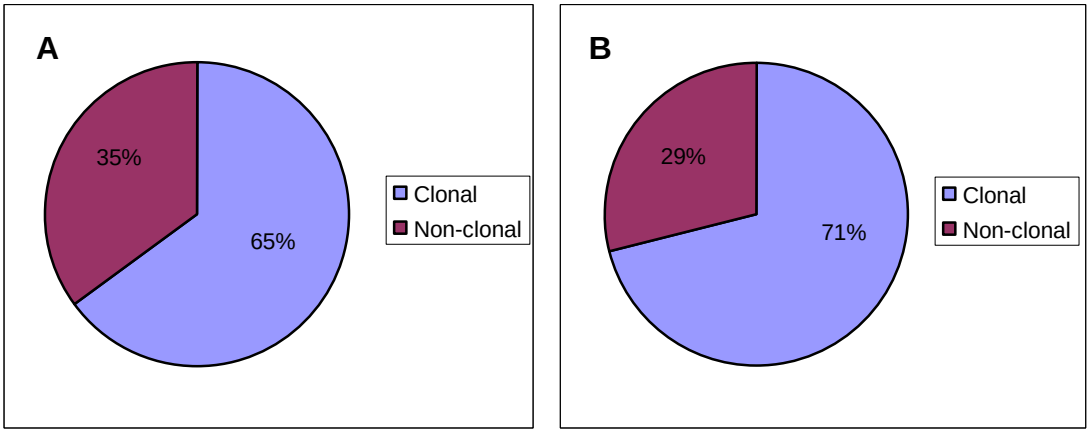


Figure 1.8. Proportion of vascular plant species in aquatic vegetation with a clonal growth habit, calculated from A) Aston (1977) and B) Sainty and Jacobs (2003).

Clonal growth and asexual reproduction may occur via a number of methods, such as rhizomes, stolons, tubers, fragmentation of the plant body, node rooting and asexual propagules (Table 1.1).

Table 1.1. Methods, definitions and examples of clonal or vegetative growth in wetland plant species.

Clonal reproduction	Definition	Example
Rhizomes	Underground horizontal storage and transport organ between ramets. Rhizomes may be fleshy, cordlike or scaly and may bear roots, stems and leaves	*Carex gaudichaudiana*, Cyperaceae *Juncus pallidus* (Pale Rush), Juncaceae *Phragmites australis* (Common Reed), Poaceae *Phalaris aquatica* (Toowoomba Canary-grass), Poaceae *Typha domingensis* (Cumbungi), Typhaceae
Stolons or runners	Aboveground horizontal branch (stolon) or stem (runner) between ramets that root in at nodes and form new plants.	*Disphyma crassifolium* (Rounded Noon-flower), Aizoaceae *Leptinella longipes* (Coast Buttons), Asteraceae *Mimulus repens* (Creeping Monkey-flower), Scrophulariaceae
Tubers, Corms and Bulbs	Underground storage and perennation organs used to oversee unfavourable growth periods	*Bolboschoenus medianus* (Club Rush), Cyperaceae *Triglochin procerum* (Streaked Arrow-grass), Juncaginaceae *Drosera binata* (Forked Sundew), Droseraceae
Root suckering	Emergent shoots from creeping roots and tap roots	*Melaleuca ericifolia* (Swamp Paperbark), Myrtaceae
Bulbils and plantlets or pseudo vivipary	Asexual plantlets formed in the axil inflorescence of branches and culm nodes	*Nymphaea mexicana* (Yellow Waterlily), Nymphaceae *Scirpus polyphyllus* (Leafy Bullrush), Cyperaceae
Fragmentation	Fragments of the plant body that disperse via water and re-establish new root systems	*Ceratophyllum demersum* (Hornwort), Ceratophyllaceae *Myriophyllum spicatum* (Eurasian Milfoil), Haloragaceae *Lemna disperma* (Common Duckweed), Lemnaceae
Turions and apomictic seeds	Asexual dormant buds or seeds that do not undergo meiosis and genetic recombination	*Hydrilla verticillata* (Water Thyme), Hydrocharitaceae *Najas marina* (Prickly Water-nymph), Najadaceae *Taraxacum officinale* (Dandelion), Asteraceae

Asexual growth in plants has traditionally been seen as an adaptation to complement sexual reproduction when conditions for germination are unfavourable (Grace 1993). Plant ecologists, however, have long recognised that many species that display vegetative growth rarely recruit sexually (via seeds) but, instead, persist largely through clonal or asexual reproduction (Harper 1977; Abrahamson 1980; Cook 1985; Eriksson 1997). The ubiquity of the clonal growth habit among wetland plant species around the world suggests that conditions for sexual recruitment are largely unreliable or infrequent, and that plant fitness and community organisation in these habitats are primarily determined by clonal growth (Oborny and Bartha 1995;

Klimeš and Klimešová 1999; Barsoum 2002; Song and Dong 2002, Song *et al.* 2002). For many clonal species, it may be more pragmatic to view sexual reproduction as being complementary to asexual reproduction rather than the other way around (Grace 1993; Nishihiro *et al.* 2004).

Reproduction via asexual pathways has many advantages over sexual reproduction (Table 1.2). In contrast to the various embryonic and seedling development phases faced by a germinating seed, asexually produced ramets bypass seedling stages and rapidly mature. The parameters for vegetative recruitment, therefore, are much wider than those for seed germination (Williams 1975).

Table 1.2. Differences in fitness between asexually and sexually reproduced offspring (adapted from Williams 1975 and Swanton 2003).

Asexually reproduced offspring	**Sexually reproduced offspring**
Larger initial size and increased growth rate as new ramets bypass the seedling stage	Small initial size as growth begins from the embryo stage
No 'cost of sex' - Resources invested into ramets	High 'cost of sex', i.e. flower and seed production
Ramet recruitment parameters are wide – High invasive potential	Seed germination parameters are narrow – Low invasive potential
Develop close to parent – Selective placement into a favourable environment – Consolidation and exploration of space	Dispersal largely random – Non-selective placement of offspring
Favourable genotype optimised	Favourable genotype unpredictable
Low mortality rate – Natural selection mild – Supported by parental ramets – Fewer environmental sieves to negotiate	High mortality rate – Natural selection intense – no support from parents – Seeds must negotiate all environmental sieves
Resource foraging, resource sharing and division of labour – Enables clones to inhabit otherwise unavailable niches	Distribution restricted by the most limiting resource as no division of labour
Genet formation increases resource acquisition and storage capacity – Buffering against temporal variability – risk spreading across genet	Unitary existence – Limited storage and risk spreading
Long lifespan and large size – Facilitation and selection of cohabitant species	Limited lifespan – Limited influence on neighbouring species
Commensal – Spatial organisation – Self non-self recognition – Prevention of intra-specific competition	Solitary – High competition

Asexual reproduction avoids the added resource costs of sex (i.e. flower and seed production) and the dispersal of propagules is not entirely random (Bell and Tomlinson 1980). For example, many clonal plants, such as *Fragaria chiloensis* (Strawberries), are able to selectively place individual ramets into preferable resource patches of light, nutrient and water availability by plastic alterations to their basic architecture (Alpert and Mooney 1986; Hutchings and De Kroon 1994). Rather than letting environmental sieve(s) impose habitat positions on their offspring by constraining seed germination, clonal growth enables active and direct exploitation of local habitat. As genetically identical modules, new ramets possess optimised genotypes for their local environment, and so the mortality rate of vegetatively produced plants is low (Wikberg 1995; Pan and Price 2002) (Fig. 1.9).

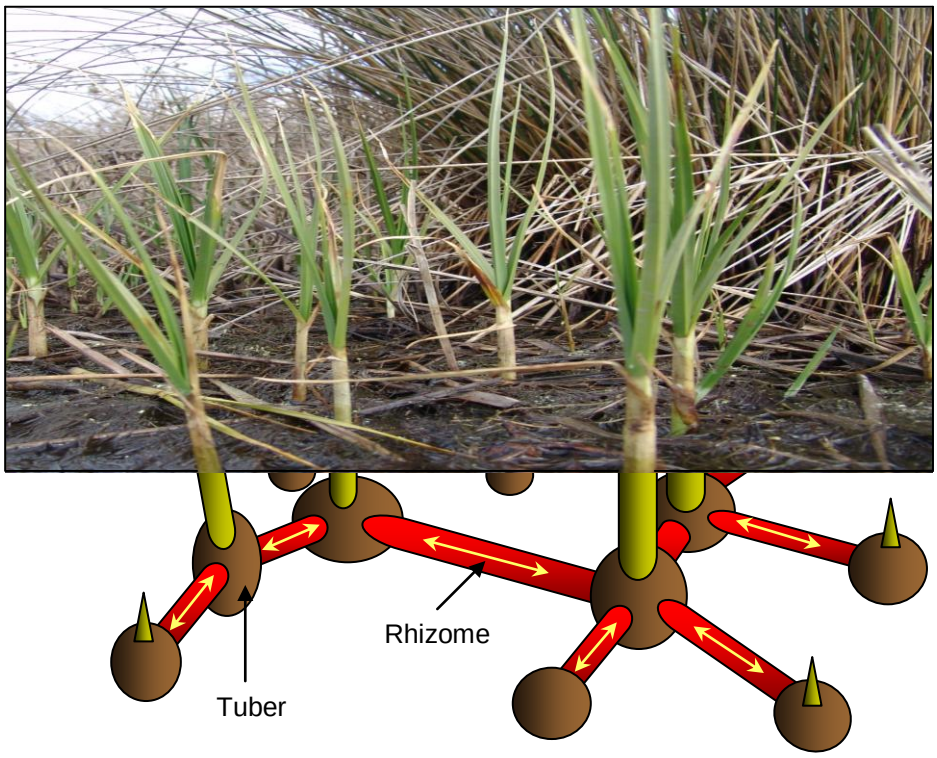


Figure 1.9. Conceptual diagram of clonal reproduction in an emergent aquatic plant (*Bolboschoenus caldwellii*). New ramets possess optimised genotypes for their local environment and may share resources between interconnected stems, rhizomes and tubers.

When integrated with their parent plant, ramets collectively form genets that may share resources between plant parts (Mágori *et al.* 2003). Resource sharing and

division of labour among individual ramets allow clonal plants to specialise in the uptake of the most available resource rather than the most limiting; this enables clones to inhabit otherwise unavailable niches (Alpert 1996; Hutchings and Wijesinghe 1997; Hutchings 1999). The perennating organs used by many clonal species, such as tubers, corms and rhizomes, also offer high resource storage capacity, which enables clones to repeatedly recover from disturbance events (Pitelka and Ashmun 1985; Schmid *et al.* 1988; de Kroon and van Groenendael 1990; Hroudová and Zákravský 1995). By definition, clonality confers the ability for rapid numerical increases in biomass; clonal plants are highly efficient at consolidating space and resisting invasion, as well as exploring new territory (Cook 1985; Grace 1993). The cooperative growth mechanisms of clonal plants allow them a capacity to buffer against environmental heterogeneity and offer a level of fitness that is otherwise unavailable to non-clonal species (Eriksson and Jerling 1990; Callaghan *et al.* 1992; Pan and Price 2002).

Expansion and consolidation of space via vegetative growth can be of considerable competitive benefit to a species in the short-term, though in the long-term a lack of sexual recruitment may incur several costs to clonal populations. The formation of large, long-lived clones can limit outcrossing opportunities and increase geitonogamy (pollination between flowers of the same plant) and so prevent genetic recombination whilst promoting genetic uniformity within populations (Charpentier 2002; Eckert 2002; Eckert *et al.* 2003). The loss of novel genotypes may decrease population fitness, while increasing the level of inbreeding, which in turn makes populations more susceptible to disease (Charpentier *et al.* 2000). When networked, diseases can easily be transmitted between ramets, though recent research suggests that clonal plants use a number of defence signals when under invasion by pathogens (Stuefer *et al.* 2004). Individual ramets within a given genet may also become physiologically independent through the severing or decay of rhizomes (sectoriality), which may offer potential escape from infection for the rest of the clone (Marshall and Price 1997).

The efficiency of various clonal growth methods, independent of sexual reproduction, is illustrated by the successful spread of many aquatic invasive (weed) species, such as *Eichhornia crassipes* (Water Hyacinth), *Fallopia japonica* (Japanese

Knotweed), *Myriophyllum* spp. (Milfoils), *Pistia stratioides* (Water Lettuce), *Salvinia molesta* (Salvinia) and *Salix* spp. (Willows) (e.g. Barrett 1980; Coble and Vance 1987; Oliver 1993; Hollingsworth and Bailey 2000). Though clonal growth is a common factor for these species, their ecology and reproductive strategies may differ substantially between populations (e.g. *Utricularia* spp., Kameyama and Ohara 2006). It is important, therefore, to understand the growth mechanisms and responses of individual species, as closely related organisms may display contrasting strategies under different environmental conditions, especially in terms of the relative contribution of sexual and asexual reproduction to population dynamics.

1.5 Environmental sieves and the role of salinity in south-eastern Australia

Van der Valk (1981) proposed a theoretical rationale for recruitment in wetland plants based on a Gleasonian model of succession. In Van der Valk's seminal work, a model for freshwater wetlands was created to predict species presence or absence under changing hydrological conditions. Annual, perennial and clonal species intermittently occur as adult plants, dormant seeds or become locally extinct, depending on the water level of the wetland and the life-history strategy of the species involved. The entire wetland, therefore, was posited to act as an environmental sieve, and only those species with characteristics that match those of the sieve or filter may establish at any given time (van der Valk 1981). Predictions of recruitment dynamics are highly accurate under freshwater conditions, such as prairie glacial marshes (e.g. van der Valk and Davis 1979), though in brackish or saline systems, the modelling of recruitment or succession is more complex (van der Valk 1981). For example, landscape-scale modifications to the hydrological regimes of rivers and wetlands in south-eastern Australia have not only changed the timing, depth and duration of wetting and drying events, but have also resulted in many freshwater bodies becoming increasingly at risk from secondary salinisation (Davis *et al.* 2003, Boon *et al.* 2007). Since salinity is such an over-riding factor for many Australian wetlands, it is considered in detail in a later segment of this chapter.

Secondary salinisation is one of the most serious and potentially irreversible threats to freshwater wetlands across the southern half of Australia (Hart *et al.* 1990; 1991; National Land and Water Resources Audit 2001; Halse *et al.* 2003; Bailey *et al.*

2002). The problem arises from a number of human-induced activities, which alter the salt and water balance of the landscape. For example, the removal of all deep-rooted perennial species through land-clearing reduces evapo-transpiration rates and causes groundwater tables to rise, which in turn bring salt to the surface (Cramer and Hobbs 2002). Similarly, long-term irrigation leads to excessive groundwater recharge and the transportation of salt to the soil surface. Inevitably, this liberated salt finds its way into wetlands via surface runoff, as they are the lowest drainage points of the landscape. Salt will accumulate in wetlands where groundwater is unable to discharge, which is often the case in areas with high water tables. In such a scenario, additional water volumes will increase the pressurisation of aquifers and hinder the vertical movement of water and exchange of ions (Paijmans *et al.* 1985). Salinity will also increase if evaporation rates exceed precipitation rates, by concentrating surface-water salts. Furthermore, prolonged draw-down conditions will confound salinity problems by promoting the capillary rise of saline groundwater. The leaching or flushing of salts from wetland systems via lateral surface water flow, therefore, becomes critical and is often the only mechanism available to lower salinity.

In heavily irrigated regions, such as the Macalister irrigation district (which surrounds much of the Gippsland Lakes: see Fig. 1.1), stream flows are often no longer sufficient to overtop river banks and inundate the surrounding floodplain and its wetlands. This problem is further confounded by river regulation such as dams, weirs and levee banks that prevent the flushing of salts from wetlands and the occurrence of regular wetting and drying cycles. It is these wet and dry cycles that characterise ephemeral wetlands and effectively open and close recruitment windows. Ephemeral water regimes maintain biodiversity and interference to theses patterns through prolonged flooding, drying or increased salinity can significantly influence the organisation and structure of wetland vegetation communities.

The negative effects of salinity and or flooding on seed germination across genera have been well documented (e.g. Ungar 1982; Partridge and Wilson 1987; Koslowski 1997; Brock *et al.* 2005; Salter *et al.* 2007). Sexual recruitment for species in highly altered wetlands must therefore occur under conditions that may never have been experienced before and vegetation communities must either adapt or shift to a different successional stage. A number of studies have shown that increases in

salinity will not only cause the composition of wetland floristics to shift towards more salt-tolerant species, but that salinity will also act as a significant environmental sieve to sexual reproduction (Nielsen *et al.* 2003; Brock *et al.* 2005; Boon *et al.* 2007; Boon *et al.* 2008). Similarly, prolonged flooding in a central Florida hardwood swamp was shown to shift the composition of the vegetation community to one dominated by clonal species (Ernst and Brooks 2003). The underlying implication of these findings is that environmental sieves over recent periods may have been actively selecting and favouring species with clonal growth habits, as they are not dependent on sexual reproduction to survive.

A large number of finely scaled environmental variables need to coincide to create the ideal conditions ('safe sites') for seed germination, and include water regime, salinity, temperature, pH, light levels and sediment type (Harper 1977; Britton and Brock 1994). Additional biotic interactions, such as predation, competition and allelopathy are also highly influential to early germination phases (Baskin and Baskin 2001). Sexual reproduction is dependent on the ability of an individual seed to progress through not one, but a series of environmental sieves, and is thus an unlikely occurrence (Van der Valk 1981; Schupp 1995; Robinson *et al.* 2006). Recent models on the sexual recruitment dynamics of clonal plants have suggested three scenarios: 1) the establishment of a population occurs via an isolated initial seed recruitment event only, 2) regular seedling recruitment occurs into existing populations, or 3) seed recruitment events are restricted to rare windows of opportunity and may occur at intervals of decades, centuries or even millennia (Eriksson 1989; Jelinski and Cheliak 1992; Eriksson and Fröborg 1996).

1.6 The importance of ascertaining sexual recruitment dynamics in clonal species

Aquatic plants often have wide distributions and are commonly found growing in large stands (Santamaría 2002). Many aquatic species are cryptic in appearance, as the abundance of separate stems in a population may give the impression of a high number of individuals and wide genetic diversity. The clonal capacity of wetland species, however, allows them to colonise large areas, irrespective of sexual reproduction, as conditions that are harmful for germination may not hinder vegetative expansion. Consequently populations may only consist of a small number

of genetic individuals, or in fact a single clonal genet (Hollingsworth and Bailey 2000; Araki and Kodono 2003; Eckert *et al.* 2003). Thus, it is important to determine the recruitment patterns of clonal species in terms of their conservation and sustainable management. There is, for example, great value in determining whether clonal species repeatedly recruit new seedlings into existing populations, or whether seed recruitment events are either a one off occurrence or limited to rare recruitment windows of opportunity (see Eriksson 1989; Jelinski and Cheliak 1992; Eriksson and Fröborg 1996). Answers to such questions allow for a more accurate assessment of population size, age, structure and fitness.

A wide variety of wetland plant species are frequently documented to fail to recruit sexually, e.g. *Phragmites australis* (Mauchamp *et al*. 2001), *Triglochin bulbosa* and *Triglochin striata* (Naidoo and Naicker 1992), *Typha angustata* (Gopal and Sharma 1983), *Typha latifolia* (Lombardi *et al*. 1997). These species nonetheless, persist over time, indicating that vegetative recruitment from over-wintering propagules (sprout-banks of rhizomes and tubers) is critical to the ongoing survival of populations and is responsible for the majority of above-ground biomass[1]. In addition to the various environmental sieves that prevent seed germination, resource allocation or investment theory suggests that reduced sexual recruitment may also be due to poor seed production and viability, which results from trade-offs in resource investment between vegetative expansion and sexual reproduction (Sutherland and Vickery 1988; Sun *et al.* 2001). As all growth is from the one resource pool, vigorous vegetative growth decreases the amount of resources available for the production of flowers and seed (Sculthorpe 1967). Plant species (across genera) are thought to adopt trade-offs in biomass allocation in an attempt to attain higher fitness (Gardner and Mangel 1999; Vretare *et al.* 2001). For example, annual and semi-perennial species expend high levels of resources on sexual reproduction to increase the likelihood of forming a viable seed bank, thereby raising the probability of success for the next generation. Long-lived perennial or clonal species in contrast, maintain fitness through adult survival and investment into vegetative growth (Shefferson and Tali 2007).

[1] It should be noted that many of these emergent aquatic plants also grow in the tropics, where they are not reliant on over-wintering propagules, as they grow unimpeded by climate all-year-round.

Trade-offs such as reduced flowering, seed production and seed viability, in favour of asexual growth have been reported for a range of clonal plants, from herbaceous groundcovers, such as *Mimulus* spp. (Sutherland and Vickery 1988) to large woody species such as *Melaleuca ericifolia* (Robinson *et al.* 2006). Resource expenditure on sex (flower and fruit production) can be a considerable investment for plants. For example, Karlsson (1988) found 27-240% higher N and P concentrations in *Pinguicula vulgaris* shoots that had not flowered or produced fruit, than in shoots that had invested in sexual reproduction. Investment into sexual reproduction may also have long-term effects. For example, Primack and Stacey (1998) demonstrated through pollen manipulation experiments of *Cypripedium acaule* (Orchidaceae) that prolonged dormancy can result from expenditure on flower and fruit production.

In highly unpredictable environments, such as wetlands, resources may be better spent on asexual expansion and consolidation of space, which buffer clones against spatial and temporal heterogeneity and increase survival or fitness, especially for long-lived clonal plants (Harper 1977; Cook 1985; Silvertown *et al.* 2001). For emergent species the benefits of asexual growth are substantial, as when a clone senesces over winter, for every ramet that has foregone sexual reproduction a higher level of resources may be re-sequestered back into tubers and rhizomes for storage. In theory, greater investment into storage (i.e. dormancy) should increase future sprouting success and vegetative expansion and, therefore, also long-term genet survival (Hroudová and Zákravský 1995). A range of abiotic and biotic factors are known to influence trade-offs between sexual and asexual growth. These factors include: 1) resource levels (e.g. *Mimulus* spp., Sutherland and Vickery 1988); 2) water level (e.g. *Chara australis*, Casanova 1994); 3) plant size (e.g. *Pinguicula vulgaris*, Worley and Harder 1996); 4) ramet density (e.g. *Elymus lanceolatus,* Humphrey and Pyke 1998); 5) age and successional status (e.g. *Sparganium erectum*, Piquot *et al.* 1998); 6) gradient elevation (e.g. *Scirpus mariqueter*, Sun *et al.* 2001). Studies examining trade-offs between sexual and asexual reproduction in relation to salinity are lacking in scientific literature.

1.7 Models of resource trade-offs and plasticity in clonal species

Several analytical and simulation models have been proposed to predict the responses of clonal plants to changing environmental conditions (e.g. Loehle 1987; Sakai 1995; Gardner and Mangel 1999; Olejniczak 2001). As with all models, the outcomes generated can vary substantially depending on the number of variables operating and the species under consideration. The models of Sakai (1995) and Gardner and Mangel (1999) predict that asexual reproduction should be favoured over sexual reproduction in favourable or productive habitats (in this case low salinity). Alternative models, such as those of Loehle (1987) and Olejniczak (2001) predict that allocation to sexual reproduction should increase with respect to asexual growth in productive environments. All of the models agree, however, that allocation to seed production is greatest when the mortality risk to the whole clone is highest (e.g. when faced with high salinity). While these models are adequate for the division of ramets into sexual and non-sexual modules, they do not consider plasticity or trade-offs between individual ramet components (tubers, rhizomes, roots and culms) or above and belowground biomass ratios.

A growing body of research has focused on the significance of morphological plasticity and biomass trade-offs in clonal plants (e.g. Li *et al.* 2001). The major concern of these studies is whether or not the architecture of a species' growth form alters between sites with different environmental conditions, or in environments where resources are unequally distributed into discrete patches. In other words, do clones ramify or enlarge their growth form under one regime compared to another and how is their biomass organised? These questions are addressed in Section III of the thesis, which examines the clonal growth mechanisms and responses of *B. caldwellii* and *B. medianus*.

Morphological plasticity in the rhizomes and stolons (spacers) of clonal species, such as alterations to spacer length, branching angle and branching intensity, enable clonal plants to selectively place ramets in more favourable resource patches within a heterogeneous or changing landscape. This concept is known as resource foraging (Slade and Hutchings 1987a and 1987b). While stoloniferous species such as *Fragaria chiloensis* (Strawberry) and *Glechoma hederacea* (Ground Ivy) display a

distinct ability to alter their spacer lengths in response to variables such as shading, it appears that rhizomatous species are largely unresponsive to environmental variation in terms of the shortening or lengthening of rhizomes (see de Kroon and Hutchings 1995). Instead of showing variation in the plagiotropic or horizontal plane, rhizomatous species appear to show a greater capacity for plasticity in the orthotropic (vertical) dimension (de Kroon and Hutchings 1995; Clevering and Hundscheid 1998). Orthotropic plasticity includes altering growth from tubers to produce more roots and stems, or for the tubers to remain dormant.

Morphological plasticity and resource trade-offs for clonal species are a function of the interaction between developmental patterns and environmental variability (Bradshaw 1965; Watson *et al.* 1997). When trying to interpret variation in the growth behaviour of individual modules within a larger genet, it is important to distinguish between non-plastic (internal or developmental) variation and plastic (external or environmental) variation (Clevering and Hundscheid 1998). While a great deal of variation in the length of stolons and rhizomes can occur within an individual plant, it may be that very little of this variation is truly 'plastic' as environmental conditions may not cause a degree of plasticity beyond normal phenotypic ranges for type specimens (de Kroon *et al.* 1994). Previous criticisms of plasticity assessments for clonal species include the fact that most studies are carried out with no knowledge of the genotypes used, or with only one or two genotypes (de Kroon *et al.* 1994). Both issues have lead to uncertainty over the accuracy of plasticity estimates within populations. In addition, while resource trade-offs and plasticity have been described in relation to variations in water depth, nutrients and light, trade-offs and plasticity may also arise from changes in salinity, though as stressed by Van Zandt *et al.* (2003) these strategies are poorly understood in wetland plants.

Both *B. caldwellii* and *B. medianus* can display morphological plasticity, such as the extension of culm length in response to flooding (Blanch *et al.* 1999a; 1999b; Siebentritt and Ganf 2000). A high proportion of resources are directed into the production of emergent leaves in both species when flooding occurs, in an attempt to maintain photosynthetic leaf area ratios (LAR) and net assimilation rates (NAR) (Siebentritt and Ganf 2000). Culm and tuber biomass have been shown to be

inversely related across a range of water depths (+20 to –60cm) for both *B. caldwellii* and *B. medianus*, suggesting that culm extension in response to flooding is supported by the translocation of resources from tubers (Blanch *et al.* 1999a; Siebentritt and Ganf 2000). The ability to alter biomass allocation, as a response to flooding has obvious benefits for wetland plant species in terms of maintaining gas exchange and photosynthetic capacity and has been documented in a number of common macrophytes including: *Scirpus* (now *Bolboschoenus*) *maritimus* (Lieffers and Shay 1982; Clevering *et al.* 1995; Coops *et al.* 1996), *Typha latifolia* and *Typha domingensis* (Grace 1989), *Cyperus esculentus* (Li *et al.* 2001) and *Phragmites australis* (Coops *et al.* 1996; Vretare *et al.* 2001).

Although the resources stored in tubers and rhizomes provide an effective buffer against stresses such as flooding, their expenditure may compromise future asexual recruitment by exhausting energy reserves that would normally be allocated to new ramet production. Eventually, a trade-off must occur between above-ground and below-ground biomass under flooded conditions, in order to ensure that resource reserves remain available for future sprouting (Sculthorpe 1967). Prolonged submergence will generally lower the activity and strengthen the dormancy of tubers, as found for *B. maritimus* (Zákravský and Hroudová 1994). Dormant tuber banks are highly important for population renewal in *Bolboschoenus* species, as they allow recovery after severe disturbances and long-term floods and droughts (Hroudová and Zákravský 1995). Tubers also play a strong role in density regulation. For example, despite vegetative reproduction leading to increased density of underground biomass, species such as *B. maritimus* usually maintain shoot sizes and density through the utilisation of dormant tubers (Hroudová and Zákravský 1995). Although flooding typically causes a shift in biomass allocation from tubers to culms in *B. caldwellii* and *B. medianus* (Blanch *et al.* 1999a; Siebentritt and Ganf 2000), increasing salinity has been shown to cause a shift in biomass allocation from culms to tubers in *B. medianus* (Morris and Ganf 2001). The responses of both species to flooding and salinity are therefore likely to be antagonistic (Morris and Ganf 2001). While the effects of flooding on the growth responses of *B. caldwellii* and *B. medianus* have been well documented, the effects of increased salinity and prolonged exposure to salt, remains largely unstudied in both species.

1.8 Effects of salt on wetland plant growth

The effects of salt on plant growth are recognised as being regulated by three mechanisms: 1) low external water potential (osmotic); 2) ion toxicity; and 3) nutrient imbalance (Taiz and Zeiger 2002). The chemical signalling of plant roots is thought to change under low water potential, altering growth patterns such as leaf expansion via hormonal signals (Munns and Termaat 1986). Low external water potential resulting from the dissolved ions in saline water, is problematic for many wetland plants, as their leaves must maintain an even lower internal water potential to retain osmotic flow between the soil and the plant (Taiz and Zeiger 2002). Plants may adjust their osmotic capacity by using a range of intracellular organic compounds, such as glycine betaine and other compatible osmotica to regulate salts and maintain equal water potentials within the cytoplasm and vacuoles (Greenway and Munns 1980). The intracellular compounds may compensate for increases in external salinity to an extent, though continued exposure to salt will lead to ionic effects appearing. Specific-ion effects reduce growth much more directly, producing visual signs such as leaf yellowing and premature senescence. While many mangrove and saltmarsh species may offset the effects of salt through specialised excretory glands, non-halophytic species such as *Bolboschoenus* must sequester salt in the vacuoles of their cells, which under high salt loads can quickly become saturated. Excess salt must then accumulate either in the cytoplasm or the cell walls where it inhibits cellular metabolism and induces desiccation (Munns and Termaat 1986; Silvertown *et al.* 2001).

One critical factor for plants under all conditions is to ensure that the rate of leaf production remains greater than that of leaf senescence. By inducing leaf desiccation, salinity also decreases the photosynthetic capacity of a plant through such effects as stomata closure, which limits gas exchange and therefore CO_2 fixation, and ultimately leads to nutrient deficiency (Lynch *et al.* 1987). Morris and Ganf (2001) demonstrated that declines in the relative growth rates of *B. medianus* under low salinity could be counteracted by the addition of nitrogen fertiliser, though as salinity increased (up to 13 d S m^{-1} or ~ 8 g L^{-1}), *B. medianus* responded by allocating greater biomass to tubers at the expense of all other ramet components, irrespective of nutrient addition. The strategy to increase resource storage into tubers under high

salinity is likely to maintain the potential for asexual reproduction, though it will occur at the expense of seed production due to a corresponding lack of above-ground biomass. Given that tubers may re-sprout at greater than three times the salinity reported for achene germination in *Bolboschoenus / Scirpus* species (Kantrud 1996), the effects of prolonged exposure to high salinity will lead to a dependence on asexual reproduction for future recruitment and thereby prevent further genetic diversity.

1.9 Genetic diversity in clonal wetland plants

A large number of studies have demonstrated the dominance and importance of clonal growth to the structure and organisation of wetland and other plant communities (Klimeš *et al.* 1997; Song *et al.* 2002; Hatton *et al.* 2008). With greater awareness of the ubiquity and influence of clonal reproduction to community ecology, demography, diversity, gene flow and evolutionary potential, the quantification of clonal structure in remnant or extant populations has become an important aspect of ecological inquiry (Douhovnikoff and Dodd 2003). A number of researchers have reported that the persistence of genetic lineages and, therefore, also the fitness of many wetland plants, results more from vegetative growth rather than seed production (Schmid 1990; Wikberg 1995; Watson *et al.* 1997). As succinctly stated by Eckert *et al.* (2003, p.331), "most plants combine sexual reproduction with asexual clonal reproduction in varying degrees, yet the genetic consequences of reproductive variation remain poorly understood". A widely held assumption for plants in harsh, unpredictable or unstable environments, such as wetlands and salt marshes, is that sexual recruitment is rare in clonal plants and that populations become dominated by few large clones (Adam 1990; Widen *et al.* 1994). Reasons behind this assumption include poor seed bank formation (Chong and Walker 2005), inhibited germination (Shumway and Bertness 1992) and high seedling mortality (Ungar 1987).

Early genetic research, using isozyme and allozyme analysis, appeared to confirm that genetic diversity was low in aquatic clonal plants (McMillan 1982; Wain *et al.* 1985; Triest 1991). In contrast, more recent evidence, using a number of different DNA fingerprinting techniques, has demonstrated that populations of clonal plants often have levels of genetic diversity that are similar to that of non-clonal or obligate out-crossing species (Ellstrand and Roose 1987; Widen *et al.* 1994; Philbrick

and Les 1996; Waycott and Barnes 2001; Richards *et al.* 2004; Shibayama and Kadono 2007). These studies reinforce the notion that few generalisations can be made regarding the genetics of clonal plants and that each species and indeed each population is worthy of individual inquiry. Reusch *et al.* (2000), for example, found wide differences in the genetic diversity of twelve *Zostera marina* stands, with the identification of ramets ranging from totally homogenous to totally heterogeneous. Stenström *et al.* (2001) examined genetic variation and clonal diversity in four closely related clonal sedges along the Arctic coast of Eurasia and reported that, while *Carex bigelowii* and *Carex lugens* were outbreeding with relatively high genetic diversity, *Carex ensifolia* and *Carex stans* displayed mixed mating systems, with different populations being mono and multi-clonal. Table 1.3 highlights the variation in genetic diversity that have been reported between different clonal aquatic plant species across scientific literature.

Table 1.3. Variation in genetic diversity in clonal aquatic plant species

Low genetic diversity or uniformity	**High genetic diversity**
Carex crinita (Bruederle and Fairbrothers 1986)	*Posidonia australis* (Waycott 1995)
Howellia aquatica (Lesica *et al.* 1988)	*Potamogeton pectinatus* (Mader *et al.* 1998)
Ceratophyllum spp. (Les 1991)	*Pedicularis palustris* (Schmidt and Jensen 2000)
Amphibolis antarctica (Waycott *et al.* 1996)	*Carex lugens* (Stenström *et al.* 2001)
Scirpus tabernaemontani and *Scirpus triqueter* (Triest and de Greef 1999)	*Thalassia testudinum* (Waycott and Barnes 2001)
Fallopia japonica (Hollingsworth and Bailey 2000)	*Iris pseudacorus* (Lamote *et al.* 2002)
Phragmites australis (Keller 2000)	*Spartina alterniflora* (Travis *et al.* 2002)
Cladium jamaicense (Ivey and Richards 2001)	*Borrichia frutescens* (Richards *et al.* 2004)
Alternanthera philoxeroides (Cheng-Yuan *et al.* 2003)	*Lychnis flos-cuculi* (Galeuchet *et al.* 2005)
Typha latifolia (Lamote *et al.* 2005)	*Luronium natans* (Nielsen *et al.* 2006)
Muehlenbeckia florulenta (Chong and Walker 2005)	*Nymphoides indica* (Shibayama and Kadono 2007)
Utricularia spp. (Kameyama and Ohara 2006)	

Before the advent of molecular genetics, the identification of clones within populations was determined by root, rhizome and stolon connectivity, or by

comparison of phenotypes and morphologies. Apart from being impractical, the study of root connectivity is further confounded by the fact that sister ramets (clones), can become physiologically independent due to the decay or disturbance of their rhizomes and or stolons (Marshall and Price 1997). Conversely, plants of different genotypic origin may exhibit underground root and rhizome connections due to natural grafting processes, as noted for *Salix exigua* (Douhovnikoff and Dodd 2003). The accuracy of phenotypical comparisons for the delineation of clones is also questionable, as decisions are ultimately subjective and fraught with error due to (intra-clonal) morphological plasticity. In contrast, recent advances in genetic fingerprinting techniques such as amplified fragment length polymorphisms (AFLP) (Vos *et al.* 1995), have allowed clones, hybrids and cultivars to be distinguished from one another with relative ease and permitted more accurate assessments of the levels of sexual versus asexual reproduction within plant populations. These techniques have been instrumental to a succession of new research determining the age and size of clones, population genetic diversity, evolutionary relationships, recruitment behaviour and the competitive relationships of clonal plants (e.g. Whittall *et al.* 2004; Kameyama and Ohara 2006).

Although generalisations must be made with caution, the amount of genetic variation in aquatic plant populations appears to depend largely on the biological characteristics of a given species (i.e. obligate out-crossers versus self compatible species or emergent versus submerged species) as well as their geographical location. For example, studies at a broad or regional scale indicate that most of the genetic variation in aquatic angiosperms is primarily distributed between populations and between sites of significantly different environmental conditions, such as freshwater, brackish and ephemeral habitats (Hettiarachchi and Triest 1991; Triest 1991). This is of particular concern to aquatic plant conservation as wetland habitats are becoming increasingly fragmented and altered at both a local and landscape level.

Other factors likely to influence the genetic diversity of populations include: growth form, environmental conditions, trade-offs between sexual and asexual reproductive investment and seed dispersal ability. For example, the clonal growth strategy employed by a species (from phalanx species with regularly arranged, closed growth forms, to guerilla species with irregular, open growth) has major implications

for survival and competitive ability, as well as future recruitment (Lovett-Doust 1981). Both *B. caldwellii* and *B. medianus* are putative phalanx species, as they form characteristic dome shapes and increase in size with a leading front of regularly arranged ramets. While clonal growth is well recognised and common to all *Bolboschoenus* species, few studies if any, have attempted to quantify the extent and nature of asexual reproduction in existing populations. Individual genets are generally presumed to grow no more than ~20-25 m in diameter (Kantrud 1996), yet *Bolboschoenus* may form extensive stands. As competitive exclusion is a characteristic of the phalanx growth form (Lovett-Doust 1981; Grace 1993), *Bolboschoenus* clones should not intermingle and should be relatively easy to discriminate using simple sampling strategies.

It may be that *B. caldwellii* and *B. medianus* clones are capable of far greater expansion than previously recorded. If so, instead of large stands consisting of thousands of unique individuals with wide genetic heterogeneity, a large 'population' may in fact be a single genet and display virtual genetic homogeneity. Such a pattern has been recorded for many invasive aquatic weed species, such as *Salvinia molesta* (Oliver 1993) and *Fallopia japonica* (Hollingsworth and Bailey 2000). This possibility is of great interest and concern to wetland managers for a number of reasons. First, if populations consist of few clones, it would imply that current conservation methodologies are preserving very little genetic diversity. Second, seed collection – albeit over a large distance – may inadvertently be taken from one or few clones, limiting the genetic contribution of future revegetation trials. Third, species-specific information regarding the capacity of asexual spread in clonal species may influence common revegetation practices in which large numbers of individuals are planted at close densities (<1-2 m), as such planting schemes are likely to limit or reduce the competitive advantages of clonal growth mechanisms such as resource sharing, resource foraging and division of labour, which allow clonal plants to compensate for environmental heterogeneity (Robinson *et al*. 2006).

Although many aquatic plant species may achieve long-distance dispersal through fragmentation of the plant body or the production of turions (vegetative propagules), emergent macrophytes do not generally adopt these strategies. The dispersal capacity of asexually derived offspring for emergent species is therefore

usually limited in comparison to the dispersal potential of their seeds (Starfinger and Stöcklin 1996). Instead, the production of new ramets in close proximity to the parent, reduces the mortality risk of both daughter modules and the entire genet (Jelinski and Cheliak 1992). One consequence of this strategy is that the added density of asexually produced ramets sets up a potential conflict by effectively preventing sexual recruitment through spatial and intra-specific competition (Eriksson 1989; 1992). Aquatic species displaying clonal reproduction, therefore, may be predicted to have low levels of genetic diversity and smaller effective population sizes than their terrestrial counterparts, independent of the reproductive strategy (Triest 1991; Barrett *et al.* 1993; Kameyama and Ohara 2006).

The ability of clonal species to recruit sexually into existing populations appears to be species specific. Of the 68 species reviewed by Eriksson (1989), 40% were able to germinate seeds within existing clonal patches, while 60% showed no evidence of sexual recruitment in local populations. Of the few Cyperaceae species that have been investigated at a molecular level, most show very low intra-population genetic variation (e.g. *Carex crinita*, Bruederle and Fairbrothers 1986; *Scirpus tabernaemontani* and *Scirpus triqueter*, Triest and De Greef 1999; *Carex arenaria*, Jonsson and Prentice 2000; *Cladium jamaicense*, Ivey and Richards 2001; *Cyperus esculentus*, Dodet *et al.* 2008). Given that sedge and rush species are renowned for their lack of sexual recruitment, low genetic diversity may be expected within populations, yet the large number of Cyperaceae genera (~100) and species (~5000) suggest that speciation events are common in this family and that there may in fact be high levels of underlying genetic variation in many of its species (e.g. De Greef and Triest 1999; McClintock and Waterway 1993).

1.10 Aims

The thesis investigates differences in the sexual and asexual reproductive ecology of two sympatric *Bolboschoenus* species (*B. caldwellii* and *B. medianus*). The recruitment dynamics of *B. caldwellii* and *B. medianus* populations are examined with reference to the landscape-scale changes in the Gippsland Lakes region over the past 100-150 years. The changes are suspected to have restricted sexual recruitment of both species and negatively influenced their genetic diversity. Thus populations

that appear genetically diverse, as they contain many thousands of stems, are suspected to instead be genetically sparse, if not homogenous. Both species produce achenes capable of extended dormancy and persistence within sediment seed banks, yet intermittent surveys over three years (2005-2007) throughout each field site discovered no definitive evidence of sexual recruitment for either species. Sexually recruited stems are easily recognised when young, as they are delicate and do not arise from tubers or emerge with well-developed triangular-shaped stems as per asexually produced ramets.

This study has three main objectives: 1) to investigate the sexual reproductive ecology (particularly the germination requirements) of *B. caldwellii* and *B. medianus*, 2) to examine the asexual growth mechanisms and responses of *B. caldwellii* and *B. medianus* to increasing salinity, and 3) to assess the genetic diversity of *B. caldwellii* and *B. medianus* stands from a number of sites in the Gippsland Lakes region. Consistent with these three aims, the thesis is divided into five sections: I – General introduction and Methods, II – Sexual reproduction, III – Asexual reproduction, IV – Population genetics, and V – Summary section.

A conceptual model of *Bolboschoenus* recruitment (Fig. 1.10) is used to illustrate each of the components examined in this thesis. Eight phases/steps are included in the model to assess the sexual and asexual reproduction of *B. caldwellii* and *B. medianus*, as well as population genetics. The model covers all seasons and begins in autumn by addressing achene production, viability and dispersal (both water and bird driven). Plant senescence, dormancy and achene stratification and scarification are shown in winter, before water levels are drawn-down in spring and summer, and achenes commence germination. Specialised germination adaptations (hypocotyl hairs) are included in spring and summer. Clonal growth or asexual reproduction is then addressed via mesocosm experiments conducted in summer and the model concludes with DNA fingerprinting of newly produced leaf tips.

The current chapter outlined the conceptual background of the investigation. Chapter Two introduces the study region and specific field sites, and also includes descriptions of common methods. The remainder of the thesis aims to discuss the various steps in the recruitment model (Fig. 1.10), through successive chapters that

separately investigate each phase of reproduction. The intention is to not only determine if there is a weak link in the recruitment process at any of the stages within the model (which may help to explain poor sexual recruitment in the field) but also to determine the specific germination requirements of each species, which are unknown at present.

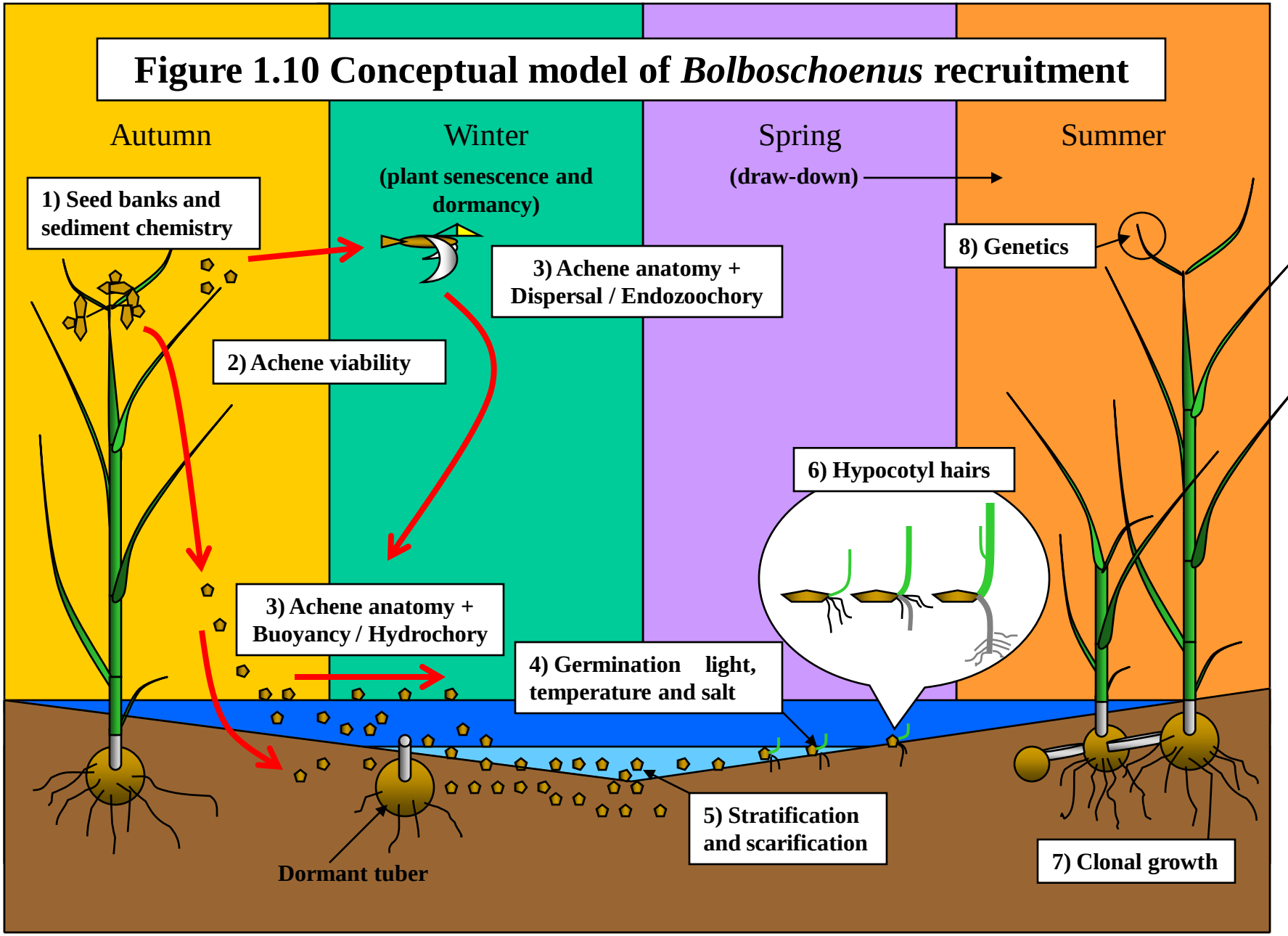


Figure 1.10 Conceptual model of *Bolboschoenus* recruitment
Autumn
Winter
(plant senescence and dormancy)
Spring
(draw-down)
Summer
1) Seed banks and sediment chemistry
2) Achene viability
3) Achene anatomy + Dispersal / Endozoochory
3) Achene anatomy + Buoyancy / Hydrochory
4) Germination light, temperature and salt
5) Stratification and scarification
6) Hypocotyl hairs
7) Clonal growth
8) Genetics
Dormant tuber

Investigation of the sexual ecology of *B. caldwellii* and *B. medianus* commences in Chapter Three, through examination of achene production and morphological variation of achenes between field sites with reference to the sediment chemistry (pH and salinity) measured beneath each population. Chapter Four asks whether poor achene viability is a contributing factor to low levels of sexual recruitment in either species at each wetland site. Chapter Five examines whether the dispersal capacity of *B. caldwellii* and *B. medianus* achenes limits recruitment, by comparing the buoyancy of achenes from each species. Buoyancy is discussed with reference to specific achene anatomical features. Chapter Six examines the individual and interactive effects of light, temperature and salinity on achene germination in each species, as well as the ability of achenes to recover from saline pre-treatments. Chapter Seven examines the ability of a range of achene stratification and scarification pre-treatments to break dormancy and improve germination in each species, as well as whether the pre-treatments are able to widen the temperature parameters for germination. Chapter Seven also discusses scarification with reference to seed passage through bird digestive tracts (endozoochory). Chapter Eight concludes the inquiry into sexual reproduction by examining the occurrence and importance of hypocotyl hairs to sexual recruitment in both *B. caldwellii* and *B. medianus* and a range of other common Cyperaceae species.

Section III addresses the asexual growth responses of *B. caldwellii* and *B. medianus* to differing salinity regimes and asks whether vegetative growth is suppressed under conditions known to inhibit sexual reproduction. Biomass allocation patterns are discussed with reference to concepts of morphological plasticity, as well as analytical models that predict the facultative responses of clonal plants to changing environmental conditions.

Section IV uses DNA fingerprinting techniques to examine the genetic diversity of populations from each of the field sites. In combination the three aspects of analysis (sexual, asexual and genetic), will enable the development of a story that describes how *Bolboschoenus* populations are established and maintained in the various wetlands used as field sites. This information is summarised in Section V of the thesis and is critical to the understanding of past and future recruitment events and the analysis of current population status in terms of genetic diversity.

Chapter 2.

Field site descriptions and general methods

2.1 Field site descriptions

Three field sites of contrasting character [Sale Common (38°07'S, 147°04'E), Clydebank Morass (38°02'S, 147°14'E) and Dowd Morass (38°09'S, 147°10'E)] were selected from among a chain of Ramsar-listed wetlands along the western edge of Lake Wellington in west Gippsland (Fig. 2.1). Sale Common is maintained as a Nature Conservation and Wildlife Reserve, while Clydebank Morass and Dowd Morass are designated State Game Reserves. Parks Victoria (Sale Office) manages all three sites and the legal status of each wetland is governed under the Crown Land (Reserves) Act of 1978 and the Wildlife Act of 1975.

Sale Common (~300 ha) is a semi-permanent freshwater wetland (Fig. 2.2). The hydrology of Sale Common is closely linked to both the Thompson and Latrobe Rivers that flank its western and southern edges. Salinity rarely exceeds 1-2 g L^{-1} in Sale Common, so the area supports a high diversity of freshwater macrophytes, waterbirds, mammals, reptiles and amphibians (DCE 1993). Gippsland/Forest Red Gum woodlands (*Eucalyptus tereticornis*) and Swamp Paperbark (*Melaleuca ericifolia*) fringe Sale Common. The ecotone contains large stands of Common Reed (*Phragmites australis*), Marsh Clubrush (*Bolboschoenus medianus*) and Tall Spike-rush (*Eleocharis sphacelata*), while submerged zones are densely carpeted with a wide variety of freshwater macrophytes including, Water Ribbons (*Triglochin procerum*), Slender Knotgrass (*Persicaria decipiens*), Mud Dock (*Rumex bidens*) and Water Primrose (*Ludwigia peploides*).

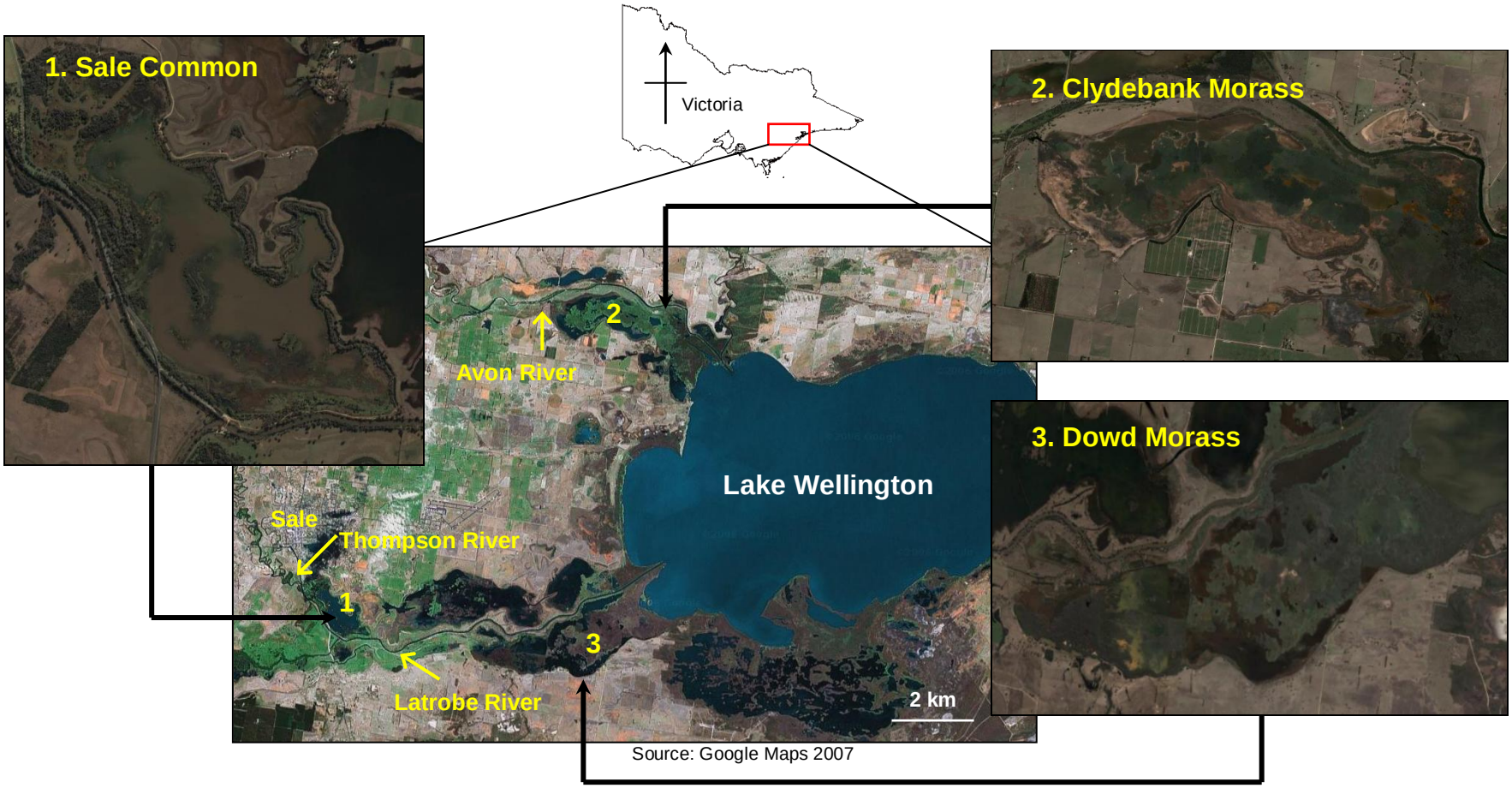


Figure 2.1. Map of the Gippsland Lakes region, south-eastern Australia, showing selected field sites for seed, sediment and vegetative sampling: 1) Sale Common, 2) Clydebank Morass and 3) Dowd Morass. Refer to Figure 1.1 for smaller scale orientation.

Figure 2.2. Sale Common in a drawn-down state, December 2006. *Bolboschoenus medianus* and *Melaleuca ericifolia* is seen on the fringes, while the background shows *Eucalyptus tereticornis* woodland.

Clydebank Morass (~1,420 ha) and Dowd Morass (~1,500 ha) are both brackish water marshes, though their hydrological and salinity regimes often differ substantially (SKM 2001; 2003). High levels of water extraction within the Macalister irrigation district have resulted in Clydebank Morass being almost entirely reliant on rainfall to lower salt levels, and recent unpublished work by DSE has indicated that this wetland is accumulating salt at a greater rate than is being exported via overbank flows from the Avon River (SKM 2001). The eastern side of Clydebank Morass is linked to Lake Wellington via a small bank breach, so surface water salinities are often greater than 10-20 g L^{-1} due to regular influxes of saline water from Lake Wellington (SKM 2001). The eastern portion of Clydebank Morass rarely dries due to the influence of Lake Wellington, though the western portion regularly dries and crystallised salts can be seen at these times on the sediment surface. Figure 2.3 shows that as water levels decline salinity in Clydebank Morass rapidly increases to greater than 40000 EC (~24 g L^{-1}) (SKM 2001).

The vegetation at Clydebank Morass is characterised by large expanses of Common Reed (*Phragmites australis*), Pale Rush (*Juncus pallidus*) and Swamp Paperbark (*Melaleuca ericifolia*). Understorey vegetation is typical of coastal saltmarsh and includes Rounded Noon Flower (*Disphyma crassifolium*), Australian

Salt-grass (*Distichlis distichophylla*), Shiny Swamp-mat (*Selliera radicans*) and Beaded Glasswort (*Sarcocornia quinqueflora*) (Fig. 2.4).

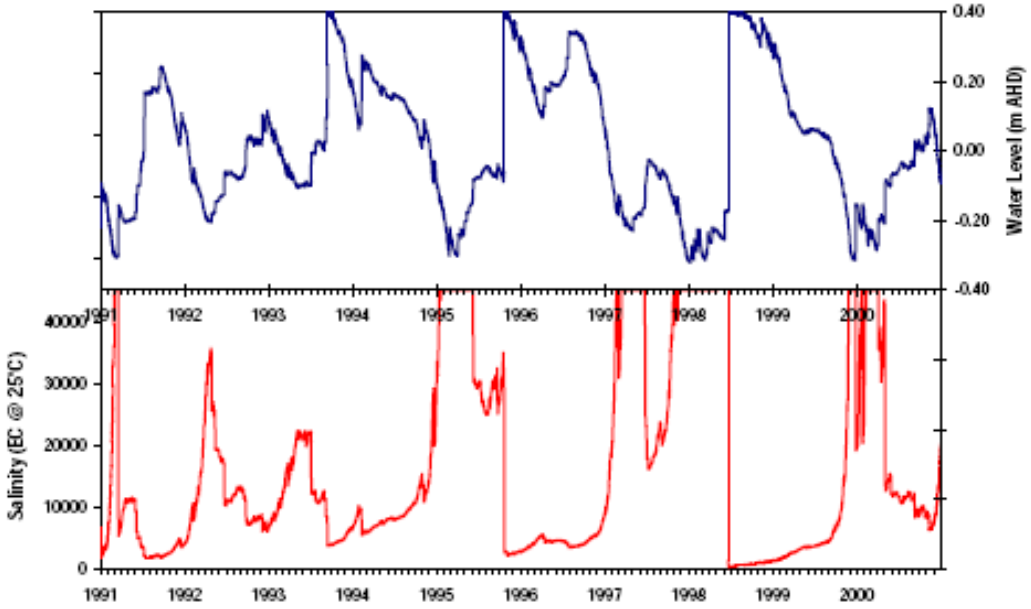


Figure 2.3. Simulated water levels and salinity in Clydebank Morass 1991-2000 (Source: SKM 2001). As the wetland dries salinity is expected to increase greater than 40,000 EC (~ 24 g L^{-1}).

Figure 2.4. Vegetation and black swans at Clydebank Morass 2005. The foreground shows *Sarcocornia quinqueflora*, while in the background a large stand of *Melaleuca ericifolia* is seen behind *Phragmites australis*.

The third site, Dowd Morass, was formerly a freshwater wetland but is now an almost permanently flooded brackish water wetland, largely due to major alterations such as the construction of levee banks during the early 1970's. With the exception of a short draw-down event in 1998, water levels in Dowd Morass have been maintained between 0.2 and 0.8 m deep for approximately the past 30 years (Grayson 2003; Boon *et al.* 2007). This permanence is in stark contrast to historical hydrological regimes, in which Dowd Morass was thought to completely dry out once in every five years (Parks Victoria 2008). Altered water regimes and landscape-scale modifications to Dowd Morass have resulted in saline water intrusions from Lake Wellington having a greater influence on water chemistry than can be compensated for by rare freshwater flushing events from the Latrobe River (SKM 2003). Average surface water levels and sediment salinities in Dowd Morass have increased over the past 20 years, in line with the predictions of Bird (1966), though salinity concentrations are highly variable and dependent on sample location (SKM 2003, Boon *et al.* 2007) (Fig.2.5) While the management objective of Parks Victoria is to maintain these wetlands at or below 1,500 EC or 1-2 g L^{-1} salt, low salinity is rarely achieved other than in Sale Common (Andrew Schulz, *pers. comm.*).

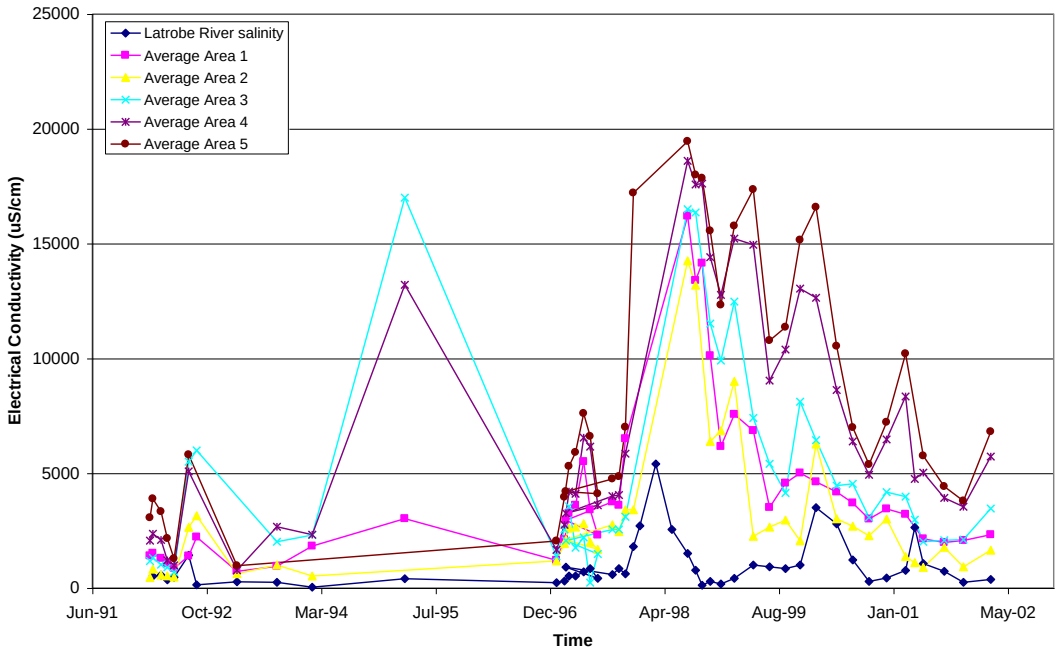


Figure 2.5. Surface water salinity of the Latrobe River and five monitoring locations at Dowd Morass 1991-2002 (Source: SKM 2003). Salinity is highly variable both spatially and temporally.

Similar to Clydebank Morass, the vegetation at Dowd Morass is dominated by large stands of Common Reed (*Phragmites australis*) fringed by Swamp Paperbark (*Melaleuca ericifolia*). Many of the formerly common freshwater macrophytes such as Eel Grass (*Vallisneria americana*), have now been largely replaced by more salt-tolerant species such as Water Buttons (*Cotula coronopifolia*), Creeping Monkey-flower (*Mimulus repens*) and Streaked Arrow-grass (*Triglochin striatum*) (Fig. 2.6).

Figure 2.6. Vegetation at Dowd Morass 2006. The foreground shows hummocks of *Paspalum distichum* (Water Couch) fringed by *Phragmites australis* and *Melaleuca ericifolia* in the background.

The contrasting environmental conditions across the selected field sites are reflected by the different affinities shown by *B. caldwellii* and *B. medianus* for each wetland. The distribution of *B. caldwellii* is restricted to Clydebank and Dowd Morass (wetlands with higher salinity) while *B. medianus* is found predominantly at Sale Common (freshwater) and to lesser extent at Dowd Morass (brackish).

2.2 General methods for Section II: Achene collection, storage and incubation

Entire flower heads were collected from several *Bolboschoenus* populations at each of the three field sites in January 2006. Where possible achenes were obtained from putatively different genetic individuals, determined on the basis of their characteristic phalanx clonal growth form (*sensu* Lovett-Doust 1981). Populations of

B. medianus at Dowd Morass appeared to consist of very few genets; most of the collected achenes came from approximately two to six putative clones. In contrast, *B. caldwellii* achenes were plentiful at Dowd Morass. Achenes were found across a broad area at Sale Common and Clydebank Morass for *B. medianus* and *B. caldwellii*, respectively, though many patches showed no sign of flowering or having produced achenes in the near past. Following collection flower heads were stored in paper bags in the dark at 20ºC for two-weeks to aid maturation. Achenes were then separated from individual bracts by breaking up the spikelets by hand and sieving away chaff and all other detritus. Achenes from each site were pooled and stored separately for a further two-weeks in the dark in sealed 10 mL plastic specimen jars before use in viability and germination trials.

To minimise infection by fungi, all achenes were surface sterilised in 10% w/v sodium hypochlorite solution for 5-minutes, then rinsed thoroughly with deionised water before plating into 90 mm petri dishes. Unless otherwise stated, treatments consisted of 4 replicate petri dishes, with 25 achenes per dish for each species, plated in a 5 x 5 grid onto one sheet of Whatmans #1 filter paper, wetted with 5 mL of deionised water. Plates were sealed with Lab film to prevent moisture loss and incubated in a single temperature-controlled chamber. A 12:12 hour light:dark cycle was employed, with a temperature regime of 30ºC during the day and 5ºC at night. This diurnal range was chosen as it has been used successfully to germinate other *Bolboschoenus* species (Clevering 1995; Moravcová *et al.* 2002). Light was provided by a bank of three *Gro-Lux*™ fluorescent tubes, which emitted PAR of ~90 μ mol m^{-2} s^{-1} at the level of the petri dishes. Replicates were inspected every two days (over a period of one month) for indications of germination and the positions of each petri dish were shuffled after each assessment, to randomise possible temperature and light inconsistencies within the chamber. Individual trials were stopped after one month as preliminary tests had shown negligible germination after 30 days. Germination was scored as successful where both the plumule and hypocotyl region had emerged from the seed coat.

Section II:

Sexual reproduction

Chapter 3.

Achene production, polymorphisms and sediment seed banks in relation to soil chemistry

3.1 Introduction

The first aspect of inquiry into the sexual ecology of *B. caldwellii* and *B. medianus* involved the quantification of achene production at each of the field sites, thus, this chapter examines the first component of the conceptual recruitment model proposed on p.37 of Chapter 1: achene production. Both the number of achenes and differences in size and shape were assessed via the examination of aerial and sediment seed banks at each. Achene production and sediment seed bank formation was then related to sediment chemistry conditions at each site.

The production of achenes is affected by both environmental and genetic characteristics, which may result in plasticity among individual plants and populations (Harper 1977). Hroudová *et al.* (1997) and Hroudová *et al.* (1999) demonstrated that the achenes of *Bolboschoenus maritimus* (including various subspecies), collected from different geographical locations across Europe, were highly polymorphic in shape and size. Achene polymorphism also occurs in Australasian *Bolboschoenus* species, particularly *B. medianus* (see Browning *et al.* 1997), though the question as to whether the variations confer any ecological advantages or serve a strategic role in recruitment for these species remains unknown.

A number of studies have shown that polymorphisms in the size, shape and colour of seeds produced by a species, can influence the rate and timing of germination, as well as the response of seeds to variables such as light, salinity and

temperature (Harper and Williams 1965; Khan and Ungar 1985; Ruiz de Clavijo 1994; Rocha 1996). Baskin and Baskin (2001) highlight the need for greater study into seed polymorphism as dimorphic seeds are found in a broad range of species and there is uncertainty as to whether the germination responses of the seeds differ. The effect of seed size on germination appears to be species specific (Baskin and Baskin 2001). Large or small seeds may germinate at an increased rate with respect to one another, or germination may be independent of seed size, depending on the species in question (e.g. *Rumex obtusifolius*, Cideciyan and Malloch 1982; *Atriplex triangularis*, Khan and Ungar 1985; *Erodium brachycarpum*, Stamp 1990). Nevertheless, it appears that smaller seeds found across the distribution of a species are generally correlated to saltier environments (Krauss *et al*. 1998), which suggests that larger seeds should be produced under fresh water conditions. However, the ability of clonal plants to trade-off resource allocation between sexual and asexual reproduction, can also lead to the production of smaller seeds when environment conditions are fresh or conducive to vegetative growth. With respect to *Bolboschoenus* species, patterns between achene production and sediment salinity may be predicted through a seed continuum / trade-off model that represents smaller seed size production and lower quality (i.e. viability) at both extremes of a salinity gradient (Fig. 3.1).

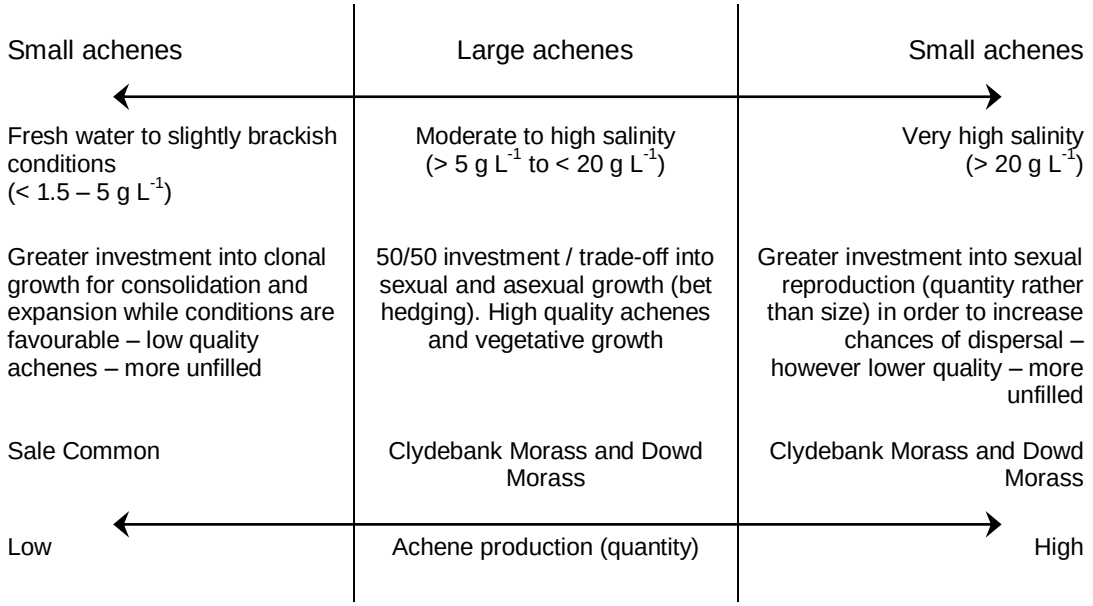


Figure. 3.1. Continuum model, predicting achene sizes in relation to salinity and resource trade-offs between sexual and asexual growth.

With respect to the overriding question of this thesis (why are sexual reproduction events rare for *B. caldwellii* and *B. medianus*), the aims of this chapter were four-fold: 1) to quantify salinity and pH of sediment cores taken within populations of *B. caldwellii* and *B. medianus* from contrasting wetland sites, in order to highlight the different environmental conditions faced by each population during achene production and recruitment, 2) compare differences in achene production among wetlands, both quantitatively and qualitatively, with respect to the seed continuum / trade-off model proposed in Fig. 3.1, 3) assess the adaptive significance of different achene polymorphs from each site by testing them under a range of germination conditions, and 4) compare sediment seed bank densities and the germinability of achenes recovered from soil cores across each wetland.

3.2 Methods

3.2.1 Field sites and achene collection

See Chapter 2 for field site descriptions and achene collection locations. The discovery of distinct achene size differences between sites prompted the decision to sample seeds from disparate populations within each wetland in order to examine whether the polymorphisms were consistent within each site (entire wetland effect) or whether the achene differences were restricted to single populations (local effect).

3.2.2 Achene production

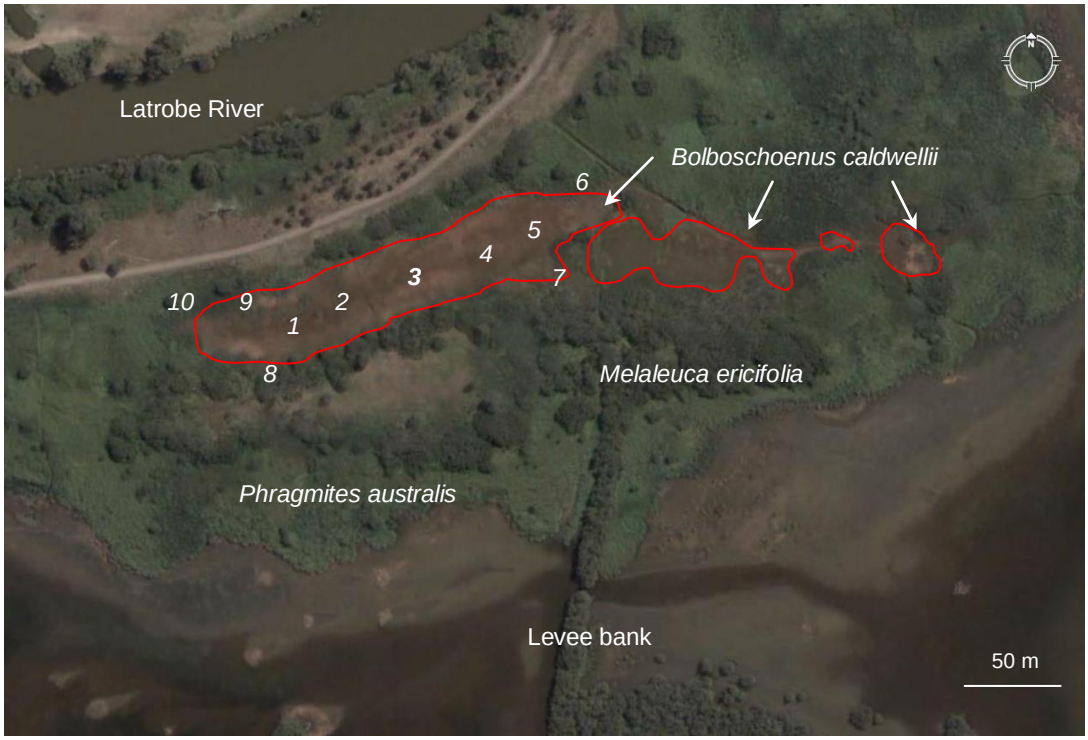
Ten entire flower heads were randomly selected from each species at each site in February 2006 in order to determine whether investment into sexual reproduction differed between the populations. Flower heads were selected from the centre and edges of populations in order to minimise within-genet sampling repetition, then stored in paper bags for later inspection. Individual flower heads (spikelets) were dissected glume by glume to quantify achene production for each site. Five randomly placed 1 m^2 quadrats were used to estimate the number of flower heads produced by each population in order to estimate the number of achenes produced per square metre.

3.2.3 Achene sizes and weights

Electronic callipers were used to measure the width and length (micropyle to stigma end) of 100 achenes of each species from each site. Ten samples of 100 achenes per site were weighed then averaged and further divided by 100 to determine the approximate weight per achene at each site.

3.2.4 Seed banks and soil core sampling

Sediment cores from each site were obtained in the last week of September and the first week of October 2006, as indicated in Figure. 3.2 (see Appendix A1 for complete list soil core GPS coordinates). Four replicate 5 cm diameter x 15 cm deep soil cores were taken at each of 10 sampling locations, giving a total of 40 cores per site. Five sampling points were taken within the middle of each *Bolboschoenus* patch, another four were taken around the perimeter of the population while a tenth sample point was taken ~5-10 m from the north-west edge of the population. Individual cores were placed into plastic zip-lock bags and stored within an esky before being transferred to the university cool-room prior to testing. Of the four cores taken at each sample point, one core was used to examine and estimate soil seed banks, while each of the remaining cores were analysed for salinity and pH.



Source: Google Maps 2007

Figure 3.2. Example of sediment core sampling regime. Freehand shapes represent *Bolboschoenus caldwellii* populations found in topographical depressions on the northern edge of Dowd Morass. Ten soil core sampling points are numbered. Five sampling points were located within the interior of populations (# 1-5), another four were located around the perimeter of the population (# 6-9), while the last sample point (# 10) was located ~10 m from the north-west corner of the patch. The scale of the sampling strategy at each site was relevant to the patch or population size. At each sampling location 4 (5 x 15 cm) soil cores were extracted and used to analyse, sediment chemistry conditions (salinity and pH) as well as soil seed bank densities.

3.2.5 Analysis of sediment seed banks

Soil cores were placed into buckets and sprayed gently with 1-2 litres of water to loosen soil mass and other matter. Broken up cores were passed through a series of different laboratory test sieves with mesh sizes 8.00 mm, 4.00 mm and 500 µm. Collected material from each sieve was first sorted by eye and secondly by dissecting microscope to separate *Bolboschoenus* achenes from other seeds, vegetative matter and particulates. Recovered achenes were inspected under a dissecting microscope, assessed as either damaged or undamaged and measured for length and width (as above) before storage in sealable plastic specimen jars for later germination trials.

3.2.6 Sediment chemistry

Soil cores were weighed and oven dried at 107ºC for 16 hours to determine moisture content (expressed as a percentage of dry soil weight). A sub-sample of 10 g of dry soil per core was ground and made into slurries (1:5 soil:deionised water mixture) then shaken by inversion for 1 hour at 25ºC following the procedure of Rayment and Higginson (1992). Both pH and electrical conductivity (EC) were measured using a TPS-LC81 water quality meter (TPS Pty Ltd, Brisbane, Qld., Australia) in order to compare sediment conditions beneath clones from different sites. Final conductivity readings ($\mu S\ cm^{-1}$) were converted to salt concentrations ($mg\ L^{-1}$) using a conversion factor of 0.6 (Close 1990) then stated in units of $g\ L^{-1}$ of soil water.

3.2.7 Germination trials

Germination trials in fresh and saline conditions (2, 4, 8 and 16 $g\ L^{-1}$) were used to compare the responses of achene size polymorphs for each species, as well as shape polymorphism in *B. medianus* achenes. Four replicates of 25 achenes per polymorph were used for each species. Germinability of achenes recovered form sediment cores was assessed in freshwater conditions only. Undamaged achenes were pooled for each site and incubated together in the same petri dish. Incubation conditions followed those outlined in the general methods description (Chapter 2). In order to assess whether differences existed in the rate of establishment between achene size polymorphs, a selection of the healthiest looking seedlings from polymorphism germination trials were transferred to 90 mm^2 punnets containing a 2:1 mixture of peat and perlite and ~0.5 g of Osmocote™ low phosphorus slow release fertiliser. Punnets were then flooded to the sediment surface and grown under a bank of GroLux™ fluorescent light tubes for approximately 8 weeks (equal to a 2 month draw-down period).

3.2.8 Statistical analysis

One-way ANOVA and Tukey *post-hoc* tests were used to determine significant differences ($p < 0.05$) in sediment core salinity and pH levels between sites for each species, as well as the quantity of achenes produced, achene dimensions and weights and the germinability of achenes recovered from sediment cores. Aerial and sediment seed banks were compared between sites using *T*-test procedures. The germination response of achene polymorphs from each species to a range of salinity concentrations were analysed via 2-way ANOVA. Data were checked for normality (Kolmogorov-Smirnov and Shapiro-Wilks tests) and homogeneity of variances (Levene's test). Percentage germination results were converted to proportional data then arcsine-sqrt transformed prior to statistical analysis (Zar 1996). All statistical analyses were performed using SPSS (Version 14) statistical package.

3.3 Results

3.3.1 Soil chemistry in the three wetlands

One-way ANOVA highlighted significant differences in soil salinity ($F_{7, 232} = 338.45$, $p < 0.05$) and pH ($F_{7, 232} = 232.67$, $p < 0.05$) between all wetlands, as well as among Dowd Morass collection sites (Figures 3.3 & 3.4). The salinities recorded in this study fell within known levels for most sites (Sinclair Knight Merz 2001; 2003) and support the classification of Sale Common as a freshwater wetland, and Clydebank and Dowd Morass as brackish water wetlands. The highest soil salinity (~ 43 g L^{-1}) found beneath *B. caldwellii* at Dowd Morass sites 1 and 2, were much higher than expected though, as this range would lend a wetland to be classified as hyper-saline rather than brackish (Mitsch and Gosselink 2000). Lowest pH (3.6) was recorded beneath patches of *B. medianus* at Sale Common.

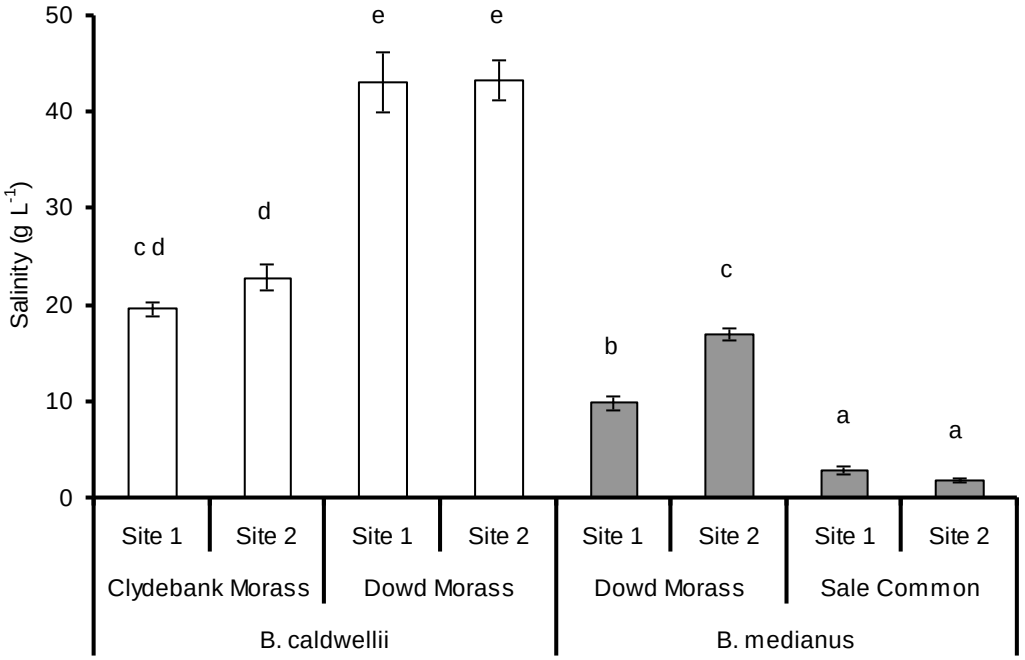


Figure 3.3. Salinity of sediment cores from separate populations of *B. caldwellii* (white bars) and *B. medianus* (grey bars) at three different wetlands: Clydebank Morass, Dowd Morass and Sale Common. Letters indicate significant differences ($p < 0.05$) following 1-way ANOVA and Tukey *post-hoc* testing. Standard error bars are shown (n = 30).

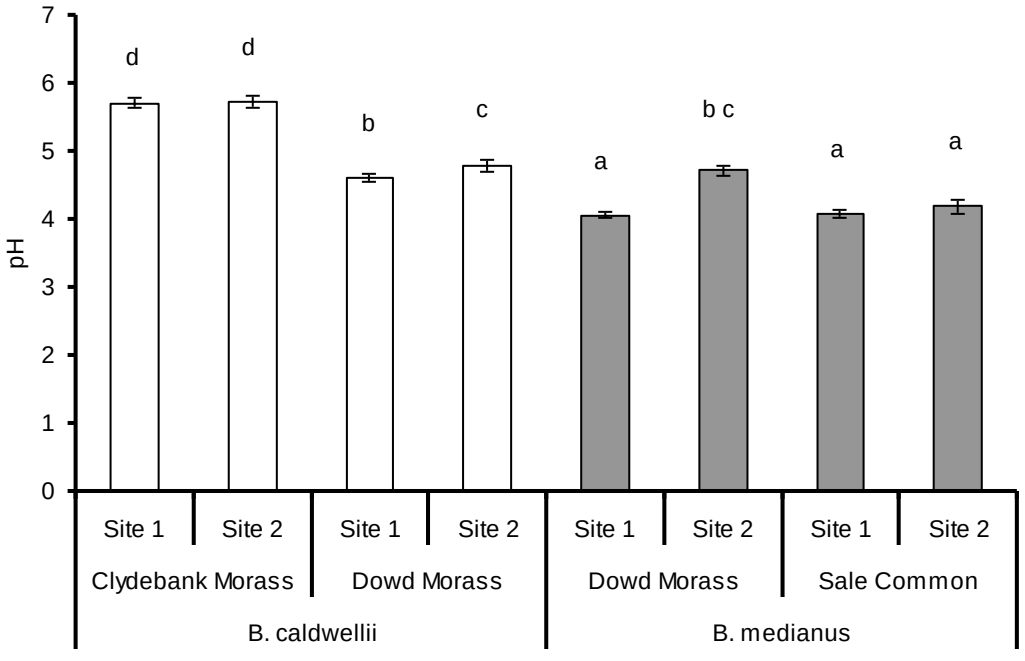


Figure 3.4. pH of dried sediments from separate populations of *B. caldwellii* (white bars) and *B. medianus* (grey bars) at three different wetlands: Clydebank Morass, Dowd Morass and Sale Common. Letters indicate significant differences ($p < 0.05$) following 1-way ANOVA and Tukey *post-hoc* testing. Standard error bars are shown (n = 30).

Table 3.1. Mean achene production results for ten randomly sampled flower heads of *B. caldwellii* and *B. medianus* from three different wetlands: Clydebank Morass, Dowd Morass and Sale Common. Letters indicate significant differences ($p < 0.05$) between sample locations for each species, following 1-way ANOVA and Tukey *post-hoc* tests. Note: no measurements are shown for *B. medianus* Dowd Morass population 1 as no flower heads were produced.

Species	Site	Population	No. flower heads / m^2	Spikelets / flower head	Avg. No. achenes / spikelet	Achenes / flower head	Total No. achenes	N. achenes / m^2	% Empty spikelets
B. caldwellii	Clydebank Morass	1	35	7.3 [a]	4.4	32.7	327	1145	23
	Clydebank Morass	2	28	6.5 [a]	4.4	29.1	291	815	22
	Dowd Morass	1	63	9.7 [b]	3.7	36.8	368	2282	42
	Dowd Morass	2	68	9.8 [b]	3.9	39.1	391	2072	38
B. medianus	Dowd Morass	1	0	-	-	-	-	-	-
	Dowd Morass	2	35	9.7 [b]	5.9 [b]	56.3 [b]	563	1970	15
	Sale Common	1	40	7.1 [a]	2.7 [a]	19.5 [a]	195	780	28
	Sale Common	2	36	7.3 [a]	3.2 [a]	23.0 [a]	230	828	23

3.3.2 Aerial seed banks

Achene production for both *B. caldwellii* and *B. medianus* was low to moderate in all populations (up to 2,282 m^2) and significant differences were apparent between sites (Table 3.1). Populations of *B. caldwellii* at Dowd Morass produced a significantly greater number of spikelets per flower head ($F_{3, 36} = 15.45$, $p < 0.05$) as well as a higher total number of achenes than equivalent populations at Clydebank Morass, yet the average number of empty spikelets per flower head at Dowd Morass (40) almost doubled those recorded at Clydebank Morass (22.5). Though the strength of comparative tests were weaker for *B. medianus* data, as only one population was found to set seed at Dowd Morass, achene production was significantly greater at Dowd Morass than at Sale Common in all statistical analyses: number of spikelets per flower head ($F_{2, 27} = 10.25$, $p < 0.05$), average number of achenes per spikelet ($F_{2, 27} = 58.86$, $p < 0.05$) and average number of achenes per flower head ($F_{2, 27} = 70.30$, $p < 0.05$). The total number of *B. medianus* achenes produced per flower head at Dowd Morass was also higher than at Sale Common and correspondingly fewer of the spikelets were empty (Table 3.1).

Upon dissection, many of the empty glumes within spikelets from flower heads at all collection sites harboured invertebrate larvae. A number of species inhabited *B. caldwellii* and *B. medianus*, including Melaleuca weevil (*Oxyops vitiosa*)[1] from the family Curculionidae (Coleoptera) and several species of gall midge larvae from the family Cecidomyiidae (Diptera) (Fig. 3.5 & 3.6). Approximately 10-15% of glumes in each flower spikelet contained insect larvae.

[1] To my knowledge *Oxyops vitosa* has not previously been recorded on *Bolboschoenus* species.

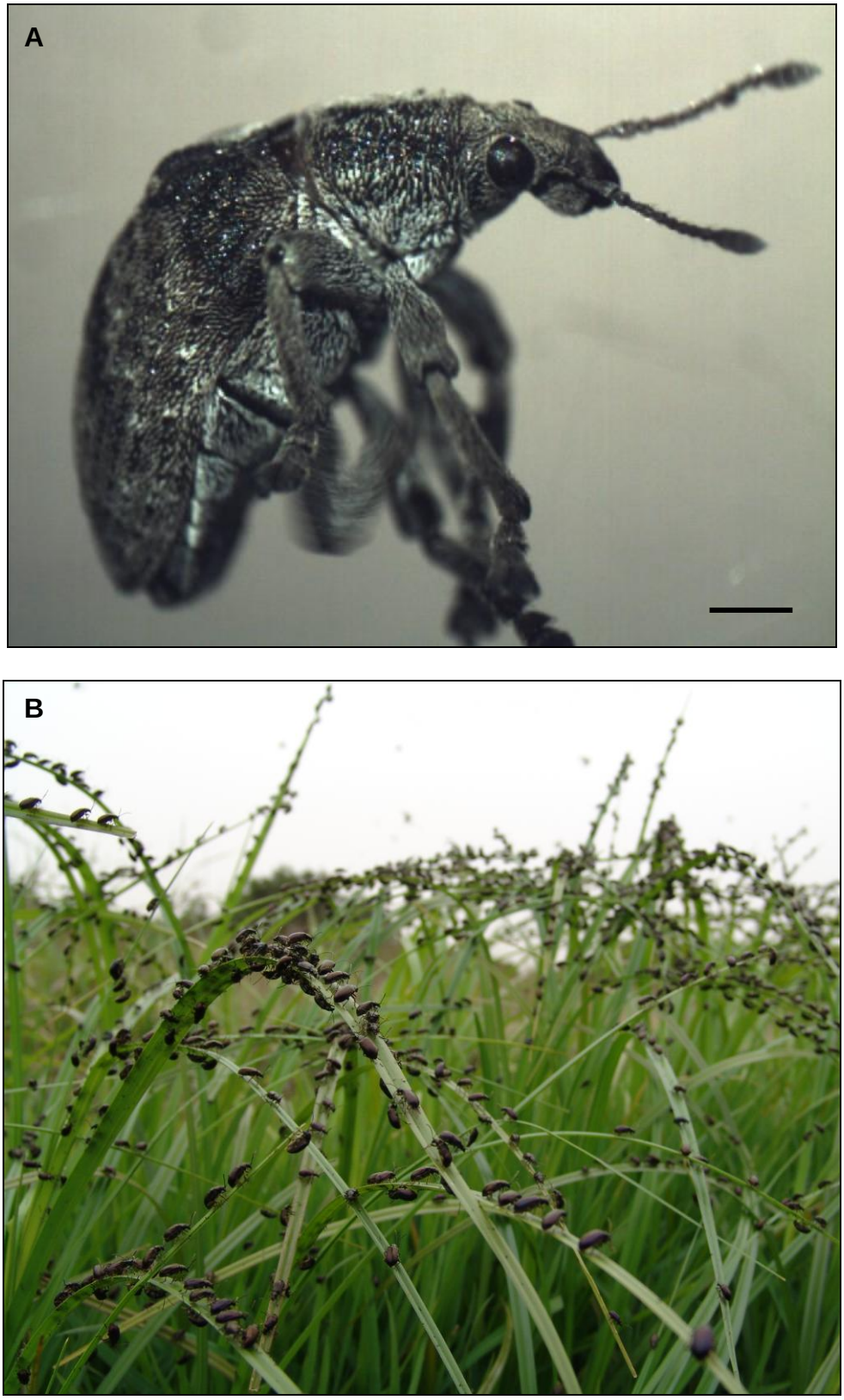


Figure 3.5. Insects and *Bolboschoenus*: A) *Oxyops vitiosa* (Melaleuca Snout Beetle) found living in the flower heads of *B. caldwellii* and *B. medianus* – Scale bar = 1 mm. B) Unidentified Coleoptera species utilising *B. medianus*.

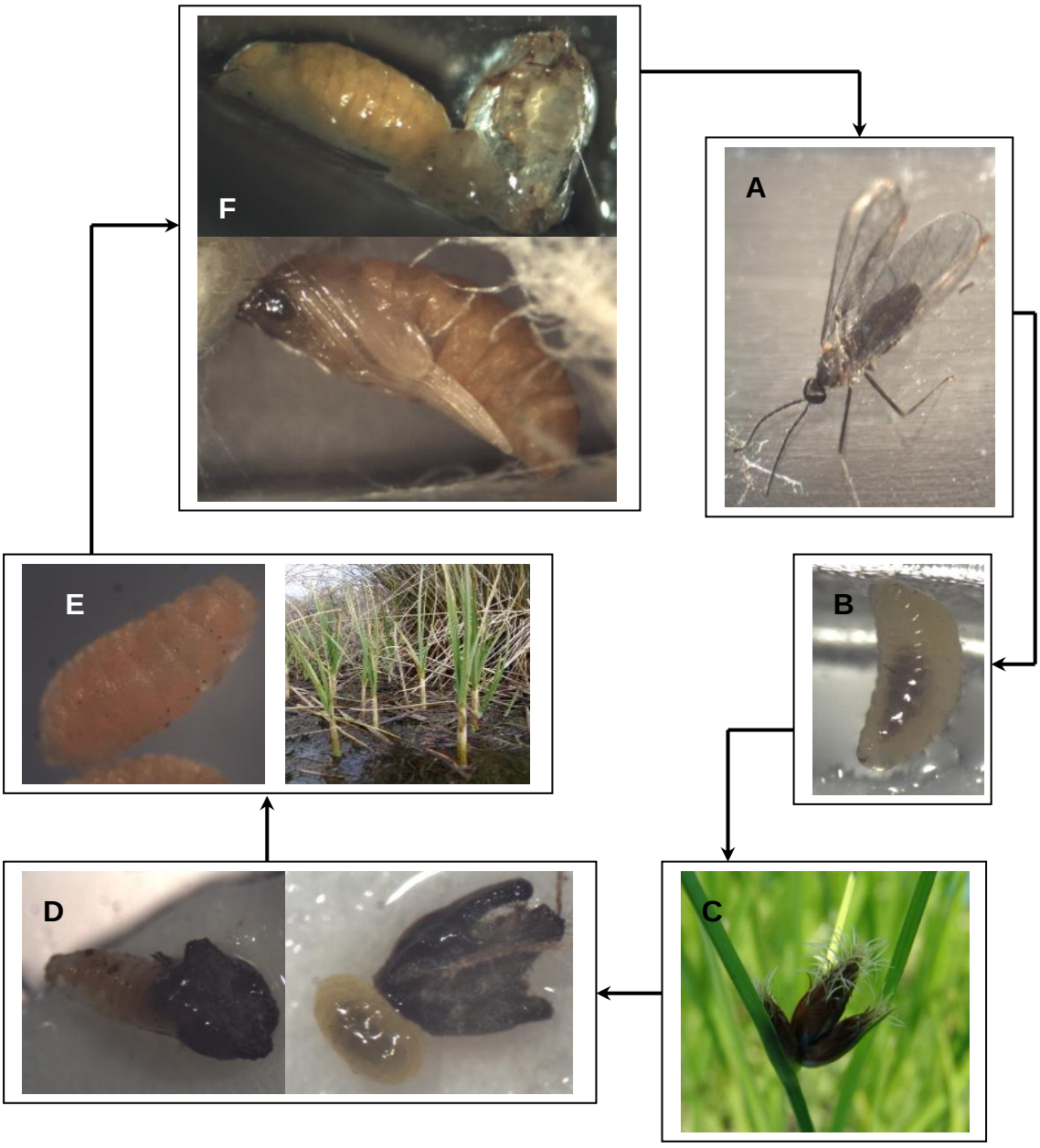


Figure 3.6. Life-cycle of Gall Midge (exact species unknown) living on *B. caldwellii* and *B. medianus* in the Gippsland Lakes region: A) Adult Gall Midge Fly – emerge in spring and females lay eggs on flowers and leaf buds. B) Maggots or larvae hatch and begin to feed on immature seeds. C) Flower spikelets of *B. caldwellii* - home to gall midge larvae. D) Juvenile larvae emerging from achene remnants of *B. caldwellii*. E) Mature larvae then fall to the ground to over-winter and pupate underground. F) Two stages of pupation prior to emergence in spring as adult Gall Midge Flies.

3.3.3 Achene polymorphisms and germination

One-way ANOVA of achene sizes from aerial seed banks indicated that significant differences in achene size (length: $F_{6, 393} = 243.91$, $p < 0.05$ and width: $F_{6, 393} = 301.53$, $p < 0.05$) existed for both test species between wetlands, though not within each wetland (Fig. 3.7.A & B and Table 3.2).

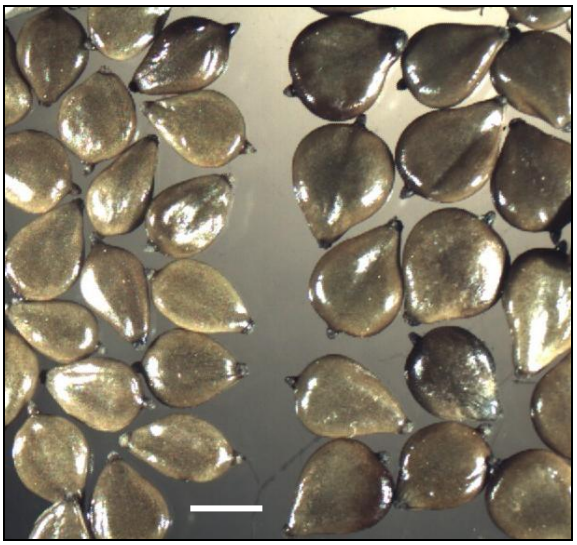


Figure 3.7.A. Achene size polymorphism in *B. caldwellii*: smaller achenes from Dowd Morass (Left hand side) compared to larger achenes from Clydebank Morass (Right hand side). Scale bar = 2 mm.

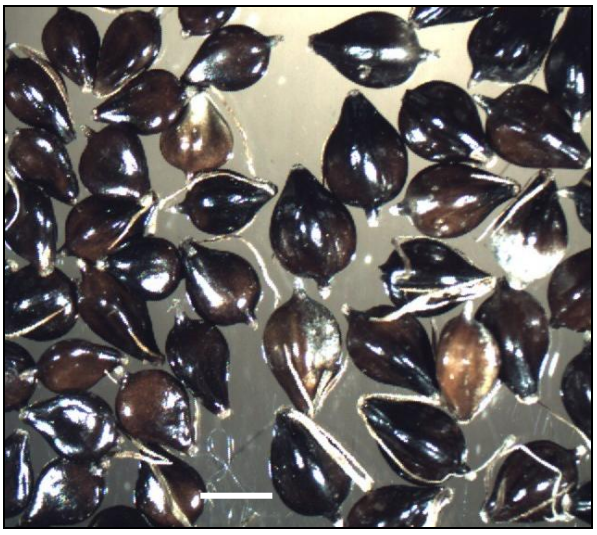


Figure 3.7.B. Achene size polymorphism in *B. medianus*: smaller achenes from Sale Common (Left hand side) compared to larger achenes from Dowd Morass (Right hand side). Scale bar = 2 mm.

Table 3.2. Mean dimensions (mm) ± standard error of *B. caldwellii* and *B. medianus* achenes collected from aerial seed banks at different field sites (n = 100). Letters within each column indicate significant differences ($p < 0.05$) following 1-way ANOVA and Tukey *post-hoc* tests. No flowers or achenes were found at Dowd Morass site 1.

		Mean achene dimensions (mm)	
Species	Site	Length	Width
B. caldwellii	Clydebank Morass site 1	3.81 ± 0.02 [a b]	2.79 ± 0.02 [a]
	Clydebank Morass site 2	3.85 ± 0.02 [a]	2.82 ± 0.02 [a]
	Dowd Morass site 1	3.30 ± 0.02 [c]	2.17 ± 0.02 [c]
	Dowd Morass site 2	3.29 ± 0.02 [c]	2.12 ± 0.02 [c]
B. medianus	Dowd Morass site 1	Not present	Not present
	Dowd Morass site 2	3.78 ± 0.02 [b]	2.30 ± 0.01 [b]
	Sale Common site 1	3.20 ± 0.02 [d]	2.05 ± 0.01 [d]
	Sale Common site 2	3.22 ± 0.02 [d]	2.04 ± 0.01 [d]

In accord with achene size differences, achene weights also differed significantly between sites ($F_{3, 36} = 148.16$, $p < 0.05$) (Fig. 3.8).

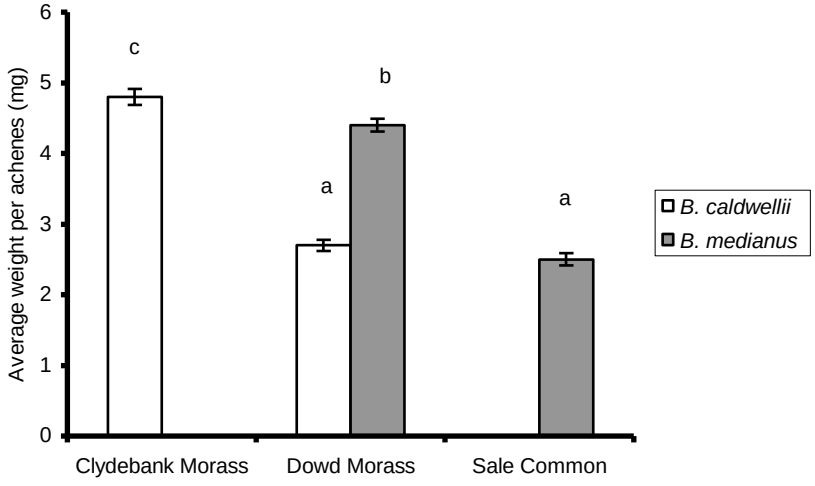


Figure 3.8. Average weight (mg) per achene for *B. caldwellii* and *B. medianus* at Clydebank Morass, Dowd Morass and Sale Common. Letters indicate significant differences ($p < 0.05$) in achene weights between sites, following 1-way ANOVA and Tukey *post-hoc* testing. Standard error bars are shown (n = 1000).

Achenes of *B. medianus* were consistently polymorphic in shape (obovate or lenticular versus trigonous), with the two seed morphs produced at an approximately equal ratio (Fig. 3.9.A & B). Although quite rare, polymorphism of achene shape was also found for *B. caldwellii* (estimated at 1 trigonous shaped achene to every 1-2,000 obovate and biconcave shaped achenes) (Fig. 3.9.C & D).

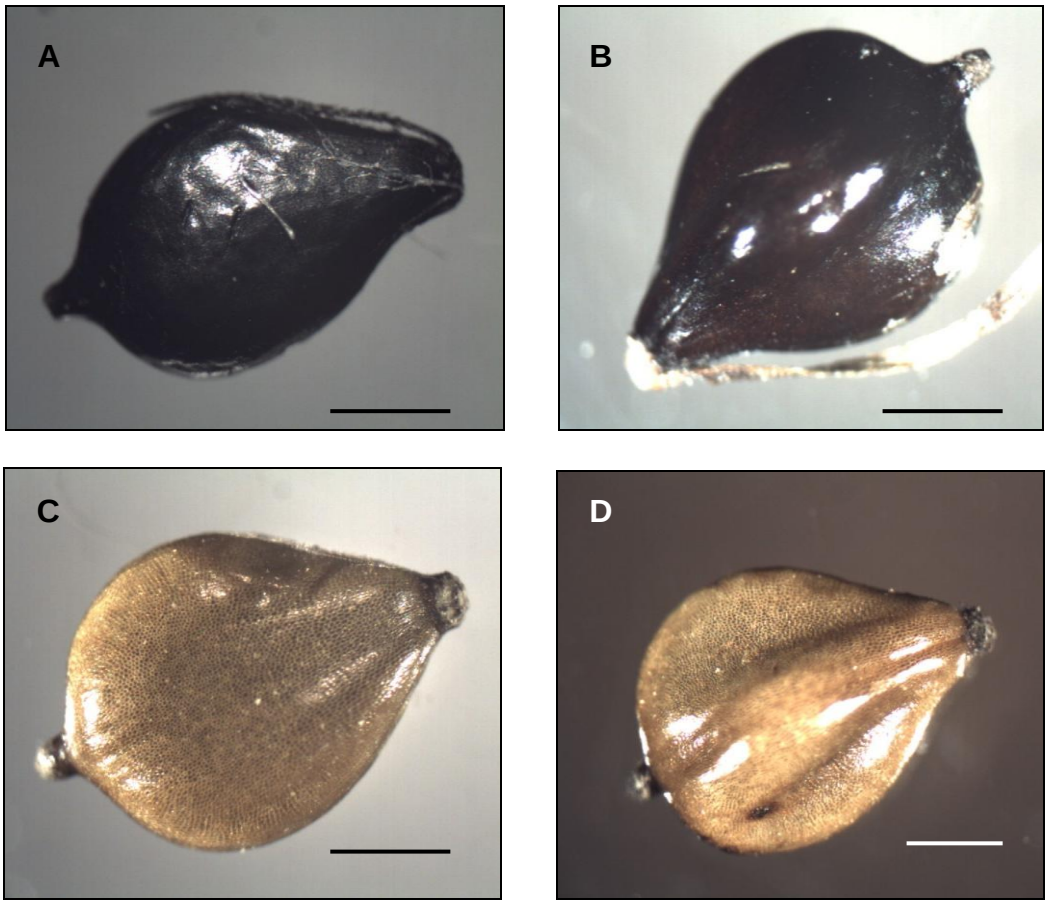


Figure 3.9. Comparative shape polymorphisms in *B. medianus* and *B. caldwellii* achenes. A) Obovate or lenticular shaped achene of *B. medianus*. B) Trigonous achene of *B. medianus*. C) Obovate or biconcave shaped achene of *B. caldwellii*. D) Rare trigonous shaped achene of *B. caldwellii*. Scale bars = 1 mm.

Achene size polymorphism did not affect total percentage germination of *B. caldwellii* ($F_{3,7} = 1.88$, $p = 0.27$) or *B. medianus* ($F_{3,7} = 3.41$, $p = 0.13$) achenes under fresh water conditions, however significant differences were recorded in the growth rate of seedlings between size morphs (Fig. 3.10.A. & B.). Germination of *B. medianus* achenes was not influenced by polymorphic shape differences (trigonous or

lenticular achene forms) ($t(6) = -0.38$, $p = 0.72$) nor was the rate of germination influenced by achene shape ($t(6) = -0.61$, $p = 0.56$). Achene size was also of no influence to germination across the range of salinities tested for both species (*B. caldwellii*: $F_{1, 32} = 0.49$, p = 0.49 and *B. medianus*: $F_{1, 32} = 0.06$, p = 0.81).

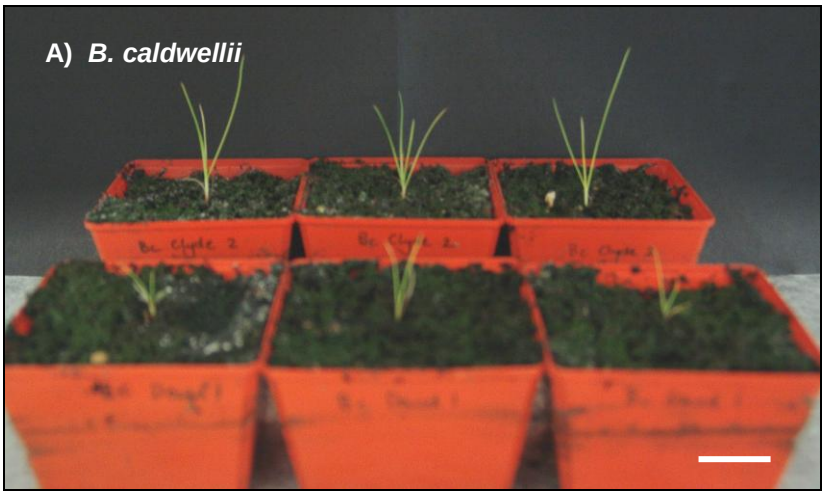


Figure 3.10.A. Growth rate differences between *B. caldwellii* achene size polymorphs, one month post germination. Seedlings in the foreground germinated from small achenes sourced from Dowd Morass, while seedlings in the background germinated from larger achenes collected from Clydebank Morass. Scale bar = 2 cm.

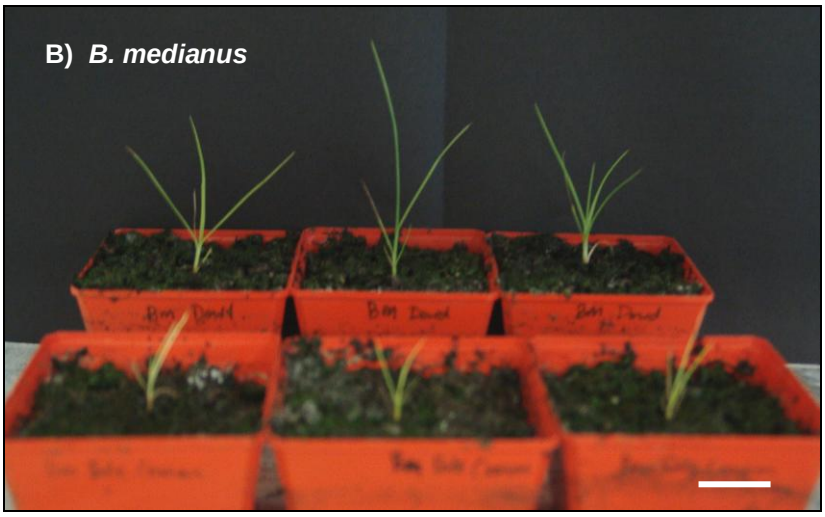


Figure 3.10.B. Growth rate differences between *B. medianus* achene size polymorphs, one month post germination. Seedlings in the foreground germinated from small achenes sourced from Sale Common, while seedlings in the background germinated from larger achenes collected from Dowd Morass. Scale bar = 2 cm.

3.3.4 Sediment seed banks and their recovery germination

Sediment seed banks (Table 3.3) were significantly greater for *B. caldwellii* at Dowd Morass than at Clydebank Morass ($t(38) = -2.28$, $p < 0.05$), whereas no significant differences were found for seed bank densities within and among *B. medianus* populations ($t(38) = 0.45$, $p = 0.66$). Greater than 70% of achenes recovered from sediment cores for either species were found within cores (#1-5) taken from the middle of populations. No achenes were found within sediment cores sampled outside the perimeter (#10) of *B. medianus* populations at either field site, whereas achenes were recovered from all sediment cores sampled outside the perimeter of each *B. caldwellii* populations. Although achenes of *B. caldwellii* were found in greater densities than those of *B. medianus*, a greater proportion of *B. medianus* achenes remained intact and potentially viable (Table 3.4). At all *B. medianus* sample locations a greater number of trigonous achenes were recovered from soil cores in comparison to lenticular shaped achenes (Table 3.5). Germination trials using achenes recovered from soil cores showed greater percentage germination for *B. caldwellii* than *B. medianus* (Fig. 3.11), most likely as a result of higher dormancy levels in the latter species (see Chapter 7 of this thesis).

In parallel with aerial seed bank results, the dimensions of achenes recovered from soil cores were statistically similar for each species within each site, though significantly different between wetlands, for both length ($F_{6, 220} = 23.27$, $p < 0.05$) and width ($F_{6, 393} = 20.18$, $p < 0.05$) (Table 3.6). A comparison of the dimensions of achenes from aerial and sediment seed banks revealed significant differences for most collection sites. Achenes of *B. caldwellii* recovered from the soil seed bank at Clydebank Morass were significantly smaller than those collected from aerial seed banks in length ($t(125) = 4.88$, $p < 0.05$) and width ($t(125) = 23.49$, $p < 0.05$), while at Dowd Morass achenes recovered from soil cores were significantly larger than those from aerial seed banks in both length ($t(198) = -4.45$, $p < 0.05$) and width ($t(198) = -7.22$, $p < 0.05$). Achenes of *B. medianus* from Dowd Morass only differed statistically in length between aerial and soil seed banks ($t(146) = 3.94$, $p < 0.05$), while the dimensions of *B. medianus* achenes at Sale Common were statistically similar.

Table 3.3. Seed bank density estimations (per m^2) for *B. caldwellii* and *B. medianus* based on total number of achenes recovered from sediment cores at three different wetlands in the Gippsland Lakes region.

	Bolboschoenus caldwellii				*Bolboschoenus medianus*			
	Clydebank Morass		Dowd Morass		Dowd Morass		Sale Common	
Sample position	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
1	10,689	19,342	13,234	4,581	509	1,527	1527	6617
2	5,090	14,761	13,234	7,635	1,018	2,036	3054	1527
3	9,671	8,653	29,522	25,959	0	4,072	0	3563
4	3,563	11,707	13,743	16,797	2,036	7,126	6617	0
5	24,432	4,072	25,450	58,535	1,018	6,108	8653	1018
Average (1-5)	10,689.0	11,707.0	19,036.6	22,701.4	916.2	4,173.8	3970.2	2545.0
6	509	0	6,617	8,144	0	1,018	0	509
7	1,527	1,527	4,072	14,761	0	3,563	3563	1018
8	1,018	3,054	4,581	1,527	0	2,545	2036	0
9	1,527	1,018	10,180	9,671	0	1,527	1018	0
10*	1,018	509	3,054	4,581	0	0	0	0
Average (6-10)	1,119.8	1,221.6	5,700.8	7,736.8	0	1,730.6	1323.4	305.4
Average (1-10)	5,904.4	6,464.3	12,368.7	15,219.1	458.1	2,952.2	2646.8	1425.2

* Note core number 10 was taken ~10 m from the north-west corner of each *Bolboschoenus* population.

Table 3.4. Seed bank density estimations (per m^2) for *B. caldwellii* and *B. medianus* based on the number of intact (potentially viable) achenes recovered from sediment cores at different wetlands.

	Bolboschoenus caldwellii				*Bolboschoenus medianus*			
	Clydebank Morass		Dowd Morass		Dowd Morass		Sale Common	
Sample position	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2	Site 1	Site 2
1	509	2,036	2,545	1,527	0	1,018	1,018	6,108
2	0	1,018	3,054	2,545	0	1,018	2,036	1,527
3	1,018	509	5,090	4,581	0	2,545	0	3,054
4	1,018	1,527	1,527	3,054	0	5,599	4,072	0
5	2,545	509	1,527	11,198	0	5,090	5,599	1,018
Average (1-5)	1,018.0	1,119.8	2,748.6	4,581.0	0.0	3,054.0	2,545.0	2,341.4
6	509	0	1,527	3,054	0	0	0	509
7	509	0	509	4,581	0	2,545	1,527	1,018
8	0	1,527	1,018	0	0	2,545	1,527	0
9	509	0	509	1,527	0	1,018	509	0
10*	0	0	509	1,018	0	0	0	0
Average (6-10)	305.4	305.4	814.4	2,036.0	0.0	1,221.6	712.6	305.4
Average (1-10)	661.7	712.6	1,781.5	3,308.5	0.0	2,137.8	1,628.8	1,323.4
% Total achenes from Table 3	11.2	11.0	26.7	11.7	n/a	72.0	61.5	92.9

* Note core number 10 was taken ~10 m from the north-west corner of each *Bolboschoenus* population.

Table 3.5. Number of intact trigonous versus lenticular achenes found within soil core samples taken among *B. medianus* populations at two different wetlands. No intact achenes were found at Dowd Morass site 1.

		Achene shape polymorphism		
Species	Site	Trigonous	Lenticular	% Trigonous
B. medianus	Dowd Morass site 1	0	0	n/a
	Dowd Morass site 2	38	4	90
	Sale Common site 1	15	11	58
	Sale Common site 2	22	10	69

Table 3.6. Mean dimensions (mm) ± standard error of *B. caldwellii* and *B. medianus* achenes recovered from soil cores at each sampling location. Letters within each column indicate significant differences ($p < 0.05$) following 1-way ANOVA and Tukey *post-hoc* tests. No intact achenes were found in soil cores taken from Dowd Morass site 1.

		Mean achene dimensions (mm)	
Species	Site	Length	Width
B. caldwellii	Clydebank Morass site 1	3.65 ± 0.04 [a]	2.18 ± 0.02 [b]
	Clydebank Morass site 2	3.63 ± 0.04 [a]	2.09 ± 0.02 [b c]
	Dowd Morass site 1	3.39 ± 0.03 [b]	2.33 ± 0.02 [a]
	Dowd Morass site 2	3.44 ± 0.02 [b]	2.30 ± 0.02 [a]
B. medianus	Dowd Morass site 1	Not present	Not present
	Dowd Morass site 2	3.65 ± 0.03 [a]	2.27 ± 0.01 [a]
	Sale Common site 1	3.23 ± 0.04 [c]	2.10 ± 0.02 [b c]
	Sale Common site 2	3.13 ± 0.03 [c]	2.03 ± 0.02 [c]

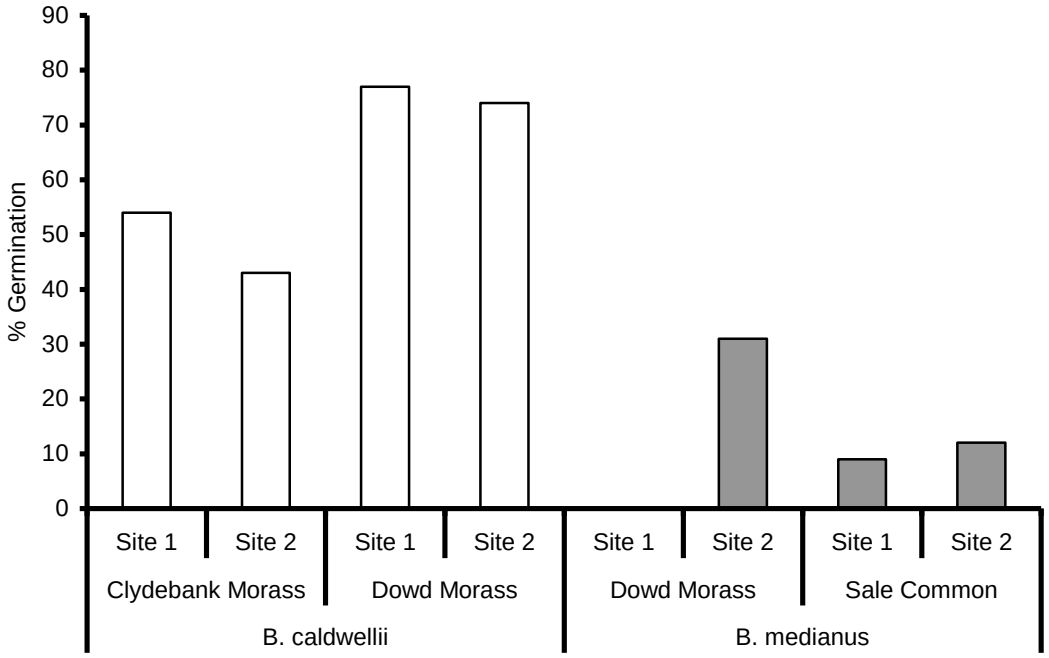


Figure 3.11. Percentage germination of *B. caldwellii* (white bars) and *B. medianus* (grey bars) achenes recovered from sediment seed banks at three different wetlands: Clydebank Morass, Dowd Morass and Sale Common. Zero germination is shown for *B. medianus* at Dowd Morass site 1 as no intact achenes were found in any of the soil cores. Standard error bars could not be included as recovered achenes within each site were pooled and placed into a single petri dish for incubation.

3.4 Discussion

3.4.1 Patterns between achene production and soil chemistry

Achene production figures for both *B. caldwellii* and *B. medianus* followed predictions made in the achene continuum / resource trade-off model proposed in the introduction to this chapter. A greater number of small achenes per spikelet were produced under either highly saline conditions (> 40g L^{-1} for *B. caldwellii* at Dowd Morass) or near freshwater conditions (< 5 g L^{-1} salt for *B. medianus* at Sale Common), while fewer (though larger) achenes were produced under brackish conditions. Results for both species suggested that as salinity levels increased, the proportion of resources allocated to the production of seeds (sexual investment) also increased, most likely as a strategy to increase the chances of achene dispersal to a

less stressful environment. The greater investment into achene production with increasing salt, did not necessarily equate to better quality seeds, as reflected by the high number of unfilled achenes produced by *B. caldwellii* (42%) under hyper-saline conditions at Dowd Morass. Smaller achenes often of inferior quality were also produced when conditions were near fresh (Sale Common), most likely as resource investment favoured vegetative growth for the consolidation and expansion of space. Resource trade-offs of this nature between asexual and sexual reproduction have been reported for a number of aquatic clonal genera including *Mimulus* and *Iris* (Sutherland and Vickery 1988; Van Zandt *et al.* 2003).

Although no quantitative measurements for asexual growth were taken in this study, variation in both achene sizes and numbers produced by *B. caldwellii* and *B. medianus* at each wetland, agree with previous analytical and simulation models that attempt to predict the changing (plastic) reproductive responses of clonal plants within environments of different productivity. For example, the models of Sakai (1995) and Gardner and Mangel (1999) predicted that asexual reproduction should be favoured over sexual reproduction in productive habitats (in this case low salinity), while the allocation of resources to sexual reproduction should increase as conditions become less favourable (high salinity) and the mortality risk to the whole genet increases.

3.4.2 The role of achene polymorphisms

Variation in achene sizes and shape between populations did not affect germinability, though seedlings emerging from larger achenes, were taller and more resilient than those originating from smaller achenes, most likely as a result of greater endosperm volumes (*pers. obs.*). In this respect, achene size differences may be of great importance in determining the sediment or water depth from which seeds can emerge (Baskin and Baskin 2001). Seed polymorphisms therefore remain an important factor for sexual recruitment, especially in unpredictable environments such as wetlands, as variation in seed size, weight and shape, widen the germination response parameters of the collective seed bank and effectively act as a risk spreading mechanism (Traveset *et al.* 2001a; Leck and Schütz 2005; Schatral and Osborne 2006).

Krahulec *et al.* (1996) examined morphological variation of commonly used taxonomical characters, such as the number of inflorescences in *Bolboschoenus maritimus* populations over ten years and concluded that temporal variations within a clone were of less importance than variations between clones or sites. The findings of this chapter agree with the above observation, as the levels of plasticity found within each population or site were insignificant compared to the level of variation found between populations or sites. Plasticity or trade-offs in seed size and other morphological parameters are generally not noticeable at the local scale, as environmental conditions such as high salinity may create uniformity of population architecture, however at the regional scale, differences in morphology become apparent in reflection of the uniqueness of each site (Hroudová *et al.* 1999). While achene polymorphisms may reflect adaptations to local conditions in terms of assimilation and production (trade-offs), the variations specific to each wetland were not found to provide any particular adaptive advantages for germination. The distinct achene size differences found for *B. caldwellii* and *B. medianus* between field sites appeared to be environmentally governed and therefore indicative of true plasticity, yet the differences were nonetheless considered non-plastic, as the range of achene sizes fell within those reported for Australasian type specimens (see Browning *et al.* 1997).

3.4.3 Sediment seed banks and implications for recruitment

Seed production for rhizomatous Cyperaceae species is reported to be relatively low, ranging between 0 and 345,000 seeds m^{-2} $year^{-1}$ (Schütz 2000; Leck and Schütz 2005). Sediment seed bank densities found for *B. caldwellii* and *B. medianus* reflected this low range, as the number of intact achenes recovered from soil cores ranged from 0-11,198 achenes m^{-2} for *B. caldwellii* and 0-8,653 achenes m^{-2} for *B. medianus*. Although estimations of sediment seed banks are rare in scientific literature for *Bolboschoenus* species, density ranges in this study accord with those from a number of previous papers examining the closely related genus *Scirpus* L. (Cyperaceae). For example, van der Valk and Davis (1978) and Poiani and Johnson (1989) recorded sediment seed bank densities of 401-4,178 achenes m^{-2} and 0-6,278 achenes m^{-2} respectively, for *Scirpus validus* in a prairie marsh; Engel (1983)

recorded density ranges of 0-18,112 achenes m^{-2} for *Scirpus americanus* and 0-16,980 achenes m^{-2} for *Scirpus robustus* in a salt marsh; while Baldwin *et al.* (2001) recorded 280-5,093 achenes m^{-2} for *Scirpus fluviatilis* in a tidal freshwater marsh.

The complete lack of achenes found in soil cores taken from outside the perimeter of *B. medianus* populations and the high concentration of achenes within the centre of populations, suggested that achenes were only produced locally and that sexually recruited seedlings would be in direct competition with standing vegetation. In contrast *B. caldwellii* achenes were found in much greater numbers both around and outside the perimeter of each population, suggesting that they may not all be produced locally, that they have a greater capacity for dispersal and that sexual recruitment may occur with less direct competition from extant vegetation.

The low number of achenes produced by both test species and poor sediment seed bank formation across wetland sites, suggested that both of these factors may contribute to rare sexual recruitment events for *B. caldwellii* and *B. medianus*. Nevertheless, the successful germination of achenes recovered from sediment cores from all wetlands indicated that *B. caldwellii* and *B. medianus* are capable of producing persistent seed banks; a finding consistent with the majority of other Cyperaceae species (Leck and Schütz 2005).

Chapter 4.

Does poor achene viability contribute to low levels of sexual recruitment in *B. caldwellii* and *B. medianus*?

4.1 Introduction

Having quantified achene production at each of the field sites, the next progression into the inquiry of the sexual ecology of *B. caldwellii* and *B. medianus* was to examine the germinability of achenes. Thus the following chapter examines the second component of the conceptual recruitment model: achene viability.

Two distinct strategies of seed production appear to be used by wetland plants. At one extreme, species such as *Phragmites australis, Typha domingensis* and *Melaleuca ericifolia* produce large quantities of small seeds with low or short-term viability. Persistent seed banks do not usually form in these species and sexual recruitment events are generally rare (Sharma and Gopal 1978; Ishii and Kadono 2002; Robinson *et al.* 2006). In contrast, many other wetland plant species, especially those belonging to the Cyperaceae family, produce a small quantity of high-quality seeds with a well developed pericarp that provides strong dormancy capacity and allows for the formation of persistent seed banks (Clevering 1995; Moravcová *et al.* 2002; Leck and Schutz 2005). Despite this adaptation, the majority of Cyperaceae species (especially those inhabiting wetland margins) are reported to rarely recruit sexually into existing populations and instead rely on vegetative recruitment for consolidation and expansion of territory (Clevering 1995; Kantrud 1996; Schütz 2000; Moravcová *et al.* 2002; Leck and Schütz 2005).

It is unclear whether poor seed viability contributes to the lack of sexual recruitment commonly reported for many Cyperaceae species. Leck and Schütz (2005) noted that there are generally low correlations between laboratory and field germination results for Cyperaceae species, which suggests that seed banks usually have high viability, but that germination is compromised by unfavourable environmental conditions during normal recruitment periods. As a result seeds must remain in dormancy and wait for germination conditions to improve, which may take many years. Given that seed viability is negatively correlated with age, questions arise also regarding the long-term viability of seed for wetland plant species (Baskin and Baskin 2001; Fenner and Thompson 2005). In addition, seed viability is known to vary between populations of the same species, largely as a result of environmental differences between sites causing trade-offs in resource allocation between sexual and asexual reproduction (Robinson *et al.* 2006).

As the seeds of Cyperaceae species typically have complex anatomy and hard seed coats, determining their viability can be difficult. Simple, reliable and cost-effective viability tests are needed to assess the recruitment potential of seed lots with complex anatomy. Though time consuming, the use of specialised chemicals such as 2,3,5-triphenyl-tetrazolium-chloride (TTC) to determine seed viability are generally more accurate than visual or cut-test inspection methods (Sawma and Mohler 2002). Recent research that examined *Leucopogon* species, which produce hard seeds with woody pericarps, however, revealed similar estimates for both cut and TTC viability test methods (Ooi *et al.* 2004).

The aims of this chapter were: 1) to describe the anatomy of *B. caldwellii* and *B. medianus* achenes and 2) to examine the viability of their aerial seed banks from populations at Dowd Morass, Clydebank Morass and Sale Common. Several hypotheses were addressed: 1) the simple cut test is as effective as the TTC method for determining seed viability, 2) sexual recruitment in *B. caldwellii* and *B. medianus* is not compromised by poor achene viability, 3) viability is not negatively affected by short-term (1-year) storage in dry or wet conditions, and 4) achene viability does not differ between populations of the same species in contrasting wetlands. A total of 3,200 achenes (1,600 per species) were tested in viability trials.

4.2 Methods

4.2.1 Achene collection and storage

Achene collection and storage followed the procedures outlined in introductory methods Chapter 2, with the following exceptions. After the initial two week maturation period, half of the achenes were assigned for immediate use in germination trials; one quarter were committed to a further year of dry-dark storage; and one quarter were cold-wet stratified in the dark at 4ºC in deionised water for 52 weeks, prior to germination testing, in order to test the effects of extended dormancy.

4.2.2 Germination conditions

As imbibition processes are slow in *Bolboschoenus* achenes due to their complex pericarp anatomy (see Fig. 4.1) it was appropriate to conduct germination tests prior to viability testing. An initial 30-day germination trial allowed both the active component of sampled achenes to be calculated separately, as well as giving time for the remaining achenes to have imbibed water before testing with TTC commenced. Germination conditions followed those outlined in introductory methods (Chapter 2) with the following exceptions: four replicates of 100 achenes per species were used for each viability trial (2 replicates x 2 sites) and germination trials were stopped after 30 days.

4.2.3 Viability testing

Ungerminated achenes from each replicate (petri-dish) were dissected longitudinally to determine whether the embryo and endosperm regions were intact. Achenes significantly affected by fungus, or with an obvious lack of endosperm and embryo were scored and omitted from further viability testing. Half of the replicates used in fresh germination trials were scored via cut testing only, and half tested with TTC; all 1-year-old dry and wet stored achenes were tested using TTC procedures. A 1% aqueous solution of 2,3,5-Triphenyl-Tetrazolium-Chloride and deionised water was used in all trials, as this concentration has proven reliable at determining seed viability levels for a wide range of species (Van Waes and Debergh 1986; Association of Official Seed Analysts 1990; Lacroix and Mosher 1995; Sawma and Mohler 2002;

Rubio-Casal *et al.* 2003). TTC is light sensitive therefore exposure to direct or prolonged diffuse sunlight was avoided during preparation. Final TTC solutions were tested for correct pH, as a value between six and seven was required for the compound to be effective (Lakon 1948).

Both halves of all dissected achenes used in TTC tests were assigned to separate lightproof vials. Each vial was filled with enough solution to ensure continuous submersion of embryonic tissue, as prolonged contact with the air is known to negatively affect results. All vials were stored in a dark laboratory cupboard at room temperature (20°C ± 2°C) until either the embryos had stained red in colour or more than 12 days had elapsed. A separate vial was also included, which contained *Nassella neesiana* (Chilean Needle-grass) seeds as a control. This species is known to stain within a few hours and this showed that the TTC solution was effective. On each day of the trial period a sample of achenes from each vial (population) were quickly photographed in as low light as possible, using a Carl Zeiss dissection microscope fitted with a digital camera (Moticam 2000 ^{TM}). Following the completion of germination trials and viability tests, mean cumulative percentages were calculated for each species using the following categories for achene results: germination, loss to fungus, unfilled, positive TTC, and negative TTC. In this chapter the term 'potential germinability' refers to combined totals of successfully germinated achenes and positive viability assessments from either cut or TTC testing.

4.2.4 Statistical analysis

T-tests were used to compare whether there were achene viability differences between sites (i.e. *B. caldwellii* from Dowd Morass compared to Clydebank Morass and *B. medianus* from Dowd Morass compared to Sale Common). One-way ANOVA was used to determine significant differences ($p < 0.05$) in achene viability for each species between treatments (cut or TTC tests) and achene age (fresh or 1-year old). Percentage data (germination, loss to mould, unfilled achenes, viable and non-viable achenes) were converted to proportions then arcsine(sqrt) transformed after checking for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity of variances (Levene's test). All statistical analyses were performed using SPSS (Version 14) statistical package.

4.3 Results

4.3.1 Anatomy of Bolboschoenus achenes

Bolboschoenus achenes consist of multiple layers of different tissue types, which make them very impermeable to water (Fig. 4.1). The pericarp consists of an outer waxy layer surrounding a row of radially arranged cells known as the exocarp. Below the exocarp is a thick layer of mesophyll cells – the mesocarp tissue – beyond which sits the last thin inner layer of the pericarp, the endocarp tissue. The entire pericarp structure surrounds a final protective layer, the seed coat or testa. The multiple pericarp layers inhibit the movement of water, therefore, imbibition to the central endosperm and embryo regions was largely assumed to occur through the micropyle region rather than the seed coat, unless the structural integrity of the pericarp was damaged.

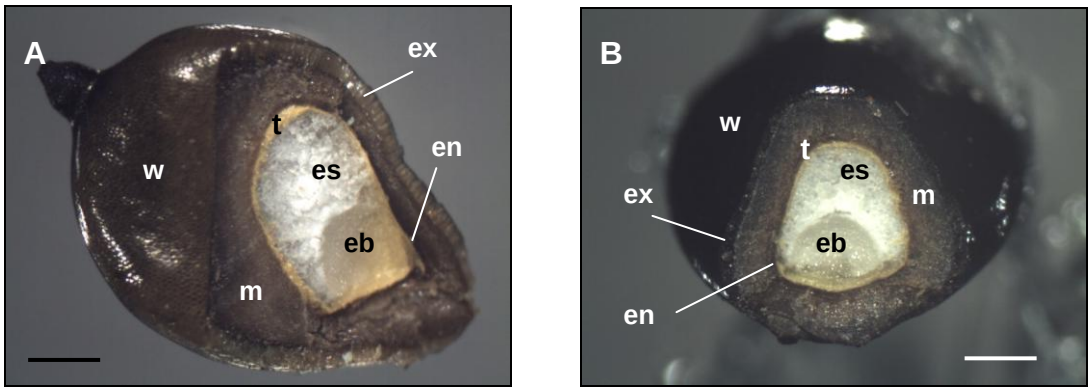


Figure 4.1. Diagonal cross-sections of A) *B. caldwellii* and B) *B. medianus* showing multilayered achene structures including: waxy outer surface layer (w), exocarp (ex), mesocarp (m), endocarp (en), testa (yellow layer) (t), endosperm (es) and embryo (eb). Scale bars = 500 µm.

4.3.2 Germination and viability assessments

Though total germination differed significantly for each species among treatments, no statistical differences were found for total germinability / viability using either the cut or TTC methods (*B. caldwellii*: $F_{3, 12} = 0.92$, $p = 0.46$ and *B. medianus*: $F_{3, 12} = 2.01$, $p = 0.17$). Total germinability / viability was high (>70%) in all treatments for achenes of both species and no significant differences were apparent

between fresh and aged achenes (*B. caldwellii*: $F_{2, 13} = 1.45$, $p = 0.27$ and *B. medianus*: $F_{2, 13} = 1.75$, $p = 0.21$) (Table 4.1). Germination was significantly greater for *B. caldwellii* compared to *B. medianus* in both cut and TTC trials and substantial germination (>50%) was only achieved for *B. medianus* following a 1-year stratification period.

Table 4.1. Mean percentage of germinated achenes, those lost to fungus, unfilled, filled or TTC positive, TTC negative and estimates of total germinability/viability for *B. caldwellii* and *B. medianus,* using fresh and 1 year old achenes, following cut or TTC testing. Significant differences ($p < 0.05$) are indicated by different superscript letters, following 1-way ANOVA and Tukey *post-hoc* tests (n = 4). Means and standard errors are shown.

		% of trialled achenes			
		Treatment			
		Fresh CUT	Fresh TTC	1 yr dry TTC	1 yr wet TTC
B. caldwellii	Germinated	66 ± 3 [a]	70 ± 4 [a]	81 ± 7 [b]	86 ± 7 [b]
	Lost to fungus	10 ± 2	8 ± 2	4 ± 3	6 ± 4
	Unfilled	9 ± 3	7 ± 5	5 ± 2	3 ± 1
	TTC +ve (*Filled)	*15 ± 4	9 ± 4	3 ± 2	0
	TTC –ve	n/a	6 ± 3	7 ± 2	5 ± 3
	Total%	100	100	100	100
Estimate of total germinability / viability		**81 ± 3**	**79 ± 8**	**84 ± 6**	**86 ± 7**
B. medianus	Germinated	6 ± 2 [a]	5 ± 1 [a]	23 ± 5 [b]	59 ± 7 [c]
	Lost to fungus	15 ± 3	16 ± 4	9 ± 5	8 ± 5
	Unfilled	7 ± 2	7 ± 3	3 ± 1	7 ± 3
	TTC +ve (*Filled)	*72 ± 4	65 ± 7	58 ± 6	19 ± 5
	TTC –ve	n/a	7 ± 4	7 ± 5	7 ± 3
	Total%	100	100	100	100
Estimate of total germinability / viability		**78 ± 5**	**70 ± 5**	**81 ± 10**	**78 ± 5**

* Filled results only relevant to fresh cut tests.

No significant differences were found for total germinability / viability among sites for each species: *B. caldwellii* ($t(14) = 1.86$, $p = 0.08$) and *B. medianus* ($t(14) = 0.19$, $p = 0.86$). The number of unfilled achenes did not differ between sites for *B. medianus* ($t(14) = 0.08$, $p = 0.93$), however, a significantly greater number of unfilled *B. caldwellii* achenes were found at Dowd Morass compared to Clydebank Morass ($t(14) = -3.17$, $p < 0.05$) (Fig. 4.2.A), most likely due to differences in soil sediment

conditions as outlined in Chapter 3. Although seeds were surface sterilised in bleach, more than 5% of achenes in all trials were infected by fungi, which in most cases prevented germination (Fig. 4.2.B).

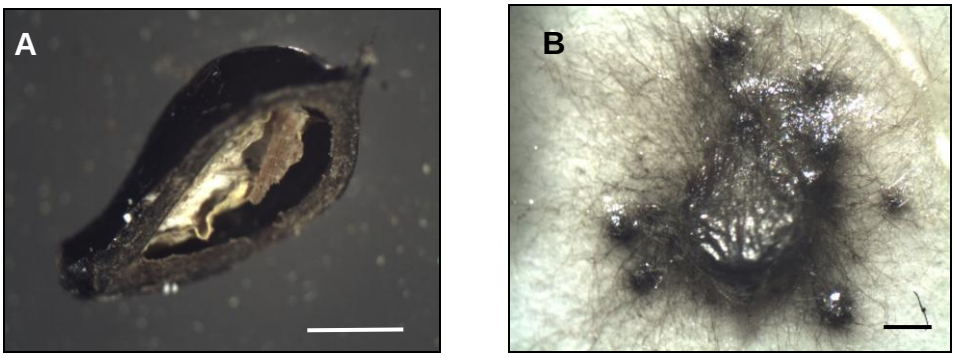


Figure 4.2. A) Longitudinal dissection of a *B. medianus* achene showing malformed endosperm and embryo tissue, and B) *B. medianus* achene badly damaged by fungal infection. Scale bars = 1 mm.

No visible embryo staining occurred for either *Bolboschoenus* species within the first 24 hours of immersion in TTC solution. In contrast, the *Nassella neesiana* seeds, used as controls, stained deeply within 3-4 hours (Fig. 4.3). Faint pink coloured embryo tissue could be detected after 48 hours in *B. caldwellii*, while the first indication of embryo viability in *B. medianus* was only observed after 72 hours immersion in TTC. Substantial staining did not occur for either species within the first week and the majority of achenes required at least 10-12 days immersion for a definitive indication of TTC embryo staining.

Figure 4.3. Example of a *Nassella neesiana* (Chilean Needle Grass) seed used as a control in viability testing, which exhibited deep red staining of the embryo after just 3 hours immersion in TTC solution. Scale bar = 1 mm.

Once achenes did take up TTC, however, the strength of the staining differed between treatments and species. A greater number of red stained embryos were recorded for *B. caldwellii* within fresh achene trials and a higher number of pink embryos within aged achenes (data not shown). In contrast, *B. medianus* embryos stained darker in aged achenes compared to those freshly harvested (Figure 4.4). The findings indicated that embryo metabolism in *B. caldwellii* was higher in freshly produced achenes than aged, while for *B. medianus* embryo metabolism was greater in aged achenes compared to fresh achenes possibly due to dormancy or after ripening requirements. The rate of germination in relation to seed age is therefore likely to be inversely proportional between species and may be highly significant to the success of sexual reproduction when recruitment windows are narrow and infrequent.

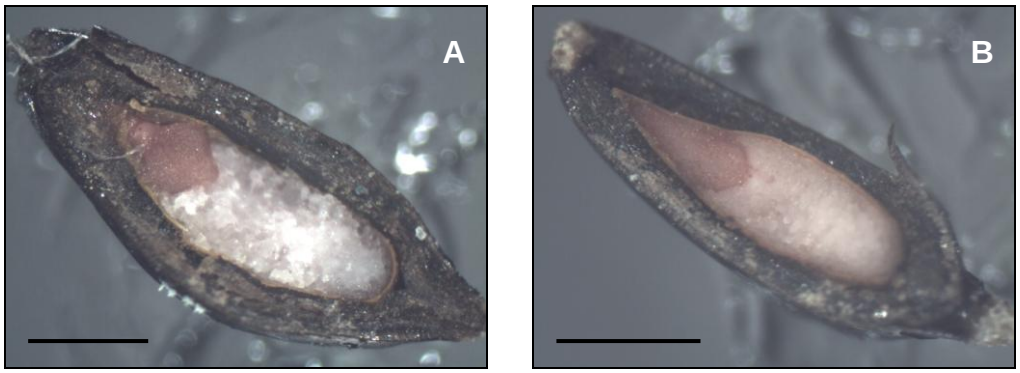


Figure 4.4. Viable (red-stained) embryos of A) *B. medianus* and B) *B. caldwellii* after 12 days immersion in 1% TTC solution. Scale bars = 1 mm.

4.4 Discussion

4.4.1 Comparison of cut and tetrazolium tests

Flemion and Poole (1948, cited in Baskin and Baskin 2001) stated that TTC was adequate for staining dormant seeds, though other authors have shown that low-intensity staining may be correlated with dormancy mechanisms such as low respiration or metabolism (Ooi *et al.* 2004). TTC is a colourless compound that changes to a pink or red colour when reduced or hydrated by living tissue (i.e. metabolizing embryo cells). Necrotic embryonic cells cannot reduce the compound and therefore change its colour; hence the compound is a highly accurate indicator of

viability (Cottrell 1947; Lakon 1948; Smith 1951). Nevertheless, achene viability in TTC testing was only judged as positive if a substantial proportion of the embryo tissue was well-stained dark pink or red. Lacroix and Mosher (1995) noted that for *Scirpus acutus* achenes, the stem and root meristematic portions of the embryo, as well as the proximal portion of the scutellum needed to be fully stained for the embryo to be considered viable. Actively metabolising meristematic regions and scutellum are all required for successful germination, hence, *B. caldwellii* and *B. medianus* achene viability was scored as positive only where all embryo components were obviously stained (Fig. 4.5).

The major deficiency of the cut method, compared with that of TTC testing for determining viability, is illustrated in Figure 4.5. Both embryos appeared healthy and would have passed the inspection criteria of cut testing, yet the discriminatory power of TTC to recognise metabolising tissue highlighted that only one embryo was truly viable.

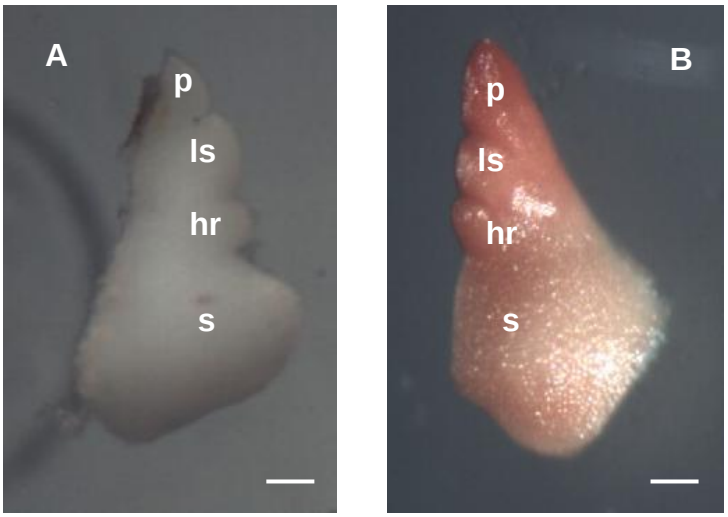


Figure 4.5. Excised *B. caldwellii* embryos showing pre-germination plumule (p), leaf sheath (ls), hypocotyl and radicle (hr) and scutellum (s) regions. A) a healthy looking but unviable (unstained) embryo after 10 days immersion in 1% TTC solution (only the faintest traces of pink can be seen around the margins), and B) a viable (red stained) embryo after 10 days immersion in 1% TTC solution. Scale bars = 100 µm.

The drawback with TTC staining of achene-bearing species was that the process was extremely slow; the time required for substantial TTC staining of both *Bolboschoenus* species was equivalent to the time it takes for their achenes to germinate. The long TTC staining periods required by both species highlighted the strong dormancy capacity and low metabolism of *Bolboschoenus* embryos. Though the more subjective cut test did not allow the viability of seed lots to be diagnosed with the explicit accuracy of TTC testing, comparative statistical analysis found no significant differences between the two methods. This is encouraging as TTC testing, though highly accurate, is much more time consuming especially when dealing with species with seed dormancy. While substantial staining with TTC can be obtained in seeds with no primary dormancy within 24-hours (e.g. *Zostera capricorni*, Conacher *et al.* 1994) the results of this chapter concur with previous studies that species producing hard coated seeds with strong dormancy mechanisms, require greater than 7 days immersion in TTC for accurate assessment (e.g. Ooi *et al.* 2004).

4.4.2 Viability of B. caldwellii and B. medianus achenes

The consistently high results (>70%) for total germinability of both species across sites, achene ages (fresh or 1-year-old) and storage treatments (dry or wet), clearly demonstrated that poor achene viability is not contributing to the lack of sexual recruitment for *B. caldwellii* and *B. medianus* in the Gippsland Lakes region. Estimates of seed viability in Cyperaceae species are rarely reported among the literature, though viability levels for *B. caldwellii* and *B. medianus* in this study were similar to those found for the closely related species *Scirpus acutus* (Lacroix and Mosher 1995).

Short periods of dry storage have been shown to reduce seed viability of *Carex* species by as much as 95% (Budelsky and Galatowitsch 1999; van der Valk *et al.* 1999; Jones *et al.* 2004). The high viability found for both species after 1-year cold-wet stratification were not as surprising as those for 1-year dry storage, as the former treatment allowed achenes to imbibe water (supporting viability) while the latter treatment accelerated desiccation (decreasing the probability of maintaining viable embryos). Though environmental conditions differ greatly between collection sites (Chapter 3) the results of this chapter suggest that achene viability levels are not

reduced by site-specific trade-offs in resource investment between sexual and asexual reproduction. Given that achene viability levels were high (>70%) for each species and did not differ between sites and treatments, the next line of inquiry in this thesis addressed the issue of seed dispersal mechanisms.

Chapter 5.

Achene anatomy and buoyancy

5.1 Introduction

Having examined both aerial and sediment seed banks, as well as the viability of *B. caldwellii* and *B. medianus* achenes, the following chapter discusses the third component of the conceptual recruitment model relevant to achene dispersal: achene anatomy and buoyancy.

Mechanisms that disperse seeds away from parent populations reduce seedling competition with established adults and offer seeds an opportunity to find unoccupied germination niches (Eriksson and Ehrlen 1992; Huckle *et al.* 2002). The transportation of buoyant seeds by water (hydrochory) is one of the most common and important vectors of dispersal in aquatic plant species (Nilsson *et al.* 1991; Huiskes *et al.* 1995; Hroudová 1997; Middleton 2000; Wolters and Bakker 2002). Several studies have demonstrated a correlation between seed buoyancy and the spatial distribution of plant populations along hydrological gradients (Ozinga *et al.* 2004; van den Broek *et al.* 2005; Kudoh *et al.* 2006). Hydrochory is a particularly important seed adaptation of salt-marsh species, as an ability to float enables the formation of 'seed-rafts' that, with time, deposit themselves as drift or strandlines at higher elevations (Wolters and Bakker 2002; Nicol 2004). Furthermore, hydrochory is an efficient adaptation for long-distance seed dispersal during extreme flood or flood-pulse events, though propagule dispersal is likely to be higher for all species on such occasions (Middleton 2002; Vogt *et al.* 2006).

Previous work on the anatomical structure of two subspecies of *Bolboschoenus* in the Czech Republic showed that closely related and sympatric

species displayed contrasting strategies (long versus short-term hydrochory) for dispersal and recruitment (Hroudová *et al.* 1997). Preliminary testing of buoyancy in *B. caldwellii* and *B. medianus* achenes for this study indicated a similar divergence in dispersal strategies for sympatric species in Australasia. The different buoyancy strategies evolved by *B. caldwellii* and *B. medianus* may aid in explaining known gradient or zonation differences for these two species, as *B. caldwellii* generally occurs at higher gradient elevations where flooding is episodic, while the distribution of *B. medianus* is restricted to littoral zones with greater periods of flooding (Siebentritt and Ganf 2000).

This chapter compared the floatation times of *B. caldwellii* and *B. medianus* achenes from separate wetlands and examined whether buoyancy capacity was related to specific achene anatomical features. More specifically the study aimed to: 1) highlight the contrasting achene dispersal techniques adopted by *B. caldwellii* and *B. medianus*, and 2) discuss the implications of the different dispersal strategies to the spatial differentiation and ecological position of each species and whether poor dispersal capacity may be contributing to poor sexual recruitment.

5.2 Methods

5.2.1 Buoyancy testing

Buoyancy tests began on 7th April 2006. Four replicates of 25 achenes from each species per site were placed into sealable plastic specimen jars (50 mL) with deionised water and shaken for 1 minute. Shaking was repeated 6 times in the first day and once daily thereafter. Immediately following shaking the number of floating and sunken achenes was determined for each replicate and totalled. Replicates were examined after 5, 30 and 60 minutes, 2, 4 and 8 hours then every day until all achenes had sunk or 100 days had elapsed. The periods after which 50% and 90% of the achenes had sunk (Ft_{50} and Ft_{90}) were determined and used to analyse differences between species and sites. Particular notice was taken to examine whether polymorphic differences in achene sizes and shapes (as discussed in Chapter 3) were influential to buoyancy.

A small sample of achenes were dissected in transverse section and photographed under a dissection microscope fitted with a digital camera to examine whether specific anatomical features of their pericarp construction could be correlated with differences in achene buoyancy between the two species.

5.2.2 Statistical analysis

T-test procedures were used to examine whether significant differences in buoyancy existed between each species or between collection sites. Where data were not normally distributed (Shapiro-Wilk) or variances homogenous (Levene's test), the non-parametric Mann-Whitney test was used to test for significant differences. Comparisons between the two species were complicated by a number of ties in the data sets disallowing both T-testing and Mann-Whitney appraisals therefore the Wilcoxon signed ranks test was applied (Zar 1996). All statistical analyses were conducted using SPSS (Version 15).

5.3 Results

5.3.1 Buoyancy

Buoyancy results were different for *B. caldwellii* and *B. medianus*, with the achenes of the former species displaying a high capacity for floatation and a gradual or staggered regimen of sinking (Fig. 5.1). In contrast, the majority of *B. medianus* achenes sank rapidly upon contact with water (Fig. 5.2). Despite the size differences of achenes from each field site, buoyancy was consistent for each species. Statistical T-tests indicated no significant differences in achene buoyancy between *B. caldwellii* populations from Clydebank Morass and Dowd Morass (Ft_{50}: $t(6) = 0.58$, $p = 0.58$ and Ft_{90}: $t(6) = 0.13$, $p = 0.91$). A *t*-statistic could not be calculated for *B. medianus* as the standard deviation of both groups was zero; therefore the non-parametric Mann-Whitney test was employed. No significant differences in achene buoyancy were found between *B. medianus* populations from Dowd Morass and Sale Common (Ft_{50}: $U = 8.00$, $p = 1.00$ and Ft_{90}: $U = 6.00$, $p = 0.56$).

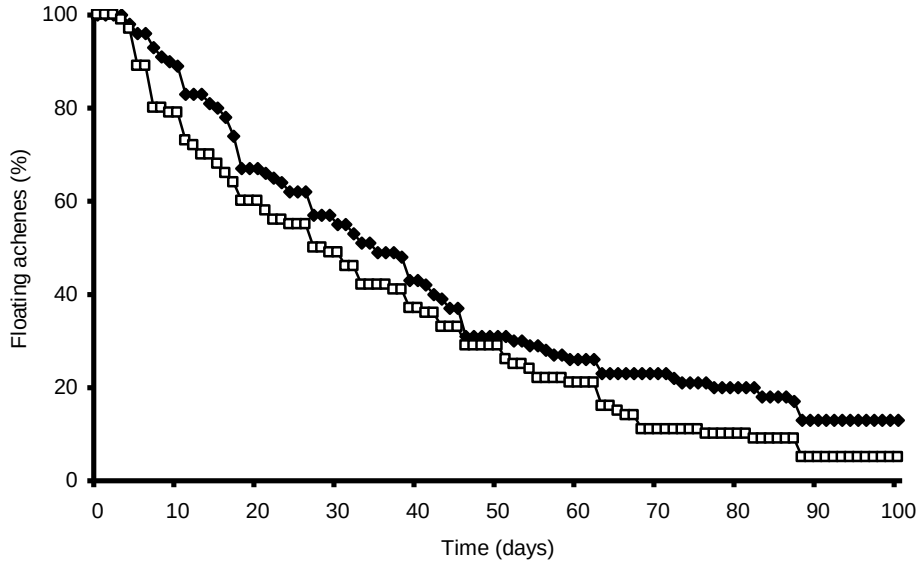


Figure 5.1. Floatation time (days) of *B. caldwellii* achenes from two different sites: larger achenes from Clydebank Morass (closed diamonds), and smaller achenes from Dowd Morass (open squares).

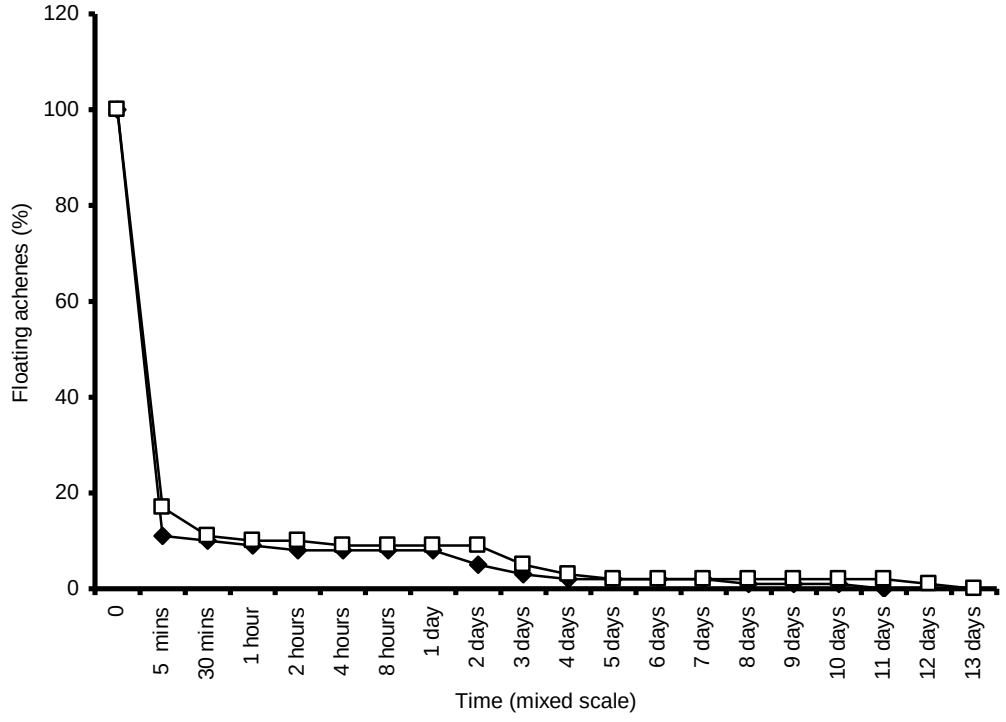


Figure 5.2. Floatation time (minutes, hours and days) of *B. medianus* achenes from two different sites: larger achenes from Dowd Morass (closed diamonds), and smaller achenes from Sale Common (open squares).

The uniformity of achene buoyancy results between sites allowed data to be pooled for each species and analysed via Wilcoxon signed rank tests. Significant differences were found between *B. caldwellii* and *B. medianus* for both Ft_{50} ($Z = 2.53$, $p < 0.05$) and Ft_{90} ($Z = 2.52$, $p < 0.05$) (Table 5.1).

Table 5.1. Comparison of floatation period (days) for 50% (Ft_{50}) and 90% (Ft_{90}) of *B. caldwellii* and *B. medianus* achenes. Means and standard errors are shown (n = 8) and significant differences indicated by different superscript letters for each column.

Species	Ft_{50} (days)	Ft_{90} (days)
Bolboschoenus caldwellii	29 ± 1^{a}	69 ± 1^{a}
Bolboschoenus medianus	0.004 ± 0^{b}	1 ± 0^{b}

Achenes of *B. caldwellii* were highly buoyant, with 9% of the total number of achenes remaining afloat after 100 days. The first *B. caldwellii* achene sank after 3 days, though 90% of achenes remained buoyant after 1 week. Ft_{50} was not attained until 27 days and Ft_{90} in 68 days, hence a significant proportion of *B. caldwellii* achenes may float for greater than 1-2 months. In contrast, Ft_{50} was attained rapidly for *B. medianus* with 86% of achenes sinking in the first 5 minutes of the experiment and Ft_{90} in all replicates within the first hour. Of the remaining floating *B. medianus* achenes, a further 5% sank within 3 days, though approximately 2-3% floated for up to 13 days. It should be noted, that the few anomalous *B. medianus* achenes that did not sink within the first hour, were all smaller, slightly less developed and dark brown in colour, in comparison to the larger, black, mature achenes which sank almost immediately. Upon inspection via 'cut testing' the embryo and endosperm region of the anomalous achenes were discovered to be incomplete with large air cavities, hence the longer floatation period. No achenes of either species germinated during the experiments while floating or having sunk to the bottom of vials.

5.3.2 Differences in pericarp anatomy

Dissection tests indicated that achene buoyancy differences between the two species were probably a consequence of their different pericarp anatomy (Fig. 5.3). The achenes of *B. caldwellii* had a waxy, water repellent surface and a substantial amount of aeriferous tissue in their exocarp cells, which were elongated (up to 200 μm) and arranged in a radial fashion. *Bolboschoenus medianus* achenes, in contrast, did not have the same waxy surface and the aeriferous pericarp layer was substantially reduced, measuring a maximum of ~50 μm.

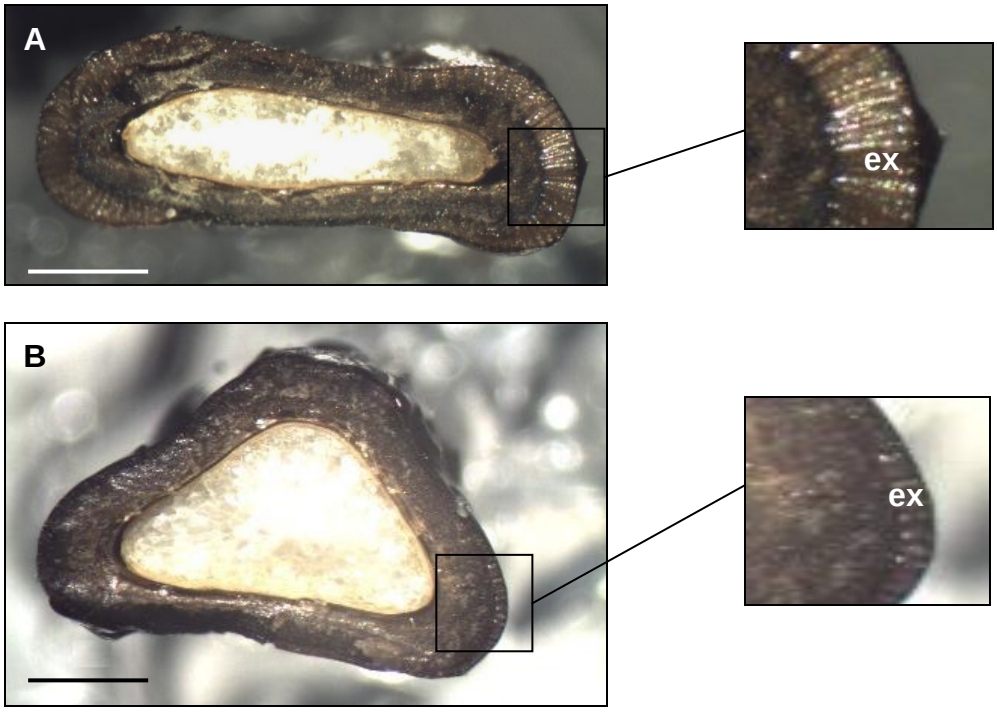


Figure 5.3. A) Transverse section of *B. caldwellii* achene showing large aeriferous exocarp (ex) cells used for buoyancy, B) Transverse section of *B. medianus* achene showing very small aeriferous cells in the exocarp layer, which are largely ineffective at buoyancy. Scale bars = 500 μm.

5.4 Discussion

5.4.1 Correlations between pericarp anatomy and hydrochory

The sexual life-history dynamics of the two *Bolboschoenus* species were in stark contrast with each other. While greater than 50% of *B. caldwellii* achenes remained afloat for more than one month, the majority of *B. medianus* achenes (~90%) sank within 5 minutes. These significantly different outcomes are directly linked to differences in the achene pericarp anatomy of each species. The water repellent surface and long, radially aligned aeriferous cells/pore spaces within *B. caldwellii* achenes give them a low specific weight, making them lighter than water and allowing for excellent buoyancy and therefore dispersal. Over time the gases within the pore spaces begin to escape and are replaced by water, making the achene heavier and denser than water. In contrast, the small amount of aeriferous tissue within the exocarp cell layer of *B. medianus* achenes means that they are already denser than water and therefore able to sink rapidly (see Browning *et al.* 1997 for detailed scanning electron microscope images of type specimens). No evidence was found to suggest that differences in achene size could influence buoyancy periodicity, although it was noted that trigonous shaped *B. medianus* achenes sank at a faster rate than their equivalent lenticular forms, which may help to explain the maintenance of the shape dimorphism.

5.4.2 Hydrochory, distribution pattens and recruitment implications

Buoyancy results for *B. caldwellii* and *B. medianus* are similar to previous observations made for two subspecies of European *Bolboschoenus maritimus* by Hroudová *et al.* (1997). Like *B. caldwellii,* the exocarp cells of *B. maritimus* subsp. *compactus* were elongated and aeriferous, which offered strong buoyancy ability, whilst the achenes of *B. maritimus* subsp. *maritimus* (like *B. medianus*) had little or no aeriferous cells in the exocarp layer and sank almost immediately. The differences in pericarp anatomy (buoyancy ability) noted by Hroudová *et al.* (1997) also correspond with the distribution patterns of the two subspecies; *Bolboschoenus maritimus* subsp. *compactus* inhabited saline areas in shallow water zones or non-aquatic areas, whereas *B. maritimus* subsp. *maritimus* was restricted to acidic substrates in standing or slow running water. Thus the former species has adopted a

high capacity for hydrochory (long-distance dispersal), while the latter species disperses seeds directly into the local seed bank. These findings are consistent with known distribution patterns for *B. caldwellii* and *B. medianus* respectively, in south-eastern Australia (Blanch *et al.* 1999; Siebentritt and Ganf 2000).

The observation that achenes could not germinate whilst underwater was significant for both species, as it demonstrated the need to germinate on exposed substrata. A similar result was recently demonstrated for the woody species *Melaleuca ericifolia* within the Gippsland Lakes (Robinson *et al.* 2006; Robinson *et al.* 2008). The rapid sinking of *B. medianus* achenes mean that the majority become incorporated into the local seed bank and remain in the vicinity of the parental population, where they must adopt a sit and wait strategy for an appropriate recruitment window in time. When conditions conducive to recruitment occur, seedlings are likely to be out-competed by established plants, leading to speculation that short transportation / dispersal capacity of *B. medianus* achenes could be contributing to the lack of recruitment observed for this species in the field. The high capacity for hydrochory in *B. caldwellii* achenes on the other hand, offers them a far greater chance of being dispersed over longer distances than *B. medianus,* or indeed to higher and drier elevations through wave action and flood-pulse events (Junk *et al.* 1989; Middleton 2002). Greater buoyancy capacity effectively opens a wider range of recruitment windows in both time and space. The probability that dispersal limitation was contributing to poor sexual recruitment of *B. caldwellii* in the field, therefore, was considered low. The variation in achene size and shape (and therefore aeriferous tissue) for *B. caldwellii* broadened the range of specific gravities between achenes, causing them to sink at random over several months. This kind of polymorphism in buoyancy capacity can be seen as a bet hedging strategy for both long and short-distance dispersal and has been reported for several other species, such as *Swartzia polyphylla* (Williamson et al. 1999) and *Penthorum chinense* (Ikeda and Itoh 2001).

Although seed floatation time is not equal to transportation time, as seeds may be caught in eddies or float motionlessly in stagnant water, it is nonetheless a relevant factor for dispersal (Nilsson *et al.* 1991). For example, Clevering (1995) noted that the achenes of *Scirpus* species that were capable of floating for several weeks could

disperse further than 10 km; Danvind and Nilsson (1997) highlighted the capacity for long-distance seed dispersal by water, by releasing small wooden cubes (pseudo seeds) into a Swedish river and collecting them up to 20 km downstream. Short-floating seeds may require currents for dispersal or additional assistance such as endozoochory by waterbirds (Hroudová *et al.* 1999; Charalambidou and Santamaría 2002). Achene morphology in the genus *Bolboschoenus* (formerly *Scirpus* L.) has been demonstrated to be highly suitable for endozoochory, as their hard integuments or multiple layered pericarp anatomy offer high resistance to vertebrate gut passage. Furthermore achenes may actually be stimulated to germinate more successfully after passage through the digestive tract of an animal. De Vlaming & Proctor (1968) noted a maximum retention time of *Scirpus paludosus* achenes in Killdeer ducks of more than 58 hours or almost 2.5 days, and Mueller and van der Valk (2002) reported that *Scirpus acutus* and *Scirpus validus* achenes may be retained by Mallard ducks for up to 30 hours. Both time periods allow considerable scope for the dispersal of achenes to new geographical areas. Krahulec and Lepš (1994) provided some evidence for both hydrochory and endozoochory whilst tracking vegetation changes in a constructed reservoir in the Czech Republic over a 21-year period. Though the nearest populations were between 10-50 km distant, *Bolboschoenus compactus* was noted to immigrate and successfully establish on at least two occasions.

Although hydrochory was restricted for *B. medianus* achenes due to the small amount of aeriferous tissue they contain, fewer dispersal limitations should hinder the movement of *B. caldwellii* achenes, as they contain a substantial aeriferous layer, which allows them to float for up to 3 months. Provided there are adequate water flows to aid in the dispersal of achenes, such as flood pulse events, the recruitment potential of *B. caldwellii* is almost unrestricted in comparison to *B. medianus*.

Chapter 6.

The effects of light, temperature and salinity on germination

6.1 Introduction

Having examined achene production, viability and dispersal, the following chapter investigates the fourth component of the *Bolboschoenus* recruitment model: the effects of light, temperature and salinity on germination, in order to determine the ideal sexual recruitment conditions for *B. caldwellii* and *B. medianus*.

In unpredictable wetland environments, the timing of germination is crucial to seedling establishment. Many aquatic plants have therefore evolved to produce seeds with mechanisms to detect environmental signals that coincide with conditions favourable for germination. One commonly used signal is that of the duration and intensity of light (Baskin and Baskin 2001). For example, the germination responses of several *Carex* species are known to be dependent on seasonal changes in light and dark ratios (Schütz 1997). The detection system used by seeds to recognise light level variations, involves phytochrome or light sensitive molecules that act as photoreceptors (Baskin and Baskin 2001). Typically, phytochrome molecules detecting far-red and blue light will inhibit germination, whereas those adsorbing or detecting red light will promote germination (Shinomura 1997; Casal and Sanchez 1998). Both water depth and burial inhibit red light, though not far-red or blue light penetration to seeds, therefore, germination is generally greatest when seeds are exposed to bright light near to or on the sediment surface (Coble and Vance 1987; Jurik *et al.* 1994; Clevering and Van der Putten 1995).

As well as detecting variations in light intensity and quality, seeds can recognise seasonal changes in mean air temperatures, due to a special class of phytochrome receptor called P_{fr} (Probert 2000). Temperature-sensitive seeds are particularly common in wetland environments, as the mechanism ensures that germination commences at an appropriate time of year (Thompson and Grime 1983; Probert 1992). The inability to germinate during narrow temperature fluctuations (i.e. winter) is an important adaptive mechanism, and as a result many wetland plant species strongly depend on the warmer temperature cues of spring for germination (e.g. *Lycopus europaeus,* Thompson 1969; *Fimbristylis littoralis* and *Scirpus juncoides,* Pons and Schröder 1986; *Rumex palustris,* Voesenek *et al*. 1992; *Scirpus* spp., Clevering 1995; *Typha latifolia,* Lombardi *et al*. 1997; *Cladium jamaicense,* Lorenzen *et al*. 2000; *Bolboschoenus* spp., Moracová *et al*. 2002).

In addition to adequate light and temperature signals, the majority of aquatic plant species, from glycophytes (freshwater species) to halophytes (salt tolerant species) also require low salinity for sexual recruitment events (Ungar 1978; Baskin and Baskin 2001). A number of studies have reported that seed germination in wetland and saltmarsh communities is restricted due to high soil salinity levels in bare patches (i.e. colonisation sites) (e.g. Shumway & Bertness 1992). Surface soil salinity levels in salt marshes may be up to 100 times greater than that of the subsoil (Ungar 1978). High salt loads result in low soil water potentials, which restrict seed imbibition and germination by inhibiting membrane bound proteins and other cytosol enzymes (Evans and Etherington 1990; Khan *et al.* 2002). The timing of germination is largely dependent on sediment salinity, which in turn is determined by hydrological regimes (Noe and Zedler 2001). Although germination responses are generally species specific, increasing salinity has been widely reported to delay germination and decrease total percentage germination in plant species from all life-history types (Waisel 1972; Chapman 1974; Ungar 1982; Joshi *et al*. 2005; Greenwood and DuBowy 2005). One major discrimination between wetland plants, however, is that the seeds of many salt tolerant species can remain viable following extended exposure to highly saline conditions and still germinate upon subsequent transference to freshwater conditions (Williams and Ungar 1972; Woodell 1985; Khan *et al*. 2000; Huang *et al*. 2003; Naidoo and Kift 2006; Robinson *et al.* 2006). This process is known as "reversible osmotic inhibition" (Ungar 1982; Woodell, 1985, Keiffer and

Ungar 1997). Furthermore, instead of having a negative effect on germination, prior stratification treatments of seeds in saline solutions may significantly accelerate the germination rate and success of many species when transferred to freshwater (Heydecker *et al*. 1973). For example, Woodell (1985) examined 31 British coastal marsh species and concluded that although the majority of seeds had highest germination in freshwater conditions, the seeds of many salt marsh species exhibited 'salt stimulation', as final germination percentages upon transference to fresh water conditions were generally greater from replicates exposed to higher salinity pre-treatments.

The inhibition of seed germination by high salinity is of particular concern to wetland plants in the Gippsland Lakes region of Victoria. As predicted by Bird (1966) salinity in Lake Wellington and its surrounding wetlands has gradually increased over time due to the permanent opening of Lakes Entrance to Bass Strait (Navanteri and Kambouris 2008; Parks Victoria 2008). One implication of this landscape-scale change is that even if appropriate light and temperature signals occur, high salinity levels often prevent sexual recruitment (Robinson *et al.* 2006; Salter *et al.* 2007). The high sediment salinity (19-43 g L^{-1}) recorded in spring of 2006 for Clydebank Morass and Dowd Morass (see Chapter 3), highlighted that salt probably has a major influence on the recruitment patterns of these communities, both in terms of absolute effects and short-term exposure effects.

This chapter examines the germination of *B. caldwellii* and *B. medianus* achenes under different light, temperature and salinity regimes, as well as the ability of achenes to recover from saline pre-treatments. Specific germination requirements for *B. caldwellii* and *B. medianus* remain unstudied and it is uncertain as to whether their needs are similar, or whether germination differences exist between the two species that reflect their typical habitat positions.

6.2 Methods

6.2.1 Effects of light and temperature on germination

Temperature and light germination trials ran from February 2006 to February 2007. Achenes of each species were tested for germination at five constant temperature regimes (10, 15, 20, 25 and 30ºC) and 4 fluctuating temperature regimes (30-5°C, 27.5-7.5ºC, 25-10°C and 20-15ºC day-night temperatures respectively) under 12:12 hour light:dark conditions, in order to determine the preferred germination temperatures for each species. Germination was also tested under 24-hour darkness (in order to mimic the effects of achene burial) at two different temperature regimes (30-5°C and 25-10°C). Treatments without light were conducted for one month and achenes were only checked for germination at the end of 30 days. In order to assess the likelihood of sexual recruitment events occurring in the field, germination results gained from temperature trials were compared to mean monthly temperature ranges for the surrounding study region for approximately the last 60 years (Bureau of Meteorology, Climate Station 085072, East Sale airport, Victoria). A total of 2,600 achenes were tested in temperature and light trials.

6.2.2 Effects of exposure to salt on germination

Salinity trials ran from February to August 2006 and a total of 2,800 achenes were tested. Filter papers used as substrata in each replicate were wetted with 5 mL of solution from one of seven different treatment levels: a control (deionised water) and 6 salinity concentrations (1, 2, 4, 8, 16 and 32 g L^{-1}). Salinity concentrations used in germination trials ranged from < 0.2 g L^{-1} salt (in mimic of freshwater conditions at Sale Common) through to a full seawater treatment (~ 32 g L^{-1}) as sediment salinity levels at Dowd Morass and Clydebank Morass are commonly recorded at or above 20 g L^{-1}. Saline solutions were made of pure Red Sea salt – Israel (Coburg Aquarium Supplies) and deionised water. Germination was assessed under two temperature regimes (30-5ºC and 25-10ºC day-night temperatures respectively). After 6 weeks, ungerminated achenes were transferred to clean petri dishes with new filter paper and 5 mL of deionised water, then incubated at the same temperature regime for a further 30 days. Following the methodology of Khan *et al*. (2002) recovery percentages were

calculated using the formula: $(a - b)/(c - b)$ x 100, whereby a is the total number of seeds that successfully germinated after transference to freshwater, b is the total number of seeds that germinated in saline solution, and c is the total number of seeds.

Note: germination trials for each treatment (1) temperature and light, and (2) salinity, were conducted separately due to logistic constraints with the number of available (working) incubators. Unforseen incubator malfunctions meant that the duration of germination trials was extended from around six months to approximately 1 year. Some concern was therefore held over the influence of achene aging (maturation) affecting germination outcomes and the validity of comparing results between treatments. Both the dormancy and viability of achenes can change over time, hence the germination results generated for achenes < 6-9 months old may be radically different to those obtained from achenes > 12 months old. In order to check whether the aging process was an influential factor on germination for either species, the initial 30-5°C and 25-10°C treatments were repeated at the completion of all other trials, using 1-year old achenes from the same collection batch.

6.2.3 Statistical analysis

Percentage germination results were converted to proportional data, then arcsine-sqrt transformed prior to statistical analysis (Zar 1996). Two or three-way ANOVA procedures were used to examine the significance of individual factors (species type, temperature regime and salinity) and their interactions on the germination percentage, germination rate and recovery germination percentage of achenes. As per Clevering (1995) treatments without germination were omitted from statistical analysis, otherwise homogeneity of variances could not be achieved. All data were analysed using SPSS version 14.

6.3 Results

6.3.1 Effects of light and temperature on germination

No germination occurred for either species in a range of trials using constant temperatures. Germination was also unsuccessful for both species in 24-hour dark treatments at the 25-10°C temperature range, however ~40% of *B. caldwellii* achenes and 5% of *B. medianus* achenes commenced germination at the wider 30-5°C temperature regime (Fig. 6.1). In all cases however, the germination process in darkness was incomplete, as seedling development failed to move pass the initial phase of plumule extension. Inhibited phytochrome activity and prevention of photosynthesis were suspected as the most likely cause of germination failure, as plumules were unusually long and white rather than green in colour. Minimal or complete absence of germination prevented statistical analysis of 24-hour dark or constant temperature trials.

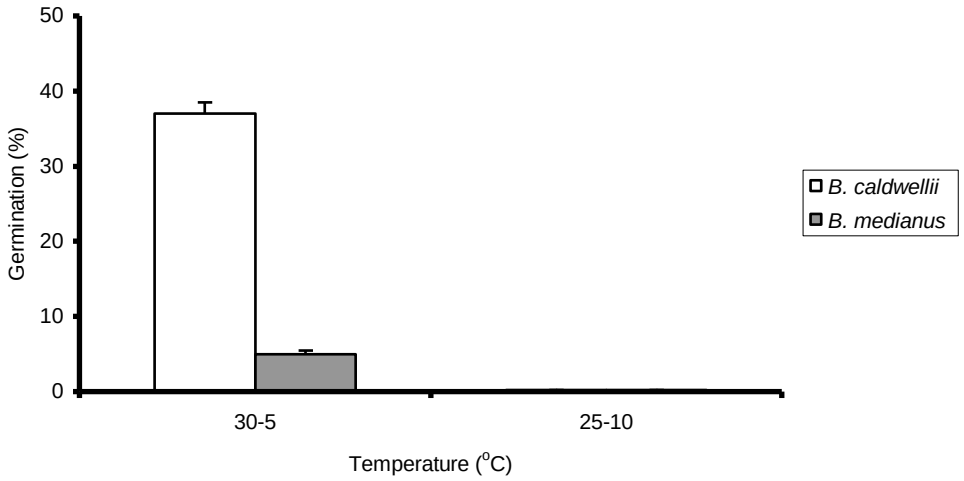


Figure 6.1. Percentage of *B. caldwellii* and *B. medianus* achenes commencing germination under 24-hour darkness at two temperature regimes. Standard error bars are shown (n = 4). No germination was recorded at the narrower 25-10°C temperature range for both test species.

Experiments using a range of alternating day-night temperatures illustrated that both *Bolboschoenus* species are adapted to germinate under temperature regimes with wide diurnal differences of up to 20-25°C (Fig. 6.2). Highest recorded

germination occurred for *B. caldwellii* (79%) at the 30-5°C temperature regime, while highest germination of *B. medianus* achenes (10%) was recorded at the 27.5-7.5°C temperature range. No germination occurred at the narrowest temperature regime (20-15°C) while less than 5% germination was recorded at the 25-10°C temperature range for either species. It should be noted that the achenes used in the 27.5-7.5°C trials were six months older than those used in the initial 30-5°C replicates, therefore an aging effect (such as the lessening of dormancy) may have gone undetected for both species at this level. Statistical comparisons of germination re-test results (1-year-old achenes) with original trials (1-3 month old achenes) at 25-10°C and 30-5°C temperatures, revealed a significant difference for *B. medianus* at the 30-5°C temperature range only ($t(6) = 2.53$, $p < 0.05$). The aging process may therefore allow slightly better germination for *B. medianus* under close to ideal germination conditions, however the insignificant gains in germination re-tests recorded for both species within 25-10°C replicates highlighted that seed aging was not able to widen the parameters at which germination could occur.

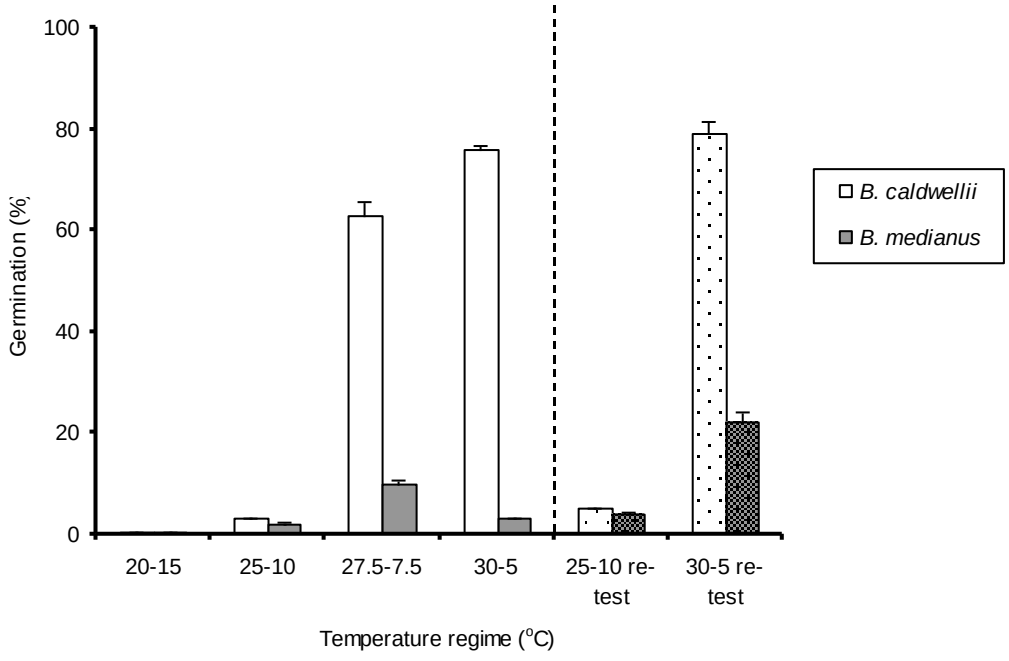


Figure 6.2. Percentage germination of *B. caldwellii* and *B. medianus* achenes under 4 different diurnal temperature regimes. Trials at 25-10°C and 30-5°C were re-conducted (dotted bars) using 1-year-old achenes at the end of all other experiments. Standard error bars are shown (n = 4). Note: no germination was found at the 20-15°C temperature range for either species.

The different responses of *B. caldwellii* and *B. medianus* achenes across the range of temperatures tested were highlighted by 2-way ANOVA, which revealed a strong influence on germination success of species ($F_{1, 18}$ = 98.37, $p < 0.001$) and temperature ($F_{2, 18}$ = 37.73, $p < 0.001$), as well as a significant interaction between the two factors ($F_{2, 18}$ = 22.66, $p < 0.001$) that accounted for ~72% of the variation found among percentage germination results (Table 6.1). Two-way ANOVA also revealed a significant interaction between species and temperature ($F_{2, 18}$ = 14.95, $p < 0.001$) that accounted for ~62% of the variation found in germination rates.

Table 6.1. Two-way ANOVA of percentage germination and germination rate data in relation to species, temperature and species x temperature interactions. Note: as no germination occurred in any replicates within the 20-15°C temperature treatments, this level was omitted from statistical analysis.

	Germination response			
	Independent variable	df	F	Sig.
Percentage germination	Species	1	98.37	.000
	Temperature	2	37.73	.000
	Species x Temperature	2	22.66	.000
Rate of germination	Species	1	64.06	.000
	Temperature	2	25.88	.000
	Species x Temperature	2	14.95	.000

6.3.2 Effects of exposure to salt on germination

Greater than 70% germination was attained by *B. caldwellii* at 30-5°C in control treatments and salinity concentrations of up to 2 g L^{-1} though germination was negligible for this species under the same treatment levels at the 25-10°C temperature regime (Fig. 6.3). No germination was observed for *B. caldwellii* at or above 16 g L^{-1}. Germination of *B. medianus* was poor in control replicates and across salinity treatments, peaking at 14% in 2 g L^{-1} trials at the 30-5°C temperature regime and 3% germination in 1 g L^{-1} trials at the 25-10°C temperature range. No germination was recorded for *B. medianus* at either temperature regime at or above 4 g L^{-1}.

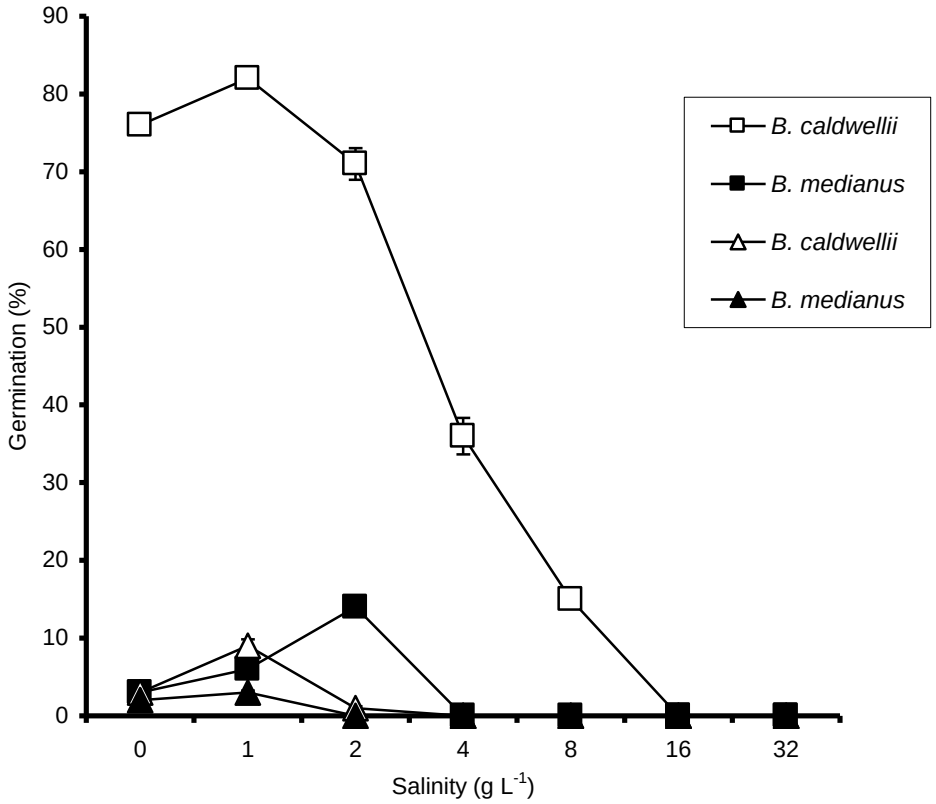


Figure 6.3. Percentage germination of *B. caldwellii* and *B. medianus* under 7 different salinity treatments and 2 temperature regimes: square icons indicate 30-5°C day and night temperatures, while triangle icons indicate 25-10°C day and night temperatures respectively. Standard error bars are shown (n = 4).

While the sexual strategy of *B. medianus* included dormancy breakage in near fresh water conditions and a slow rate of germination, *B. caldwellii* achenes in contrast showed greater tolerance to salinity, as well as an ability to readily arrest dormancy and germinate rapidly upon transference to fresh water. Strong dormancy in *B. medianus* achenes in all treatments indicated that germination was restricted not only by salt, but also by an after-ripening requirement, such as stratification (see Chapter 7). The different germination responses of *B. caldwellii* and *B. medianus* to salinity, were highlighted by 3-way ANOVA, which indicated that all individual factors were significantly influential on germination success, with temperature ($F_{1, 39}$ = 137.88, $p < 0.001$) accounting for ~78% of the variation (Table 6.2). Significant interactions also occurred between the factors of species and temperature, and species and salinity, accounting for ~67% and 15% of the variation in germination response respectively.

Table 6.2. Results of 3-way ANOVA of percentage germination, rate of germination and recovery germination in relation to species, temperature and salinity and all potential interactions.

	Germination response		
Independent variable	Percentage germination	Rate of germination	Recovery germination
Species	111.79*	417.15*	158.27*
Temperature	137.88*	436.59*	25.65*
Salinity	22.59*	69.53*	34.40*
Species x Temperature	79.07*	394.78*	0.30
Species x Salinity	3.49**	58.78*	6.17*
Temperature x Salinity	0.232	59.94*	10.12*
Species x Temperature x Salinity	0.003	54.00*	11.18*

Note: Numbers represent *F* values; *$p < 0.001$, **$p < 0.05$

Three-way ANOVA of germination rate data revealed that the individual factors of species type, temperature and salinity were all highly influential on the average time taken for achenes to germinate (Table 6.2. column 2). Significant interactions were also found between all possible combinations of individual factors, with the highest being that between species type and temperature ($F_{1, 84} = 394.78$, $p < 0.001$), which accounted for ~83% of the variation in germination rate. Mean germination rate (T_{50}) was consistently slow (>30 days) for *B. medianus* regardless of salinity or temperature. Germination rate was similarly slow for *B. caldwellii* at all salinities under the narrow (25-10°C) temperature regime, though at 1 g L^{-1} salt under the wider 30-5°C temperature range, T_{50} germination was achieved in 18 days.

Achene germination for both species required light and was highest at salinities ≤2 g L^{-1} under wide diurnal temperature ranges (30°C day and 5°C night). While total germination percentages for *B. caldwellii* achenes were consistently high (>70%) under these conditions, total germination of *B. medianus* achenes was consistently poor (<15%) and warrants further investigation.

6.3.3 Achene transference to freshwater

A percentage of ungerminated achenes from all salinity treatments were able to germinate following transference to freshwater conditions (Fig. 6.4). Achene viability was therefore not compromised by the 6-week salinity pre-treatments (i.e. germination trials) at all salt concentrations tested (including full seawater) and all osmotic effects appeared reversible. At the 30-5°C range, total recovery germination for *B. caldwellii* from all salinities were similar to those recorded in controls, whereas at 25-10°C substantial increases in recovery germination were noted from all salinity treatments compared to controls. Highest recovery germination (54%) was recorded within replicates from the lowest salinity concentration (1 g L^{-1}), though 17% of achenes germinated from 32 g L^{-1} replicates upon transference to fresh water (more than 5 times the germination found in controls).

Recovery germination was greater than control totals at almost every salinity concentration tested for *B. medianus*, though overall recovery germination at both temperature regimes was far lower than achieved by *B. caldwellii*. Highest recovery of *B. medianus* achenes (~20%) occurred from salinity concentrations at or above 8 g L^{-1}, supporting suggestions that pre-treatments in saline water may help to stimulate final germination. The only anomaly within *B. medianus* data occurred at 2 g L^{-1} salt in 25-10°C replicates, as no germination was recorded in any of the 4 replicates during the entire 74 days of testing. The lack of germination was difficult to explain as the tested achenes originated from the same seed pool as other trials. Negligible germination for *B. medianus* below 8 g L^{-1} salt at both temperature regimes suggested that low levels of salinity reinforced dormancy in this species. Three-way ANOVA of recovery germination data revealed that all of the individual test factors were highly significant to the recovery ability of achenes ($p < 0.001$) (Table 6.2. column 3). Significant interactions were also found between all possible combinations of factors with the exception of species and temperature. The highest interaction occurred between all 3 factors and accounted for ~44% of the variation found in recovery germination percentages.

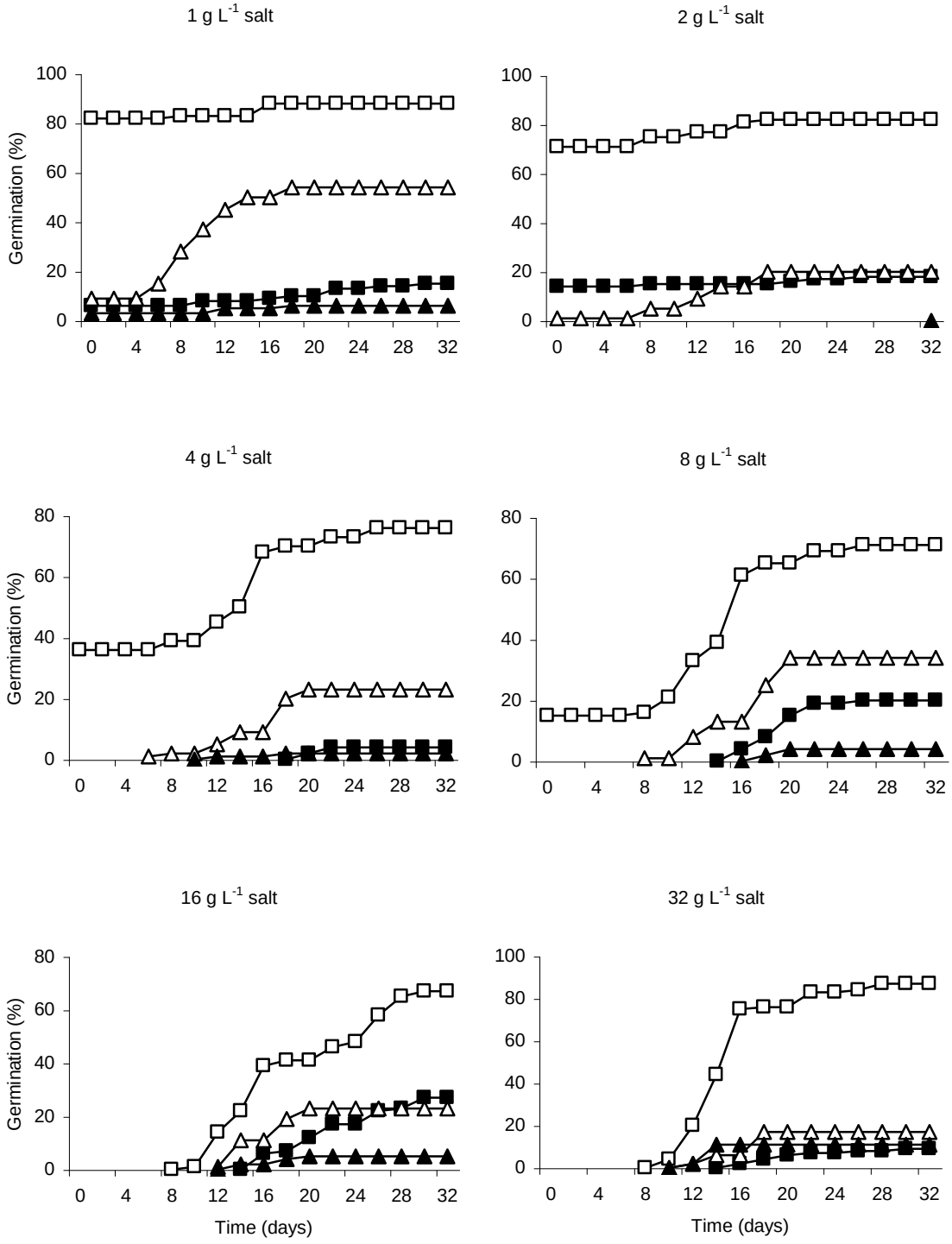


Figure 6.4. Cumulative percentage recovery germination of *B. caldwellii* (white icons) and *B. medianus* achenes (black icons) from saline pre-treatments at 30-5°C (square icons) and 25-10°C temperature regimes (triangular icons). Standard error bars are omitted for the sake of clarity (n = 4).

6.4 Discussion

6.4.1 The importance of light and wide diurnal temperature regimes to germination

The positive germination response of both *B. caldwellii* and *B. medianus* achenes to light agrees with previous findings that the majority of species belonging to the Cyperaceae family have seeds that are photoblastic, that is, they require light for germination (Leck and Schütz 2005; Fenner and Thompson 2005). Light sensitivity has been demonstrated to be common within other helophytic genera, such as *Carex* (Schütz 2000), *Triglochin* (Naidoo and Naicker 1992) and *Typha* (Gopal and Sharma 1983; Lombardi *et al*. 1997; Lorenzen *et al*. 2000). Widell and Vogelmann (1988) showed via the use of fibre optics that light was filtered as it passed through *Lactuca sativa* seed coats, though wavelengths such as far red, were still able to reach seed embryos. Photoreceptor molecules found in seed embryos, known as phytochromes, are sensitive to such wavelengths and are responsible for the mediation of germination responses (Shinomura 1997; Casal and Sánchez 1998). The seed coats of *Bolboschoenus* species are surrounded by 3-4 layers of pericarp tissue, increasing the difficulty of light penetration to embryos (see Fig. 4.1, Chapter 4). If achenes become buried or flooded the light requirement of both species means that germination is unlikely, though this may be an important strategy for the maintenance of persistent seed banks, as noted for *Triglochin* spp. (Naidoo and Naicker 1992). Some elongated, white coloured plumules emerged within 24-hour darkness trials for *B. caldwellii*, indicating that this species may not be totally dependent on light for germination, however, the failure of hypocotyl and radicle regions to emerge suggested that an interaction must occur between light and the cotyledon (plumule). The ability of *B. caldwellii* achenes to produce plumules in darkness may be of great benefit where burial or water depths are only superficial and light may be attained shortly after germination commences.

The finding that *B. caldwellii* and *B. medianus* achenes were responsive to wide temperature amplitudes agrees with previous observations made for a number of European *Bolboschoenus* and *Scirpus* species (Thompson and Grime 1983; Pons and Schröder 1986; Clevering 1995; Moracová *et al*. 2002). However, the amplitude of temperature variation (2-3°C) that produced 50% germination in species such as

Scirpus sylvaticus and *Scirpus lacustris* (Thompson and Grime 1983) was extremely low compared to the temperature variation required by *B. caldwellii* and *B. medianus* achenes. In order to commence germination, *B. caldwellii* and *B. medianus* achenes required between 2-3 weeks of wide diurnal temperature variations in the order of 25°C. This level of temperature variation is rarely attained within climatic conditions in the study region (Fig. 6.5), unless unusually high or low temperatures occur (86th and 14th percentiles), suggesting that sexual recruitment of *B. caldwellii* and *B. medianus* is likely to be restricted. However, specific conclusions regarding limited recruitment opportunities and inappropriate temperature regimes cannot be made as the parameters for successful seed germination may be widened following seed coat scarification or cold-wet stratification (see Chapter 7 of this thesis).

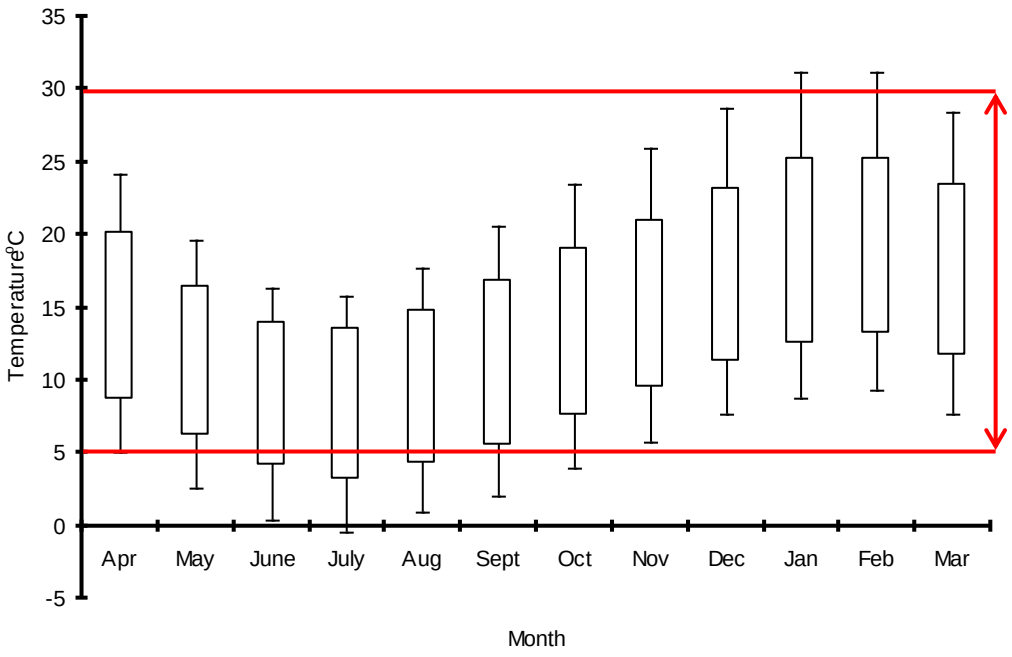


Figure 6.5. Mean monthly temperature ranges for the Gippsland Lakes region for approximately the last 60 years (Bureau of Meteorology, Climate Station 085072, East Sale airport, Victoria). Down bars represent mean maximum and mean minimum temperatures, while high and low error bars represent the 86th and 14th percentiles respectively. Overlayed red lines and red arrow indicate that the 25°C diurnal temperature difference required for germination is rare within the study region.

The germination requirements of light and wide diurnal temperature regimes support previous findings that sexual recruitment of *Bolboschoenus* species is adapted to occur on exposed substrates in the warmer temperatures of spring (Clevering 1995; Moravcová *et al.* 2002). Variations in both temperature and light provide important dormancy-breaking cues for the achenes of *B. caldwellii* and *B. medianus*. The results supported previous conclusions that the achenes of *Bolboschoenus* and *Scirpus* species are effectively equipped with depth sensing mechanisms in order to prevent germination during unfavourable conditions (Pons and Schröder 1986; Clevering 1995). As light penetration will naturally be higher in exposed achenes, compared to those buried in sediment or submerged under water, the activity of light sensitive phytochrome molecules is likely to play a major role in germination processes for *B. caldwellii* and *B. medianus* when water levels recede. Additional phytochrome molecules, such as P_{fr}, which are thought to be involved in the recognition of temperature variations (Probert 2000), were also suspected to play an active role in determining the timing of germination.

6.4.2 The importance of salt flushing events for sexual recruitment

Inhibition and delay of germination in wetland plant species from salinised sites is generally attributed to osmotic and specific-ion effects that reduce water imbibition rates and therefore embryonic metabolism and enzyme activity, which in turn induces secondary dormancy (Ungar 1978; Partridge and Wilson 1987; Evans and Etherington 1990; Naidoo and Naicker 1992). Though the influence of salinity on dormant seed banks is not entirely clear, recent research demonstrated that the exposure of seeds to saline pre-treatments of different durations could significantly influence the germination success of salt marsh species (Espinar *et al*. 2005). For example, the recovery germination of *Scirpus maritimus* (now *Bolboschoenus maritimus*) was found to significantly decrease as salinity pre-treatments increased, while germination of *S. litoralis* was found to either decrease or be stimulated following short and long exposure durations to salinity respectively (Espinar *et al*. 2005). While only one saline exposure period was used in this study (42 days), the results for *B. caldwellii* and *B. medianus* achenes were in closer alignment with findings for the achenes of *S. litoralis* than *B. maritimus,* due to evidence of reverse osmotic inhibition and salt stimulation in achenes recovering from high salinities, and

induced secondary dormancy in achenes from low salinity levels. For example, highest recovery germination for *B. caldwellii* achenes was recorded from 32 g L^{-1} replicates (0-87%), while highest recovery of *B. medianus* achenes (0-27%) occurred from 16 g L^{-1} treatments. Results for both species suggested that short pre-treatments of achenes in highly saline solutions had a positive influence on germination.

Upon transfer to freshwater, seeds initially exposed to high salinity pre-treatments often exhibit higher germination than seeds initially exposed to low or negligible salinity (e.g. *Sueda depressa,* Williams and Ungar 1972; *Salicornia europaea,* Ungar 1977; *Triglochin striata,* Naidoo and Naicker 1992; *Salicornia rubra,* Khan *et al*. 2000; *Kochia scoparia,* Khan *et al*. 2001; *Haloxylon ammodendron,* Huang *et al*. 2003; *Juncus subulatus,* Espinar *et al*. 2005; *Juncus kraussii,* Naidoo and Kift 2006). The ability of seeds to post-pone and prolong their germination period under high salinity, effectively acts as a risk-spreading mechanism (Naidoo and Kift 2006). The results of this chapter indicated that the flooding of seed banks under saline conditions during non-growth periods, may be of some benefit to *B. caldwellii* and *B. medianus*, though both species require water and salt levels to decrease by spring or early summer for sexual recruitment, and extended exposure to saline conditions is likely to reduce the viability of seed banks in the long-term.

Coordination of germination timing is crucial, as temperatures and salinity levels only marginally above or below the normal germination parameters of a species can quickly induce secondary dormancy or the abortion of embryos (Bewely and Black 1994; Kahn *et al.* 2002). The findings of this chapter agree with previous conclusions that salinity, light and temperature interact to produce a fairly specific germination optima, that is, a narrowing of the margin for recruitment (Rivers and Weber 1971; Rozema 1975; Badger and Ungar 1989; Khan and Ungar 1996; Khan *et al*. 2002). The restriction of sexual recruitment events caused by high salinity illustrates the importance of natural flooding events to periodically flush salt from brackish wetlands. Given growing water demands, increasing salinity and rare flooding events there is reason to suspect that appropriate germination conditions are now infrequent in the Gippsland Lakes region. Sexual recruitment limitations, particularly those caused by high salinity, are likely to compound dependence on clonal growth mechanisms in *Bolboschoenus* species occupying saline sites.

Chapter 7.

The importance of stratification and scarification treatments to dormancy breakage

7.1 Introduction

The previous chapter examined the specific germination requirements of *B. caldwellii* and *B. medianus* to light, temperature and salinity. One important finding of the chapter indicated that mean temperature regimes across the study region are rarely conducive to germination and may in fact promote achene dormancy. In addition, laboratory experiments in each of the previous chapters highlighted a substantial difference in the overall germination percentages of *B. caldwellii* and *B. medianus* achenes under a range of treatments, with the former species consistently responding with high germination (>60-70%) and the latter species showing consistently poor germination (<5%).

The following chapter examines the fifth component of the recruitment model: stratification and scarification. The aim of this chapter was to examine whether the differences in germination success between *B. caldwellii* and *B. medianus* could be counteracted or explained via improved permeability of the seed coat with stratification or scarification treatments. More specifically, a range of cold-wet stratification and bleach scarification periods were used to test the hypothesis that long-term stratification or short-term scarification would significantly increase germination success of *B. medianus* achenes, as well as widening the temperature parameters at which both *B. medianus* and *B. caldwellii* could germinate. Stratification (over several months) should allow both species adequate time to imbibe water into their endosperm and embryo regions, while bleach and manual

scarification treatments (applied to damage achene pericarp layers) should effectively cancel out any germination discrepancies in imbibition rates due to differences in achene pericarp anatomy.

7.2 Methods

7.2.1 Germination conditions

Germination conditions followed those outlined in the introductory method descriptions (see Chapter 2), with the exception that an additional narrower temperature regime (25°C day and 10°C night) was used in conjunction with the standard test range of 30°C day and 5°C night. The additional temperature regime was chosen as previous germination tests (Chapter 6) showed poor results for either species at the 25-10°C range and it was predicted that there would be a significant improvement at this level following stratification or scarification treatments. A total of 5,000 achenes were tested across different treatments.

7.2.2 Stratification

Stratification experiments ran from February 2006–April 2007. One hundred undamaged achenes each were placed into 10 mL plastic vials containing deionised water and stored in a refrigerator at 4°C in the dark. As the duration of stratification treatments are highly influential on germination success, one control and six stratification treatments of 2, 4, 8, 16, 32 and 55 weeks duration were employed, in order to assess their effectiveness at increasing the overall germination of both test species.

7.2.3 Scarification

Two scarification treatments were tested. 1) Bleach scarification trials ran from February to April 2006 and consisted of 1 control (0 days) and 8 bleach treatments of 1, 2, 3, 4, 5, 6, 7 and 14 days exposure of achenes to 10% w/v sodium hypochlorite. The 14-day exposure to bleach was predicted to kill embryos. 2) Manual scarification trials ran from February to March 2006. One hundred achenes

from each species were used as controls and a further 100 hundred achenes were individually scarified by manually slicing off a small section (~0.5 mm) of the micropylar end with a scalpel.

7.2.4 Statistical analysis

Mean germination rate (T_{50} – the time taken in days to reach 50% of total germination) was calculated for each replicate / petri dish. All data sets were checked for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity of variances (Levene's test) and, where appropriate, percentage germination results were converted to proportional data, then arcsine-square-root transformed prior to statistical analysis (Zar 1996). Interactions between species, temperature and stratification duration were analysed via 3-way ANOVA, while interactions between species and scarification were analysed via 2-way ANOVA. When applicable, significant differences within each analysis ($p < 0.05$) were determined by Tukey *post-hoc* tests. All statistical analyses were performed using SPSS (Version 14) statistical package.

7.3 Results

7.3.1 The effects of different stratification periods on germination

Stratification was not obligatory for breaking achene dormancy in either species, as some germination was observed in all control replicates. Germination rose as high as 92% for *B. caldwellii* at the 30-5°C temperature regime, following an 8-week stratification period, though germination was consistently high (>70%) for this species at all treatment levels under the wider temperature range (Fig. 7.1).

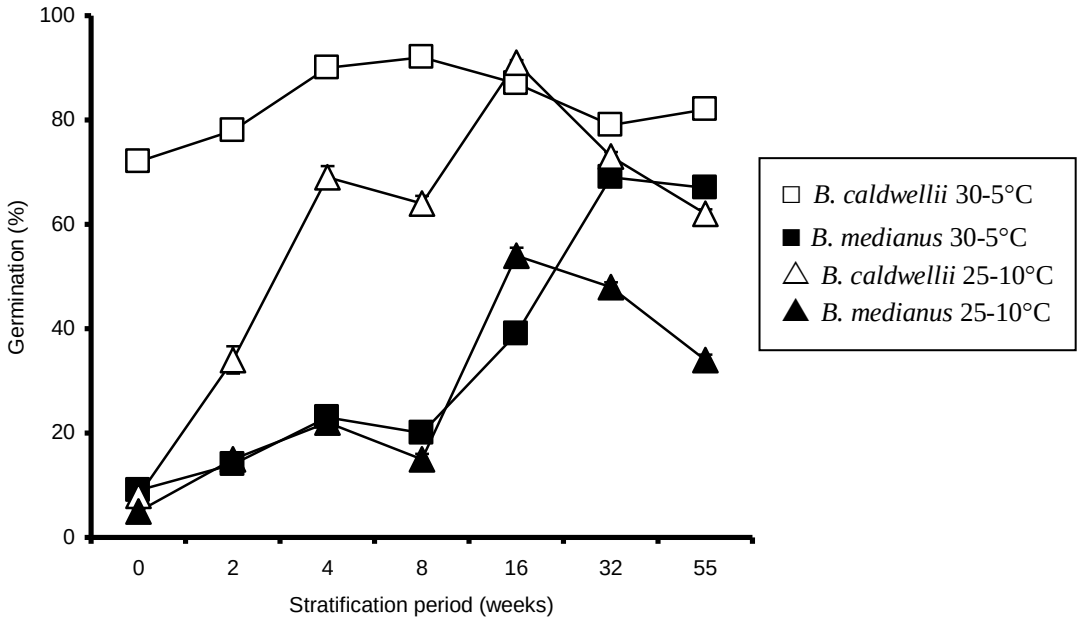


Figure 7.1. Mean percentage germination of *B. caldwellii* and *B. medianus* achenes at two different temperature regimes and 7 different stratification durations. Standard error bars are shown (n = 4).

At the narrower (25-10°C) temperature range, germination of *B. caldwellii* achenes dramatically increased from less than 10% in control replicates, to greater than 60% with a stratification period of at least one month. Germination of *B. medianus* achenes in control and short-term stratification treatments was poor, irrespective of temperature. Mean germination percentages for *B. medianus* achenes stratified for less than 2-months, were similar to control treatments at either temperature range, however, germination was significantly improved with stratification durations of 16 weeks and above. Three-way ANOVA indicated that the individual and interactive effects of species, temperature and stratification were all highly significant ($p < 0.001$) factors for germination (Table 7.1). The most significant interaction occurred between species and stratification treatment, accounting for ~48% of variation in germination.

Table 7.1. The effect of temperature and stratification and their interaction on germination of *B. caldwellii* and *B. medianus* achenes. Significant differences (* $p < 0.001$) are indicated following 3-way ANOVA.

% Germination	Effect	df	F-value	Sig.
Temperature and stratification	1 Species	1	510.16	*
	2 Temperature	1	94.35	*
	3 Stratification	6	58.14	*
	1 x 2	1	29.84	*
	1 x 3	6	13.08	*
	2 x 3	6	9.83	*
	1 x 2 x 3	6	7.80	*

The rate of germination for both *B. caldwellii* and *B. medianus* achenes decreased as stratification periods increased, though germination response times at the narrower temperature range (25-10°C) increased with short-term stratification suggesting an initial reinforcement of dormancy (Fig. 7.2). A stratification period of 16 weeks (~4 months) was required before mean germination rates at the 25-10°C temperature range dropped below those measured in controls. Substantial disruption to the integrity of achene surfaces was evident following long-term stratification (Fig. 7.3.A-C) and after 55 weeks stratification, germination rate was very similar between species and temperature regimes. Three-way ANOVA revealed that all individual factors as well as their interactions were significantly influential on achene germination rates (Table 7.2).

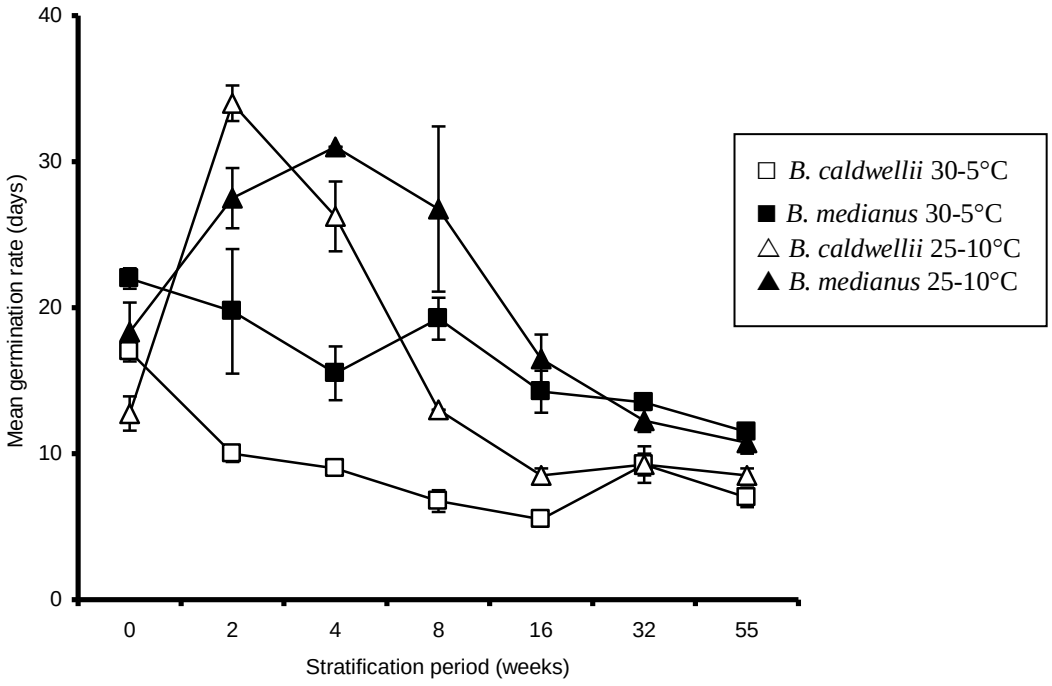


Figure 7.2. Mean germination rate of *B. caldwellii* and *B. medianus* achenes under control and 6 stratification durations (weeks) at two different temperature regimes (square markers = 30°C day and 5°C night temperature and triangle markers = 25°C day and 10°C night temperatures). Standard error bars are shown (n = 4).

Table 7.2. The effect of temperature and stratification and their interaction on germination rate in *B. caldwellii* and *B. medianus*. Significant differences (* $p < 0.001$) are indicated following 3-way ANOVA.

Germination rate	Effect	df	F-value	Sig.
Temperature and stratification	1 Species	1	970.126	*
	2 Temperature	1	255.044	*
	3 Stratification	6	117.304	*
	1 x 2	1	107.801	*
	1 x 3	6	17.693	*
	2 x 3	6	15.503	*
	1 x 2 x 3	6	15.799	*

7.3.2 The effects of bleach scarification on achene germination

Several changes in the appearance of achenes of both species were observed following 24 hours of bleach treatment. The weak acid produced by sodium hypochlorite when in solution, disrupted the waxy lining surrounding achenes, allowing small air bubbles to escape from exocarp cells and gather on the surface of achenes. The ends of achenes (micropyle and former stigma) had swollen and bleached white, indicating that the effects of the acid were particularly influential in these regions (Fig. 7.3.D). After 48 hours bleach treatment, the outer wax layer of achenes became gelatinous and was no longer impermeable to water (Fig. 7.3.E-F). Prolonged immersion in acid caused large patches of the pericarp layer to turn white and semi-translucent, or in extreme cases, resulted in severe pitting of the achene surface (Fig. 7.3.G-H).

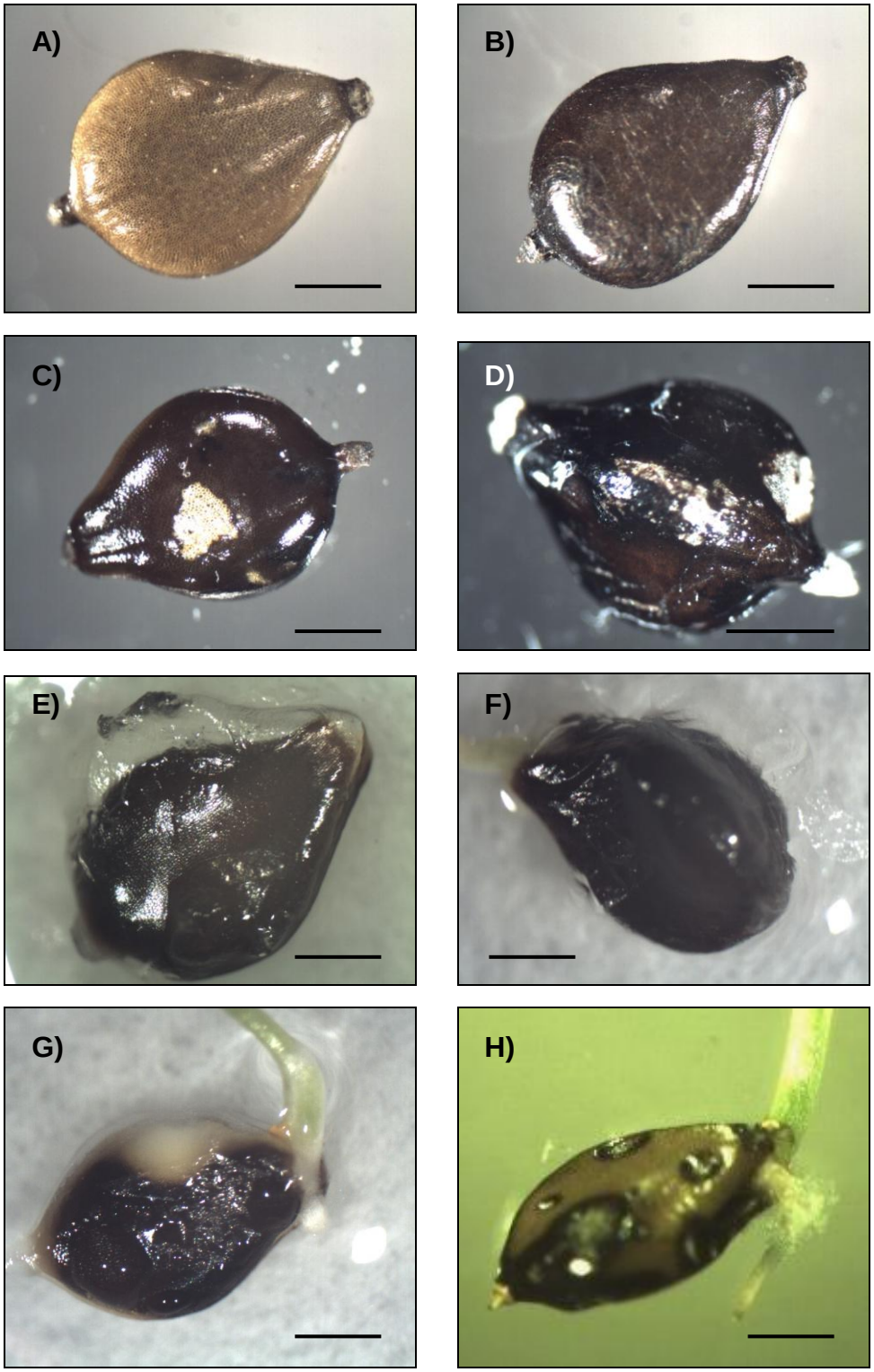


Figure 7.3. A) 1-year old *B. caldwellii* achene stored in dry-dark conditions (light brown coloured), B) Effects of 4-months cold-wet stratification on *B. caldwellii* achene (dark brown with cracked surface layer), C) Damage of the achene pericarp layer of *B. caldwellii* through long-term (~ 1 year) stratification, D) Bleaching of achene ends and pericarp damage in *B. medianus*, E) & F) Gelatinous exocarp layer forming from short-term bleach treatments in *B. caldwellii* and *B. medianus* respectively, G) & H) Germinating *B. caldwellii* achenes showing extensive pericarp damage from long-term bleach scarification. All scale bars = 1 mm.

Contrary to predictions, prolonged immersion in bleach for 2 weeks did not have a negative effect on germination for either test species (Fig. 7.4). Germination of *B. caldwellii* was consistently high across all germination treatments in comparison to *B. medianus*, though the large difference in germination response found between species in control treatments could be overcome with a 4-5 day bleach pre-treatment of achenes. Furthermore, mean germination of *B. medianus* achenes after 4 days bleach ($\bar{x}$ = 19.75, SD = 2.87, n = 4) was exactly the same as mean germination for *B. caldwellii* ($\bar{x}$ = 19.75, SD = 2.50, n = 4) after 32 weeks stratification (at 30-5°C). Two-way ANOVA revealed a strong effect of both species ($F_{1, 54} = 342.08$, $p < 0.001$) and bleach ($F_{8, 54} = 50.89$, $p < 0.001$) on germination as well as a significant interaction between both factors ($F_{8, 54} = 29.15$, $p < 0.001$) that accounted for ~81% of the observed variation.

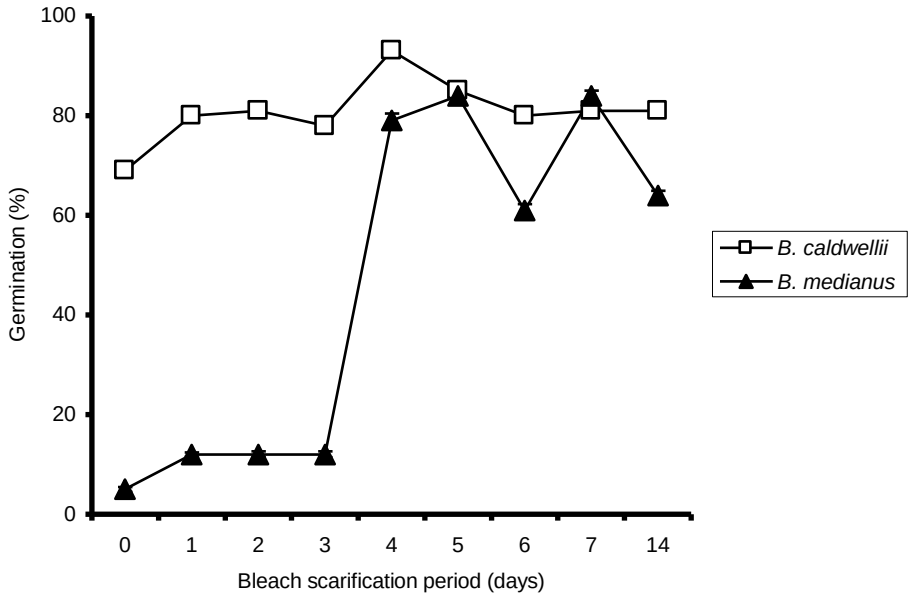


Figure 7.4. Mean percentage germination of *B. caldwellii* and *B. medianus* achenes under control (0 days) and 8 different bleach scarification periods (days). Standard error bars are shown (n = 4).

The highest germination percentages and most rapid germination rates were recorded for either species following 4-5 days bleach scarification. Mean germination time dropped by almost 50% for both species from an average of around 3 weeks in control treatments, to less than 2 weeks after 4 or more days bleach pre-treatment (Fig. 7.5). Two-way ANOVA revealed a significant influence on germination rate

from species ($F_{1, 54}$ = 392.12, p < 0.001) and bleach ($F_{8, 54}$ = 59.73, p < 0.001), as well as a significant interaction between the two terms ($F_{8, 54}$ = 24.67, p < 0.001) that accounted for ~79% of the variation in germination time.

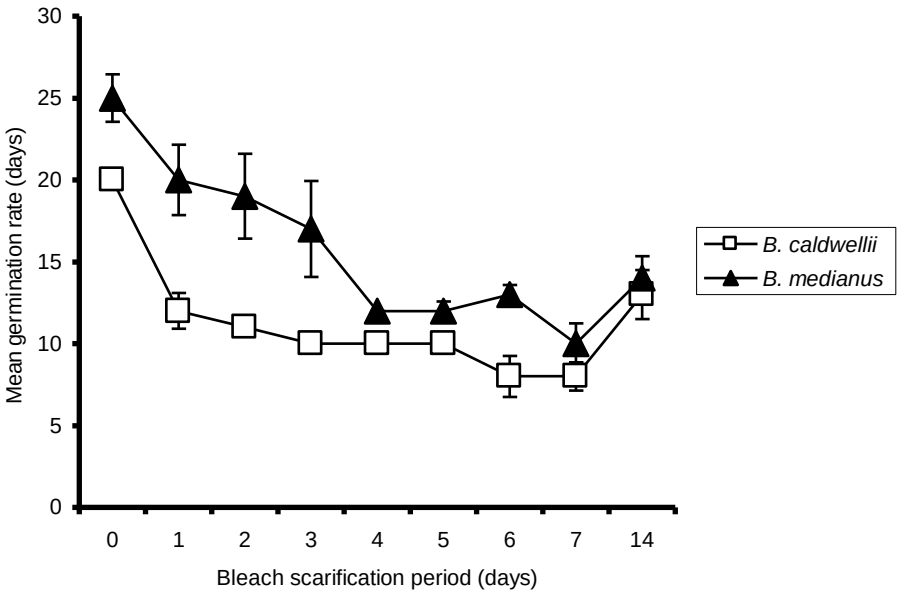


Figure 7.5. Mean germination rate of *B. caldwellii* and *B. medianus* achenes under control and 8 different bleach scarification periods (days). Standard error bars are shown (n = 4).

7.3.3 The effects of manual scarification

Contrasting germination results were recorded for *B. caldwellii* and *B. medianus* between control treatments and those with micropyle scarification (Fig. 7.6). No significant benefit in germination success was gained for *B. caldwellii* through manual scarification of the micropyle region, as overall germination was very similar for *B. caldwellii* between controls (72%) and scarified achenes (69%). In contrast, a strong positive effect of scarification was found for *B. medianus*, as germination totals rose from 7% in controls to 57% when scarified. Two-way ANOVA revealed a significant effect for both individual factors of species type ($F_{1, 12}$ = 66.69, p < 0.001) and scarification ($F_{1, 12}$ = 165.03, p < 0.001) on germination success, as well as a significant interaction between the terms ($F_{1, 12}$ = 84.68, p < 0.001) that accounted for ~88% of variation.

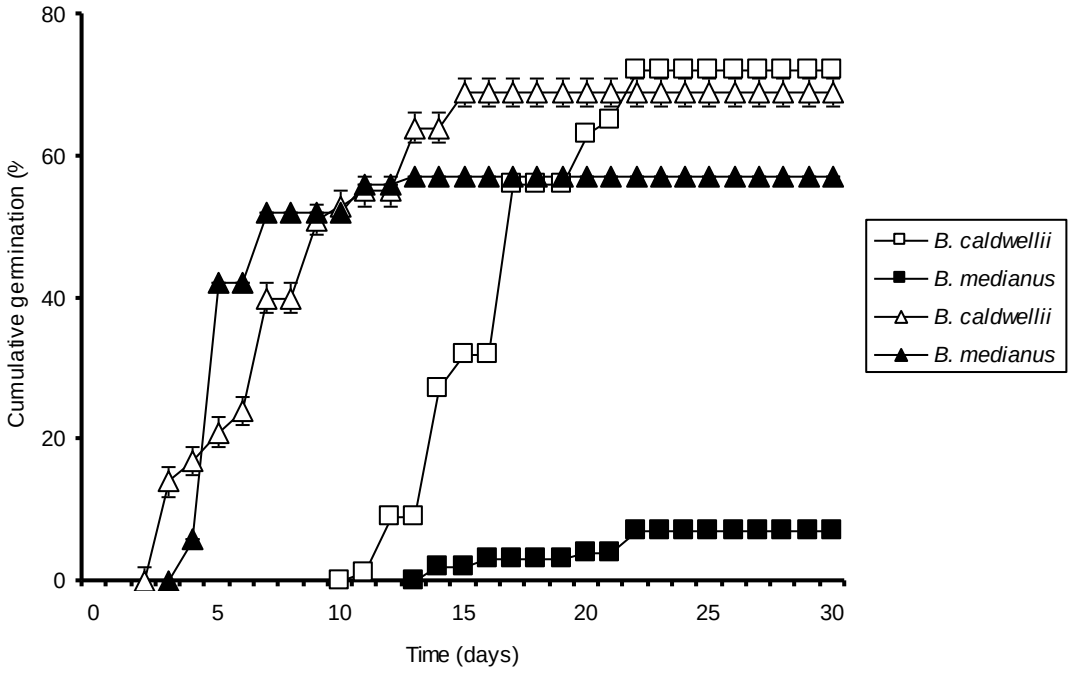


Figure 7.6. Mean germination rate and cumulative germination of *Bolboschoenus caldwellii* and *Bolboschoenus medianus* achenes in control treatments (square symbols) and following scarification of the micropyle region (triangular symbols). Standard error bars are shown (n = 4).

Removal of the micropyle significantly accelerated the rate of germination for both species, with T_{50} for *B. caldwellii* cut by less than half (7 days versus 17 days for controls) while for *B. medianus* T_{50} decreased to one quarter of the average control time (5 versus 20 days) (Fig. 7.7). Two-way ANOVA showed significance for both individual factors of species ($F_{1, 12} = 63.60$, $p < 0.001$) and scarification ($F_{1, 12} = 150.09$, $p < 0.001$), as well as a significant interaction ($F_{1, 12} = 26.82$, $p < 0.001$) between the terms that accounted for ~69% of the variation in germination rates. Of all the various achene tests conducted in this thesis, the manual scarification treatment was the only test where mean germination occurred at a faster rate in *B. medianus* in comparison to *B. caldwellii*. It should be noted that although greater than 50% of scarified achenes for either species germinated quickly, very few survived due to incomplete radicle emergence or rejection of the embryo or endosperm. When

scarifying achenes it was important to remove only a very small portion of the micropyle end (<0.5 mm).

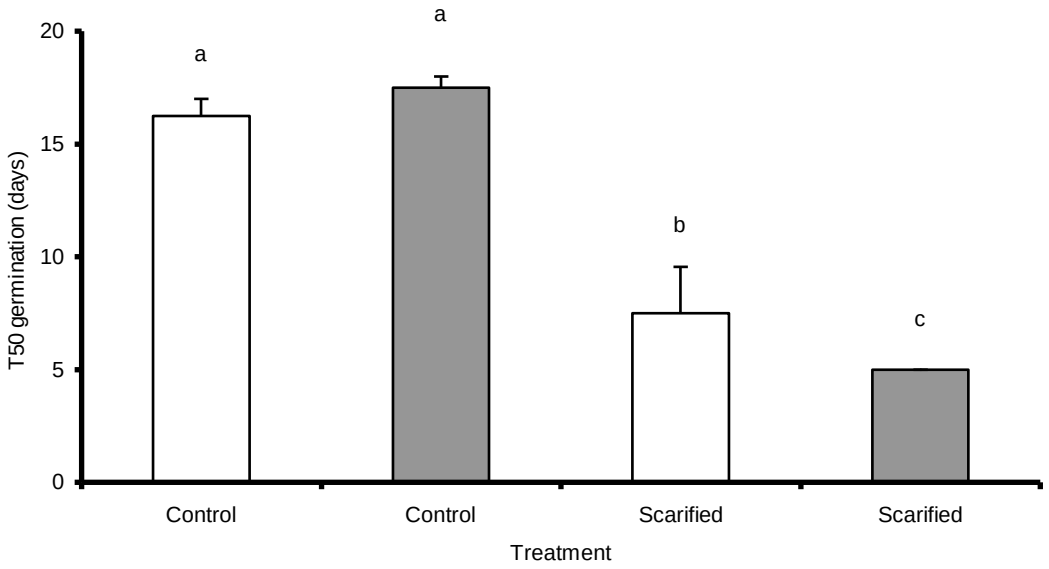


Figure 7.7. Mean germination rate for control and scarified *B. caldwellii* achenes (white bars) and *B. medianus* achenes (grey bars). Means and standard error bars are shown though error bars are not included for scarified *B. medianus* achenes, as data was constant across replicates. Significant differences ($p < 0.05$) are indicated by different letters, following 2-way ANOVA and Tukey *post hoc* tests.

7.4 Discussion

7.4.1 The benefits of stratification and scarification

Germination differences recorded between the two test species in control treatments could be resolved through long-term cold-wet stratification and scarification. Stratification and scarification accelerated the rate of germination and widened the temperature parameters at which germination could occur in both *B. caldwellii* and *B. medianus*. Analysis of mean monthly temperature ranges for the region of Sale for the past 60 years (Bureau of Meteorology, Victoria), illustrated that the average day-night temperature differences were very consistent throughout the calendar year at approximately 11.5°C. This temperature range is well below the 20-25°C diurnal temperature difference required for germination in either test species, unless achenes have been stratified or scarified (Fig. 7.8).

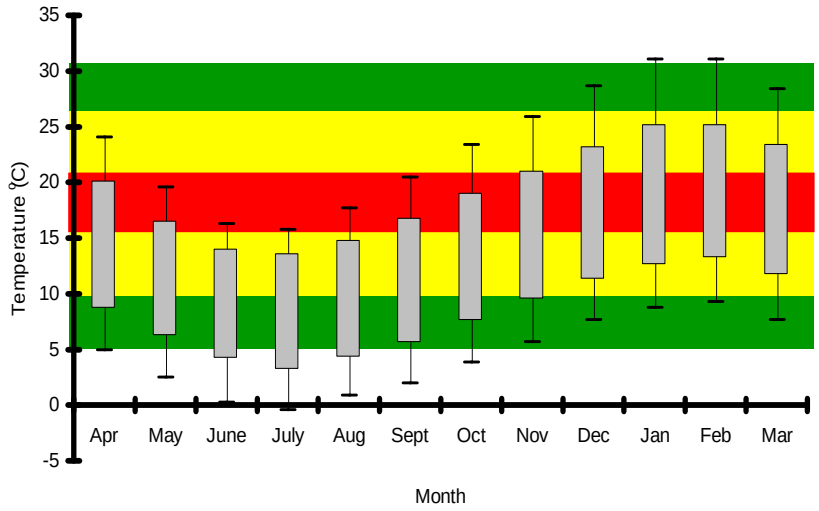


Figure 7.8. Chart of mean monthly temperature data from Sale Weather Station (085072) for the past 60 years (Bureau of Meteorology, Victoria), with superimposed colour bars representing effective and non-effective germination temperature ranges for *B. caldwellii* and *B. medianus* achenes. No germination occurred within the Red zone (20-15°C diurnal range), irrespective of achene pre-treatment. Negligible germination occurred within the Yellow zone (25-10°C diurnal range), unless achenes were stratified or scarified. Germination was successful within the Green zone (30-5°C ranges) irrespective of achene treatment. The chart shows that unless seeds are pre-treated (scarified or stratified) they require above average temperature regimes to germinate from October through March.

Achene dormancy was stronger in *B. medianus* than *B. caldwellii*, as total germination for *B. medianus* rarely exceeded 50% while *B. caldwellii* displayed great flexibility by readily arresting achene dormancy and germinating a large proportion of achenes. The results indicated that a substantial stratification (imbibition) period, as opposed to a particularly wide diurnal temperature regime, was of greater importance to germination success in *B. medianus* than *B. caldwellii*, although the higher total germination percentages of stratified achenes for either species at the 30-5°C temperature range, illustrated that achenes are directed to germinate in spring by high temperature fluctuations that buffer against germination being too early in the year (Thompson and Grime 1983; Probert 1992).

The benefits observed from long-term stratification might also be in part a reflection of seed aging. Viability and germination results from Chapters 4 & 6

revealed a slight improvement in *B. medianus* germination after 1 year of aging in dry or wet conditions, though the increases noted in earlier trials (10-20%) did not parallel those seen within stratification trials (up to 70%). Further research may include an examination of the effects of enforced long-term stratification (>2 years), as there will inevitably be a time threshold after which stratification becomes detrimental to achene viability.

The similarity of overall germination percentages between long-term stratified and bleach-treated achenes, illustrated that pre-treating seeds in weak acid effectively acts as a surrogate stratification period. The effects of achene bleach pre-treatment, such as the swelling of micropyle regions and disruption of the waxy surface layer and pericarp anatomy had major implications for the germination success of *B. caldwellii* and *B. medianus*. Damage to the integrity of achenes significantly increased their capacity for water imbibition, potentially flushing chemical growth inhibitors and thereby raising embryonic metabolism. Rapid germination responses observed for both species following manual scarification, clearly illustrated the influence of pericarp anatomy to dormancy in these species. Though the manual scarification method was time consuming and seedling survival was poor, the results confirmed that the main inhibitive factor for germination was anatomical. The findings of this chapter agree with conclusions by Clevering (1995) that germination within *Scirpus / Bolboschoenus* species appeared to be regulated by the permeability of the seed coat rather than an internal dormancy mechanism.

While germination parameters were found to be much wider for *B. caldwellii* in comparison to *B. medianus,* both species benefited from a stratification period of at least 16 weeks (4 months) or a scarification treatment of 4-5 days in 10% w/v bleach, prior to germination under light and wide diurnal temperatures regimes. Stratification and scarification results from this study were consistent with those found for a range of closely related Cyperaceae species from the northern hemisphere: *Scirpus acutus* and *S. validus* (Harris and Marshall 1960), *S. robustus* (Dietert and Shontz 1978; Prevost and Gresham 1981), *S. lacustris* and *S. maritimus* (Clevering 1995), *Bolboschoenus koshewnikowii, B. laticarpus, B. maritimus* and *B. yagara* (Moravcová *et al*. 2002).

Chapter 8.

The importance of hypocotyl hairs to sexual recruitment for *Bolboschoenus* and other Cyperaceae species

8.1 Introduction

Having examined the specific germination requirements of *B. caldwellii* and *B. medianus* achenes, as well as the ability of achene pre-treatments such as stratification to enhance germination, the next step examined whether any specialised adaptations have evolved within each species to assist the germination process. The following chapter looks at the sixth component of the recruitment model proposed in Chapter 1: hypocotyl hairs.

In highly variable environments such as wetlands, sexual recruitment opportunities in time and space are generally rare and narrow. Accordingly, rapid germination responses are crucial for seeds if they are to establish and survive (Nishihiro *et al*. 2004). Many adaptations have evolved under harsh climates to aid the germination process, including the production of fine unicellular projections from the lower hypocotyl region known as hypocotyl hairs (Kaul 1978; Morita *et al.* 1995; Aronne and De Micco 2004). Hypocotyl hairs generally form in a complete ring or collar exclusively restricted to a narrow band around the base of the hypocotyl region of emerging seedlings (Baranov 1957; Kaul 1978). They are short-lived structures that disappear soon after radicle establishment and are not associated with the true root system. This anatomical finding is supported by recent genetic research on *Arabidopsis thaliana*, which showed that epidermal cell patterning during embryogenesis and hypocotyl development is not determined by the same kinase complex that is involved in post-embryonic root or radicle epidermal development

(Kwak and Schiefelbein 2007). Though hypocotyl hairs form independently of, and prior to, the true root system, initial radicle and root hair extensions do not appear to be independent of hypocotyl hair formation and function. For example, Matsuo and Shibayama (2002) noted that *Monochoria vaginalis* seedlings failed to establish healthy radicle and root hairs or aborted when the formation of hypocotyl hairs was incomplete or interrupted. Though hypocotyl hairs are exceptionally delicate, being comprised of only a single elongated cell, they are able to perform similar functions to roots, such as the passive uptake of water making them a critical factor in preventing desiccation during early germination stages (Aronne and De Micco 2004). Hypocotyl hairs also function to anchor seeds to the soil (Debaene-Gill *et al.* 1994; Matsuo and Shibayama 2000). Sticky mucilage is produced at the tips of hypocotyl hairs that adheres seeds to the substrate, preventing emerging seedlings from being damaged by wind or wave action (Kuo and Kirkman 1992). Moreover the arrangement of hypocotyl hairs anchored to the ground in a ring, effectively act as a brace for the emerging radicle to work (grow) against, assisting geotropism and substrate penetration (Young and Martens 1991; Matsuo and Shibayama 2002).

Previous studies have indicated that hypocotyl hair function is largely reflective of habitat type (Baranov 1957; Aronne and De Micco 2004). For terrestrial species such as *Populus alba* (Salicaceae), *Artemisia tridentata* (Asteraceae) and *Myrtus communis* (Myrtaceae) the primary function of hypocotyl hairs appears to be anchorage, which may in turn assist geotropism as seeds are braced to the ground (Polya 1961; Young and Martens 1991; Aronne and De Micco 2004). The adaptive advantage of anchorage through hypocotyl hairs also has obvious benefits for aquatic and semi-aquatic species, whose seedlings may be easily damaged by wave action or currents. Seed anchorage via hypocotyl hairs has been reported for several submergent plant groups including: Hydrocharitaceae (Kaul 1978; Kuo and Kirkman 1992), Zosteraceae (Coolidge Churchill 1983) and Podostemaceae (Rutishauser and Grubert 1999). The seeds of emergent and semi-aquatic species are adapted to germinate in early spring when water levels have receded and substrates become exposed (Clevering 1995; Moravcová *et al.* 2002; Leck and Schütz 2005). Under such circumstances, the physiological capacity of hypocotyl hairs to absorb water and protect undeveloped embryos from desiccation is likely to be very important to recruitment success (Kaul 1978; Matsuo and Shibayama 2002).

Klebs (1885, cited in Kaul 1978) reported that hypocotyl hairs are typical of aquatic monocotyledonous species, yet few studies have acknowledged the presence or importance of hypocotyl hairs to aquatic and semi-aquatic plants during recruitment events. To the author's knowledge, no genera belonging to the Cyperaceae family have been identified as possessing hypocotyl hairs, despite the high prevalence of semi-aquatic species in this large group (Leck and Schütz 2005). I observed hypocotyl hairs in preliminary germination trials for two south-eastern Australian species of *Bolboschoenus* (Asch.) Palla. Furthermore, seedling survival appeared to be related to hypocotyl hair formation, leading to speculation that hypocotyl hairs may be critical to the success of sexual recruitment processes for *Bolboschoenus* and possibly many other species within the Cyperaceae family.

Clevering *et al.* (1996) concluded that seedling establishment of *Scirpus lacustris* and *S. maritimus* (now *Bolboschoenus maritimus*) was most successful on exposed sediments, with water levels acting as a major selective pressure across wetland gradients. Fine-scale hydrological variations (and their associated salinity loads) are highly relevant to the germination patterns of emergent plant species that co-inhabit wetland gradients, and it is the subtle differences in germination tolerances to flooding and salinity between species, that largely determines spatial patterns and plant distributions (Rea and Ganf 1994; Brock *et al.* 2005). Whilst the negative effects of flooding and salinity to seed germination have been well documented (e.g. Ungar 1982; Lenssen *et al.* 1998; Peterson and Baldwin 2004; Brock *et al.* 2005) it remains unclear as to what external factors are significant in controlling the formation of hypocotyl hairs.

The inconspicuous and cryptic nature of hypocotyl hairs means that they are often overlooked or misjudged as true root hairs (Robinson *et al*. 2008). The aims of this chapter were four-fold: 1) document the presence or absence of hypocotyl hairs in a range of species from different Cyperaceae genera to determine whether hypocotyl hairs commonly occur among this cosmopolitan family; 2) describe the formation and eventual disintegration of hypocotyl hairs during germination and seedling development stages of *Bolboschoenus caldwellii* and *Bolboschoenus medianus*; 3) describe the effects of a range of salinity concentrations and substrata water

availabilities on hypocotyl hair formation in *B. caldwellii* and *B. medianus*; and 4) discuss the importance of hypocotyl hairs to sexual recruitment events in *Bolboschoenus* and other wetland Cyperaceae species.

8.2 Methods

8.2.1 Seed collection

While the term 'achene' is used accurately in this investigation for *Bolboschoenus* species, the term 'seed' is used loosely to collectively describe the sexual propagules of all other Cyperaceae species tested. Cyperaceae seeds used solely in hypocotyl hair presence/absence trials [*Carex appressa, Carex fascicularis, Cyperus eragrostis, Eleocharis sphacelata, Ficinia nodosa* (formerly *Isolepis nodosa*) and *Schoenoplectus tabernaemontani* (formerly *Schoenoplectus validus*)] were collected from a wetland in western Melbourne (37°44'S, 144°47'E) in February 2006. Achenes of *Bolboschoenus caldwellii* and *B. medianus* used in both presence/absence and main experimental treatments were collected in January and February 2006 from several populations at Clydebank Morass (38°02'S, 147°14'E) and Dowd Morass (38°07'S, 147°10'E) in West Gippsland, south-eastern Australia. *Bolboschoenus* achenes were collected from wetlands within the Gippsland Lakes region, as the data generated was directly relevant to all other sections of the greater PhD project, while seeds from the remaining Cyperaceae species were harvested locally for ease of collection, as they were only used to illustrate the common occurrence of hypocotyl hairs among the Cyperaceae family.

8.2.2 Pre-germination treatment and incubation

Cleaned seeds/achenes were stored in paper bags in the dark for 2 weeks (after-ripening) prior to a cold-wet (3-4°C) stratification (imbibition) period of ~40 weeks. The stratification pre-treatment was used to suppress the influence of dormancy and increase both the rate and the probability of germination success, as well as hypocotyl hair formation. A strong tendency for hydrochory (seed dispersal via buoyancy in water) within *B. caldwellii* meant that some achenes were still

floating after 40 weeks stratification. Only achenes that had sunk were used in trials to ensure that they were always in contact with the substratum, otherwise germination and subsequent hypocotyl hair formation could be compromised, as *Bolboschoenus* achenes do not commence germination while floating in the water column. All other seed preparation and germination (incubation) conditions followed those outlined in introductory methods (Chapter 2). Experiments ran from January to April 2007 and a total of 3,600 achenes were tested.

8.2.3 Presence of hypocotyl hairs in various Cyperaceae species

Two replicates of 50 seeds/achenes each of *B. caldwellii, B. medianus, C. appressa, C. fascicularis, C. eragrostis, E. sphacelata, F. nodosa* and *S. tabernaemontani* were plated into individual petri dishes as outlined in introductory methods (Chapter 2). Observations of hypocotyl hairs in these trials were recorded, though results were not statistically analysed as the tests were conducted only to demonstrate the widespread occurrence of hypocotyl hairs across Cyperaceae genera.

8.2.4 Effects of variations in water availability

Two approaches were used to test the effects of different water availability on hypocotyl hair formation for *B. caldwellii* and *B. medianus*. The first treatment consisted of 4 replicates of 25 achenes per species, plated in a 5 x 5 grid onto 1 sheet of Whatmans # 1 filter paper in 90 mm petri dishes. Four different water volumes were employed, mimicking a gradient from saturated (2 mL and 4 mL treatments) to partially inundated (8 mL) and flooded (16 mL) conditions. The second treatment consisted of 4 replicates of 10 achenes per species plated into 90 mm petri dishes containing 5 different concentrations of agar (0.2%, 0.5%, 1%, 5% and 10% w/v in deionised water). Agar plates were prepared in a laminar flow cabinet using aseptic technique and poured on a slight angle so that approximately one quarter of the bottom of the Petri dish remained uncovered. Achenes were then positioned along the leading edge of the agar against a clear background so that the development of hypocotyl hairs could be easily followed.

Prior to their disintegration, the length of the longest hypocotyl hairs from 5 achenes per treatment were measured from final photographs using Moticam Digital Imaging Software Version 1.3 (Moticam 2000™, China) for the agar treatments only. The lengths of hypocotyl hairs were not measured in treatments using filter paper, as the hairs were difficult to distinguish from the fibres of the paper or had often grown into or through the filter paper, making measurements error prone.

8.2.5 Effects of salinity variations

One control (deionised water) and 4 different salinity concentrations (1, 2, 4 and 8 g L^{-1}) were used to test the effects of salt on hypocotyl hair development. Four replicates of 25 achenes per species were plated in a 5 x 5 grid into separate petri dishes containing 1 sheet of Whatman's #1 filter paper. Saline solutions were made from pure Red Sea Salt and deionised water (Coburg Aquarium Supplies). Three millilitres of saline solution were applied to each replicate – just enough to saturate filter paper without covering achenes in a film of water – as recommended by the Association of Official Seed Analysts (1990). An upper limit of 8 g L^{-1} was used in hypocotyl hair trials as previous tests had shown negligible germination for *B. caldwellii* and no germination for *B. medianus* at this concentration. Comparable salinity regimes have also been used in prior studies involving aquatic and semi-aquatic plant species from Dowd Morass (Robinson *et al.* 2006; Salter *et al.* 2006). Cumulative percentage germination was recorded in substrate water and salinity trials within four categories: 1) Hypocotyl hairs present, 2) Hypocotyl hairs absent, 3) No germination, and 4) Loss to mould.

8.2.6 Statistical analysis

All data sets were checked for normality (Kolmogorov-Smirnov and Shapiro-Wilk tests) and homogeneity of variances (Levene's test). Percentage data were converted to proportions and arc-sine(sqrt) transformed prior to 2-way ANOVA and Tukey *post-hoc* tests. Hypocotyl hair length data were square root (+ 0.5) transformed prior to 2-way ANOVA (Zar, 1996). All statistical procedures were calculated using SPSS statistical software (version 14).

8.3 Results

8.3.1 Presence of hypocotyl hairs in Cyperaceae

Hypocotyl hairs were present in each of the Cyperaceae species tested (Fig. 8.1), though differences were apparent between the shape, length and arrangement of

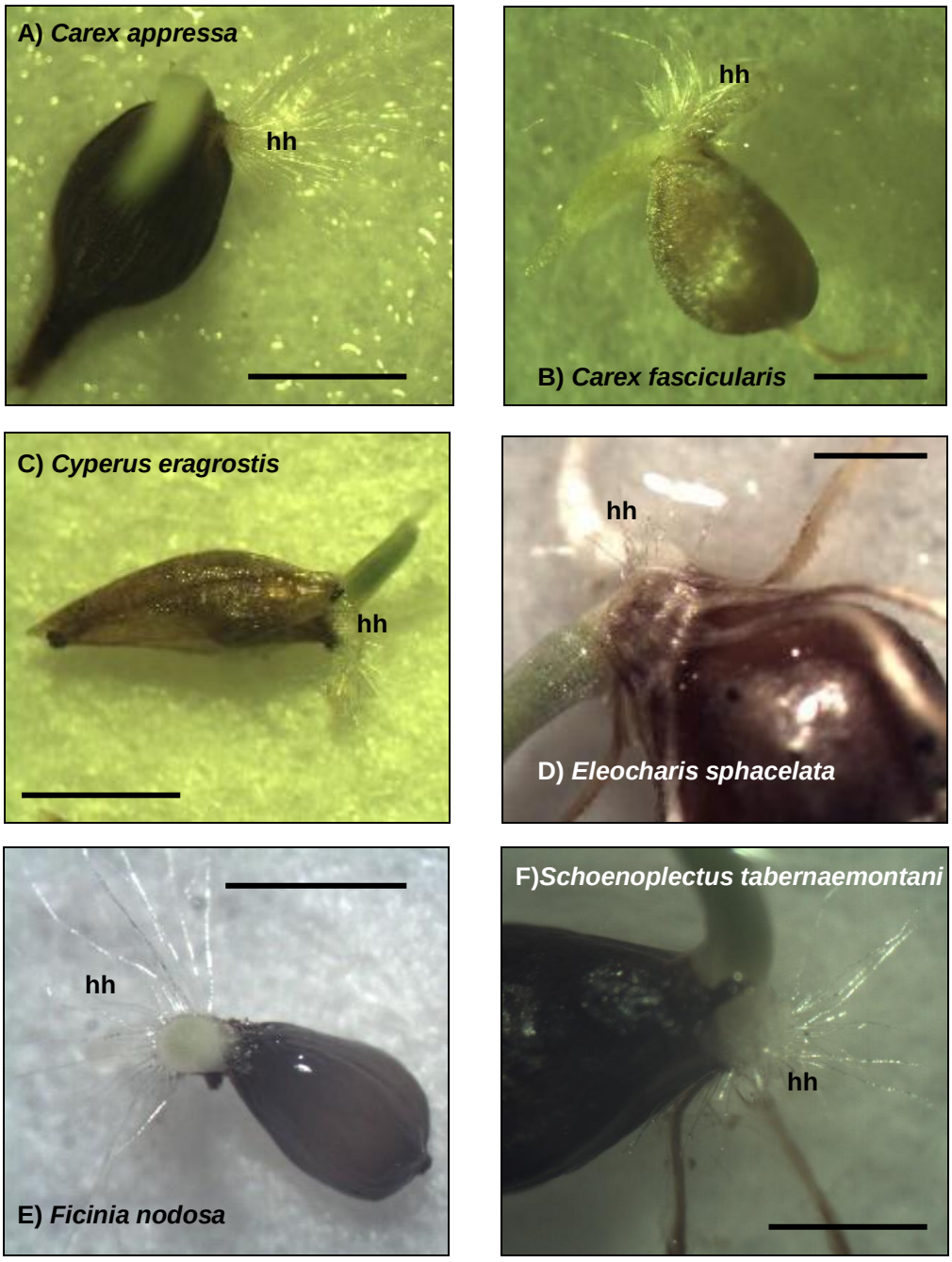


Figure 8.1. Hypocotyl hairs (hh) in various Cyperaceae species: A) *Carex appressa*, B) *Carex fascicularis*, C) *Cyperus eragrostis*, D) *Eleocharis sphacelata*, E) *Ficinia nodosa*, F) *Schoenoplectus tabernaemontani*. Scale bars = 1 mm.

hypocotyl hairs produced by each species (Table 8.1). Hypocotyl hairs did not form in a classical ring shape in species that emerged plumule first. *Ficinia nodosa* was the only species that emerged hypocotyl and radicle region first, producing the longest and most clearly arranged ring of hypocotyl hairs. Hypocotyl hairs grew as long or longer than the body of most seeds, with the exception of *C. eragrostis* and *E. sphacelata*, which instead produced hypocotyl hairs less than one quarter the length of their seed bodies. In all cases, seeds that failed to produce hypocotyl hairs also failed to produce radicles and root hairs.

Table 8.1. Hypocotyl hair presence/absence and notes for various Cyperaceae species.

Species	Emergence	Hypocotyl hairs	Length*	Appearance*	Arrangement*
B. caldwellii	Plumule first	Present	Long	Dense	Half circle
B. medianus	Plumule first	Present	Long	Dense	Half circle
C. appressa	Plumule first	Present	Long	Dense	Half circle
C. fascicularis	Plumule first	Present	Long	Dense	Half circle
C. eragrostis	Plumule first	Present	Short	Sparse	Full ring
E. sphacelata	Plumule first	Present	Short	Sparse	Half circle
F. nodosa	Radicle first	Present	Long	Sparse	Full ring
S. tabernaemontani	Plumule first	Present	Long	Sparse	Half circle

* The notation ‘Long’ refers to hypocotyl hairs being as long or longer than the respective seed body, while ‘Short’ refers to hypocotyl hairs being shorter than the seed body. The term ‘Dense’ describes hypocotyl hairs growing closely together, while ‘Sparse’ refers to hypocotyl hairs arranged with distinct or conspicuous gaps between them. ‘Half-circle refers to hypocotyl hairs only observed growing half way around the base of the hypocotyl, while ‘Full ring’ refers to hypocotyl hairs formed in a complete ring around the hypocotyl region.

8.3.2 Germination and hypocotyl hair development in Bolboschoenus

Five stages of germination were noted for both *B. caldwellii* and *B. medianus*. The first stage was the emergence of the plumule or cotyledon through the micropylar end of the achene and the consequent partial splitting of pericarp tissue along suture-lines (Fig. 8.2.A). Plumules extended more than 8 mm from many achenes prior to radicle emergence. The second stage was characterised by the lengthening of the plumule and the emergence of the radicle apex and hypocotyl region containing hypocotyl hair primordia cells (Fig. 8.2.B). Hypocotyl hairs in *B. caldwellii* and *B. medianus* usually began to form on the third or fourth day of germination and made contact with the substrata within 24-48 hours of their appearance. Rapid elongation of hypocotyl hairs in contrast to limited radicle growth, characterised the third stage (Fig. 8.2.C-E). The fourth stage involved full maturation of hypocotyl hairs along with rapid extension of the radicle and development of true root hairs (Fig. 8.2.F-G). Complete disintegration of hypocotyl hairs with ongoing formation and provision of function from the radicle and its true root hairs characterised the final stage of hypocotyl hair formation (Fig. 8.2.H).

8.3.3 Effects of water availability on hypocotyl hair formation

8.3.3.1 a) Filter paper media

Germination responses were almost identical between *B. caldwellii* and *B. medianus* over the range of substrata water availabilities used in filter media treatments (Fig. 8.3). Water availability did not compromise germinability for either species, and approximately 50% or more achenes germinated at all treatment levels. Substrata water availability did, however, strongly influence the formation and growth of hypocotyl hairs. Two-way ANOVA confirmed that substrate water volumes largely determined hypocotyl hair formation ($F_{3, 24} = 331.18$, $p < 0.001$) accounting for ~98% of variation. Hypocotyl hair formation was significantly greater on saturated substrata (2 and 4 mL treatments) compared with semi-flooded and flooded treatments (8 and 16 mL) (Fig. 8.4). A water depth of 1.2 to 3 mm (8 and 16 mL treatments respectively) was sufficient to completely arrest hypocotyl hair development for both test species. Greater than 50% of achenes either failed to germinate under flooded conditions, or aborted germination due to a lack of hypocotyl

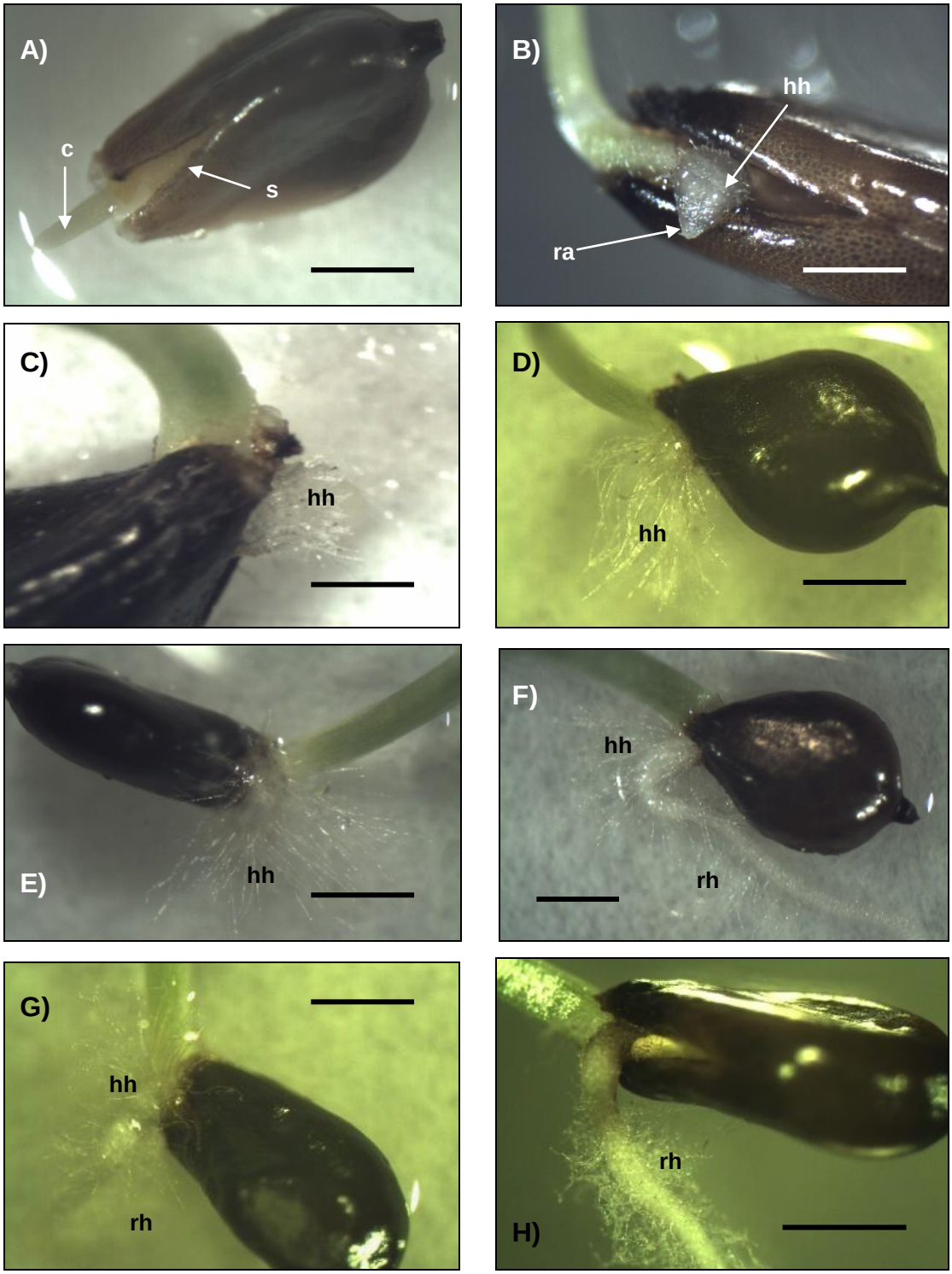
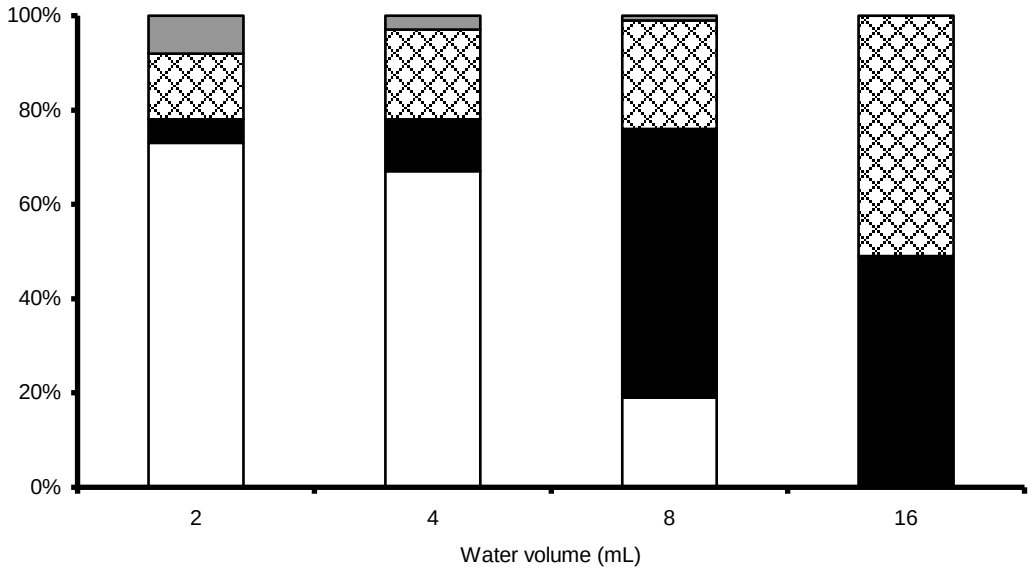


Figure 8.2. Germination development stages for *B. caldwellii* and *B. medianus*. A) Emergence of cotyledon / plumule (c) and splitting of suture-line (s) in *B. medianus*. B) Emergence of radicle apex (ra) and hypocotyl region in *B. caldwellii* showing early formation of hypocotyl hair (hh) cells. C) Emergence of hypocotylar tissue and early growth of hh in *B. medianus*. D) Lengthening of hh in *B. caldwellii*. E) Lengthening of hh in *B. medianus*. F) Radicle lengthening and formation of root hairs (rh) in *B. caldwellii*. G) Radicle lengthening and early formation of root hairs in *B. medianus*. H) Dense root hairs and complete absence of hh in *B. caldwellii*. All scale bars = 1 mm except for B) and C) which = 500 µm.

a) *B. caldwellii*



b) *B. medianus*

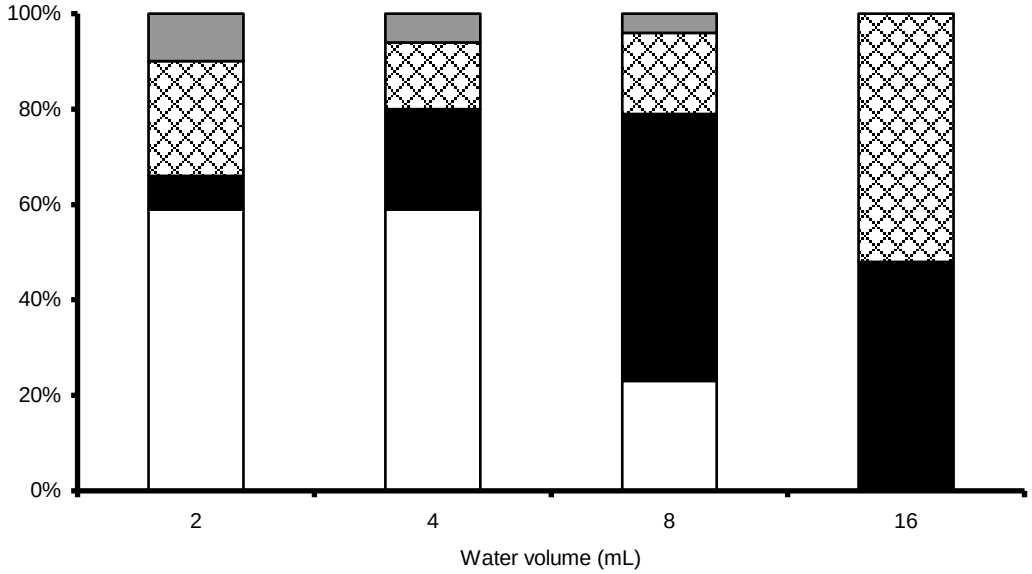


Figure 8.3. Cumulative percentage germination charts of achenes with hypocotyl hairs (white), without hypocotyl hairs (black), unsuccessful germination (cross hatching) and infected with mould (grey) at four different substrate water levels for a) *B. caldwellii* and b) *B. medianus*.

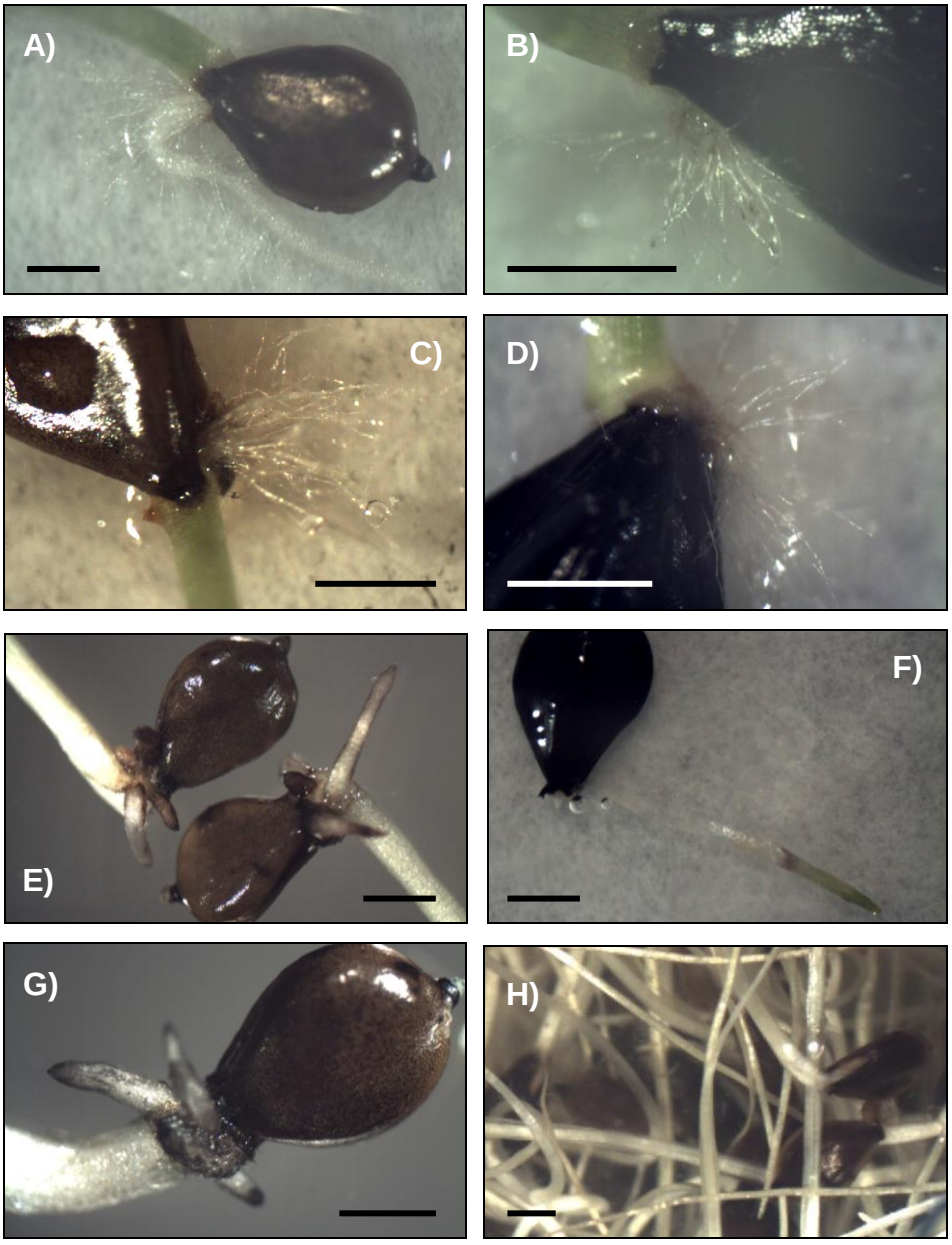


Figure 8.4. Effects of different water levels on hypocotyl hair formation in *B. caldwellii* and *B. medianus*: A) radicle extension with mature hypocotyl hairs (hh) still present in *B. caldwellii* after 5 days in 2 mL replicate. B) hh growth in *B. medianus* after 2 days at 2 mL. C) hh growth in *B. caldwellii* after 2 days at 4 mL. D) hh in *B. medianus* after 2 days at 4 mL. E) Abnormal development of radicle and hypocotyl regions in *B. caldwellii* at 8 mL, no hh or radicle hairs present, early onset of secondary roots apparent. F) Abnormal germination and no hh in *B. medianus* at 8 mL. G) Complete absence of hypocotyl and radicle hairs and early onset of secondary roots in *B. caldwellii* at 16 mL. H) Stratified *B. caldwellii* achenes exposed to light and wide diurnal temperature regimes – submergence prevented germination from moving past the initial stage of plumule emergence. All scale bars = 1 mm.

and root hair formation (Fig. 8.4.E-H). Achene differences between the two species were not significantly influential on whether hypocotyl hairs formed, though a weak significant interaction ($F_{3, 24} = 3.17$, $p < 0.05$) between species and water volume, accounted for ~28% of the variance found for hypocotyl hair formation.

8.3.3.2 b) Agar media

Two-way ANOVA revealed that hypocotyl hair length was significantly influenced by both test factors: species type ($F_{1, 24} = 10.97$, $p < 0.005$) and agar concentration ($F_{2, 24} = 107.47$, $p < 0.001$). A significant interaction also occurred between both factors ($F_{2, 24} = 13.63$, $p < 0.001$) which accounted for ~53% of the variance found in hypocotyl hair lengths. Hypocotyl hairs were not formed by each species at 0.2% agar and only *B. caldwellii* produced hypocotyl hairs at 10% agar (Fig. 8.5).

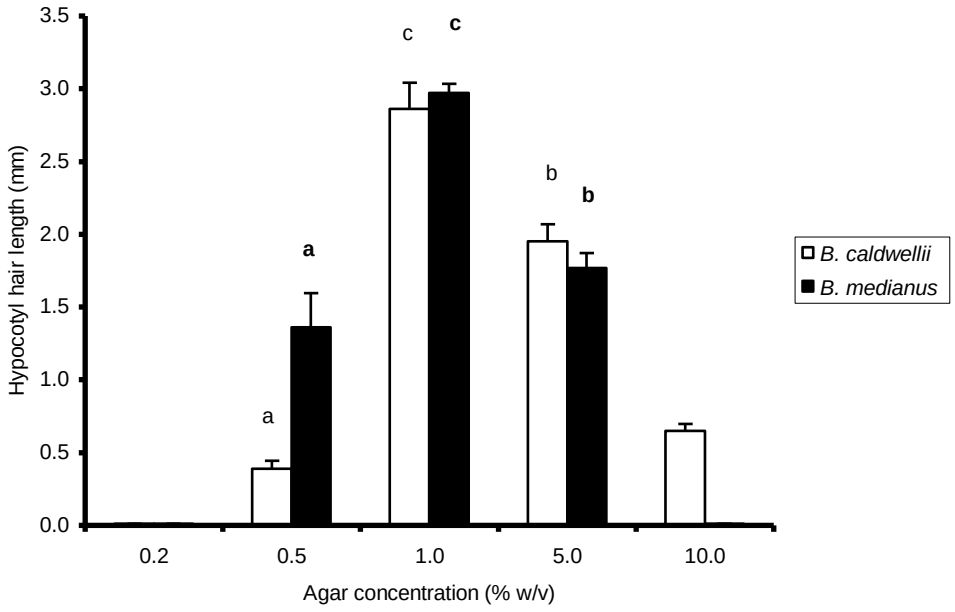


Figure 8.5. Mean maximum length of hypocotyl hairs for *B. caldwellii* and *B. medianus* at five different agar concentrations (n = 5). Significant differences ($p < 0.05$) are indicated for each species by different superscript letters, following 2-way ANOVA and Tukey *post-hoc* testing. Note: no results were recorded for either species at 0.2% w/v agar and at 10% w/v agar for *B. medianus*, therefore these levels were excluded from statistical analysis.

On 1% agar (saturated), hypocotyl hairs were deeply embedded into the media/substratum, whereas at 10% agar (effectively dry) and 0.2% agar (flooded) hypocotyl hairs were either extremely short and did not penetrate the substrate or were completely absent (Fig. 8.6). Agar treatments highlighted that *B. caldwellii* was able to produce longer hypocotyl hairs on slightly drier substrates in contrast to *B. medianus*, which in turn produced longer hypocotyl hairs on slightly wetter substrates (though not flooded).

8.3.4 Effects of salinity on hypocotyl hair formation

Up to 80% germination was recorded for *B. caldwellii* at or below 4 g L^{-1} substrate salinity and greater than 50% of achenes germinated at 8 g L^{-1} (Fig. 8.7). In contrast, germination of *B. medianus* achenes decreased substantially with minor increases in salinity, and seeds failed to germinate under the highest salinity (8 g L^{-1}). Hypocotyl hairs were recorded in greater than 90% of achenes from control treatments for both species. Although hypocotyl hairs were observed at all salinities for *B. caldwellii* and at salinities as high as 4 g L^{-1} for *B. medianus*, very few of the hypocotyl hairs produced at or above 1 g L^{-1} salt grew longer than 2-3 mm for either species. Two-way ANOVA indicated that hypocotyl hair formation was significantly influenced by both species type ($F_{1, 30}$ = 216.10, $p < 0.001$) and salinity ($F_{4, 30}$ = 103.24, $p < 0.001$). A significant interaction was also found between species and salinity ($F_{4, 30}$ = 7.72, $p < 0.001$) that accounted for ~51% of variation in hypocotyl hair formation. The negative effects of saline water on hypocotyl hair development were assumed to be osmotic, as hypocotyl hairs exhibited extremely stunted growth barely past their primordia state (Fig. 8.8). Minor radicle extension and root hair formation occurred at salinities greater than 2 g L^{-1}, while achene germination was generally aborted at or above 4 g L^{-1} salt.

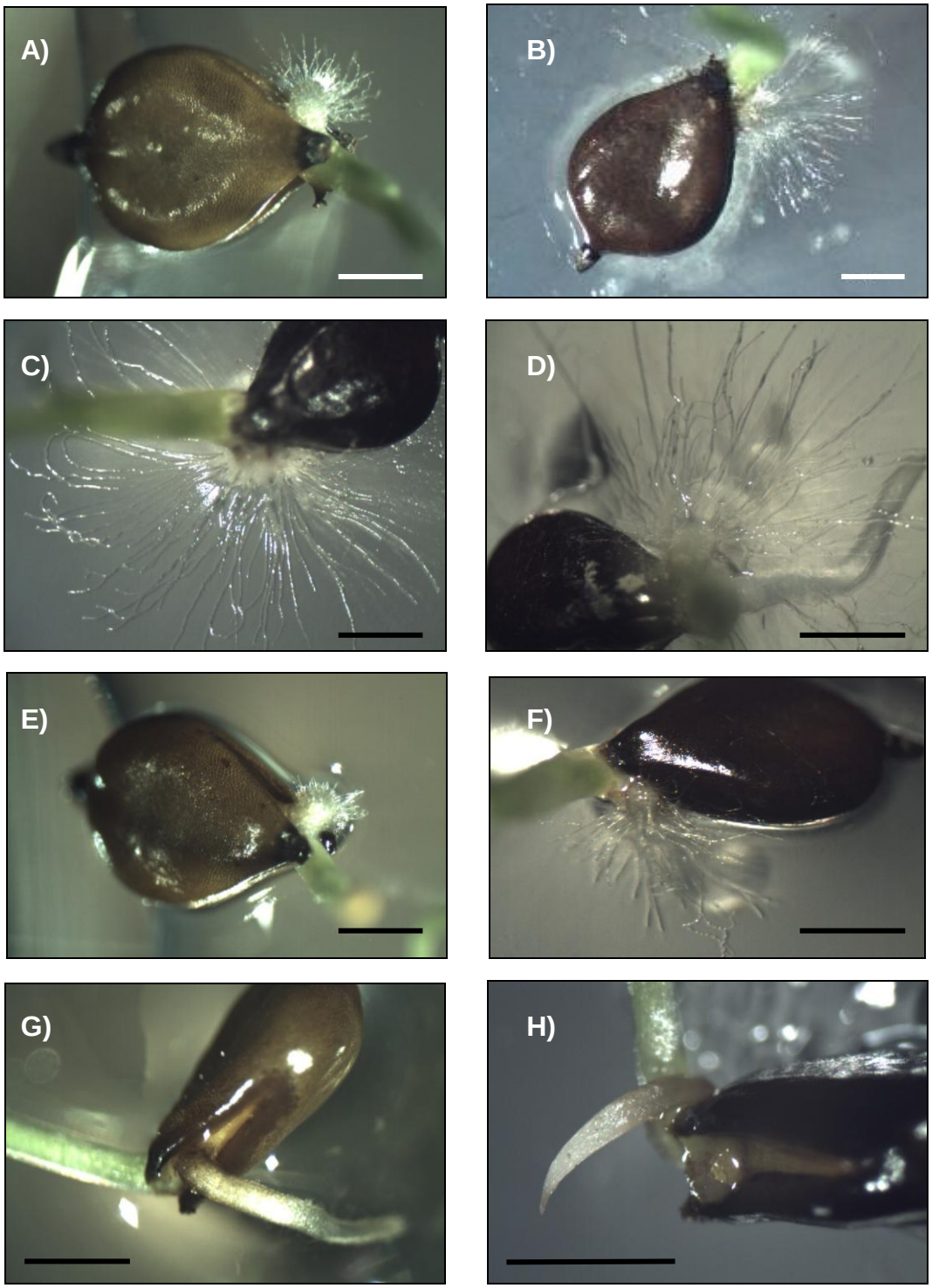
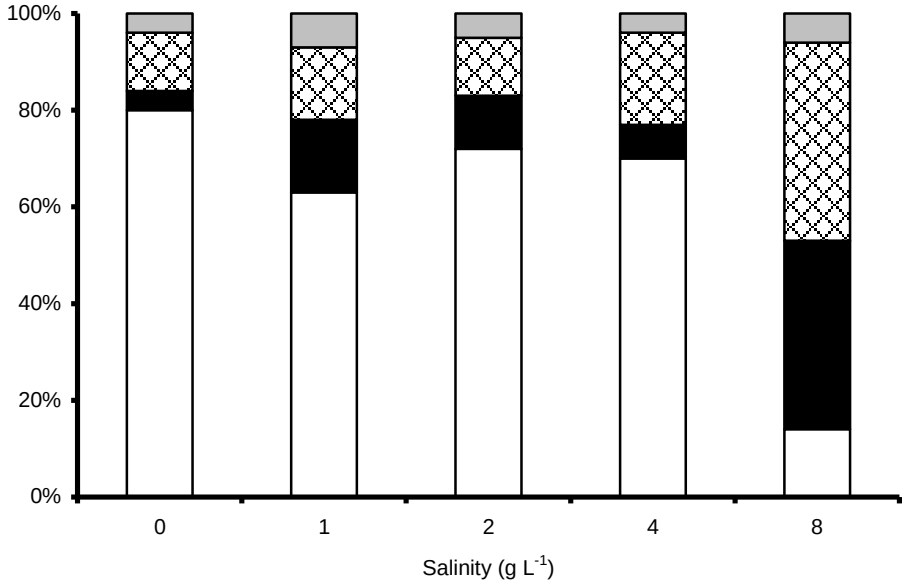


Figure 8.6. Variation of hypocotyl hair development for *B. caldwellii* and *B. medianus* at five different agar concentrations. A) *B. caldwellii* achene germinating on 10% agar with short non-penetrating hypocotyl hairs (hh). B) *B. caldwellii* on 5% agar with medium non-penetrating hh. C) *B. caldwellii* on 1% agar with long penetrating hh. D) *B. medianus* on 1% agar with long penetrating hh. E) *B. caldwellii* on 0.5% agar with short penetrating hh. F) *B. medianus* on 0.5% agar with medium penetrating hh. G) *B. caldwellii* on 0.2% agar showing complete absence of hh, H) *B. medianus* on 0.2% agar showing complete absence of hh. All scale bars = 1 mm.

a) *B. caldwellii*



b) *B. medianus*

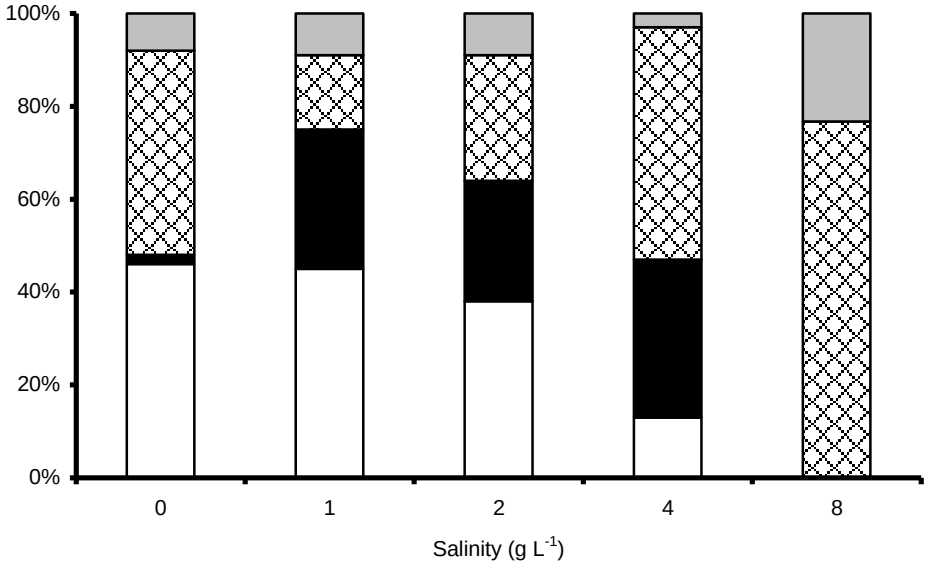


Figure 8.7. Cumulative percentage germination charts of achenes with hypocotyl hairs (white), without hypocotyl hairs (black), unsuccessful germination (cross hatching) and infected with mould (grey) at five different salinities, for a) *B. caldwellii* and b) *B. medianus*.

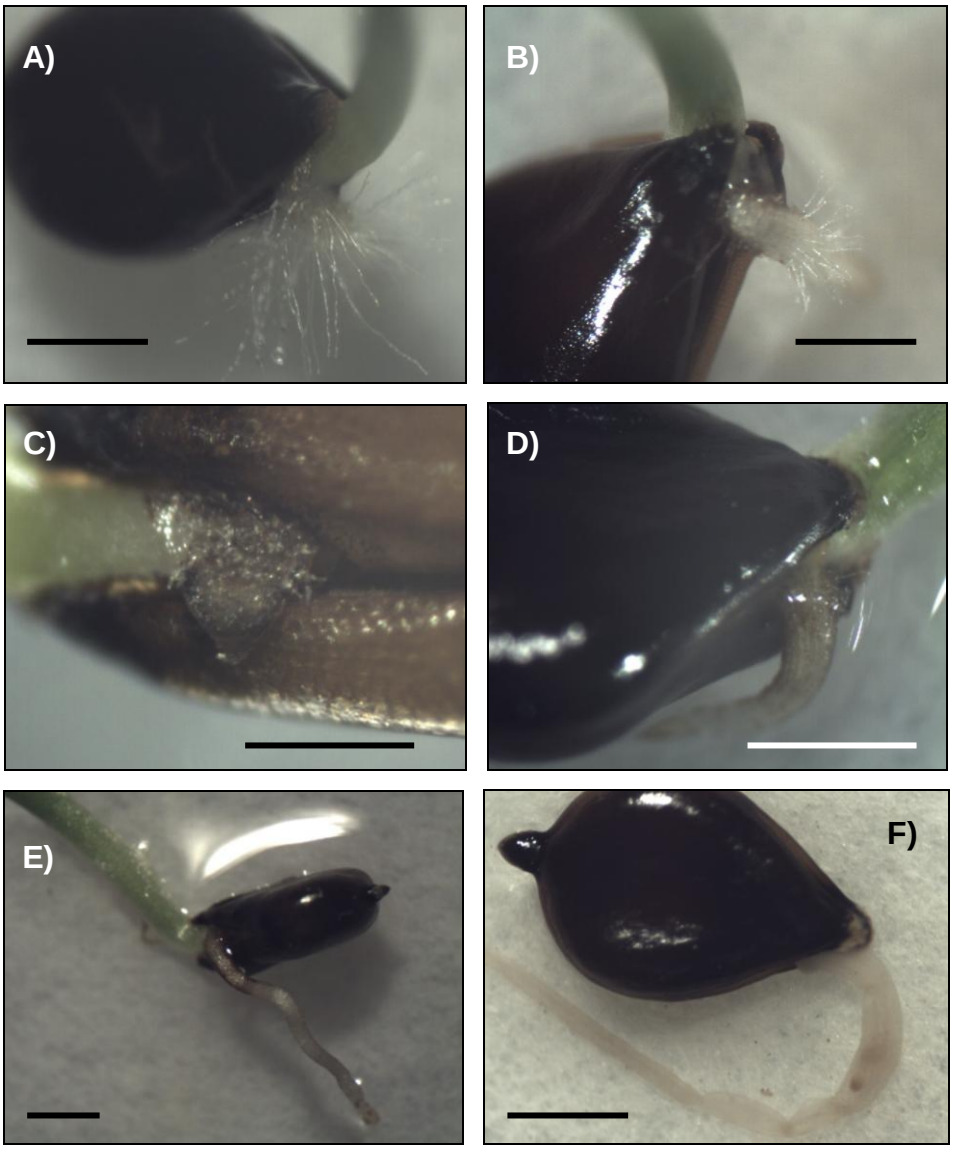


Figure 8.8. Effects of different salinities on hypocotyl hair formation in *B. caldwellii* and *B. medianus*. A) Hypocotyl hairs (hh) in *B. caldwellii* control after 3 days. B) Reduced hh growth in *B. caldwellii* after three days at 1 g L^{-1}. C) Arrested development of both radicle and hypocotyl regions in *B. caldwellii* at 2 g L^{-1}. D) Complete absence of hypocotyl and radicle hairs in *B. medianus* at 2 g L^{-1}. E) Complete absence of hypocotyl and radicle hairs in *B. caldwellii* at 4 g L^{-1}. F) Unsuccessful germination of *B. caldwellii* at 8 g L^{-1}. All scale bars = 1 mm except for plate E) which = 500 μm.

8.4 Discussion

8.4.1 Importance of hypocotyl hairs during early germination phases

The relationship between aborted germination and the failure of seeds to produce hypocotyl hairs during early germination phases, suggested that hypocotyl hair production was pivotal in overcoming the many challenges associated with sexual recruitment. Hypocotyl hairs were crucial to successful germination in all eight species from the six Cyperaceae genera tested. While these results represent only a small percentage of Cyperaceae species, they suggest that the single-celled structures are likely to be important to sexual recruitment events in other Cyperaceae genera world-wide. In all treatments the growth of radicles and the production of root hairs were more vigorous when associated with healthy hypocotyl hairs, suggesting that hypocotyl hairs play an important role in the uptake of water. The disintegration of hypocotyl hairs after approximately 10-12 days highlighted that hypocotyl hairs have a restricted life span and a specialised role in the early stages of establishment and recruitment for both *B. caldwellii* and *B. medianus.* These conclusions are consistent with observations made for other emergent aquatic genera, such as *Ottelia* and *Vallisneria* (Kaul 1978), as well as species living under similar Mediterranean conditions, such as *Lolium perenne* (Debaene-Gill *et al.* 1994), *Silene ameria* (Morita *et al.* 1995) and *Melaleuca ericifolia* (Robinson *et al.* 2008). Given that hypocotyl hairs were most prominent during the first 10-12 days of the germination process, it would seem that this period is of crucial importance to sexual recruitment events for *B. caldwellii* and *B. medianus*. Failure to meet the conditions required for hypocotyl hair formation during early establishment, may offer an additional explanation for the lack of sexual recruitment commonly reported for *Bolboschoenus* and other Cyperaceae species.

8.4.2 Hypocotyl hair responses to water availability and salinity

Though *B. caldwellii* and *B. medianus* are sympatric species their germination tolerances differed with respect to both water availability and salinity. *Bolboschoenus caldwellii* was able to germinate and develop hypocotyl hairs over a wider range of salinities and on drier substrates than *B. medianus*, which in contrast only produced long hypocotyl hairs at low salinity and on saturated (though not flooded substrata).

Results from these experiments fit well with known spatial distribution patterns of the two test species, as *B. caldwellii* is generally found at higher and drier gradient elevations that may be only periodically flooded, while *B. medianus* is restricted to lower and therefore wetter gradient positions (Blanch *et al.* 1999; Siebentritt and Ganf 2000). The tolerance differences of hypocotyl hairs from each species to salinity and water availability, may aid in explaining the typical zonation patterns formed by both species in the field.

Though the hypocotyl hairs of *B. medianus* achenes displayed greater adaptation to increased water availability than those of *B. caldwellii*, the differences were subtle, as hypocotyl hair lengths for both species were significantly reduced in flooded or dry compared to saturated (exposed) treatments. The results of this study highlighted that fine-scale surface water variation of just 1-2 mm, was enough to significantly alter the formation of hypocotyl hairs. This finding is in agreement with Matsuo and Shibayama (2000) who reported a reduction in size and adhering strength for *Monochoria vaginalis* hypocotyl hairs with just 0.5-1 mm of surface flooding. The results agree with past suggestions that hypocotyl hairs are inhibited at one extreme by excess water or flood conditions (Polya 1961) yet encouraged by dryness or conditions of water stress at the opposite extreme (Aronne and De Micco 2004). Future inquiry into the specific functional provisions of hypocotyl hairs in Cyperaceae species would be of great benefit in expanding our understanding of their sexual reproductive ecology.

Chapter 9.

Summary of sexual reproduction (Chapters 3-8)

9.1 Summary of sexual reproduction (Chapters 3-8)

Chapters 3-8 inquired into six different aspects of the sexual reproduction of *B. caldwellii* and *B. medianus,* as illustrated in the conceptual recruitment model on p. 37 of this thesis. Each of these chapters were conducted to highlight factors that may or may not explain the lack of sexual recruitment found for *Bolboschoenus* species in the wetlands of the Gippsland Lakes.

The analysis of achene production in Chapter three indicated that achenes of both *B. caldwellii* and *B. medianus* are produced in very low numbers (<2,300 m^{-2}), which is a common trait of most Cyperaceae species. The low number of achenes recovered from sediment seed banks from each field site (<11,200 m^{-2}) was consistent with poor achene production. The findings suggested that poor achene production and low numbers of achenes stored in the sediment bank are likely to contribute to rare sexual recruitment events for *B. caldwellii* and *B. medianus*. Low achene production is also likely to reduce the probability of achene dispersal to suitable germination niches. Interestingly, achene production was low in both fresh and brackish water wetlands, suggesting that production levels are unlikely to be influenced by management intervention through changes to hydrological and salinity regimes. The successful germination of achenes recovered from sediment cores from all field sites indicated that *B. caldwellii* and *B. medianus* are capable of forming persistent seed banks. Annual achene production, therefore, is not integral to future sexual reproduction of *Bolboschoenus* species.

Chapter four examined the viability of *B. caldwellii* and *B. medianus* achenes from each field site. Both fresh and 1-year-old achenes had consistently high viability

(~80%) irrespective of the source of plants. The results strongly indicate that poor viability was unlikely to be a contributing factor to the lack sexual recruitment observed for *B. caldwellii* and *B. medianus* in the wetlands of the Gippsland Lakes region.

Chapter five examined the buoyancy of *Bolboschoenus* achenes. Contrasting achene dispersal mechanisms were displayed by *B. medianus* and *B. caldwellii*. Achenes of the former species contained little aeriferous tissue in their pericarp layer and sank almost immediately. Thus, at each field site achenes probably join the local sediment seed bank only and do not disperse far from the parental population. In contrast, *B. caldwellii* achenes contained substantial aeriferous tissue, which enabled them to remain afloat for up to three months. This ability likely offers *B. caldwellii* achenes a higher probability of long-distance dispersal and allows them to find recruitment windows in both space and time. The differences in buoyancy between the two species could account for the typical differentiation reported in their landscape positions by Siebentritt and Ganf (2000).

Chapter six examined the effects of light, temperature and salinity on germination. Achene germination for both species was light dependent and required wide diurnal temperature variations of at least 20-25°C. Salinity significantly affected germinability, though achenes of both species were able to recover from salinity as high as 32 g L^{-1} when transferred to freshwater. As little germination was recorded above a salinity of about 4 g L^{-1}, salt could be a major factor in affecting achene germination and thus sexual recruitment. Unless salt is leached from wetlands (such as Clydebank Morass and Dowd Morass) by periodic flushing events, sexual recruitment is highly unlikely. Large-scale irrigation, levee banks and periodic drought restrict hydrological regimes in the majority of wetlands fringing the Gippsland Lakes, thus, salinity is likely a significant environmental sieve for the sexual reproduction of *B. caldwellii* and *B. medianus*.

Chapter seven examined additional requirements for the germination of *B. caldwellii* and *B. medianus* achenes. Cold-wet stratification of four weeks or greater and scarification of achenes, either for several days in weak acid or by manual

scarification with a razor blade, significantly improved germination for both species. Stratification and scarification pre-treatments were especially effective at improving germination of *B. medianus* achenes. Most importantly, stratification and scarification trials widened the temperature range at which germination could take place for both species. The shedding of achenes in autumn in order that they may over-winter under water appears to be a crucial factor for the sexual recruitment of *B. caldwellii* and *B. medianus*.

Chapter eight examined the presence, formation and influence of hypocotyl hairs to the sexual recruitment of *B. caldwellii* and *B. medianus* and other Cyperaceae species. The production of hypocotyl hairs was independent of the true root system and critical to early germination phases and the establishment of young seedlings. Fine-scale water variation of just 1-2 mm and low salinity (2-4 g L^{-1}) was enough to impede the development of hypocotyl hairs, and in all cases where hypocotyl hairs were absent or did not form completely, radicle formation was compromised and achenes failed to complete germination. Hypocotyl hair formation differed between species with *B. caldwellii* producing hypocotyl hairs under drier conditions than *B. medianus*, which in turn produced hypocotyl hairs under wetter conditions than *B. caldwellii*. The species-specific differences in hypocotyl hair formation may define the typical gradient differentiation noted for each species (i.e. *B. caldwellii* at higher and drier elevations than *B. medianus*, which is more often found at the waterline). Hypocotyl hairs have never been reported for Cyperaceae species in the scientific literature and may be an overlooked element that influences germination and seedling success.

In conjunction, each aspect of inquiry into the sexual reproductive ecology of *B. caldwellii* and *B. medianus* highlights the wide range of environmental sieves that must be overcome for *Bolboschoenus* achenes to be produced, germinate and survive as young seedlings. Each of these steps is in stark contrast to the asexual reproductive ecology of *Bolboschoenus* species, as discussed in the next chapter.

Section III:

Asexual reproduction

Chapter 10.

Effects of salinity on clonal growth

10.1 Introduction

Section II of this thesis examined the sexual reproductive ecology of *B. caldwellii* and *B. medianus*. A number of findings from this inquiry have critical implications to the clonal growth responses of each species. For example, low achene production and poor sediment seed bank formation, may limit sexual recruitment opportunities. In addition, the requirement of low salinity for germination and hypocotyl hair formation may prevent sexual recruitment and lead to a dependence on clonal growth for consolidation and expansion of space. Although clonality is common to all *Bolboschoenus* species, little information is available on how environmental variation affects asexual reproduction within the genus. Examinations of clonal growth mechanisms and responses are, therefore, critical to understanding the population structure and dynamics of sedge and rush dominated communities.

The objective of this chapter is to examine whether the growth, biomass allocation and ramet morphology of *B. caldwellii* and *B. medianus* genets differ across a range of salinity treatments. Rhizome and tuber data are focused on, as rhizomes provide a method of mobility and broaden the scope of resource capture and expansion into new territories, while tubers play an important role in resource storage, spatial organisation and future asexual recruitment. The specific questions asked in this chapter are: 1) is asexual growth of *B. caldwellii* and *B. medianus* compromised by moderate to high salinity (i.e. salinity known to negatively affect sexual reproduction)? 2) Do biomass allocation patterns differ between *B. caldwellii* and *B. medianus* clones across a range of salinity treatments? 3) Do *B. caldwellii* or *B.*

medianus rhizomes and tubers exhibit significant differences in size, production and dormancy between salinity treatments and are putative differences, plastic or non-plastic responses? 4) How do the biomass allocation patterns of each species fit with proposed analytical models that attempt to predict the facultative or plastic responses of clonal plants to changing environmental conditions?

10.2 Methods

10.2.1 Experimental treatments

In order to ensure the selection of unique genotypes, achenes of *B. caldwellii* and *B. medianus* were collected from several discrete populations at Dowd Morass, Clydebank Morass and Sale Common (Gippsland) in February 2006. Spatially discrete populations were sampled, as they were more likely to represent different clones and, therefore, be of different genetic composition. Likewise, achenes collected from putatively different clones were likely to contain much wider genetic diversity than those from one large patch, which may be subject to self-fertilisation and limited outcrossing opportunities. Achenes were pooled and stored in deionised water at 4°C (i.e. cold-wet stratification). In September of 2006 several hundred achenes of each species were germinated in petri dishes in temperature-controlled growth chambers (as per general methods Chapter 2). One week after germination, single seedlings were transferred to individual plastic punnets (7 x 6.8 x 4.8 cm) containing a (2:1) mixture of sieved potting soil and peat. Punnets were then placed into 30 x 50 x 10 cm plastic boxes (20 punnets per box), to allow each replicate to be flooded to the sediment surface. Boxes were positioned under banks of Gro-lux™ fluorescent tubes, which emitted PAR of ~ 90 μmol m^{-2} s^{-1} at seedling level. Both the orientation and position of each box were rotated every 2-3 days to randomise any spatial heterogeneity. After one month, forty plants of each species were chosen for uniformity, with all having at least 2 leaves and a culm height of between 50-60 mm for *B. caldwellii* and 60-70 mm for *B. medianus*. Selected seedlings were re-potted into 28 cm diameter and 12 cm deep pots, which in turn were placed into individual 52 L plastic flower buckets. Growing medium contained a (5:4:1) mixture of basic potting soil, peat (TEEM™ Canada) and coarse perlite (Chillagoe™ Australia).

Nutrients were supplied as Osmocote Plus® (8-9 months), which is a slow release, complete nutrient fertiliser containing: 16% N, 3.5% P, 10% K, 3.6% S, 2% C, 1.2% Mg, 0.15% Fe, 0.06% Mn, 0.05% Cu, 0.02% B, 0.015% Zn. Approximately 15 g of Osmocote Plus® was used in each replicate. Given that an individual pot had a surface area of 0.062 m^2 the estimated nitrogen loading for each treatment was 52 g N m^{-2} yr^{-1}. Orchid pots were lined with shade cloth to prevent soil loss while allowing efficient ion exchange between the sediment and the external solution. Water levels were maintained at the surface of pots throughout the experiment in order to mimic draw-down conditions and ensure that growth was not compromised by poor gas exchange or carbon sequestration.

Seedlings were grown under three salinity treatments (fresh water controls, 4 g L^{-1} and 12 g L^{-1}) in order to assess the capacity of each species for architectural plasticity and shifts in biomass apportionment. The three treatment levels were chosen for a number of reasons; they are not uncommon in wetlands, especially within the Gippsland Lakes region; there is minimal likelihood of any overlap occurring between treatments; and plants were likely to survive at each salinity level. Additionally, salinity levels of 15 g L^{-1} or greater are known to severely inhibit if not cause mortality in a number of closely related species from the genus *Scirpus* (Kantrud 1996; Hootsmans and Weigman 1998; Lillebø *et al.* 2003). The upper salinity level (12 g L^{-1}) used in this study was therefore intended to be high enough to cause some growth depression /alteration (such as shorter culm heights) but not so high as to entirely hamper growth and cause mortality. The experimental salt range (0-12 g L^{-1}) is also highly relevant to wetland managers as significant changes in community structure and function are known to occur between 0.3 and 10-12 g L^{-1} salt (Hart *et al.* 1991).

Of the 40 initial replicates of each species, 10 were randomly harvested following a 2-week establishment period – prior to salt addition – leaving 10 replicates at each of 3 treatment levels. The 10 harvested plants for each species were used to estimate the initial plant dry weights [(culm + tuber + rhizome + roots) x number of culms per pot], used in relative growth rate (RGR) calculations. Saline solutions were made up using seawater (from Queenscliff Marine Research Centre) diluted with tap water. Salinity, pH and water temperature were monitored weekly

using a Horiba Ltd. water quality meter (Kyoto, Japan). Salt concentrations were adjusted accordingly with either more seawater or tap water to maintain correct concentrations. Internal pots were held above the water line several times a week to drain water then resubmerged to the sediment level to assist in equalising soil and water salinity concentrations. The water from control replicates was also drained/flushed weekly, to ensure that the sediment redox conditions were similar to those of saline replicates.

Trials ran from October 2006 to January 2007 and were undertaken in an outdoor experimental area at Victoria University (Fig. 10.1). Individual mesocosms (large flower buckets) were arranged in a randomised design and shuffled at each survey to randomise any inconsistencies (such as shading) that may have been apparent in the experimental area.

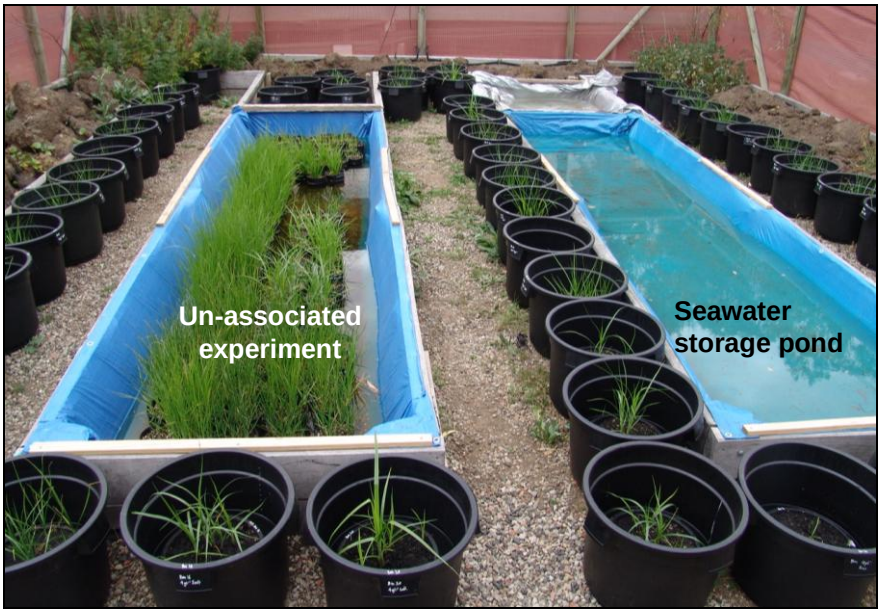


Figure 10.1. Photograph of experimental area and design layout used in asexual growth trials. Replicate plants of *B. caldwellii* and *B. medianus* each contained in individual 52 litre black plastic buckets (mesocosms) were randomly arranged as pictured and shuffled after each survey to avoid shading and other potential layout effects. Larger mesocosms (blue lined pits) were used to store seawater for adjusting salinity levels during the experiment, and also to grow additional plants in an un-associated freshwater experiment.

10.2.2 Morphological measurements

The total number of culms per pot was measured every 7-8 days. Culms and leaves were considered living if greater than 50% of their length was green. The 4th emerged culm was marked in each replicate and measured for width (using callipers to two decimal places) and height (from the sediment surface to the last node). Longest leaf measurements were also taken from the 4th culm as well as the number of leaves. Harvested plants were separated into culms (stems, leaves, flowers and seeds), roots, rhizomes and tubers. Particular note was taken of the number and weight of dormant tubers, which were defined in this study as those tubers that did not form an above-ground culm or axillary rhizomes. The definition of dormant is used loosely, because although the 'dormant' tubers were not sprouting, they were still growing in size (i.e. actively storing resources). Dry weights were obtained after drying at 70°C until constant weight was recorded. In order to assess levels of plasticity within rhizome production for *B. caldwellii* and *B. medianus*, the lengths of rhizomes from each replicate were individually measured and assigned to rhizome categories set at 20 mm incremental lengths. Relative growth rate (RGR) was calculated after Harper (1977):

$$\text{RGR} = \frac{\ln W_1 - \ln W_2}{\triangle t} \quad (\text{mg g}^{-1}\ \text{day}^{-1})$$

where t is the length of the experimental period, and W_2 and W_1 are the final and initial plant dry weights.

10.2.3 Statistical analysis

Cases where all culms died before the end of the experiment were excluded from statistical analysis. Biomass components were compared within each species via 1-way ANOVA and between species via 2-way ANOVA. Where appropriate, data were transformed (e.g. weight values with log(x), length values with square root, and percentage values with arcsine square root transformation) prior to analysis if the assumptions of normality were not met (Zar 1996). Normality was assessed via the Kolmogorov-Smirnov/Shapiro-Wilk test and homogeneity of variance via Levene's

test. Significant differences ($p < 0.05$) were assessed with Tukey HSD *post-hoc* tests and the Bonferroni correction was applied when necessary. Rhizome frequency and length differences were assessed via contingency table analysis or Chi-square tests of independence. Significant differences between the frequency and length of rhizomes, irrespective of salinity, were considered to be non-plastic (developmental) changes, while significant differences in the representation (presence or absence) of rhizome lengths categories between salinity treatments, were considered plastic (environmentally driven) changes. All analyses were conducted with SPSS v.15 statistical software.

10.3 Results

10.3.1 Environmental conditions during experimental period

Mean water temperatures were similar between replicates and treatments throughout the experimental period (~ 22 °C ± 3.5 °C). Mean salinity was maintained within ± 2 g L^{-1} of desired concentrations in all replicates and did not overlap between treatments (see Appendices A.2 and A.3). Possible inconsistencies arising from positional differences within the experimental area were considered minimal.

10.3.2 Effects of salt on above-ground growth measurements

Both test species survived under all treatment levels, though high salinity resulted in greater suppression of growth for *B. medianus* and the death of 50% of individuals. In contrast, *B. caldwellii*, while suppressed at high salinity did not perish in any treatment (Fig. 10.2). The death of two *B. medianus* replicates in control treatments was likely due to being attacked by insect larvae during the establishment period, while deceased replicates in saline treatments showed classical signs of ion toxicity, as leaves and stems yellowed and shrivelled through necrosis.

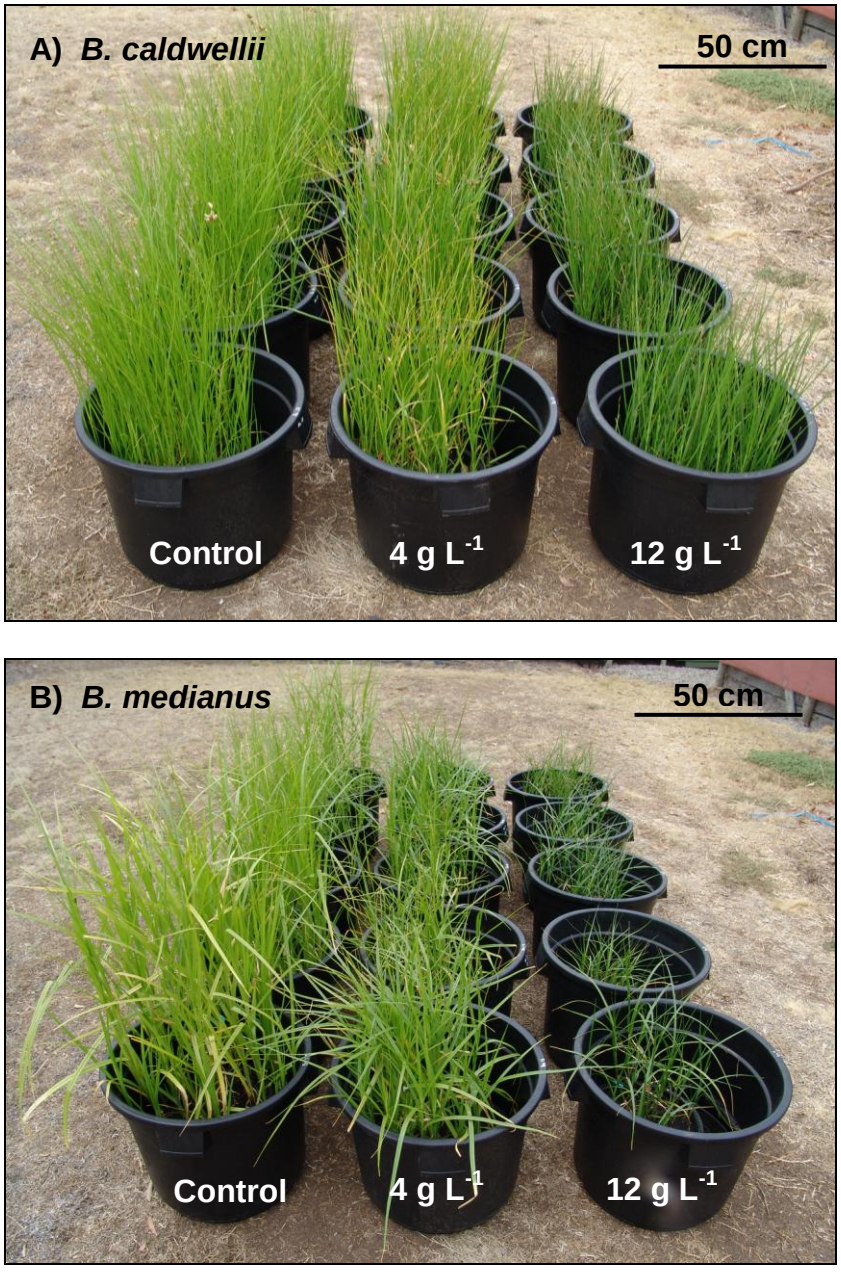


Figure 10.2. Comparative photographs of five replicates of: A) *B. caldwellii* and B) *B. medianus* grown under control (freshwater), 4 g L^{-1} salt and 12 g L^{-1} salt treatments, for four months.

In most cases, both test species were able to replace necrotic leaves with new leaves at a greater rate than they were lost. Weekly growth measurements of above ground culm morphology (taken from the fourth emerged culm) were similar for *B. caldwellii* and *B. medianus* (Fig. 10.3). Culm measurements mirrored one another in control and 4 g L^{-1} salt treatments, though were inhibited under 12 g L^{-1} salinity. Culm height, culm width and leaf length was reduced by ~25% for *B. caldwellii* at the highest salinity over the trial period, while for *B. medianus* the same measurements decreased by ~50% under high salt. The number of leaves produced by each species was also reduced under 12 g L^{-1} salinity. Investment into flowering or sexual ramets only occurred for *B. caldwellii* clones over the experimental period; differing significantly between low and high salinity treatments ($F_{2, 27} = 4.34$, $p < 0.05$) (Fig. 10.4).

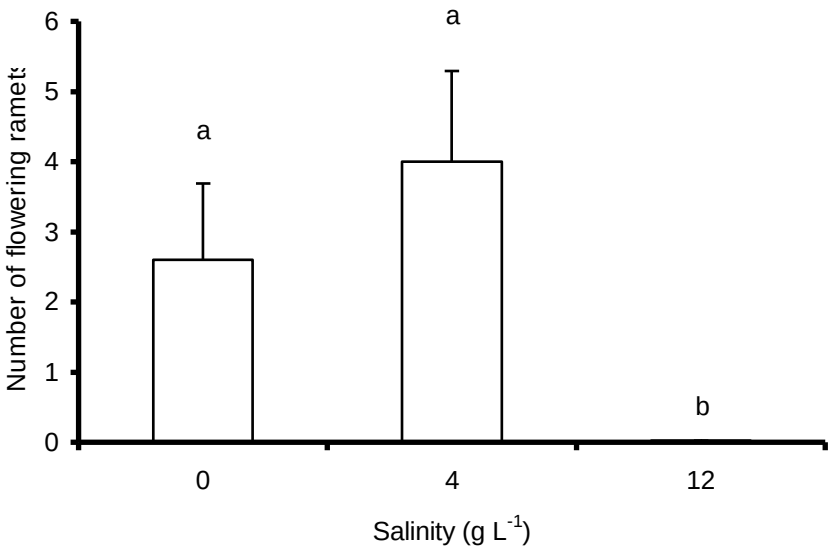


Figure 10.4. Average number of flowering ramets for *B. caldwellii* in control, 4 g L^{-1} and 12 g L^{-1} salinity treatments (n = 10). No flowers were produced at 12 g L^{-1}. Different superscript letters indicate significant differences ($p = 0.05$) following 1-way ANOVA and Tukey post hoc test.

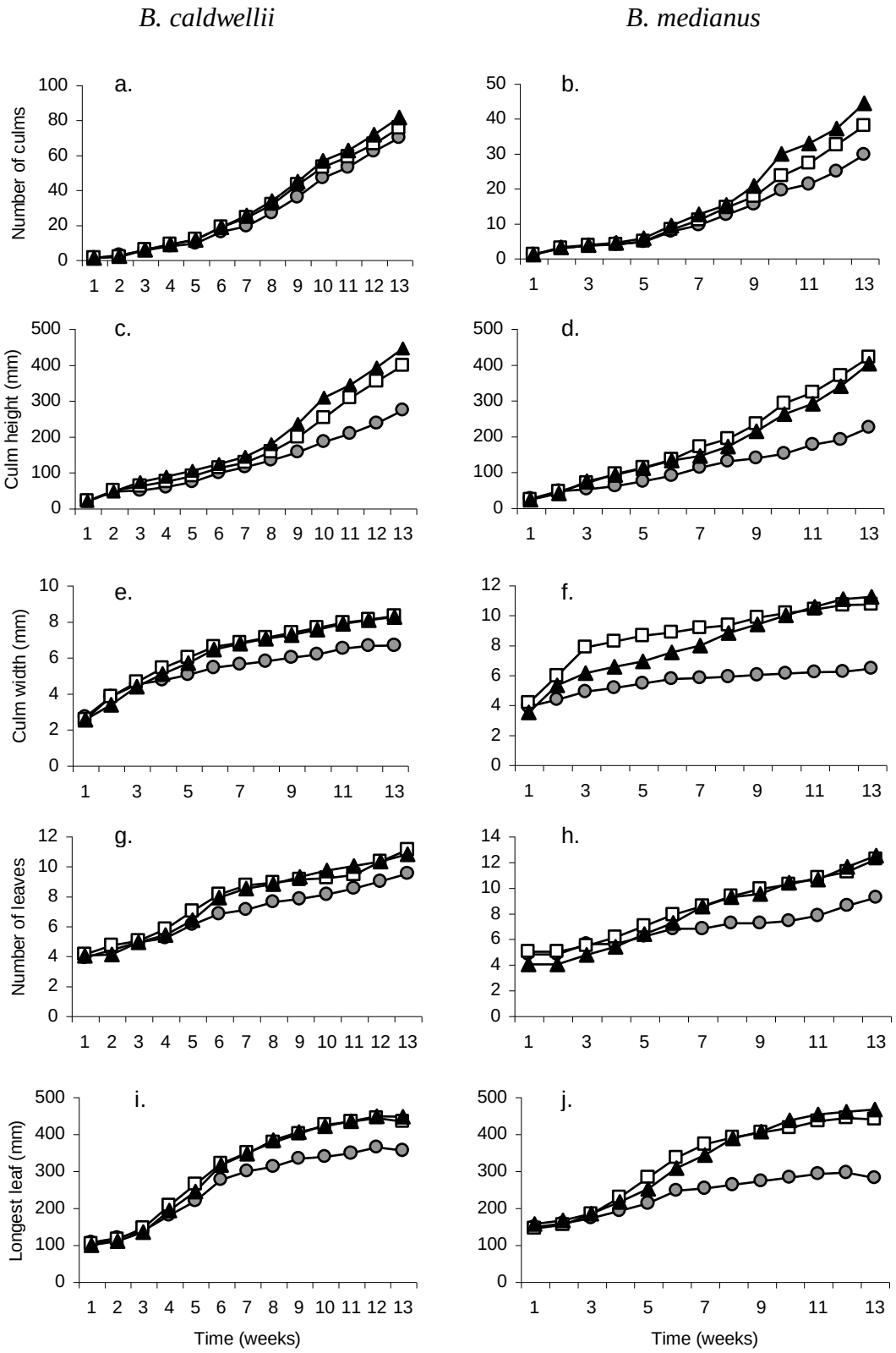


Figure 10.3. Performance of *B. caldwellii* and *B. medianus* under three contrasting salinity regimes: control conditions (black triangles), 4 g L^{-1} salt (white squares) and 12 g L^{-1} salt (grey circles). Data shown includes, average number of culms per pot (a, b), culm height (c, d), culm width (e, f), number of leaves (g, h) and longest leaf (i, j). Standard error bars are omitted for clarity, but were typically <10% of the means. All measurements other than number of culms, were taken from the fourth culm to emerge after germination.

10.3.3 Effects of salt on ramets

Though the smaller of the two test species, *B. caldwellii* produced more than double the number of culms, tubers and rhizomes compared to *B. medianus* under all treatments. Significant differences ($p < 0.05$) in the frequency of all growth components were found between treatments for both species, with the exception of culm frequency for *B. caldwellii*, ($F_{2, 27} = 2.0$, $p = 0.16$) (Table 10.1). Total rhizome length declined by more than 40% for *B. caldwellii* and greater than 65% for *B. medianus*.

Table 10.1. Mean number of culms, tubers and rhizomes, as well as total rhizome length (m) of individual *B. caldwellii* and *B. medianus* replicates (clones) grown under fresh water conditions (controls), 4 g L^{-1} and 12 g L^{-1} salinity treatments. Data shown are means and standard errors (n = 10).

	Number of ramet components per replicate / pot					
	B. caldwellii			*B. medianus*		
Component	Control	4 g L^{-1}	12 g L^{-1}	Control	4 g L^{-1}	12 g L^{-1}
Culms	82 ± 4	75 ± 4	70 ± 4	44 ± 4 ^{A}	38 ± 3 $^{A B}$	30 ± 4 ^{B}
Tubers	182 ± 11 ^{a}	152 ± 10 $^{a b}$	122 ± 7 ^{b}	81.6 ± 8 ^{A}	62 ± 7 $^{A B}$	42 ± 8 ^{B}
Rhizomes	230 ± 19 ^{a}	171 ± 12 ^{b}	133 ± 11 ^{b}	107 ± 8 ^{A}	73 ± 7 ^{B}	53 ± 9 ^{B}
Total rhizome length (m)	20.0 ± 1.8 ^{a}	13.8 ± 1 ^{b}	11.8 ± 0.9 ^{b}	10.4 ± 1.0 ^{A}	6.1 ± 1.1 ^{B}	3.6 ± 1.1 ^{B}
	n = 10	n = 10	n = 10	n = 8	n = 9	n = 5

Numbers with alternative superscripts are significantly different ($p < 0.05$) following 1-way ANOVA and Tukey's HSD *post-hoc* testing.

10.3.4 Effects of salt on the biomass of individual ramet components

Highest salinity decreased average culm weight by approximately 40% in *B. caldwellii*, whereas average culm weight for *B. medianus* dropped by approximately 65% at 12 g L^{-1} salt (Table 10.2). Average tuber weight was greatest for both species under 4 g L^{-1} salt, with *B. medianus* more than doubling its investment into tubers compared to other treatments. Average rhizome weight was maintained between treatments for *B. caldwellii*, though high salinity reduced the average weight of *B. medianus* rhizomes more than 50%.

Table 10.2. Average biomass of individual ramet components given as dry weights (g) for *B. caldwellii* and *B. medianus* grown under control, 4 g L^{-1} and 12 g L^{-1} salinity treatments (data shown are means and standard errors).

	Average weight of ramet components per replicate / pot (g)					
	B. caldwellii			*B. medianus*		
Component	Control	4 g L^{-1}	12 g L^{-1}	Control	4 g L^{-1}	12 g L^{-1}
Culm	1.08 ± 0.05 ^{a}	1.05 ± 0.04 ^{a}	0.63 ± 0.04 ^{b}	1.88 ± 0.24 ^{A}	1.75 ± 0.11 ^{A}	0.66 ± 0.07 ^{B}
Tuber	0.42 ± 0.06 ^{b}	0.48 ± 0.04 ^{b}	0.27 ± 0.04 ^{a}	0.46 ± 0.08 ^{A}	1.05 ± 0.15 ^{B}	0.42 ± 0.10 ^{A}
Rhizome	0.049 ± 0.003	0.053 ± 0.004	0.044 ± 0.004	0.087 ± 0.012 ^{A}	0.075 ± 0.015 ^{A}	0.034 ± 0.006 ^{B}
	n = 10	n = 10	n = 10	n = 8	n = 9	n = 5

Numbers with alternative superscripts indicate significant differences ($p < 0.05$) following 1-way ANOVA and Tukey's HSD *post-hoc* testing.

10.3.5 Effects of salt on biomass allocation

Biomass averages for clones of each species did not differ statistically between control and 4 g L^{-1} salt treatments, with the exception of total root weight for *B. caldwellii* ($F_{2, 27} = 41.0$, $p < 0.05$) (Table 10.3). Highest tuber biomass for both test species occurred under 4 g L^{-1} salt, while for *B. medianus* total biomass of clones was greatest under 4 g L^{-1} salt. At 12 g L^{-1} salt, the weight of ramet components of both species was significantly reduced, with total biomass decreasing by approximately 50% or greater in *B. caldwellii* and more than two thirds in *B. medianus*.

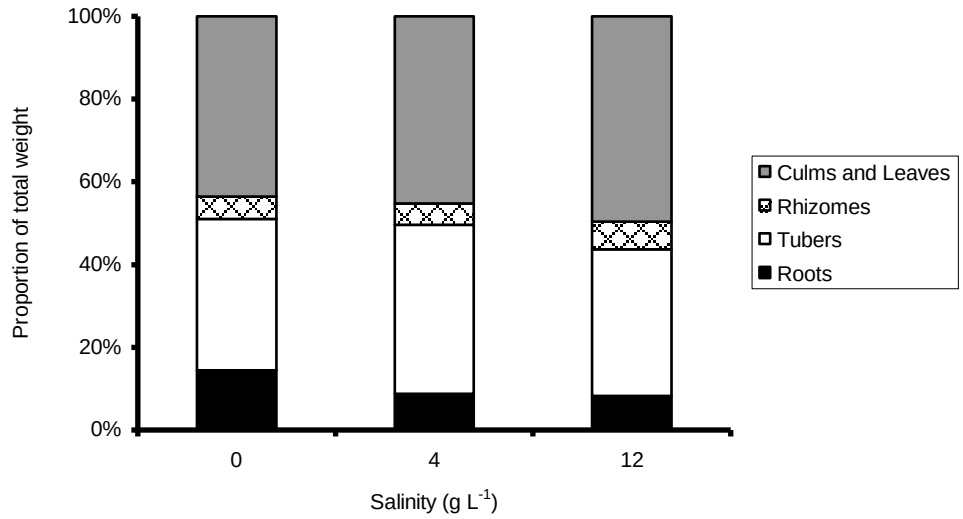
Table 10.3. Total biomass of whole clones given as dry weights (g) for *B. caldwellii* and *B. medianus* grown under fresh water conditions (controls), 4 g L^{-1} and 12 g L^{-1} salinity (data shown are means and standard errors).

Total weight (g)						
	B. caldwellii			*B. medianus*		
Genet component	Control	4 g L^{-1}	12 g L^{-1}	Control	4 g L^{-1}	12 g L^{-1}
Culms	86.9 ± 4.5 ^{a}	79.1 ± 5.2 ^{a}	43.5 ± 3.4 ^{b}	78.6 ± 6.9 ^{A}	65.3 ± 5.0 ^{A}	19.6 ± 3.2 ^{B}
Tubers	75.4 ± 10.1 ^{a}	75.6 ± 9.4 ^{a}	35.0 ± 7.6 ^{b}	35.8 ± 5.4 $^{A\ B}$	64.6 ± 9.8 ^{A}	19.0 ± 8.0 ^{B}
Rhizomes	10.9 ± 0.8 ^{a}	9.1 ± 0.9 ^{a}	5.8 ± 0.5 ^{b}	8.8 ± 1.0 ^{A}	5.7 ± 1.3 $^{A\ B}$	2.0 ± 0.6 ^{B}
Roots	27.8 ± 3.3 ^{a}*	14.5 ± 0.9 ^{b}*	7.1 ± 0.6 ^{c}*	22.2 ± 1.5 ^{A}	16.4 ± 2.7 ^{A}	4.4 ± 1.2 ^{B}
Total	200.9 ± 10.9 ^{a}	178.3 ± 14.8 ^{a}	91.3 ± 11.6 ^{b}	145.3 ± 11.7 ^{A}	151.9 ± 15.0 ^{A}	44.9 ± 11.9 ^{B}
	n = 10	n = 10	n = 10	n = 8	n = 9	n = 5

Numbers with alternative superscripts are significantly different ($p < 0.05$) following 1-way ANOVA and Tukey's HSD *post-hoc* testing. * Indicates treatments where significant differences were calculated using the non-parametric Kruskal-Wallis ANOVA ($p < 0.05$) and Nemenyi *post-hoc* tests.

The percentage biomass allocated to culms was similar between treatments for *B. caldwellii* ($F_{2, 27} = 3.30$, $p = 0.052$) and *B. medianus* ($F_{2, 19} = 2.60$, $p = 0.100$) and both species showed significantly reduced root proportions under high salinity (*B. caldwellii* $F_{2, 27} = 5.94$, $p < 0.01$ and *B. medianus* $F_{2, 19} = 3.87$, $p < 0.05$) (Fig. 10.5). Tuber biomass did not differ across treatments for *B. caldwellii* ($F_{2, 27} = 1.16$, $p = 0.33$), while *B. medianus* allocated significantly more resources to tubers (storage) as salinity increased ($F_{2, 19} = 6.48$, $p < 0.01$). Dissimilar results were also found for percentage biomass allocation to rhizomes for each species, with *B. caldwellii* allocating significantly more resources to rhizomes under high salinity ($F_{2, 27} = 4.86$, $p < 0.05$) and *B. medianus* allocating significantly fewer resources to rhizomes as salinity increased ($F_{2, 27} = 3.46$, $p < 0.05$).

a) *B. caldwellii*



b) *B. medianus*

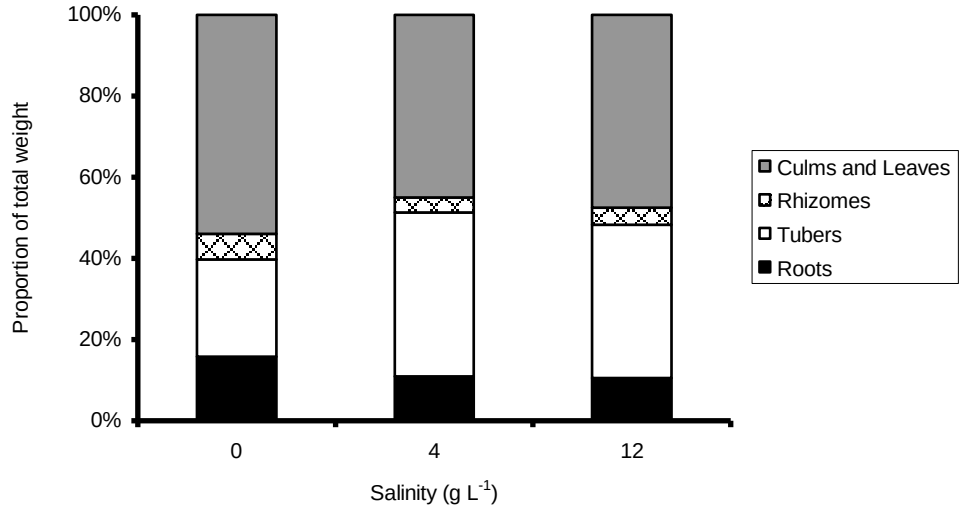


Figure 10.5. Percentage biomass allocation to various tissues as a proportion of total weight for a) *B. caldwellii* and b) *B. medianus* in control, 4 g L^{-1}, and 12 g L^{-1} salinity treatments. In all treatments n = 10 for *B. caldwellii* while for *B. medianus* n = 8, 9 and 5 for control, 4 g L^{-1}, and 12 g L^{-1} salinity treatments, respectively.

Two-way ANOVA of percentage biomass data for each species across treatments highlighted the different asexual growth strategies employed by *B. caldwellii* and *B. medianus*, when confronted with increasing salinity. No differences were found across species for biomass allocation to culms, tubers or roots, though significant differences were found for rhizomes (Table 10.4). Salinity exerted an effect on tubers, rhizomes and roots though not culms. Significant interactions were

found between species and salinity for all categories of biomass allocation with the exception of roots.

Table 10.4. Effects of salinity on total percentage (culm, tuber, rhizome and root) biomass allocation between *B. caldwellii* and *B. medianus* and potential interactions, following 2-way ANOVA (p <0.05).

	% Biomass allocation			
	Effect	df	*F*-value	Sig.
Culms	Species	1	1.86	ns
	Salinity	2	1.66	ns
	Species x Salinity	2	4.19	0.021
Tubers	Species	1	2.14	ns
	Salinity	2	6.32	0.004
	Species x Salinity	2	3.33	0.045
Rhizomes	Species	1	6.46	0.014
	Salinity	2	4.30	0.019
	Species x Salinity	2	4.14	0.022
Roots	Species	1	3.03	ns
	Salinity	2	9.29	0.000
	Species x Salinity	2	0.05	ns

10.3.6 Effects of salinity on the ratio of above-ground to below-ground biomass

Above and below-ground biomass for either species remained stable within control and low salinity treatments, though decreased significantly under 12 g L^{-1} salinity (Table 10.5). Although the ratio of above-ground to below-ground biomass for *B. caldwellii* increased significantly with increasing salinity ($F_{2, 27} = 3.92$, $p < 0.05$), no significant differences were found for *B. medianus* as salinity increased ($F_{2, 19} = 1.86$, $p = 0.18$).

Table 10.5. Total above and below-ground biomass and the ratio of above to below-ground biomass given as dry weights (g) for *B. caldwellii* and *B. medianus* grown under fresh water conditions (controls), 4 g L^{-1} and 12 g L^{-1} salinity treatments (data shown are means and standard error values).

	Biomass (g)					
	B. caldwellii			*B. medianus*		
Plant parts	Control	4 g L^{-1}	12 g L^{-1}	Control	4 g L^{-1}	12 g L^{-1}
Above-ground	86.9 ± 4.5 ^{a}	79.1 ± 5.2 ^{a}	43.5 ± 3.4 ^{b}	78.6 ± 6.9 ^{A}	65.3 ± 5.0 ^{A}	19.6 ± 3.2 ^{B}
Below-ground	114.0 ± 0.1 ^{a}	99.2 ± 10.3 ^{a}	47.9 ± 8.5 ^{b}	66.8 ± 6.0 ^{A}	86.6 ± 11.3 ^{A}	25.3 ± 8.8 ^{B}
Ratio A:B	0.79 ± 0.06 ^{a}	0.84 ± 0.05 ^{ab}	1.01 ± 0.07 ^{b}	1.20 ± 0.10	0.88 ± 0.14	0.94 ± 0.13
	N = 10	n = 10	n = 10	n = 8	N = 9	n = 5

Numbers with the alternative superscripts are significantly different (P = 0.05) following 1-way ANOVA and Tukey's HSD post hoc test.

Two-way ANOVA showed significant differences between species for percentage above and below-ground biomass allocation, though no significant difference was found for the ratio of above to below-ground biomass (Table 10.6). Likewise, salinity exerted a significant effect on above and below-ground biomass, though it did not affect the ratio of above to below-ground biomass. A significant interaction between species and salinity was only apparent for the ratio of above to below-ground biomass, which accounted for ~14% of the variation found between *B. caldwellii* and *B. medianus*. With reference to Table 10.6, the significant interaction may be explained by the fact that *B. caldwellii* clones directed more resources into above-ground biomass as salinity increased, while *B. medianus* clones maintained their above to below-ground biomass ratios.

Table 10.6. Two-way ANOVA of the effects of salinity on the ratio of above-ground to below-ground biomass in *B. caldwellii* and *B. medianus* ($p < 0.05$).

	% Biomass allocation			
	Effect	df	*F*-value	Sig.
Above-ground	Species	1	13.33	0.001
	Salinity	2	49.91	0.000
	Species x Salinity	2	1.08	ns
Below-ground	Species	1	12.15	0.001
	Salinity	2	19.60	0.000
	Species x Salinity	2	1.89	ns
Ratio A:B biomass	Species	1	2.86	ns
	Salinity	2	1.33	ns
	Species x Salinity	2	3.63	0.034

10.3.7 Effects of salinity on relative growth rates

The relative growth rate (RGR) of both species was negatively affected by increasing salinity, though remained higher for *B. caldwellii* than *B. medianus* in all treatments (Fig. 10.6).

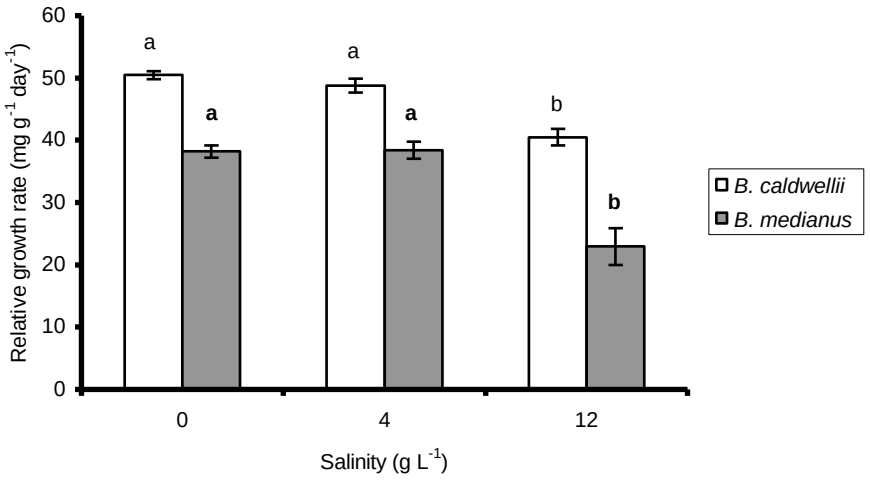


Figure 10.6. Mean relative growth rates (mg g^{-1} day^{-1}) of *B. caldwellii* and *B. medianus* grown at three different salinities. Significant differences ($p < 0.05$) are indicated by different superscript letters for each species following 1-way ANOVA and Tukey's HSD *post-hoc* tests. Standard error bars are shown.

Significant differences were revealed by 2-way ANOVA for the individual factors of species and salinity, as well as a significant interaction between the two terms that accounted for ~14% of the variation (Table 10.7).

Table 10.7. Two-way ANOVA comparing the relative growth rate (RGR) of *B. caldwellii* and *B. medianus* grown under fresh water conditions (controls), 4 g L^{-1} and 12 g L^{-1} salinity treatments ($p < 0.05$).

		RGR (mg g^{-1} day^{-1})		
	Effect	df	*F*-value	Sig.
RGR	Species	1	151.88	0.000
	Salinity	2	50.83	0.000
	Species x Salinity	2	3.63	0.034

10.3.8 Effects of salt on tuber dormancy

The percentage of dormant tubers significantly decreased for both *B. caldwellii* ($F_{2, 27} = 25.52$, $p < 0.05$) and *B. medianus* ($F_{2, 19} = 5.80$, $p < 0.05$) with increasing salinity (Fig. 10.7).

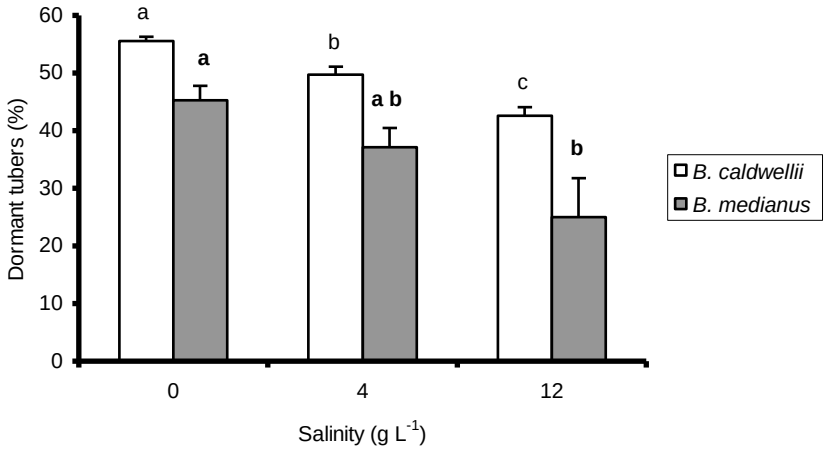


Figure 10.7. Percentage of dormant tubers for *B. caldwellii* and *B. medianus* grown under control, 4 g L^{-1} and 12 g L^{-1} salinity treatments (data shown are means and standard error values). Significant differences ($p < 0.05$) for each species are indicated by different superscripts, following 1-way ANOVA and Tukey's HSD *post-hoc* tests.

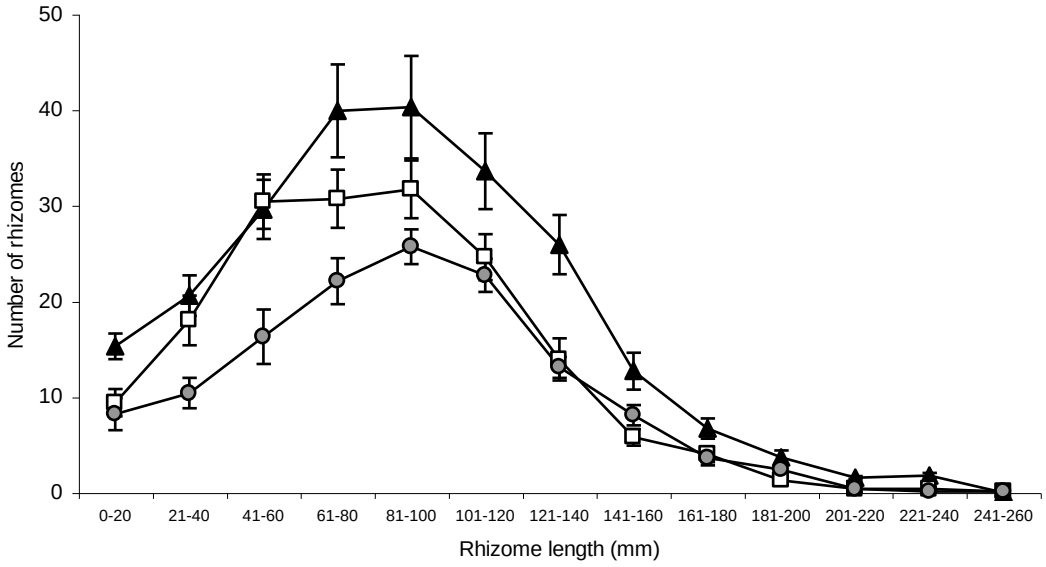
10.3.9 Effects of salt on rhizome lengths

Older tubers at the centre of clones gave rise of up to six daughter ramets, though generally only three new ramets advanced from each tuber. Typical growth patterns for *B. caldwellii* and *B. medianus* from each tuber, involved the initial production of one short axillary rhizome, followed by an intermediate rhizome, then a final long rhizome that often contained a dormant tuber and no culm.

Chi-square tests of independence within each species, revealed significant differences between salinity and the frequency of rhizomes within each 20 mm length category: *B. caldwellii* (χ^2 (24, N = 5338) = 74.25, $p < 0.05$, Cramérs V = 0.08) and *B. medianus* (χ^2 (28, N = 1779) = 96.23, $p < 0.05$, Cramérs V = 0.16). Figure 10.8 highlights the different modality of each species in response to salinity. Although significantly fewer rhizomes were produced within most rhizome length categories for *B. caldwellii* as salinity increased, the growth responses of rhizomes under all treatments displayed similar bell shaped curves. In contrast, the response of *B. medianus* to increasing salinity was irregular, as seen by the production of a greater number of very short rhizomes (0-20 mm) under high salinity in comparison to fresh water. The majority of rhizome categories for each species were still represented irrespective of treatment, however, and accordingly, no significant relationships were found between salinity and rhizome length when analysed by presence or absence: *B. caldwellii* (χ^2 (2, N = 39) = 2.05, p = 0.36, Cramérs V = 0.23) and *B. medianus* (χ^2 (2, N = 45) = 5.85, p = 0.054, Cramérs V = 0.36). The non-plastic (developmental) capacity of each species was therefore deemed to be greater than any plastic capacity to environmental differences (in this case salinity).

Chi-square tests of independence on combined data from both species, revealed that a significantly greater number of rhizomes were produced by *B. caldwellii* compared to *B. medianus* under all treatment conditions (χ^2 (14, N = 7117) = 215.41, $p < 0.05$, Cramérs V = 0.17). Differences in the frequency of rhizomes did not influence rhizome length however, as an additional Chi-square test using presence / absence data, highlighted that there were no significant differences in the lengths of rhizomes produced by each species (χ^2 (2, N = 90) = 2.31, p = 0.32, Cramérs V = 0.16).

a) *B. caldwellii*



b) *B. medianus*

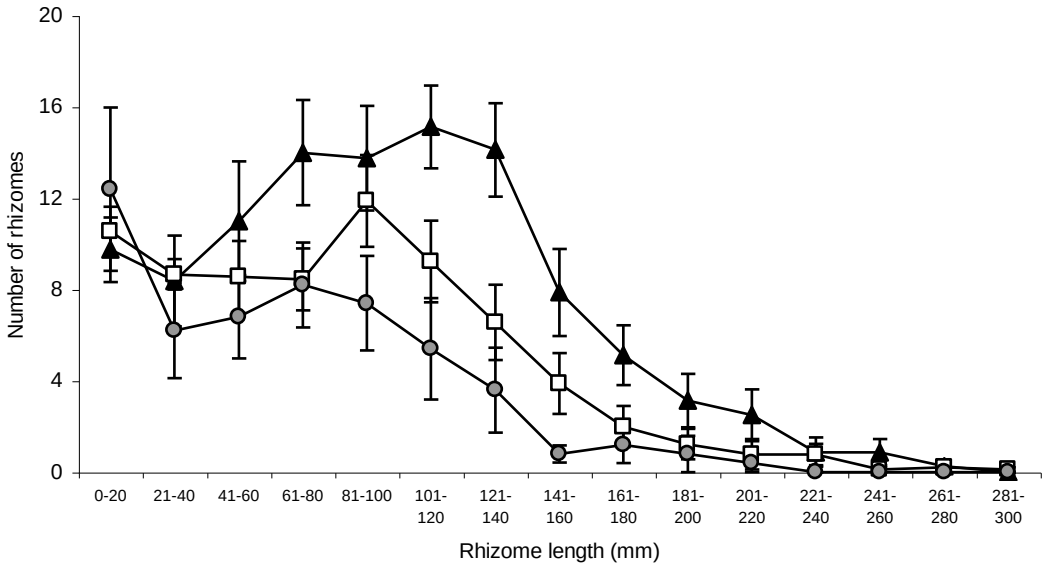


Figure 10.8. Average number of rhizomes measured at 20 mm increment lengths for a) *B. caldwellii* and, b) *B. medianus* under control or freshwater conditions (black triangles), 4 g L^{-1} salt (white squares) and 12 g L^{-1} salt (grey circles). Standard error bars are shown, (n = 10) for *B. caldwellii* in all treatments, while n = 8, 9 and 5 for *B. medianus* in control, 4 g L^{-1} salt and 12 g L^{-1} salt treatments respectively.

10.4 Discussion

10.4.1 Clonal growth responses to salinity

With reference to the first question posed at the beginning of this chapter (p.144), the survival of both test species in moderate (4 g L^{-1}) to high (12 g L^{-1}) salinity treatments highlights the importance of clonal growth mechanisms to the persistence of *B. caldwellii* and *B. medianus* in salinised habitats, such as those that occur around the Gippsland Lakes. Although 50% of *B. medianus* replicates died at the upper salt concentration (12 g L^{-1}), the growth of all remaining clones illustrated that the recruitment parameters for asexual reproduction in both species were much wider than those for sexual reproduction, as 12 g L^{-1} salt is well beyond the tolerance levels for achene germination (Chapter 6). The high biomass produced by both species within freshwater control treatments, suggested that their occurrence in brackish sites is due to ecological displacement (most likely in the form of competition from better adapted species) rather than an optimum response to the environment, as noted for *S. robustus* (Pearcy *et al.* 1982 cited in Kantrud 1996). Nevertheless, both species are well adapted to salinity, as their RGRs and above and below-ground biomass ratios remained stable under low salinity concentrations (up to 4 g L^{-1}). *Bolboschoenus* species are, therefore, well placed to fill the niche vacated by freshwater plants if salinity should rise, as few truly freshwater macrophytes persist at salinities > 4 g L^{-1} (Brock and Shiel 1983; Hart *et al.* 1991; Bailey *et al.* 2002).

Not unlike achene germination trials, which found higher germination at 1-2 g L^{-1} salt (Chapter 6), low salinity appeared to benefit asexual growth and storage, as both test species produced highest tuber biomass within 4 g L^{-1} salt treatments. This result is in agreement with previous findings, in which *B. medianus* was shown to shift biomass allocation from culms to tubers in response to increasing salinity (up to ~ 8 g L^{-1}) (Morris and Ganf 2001). Mercado *et al.* (1971) documented similar findings for *B. maritimus*, which produced larger and heavier corms (tubers) and more shoots when grown in 4-10 g L^{-1} NaCl solutions, than dilute solutions or freshwater. The trend to increase investment into underground organs as salinity increases, appears marginal however, as illustrated by the negative relationship between tuber number and 18 g L^{-1} salt for *B. maritimus* (Hootsmans and Wiegman 1998). The

cause of the decline was most likely due to a combination of osmotic and ionic effects, though the upper salt tolerance limits for active growth in *Scirpus* and *Bolboschoenus* species examined so far ranges from 15-25 g L^{-1} (Palmisano 1970; Dietert and Shontz 1978; Brock and Lane 1983; Kantrud 1996; Hootsman and Wiegman 1998). Mortality of *Bolboschoenus* species has been recorded at salinities below 11 g L^{-1} and specific ionic effects such as necrotic mottling and chlorosis (leaf yellowing) can become apparent at around 7-8 g L^{-1}, though this may depend on how well a clone has already established prior to salinisation (Kaushik 1963 cited in Kantrud 1996). With reference to the soil salinity results recorded in Chapter 3 (see Fig. 3.3), only one of the field sites sampled in this study (Sale Common) contained sediment salinities below the theoretical 4 g L^{-1} upper limit for freshwater species. Reproduction for *B. caldwellii* or *B. medianus* at either Dowd Morass or Clydebank Morass must therefore be predominantly, if not exclusively clonal, unless salt is flushed from both systems. This very pattern was recently documented at Dowd Morass for the woody species *Melaleuca ericifolia* (Swamp Paperbark), which has a strongly rhizomatous or suckering growth form and short-lived seeds (Robinson *et al.* 2006). The seeds have very specific germination requirements that are only met after rare flooding events followed by drawn-down conditions in spring, therefore populations of *M. ericifolia* are largely dependent on clonal growth for expansion (Robinson *et al.* 2006).

10.4.2 Biomass allocation in response to salt

The following text refers to the second question posed on p.144 of this chapter, which examines whether biomass allocation patterns differ between *B. caldwellii* and *B. medianus* clones across a range of salinities. Unlike different flooding depths, which affected biomass apportionment but not necessarily total ramet dry weight in *B. maritimus* (Clevering and Hundscheid 1998), high salinity affected both biomass allocation and total dry weight of *B. caldwellii* and *B. medianus* ramets. Although *B. caldwellii* and *B. medianus* survived at all treatment levels used in this study, the decreases in culm height, culm width and leaf length, as well as fewer leaves per ramet recorded in 12 g L^{-1} salt treatments, have serious recruitment implications for these species in wetlands facing secondary salinisation. Shorter culms place clones at greater risk of being over-topped during floods, which will

compromise the gas exchange and photosynthetic capacity of ramets, though these issues should not be significant in transient flood events. Thinner culms make above ground biomass more susceptible to mechanical damage, such as wave action, while the decline of both leaf length and leaf frequency per ramet, reduce leaf area ratios and therefore the net assimilation rates of plants. Total biomass reductions of ~ 50% for *B. caldwellii* and *B. medianus* at 12 g L^{-1} salt in this study were similar to those found for *S. robustus* grown at 10 g L^{-1} salt (Palmisano 1970; Dietert and Shontz 1978).

Reductions in LAR caused by salt will inevitably lead to a reduction in RGR, unless leaves are replaced faster than they are lost (Munns and Termaat 1986). The maintenance of culm proportions was therefore highly important to *B. caldwellii* and *B. medianus* across treatments and indeed both species had significantly fewer dormant tubers under high salinity. Interestingly, *B. caldwellii* and *B. medianus* produced proportionally fewer roots as salinity increased, which was contrary to both the finding of Palmisano (1970) who reported that culm growth of *S. robustus* was more restricted than root growth at high salinity, and the predictions of Munns and Termaat (1986), which stated that as the proportion of a plant's leaves decreases the proportion of its roots should increase. Trade-off responses in the *Bolboschoenus / Scirpus* complex, therefore, appear to be species specific. In agreement with the findings of Morris and Ganf (2001), the most prominent response of *B. medianus* to increasing salinity was elevated resource allocation into tubers, though in the present study (i.e. this thesis) additional tuber biomass did not appear to occur at the expense of culm production. Significantly fewer rhizomes were produced by *B. medianus* at high salinity, which may account for stability within culm proportions. Nonetheless, the ratio of above to below-ground biomass for *B. medianus* decreased with increasing salinity, indicating that the ecological strategy of this species to salt, involved trading-off exploration for storage and dormancy. Given the significant decrease in the RGR found for *B. medianus* under high salt, lateral growth responses of this species are likely to be too slow to compete with increasing salinity and may be counterproductive to the clone, hence the added biomass allocation to tubers rather than sister ramet production (Hutchings and Mogie 1990; Price *et al.* 1992; Clevering and Hundscheid 1998).

In contrast to *B. medianus*, the ratio of above-ground to below-ground biomass for *B. caldwellii* increased as salinity increased, though this was somewhat confusing to interpret, as percentage allocation to both tubers and culms was stable across treatments and proportionally more rhizomes were produced under high salt. Although the RGR of *B. caldwellii* significantly decreased under high salinity (from ~ 50 mg g^{-1} day^{-1} in controls to ~ 40 mg g^{-1} day^{-1}), RGR results in all treatments remained greater than those attained for *B. medianus* under control conditions. In comparison to *B. medianus*, the asexual strategy of *B. caldwellii* was to maintain exploration under all treatments. With a high RGR and a smaller overall growth form, sister ramets appeared to quickly become an asset for resource acquisition in *B. caldwellii* rather than being counterproductive to growth. Continuous exploration requires a high NAR, hence the reason for maintaining or increasing culm proportions, though it is likely that this strategy would be confounded by any further proportional biomass loss of roots, which may be predicted at higher salinities.

The overall range of RGRs for *B. caldwellii* and *B. medianus* found between high and low salinity treatments in this study, were similar to values obtained for these species in previous research conducted under different environmental stresses, such as flooding (Blanch *et al.* 1999a; Siebentritt and Ganf 2000) and salinity and nutrient interactions (Morris and Ganf 2001). However, while *B. medianus* was able to maintain RGR across water depths ranging from +20 to –60 cm below or above the sediment surface by elongating culms and maintaining net assimilation rates (see Siebentritt and Ganf 2000), results from this study showed that RGR declined by ~50% at 12 g L^{-1} salt in comparison to control treatments. Asexual growth of *B. medianus* is therefore more susceptible to increasing salinity than flooding. In contrast, the RGR of *B. caldwellii* appears more susceptible to flooding than salinity. For example, while *B. caldwellii* was unable to compensate for lost leaf area ratio at flooding depths greater than 20 cm and its RGR declined to zero at 60 cm flooding (see Siebentritt and Ganf 2000), the RGR of *B. caldwellii* in this study was not as susceptible to salinity, with only a 20% reduction recorded between control and 12 g L^{-1} salt treatments.

10.4.3 Plasticity assessments in tubers and rhizomes

The following text refers to the third question posed on pp.144-145 of this chapter. Little variation was found in the size of tubers between replicates within the same treatments, though tuber sizes for both *B. caldwellii* and *B. medianus* were highly variable within each replicate (data not included). In alignment with findings for *Scirpus maritimus* (see Lieffers and Shay 1982; Charpentier and Stuefer 1999), tuber size in *B. caldwellii* and *B. medianus* appeared to be related to position, as tuber size generally increased with each successive ramet generation (Fig. 10.9). This finding suggested that older ramets support newer ramets through resource sharing, rather than new ramets acting as scouts that forage for resources and support their parental ramets. Because the most recently produced ramets are likely to be found at the perimeter of a genet, the strategy to produce larger tubers in peripheral positions provides an effective method to ensure individual ramet survival and consolidation of space for the entire genet (Lieffers and Shay 1982; Hroudová and Zákravský 1995). Both *B. caldwellii* and *B. medianus* incorporate a trade-off between above and below-ground biomass over winter, therefore, dormant tuber banks represent the future potential of populations to recover during spring. The significant decrease in the number of dormant tubers for both species at 12 g L^{-1} suggested that higher salinities may have serious implications for the future recruitment potential (i.e. fitness) of populations.

Submergence is a decisive factor in enhancing tuber dormancy in *B. maritimus* (Hroudová and Zákravský 1995). Increasing salinity, therefore, was expected to similarly increase the number of dormant tubers in *B. caldwellii* and *B. medianus*, yet both species had fewer dormant tubers at high salinity than in the freshwater treatment. A decline in dormant tuber numbers did not necessarily mean that more tubers were 'activated' as such, but rather fewer ramets and therefore tubers were produced. Although tubers were highly variable in size, perhaps their greatest (plastic) response to salinity was simply not to sprout, as dormancy and resource storage are highly influential to the future survival and spatial organisation of the clone. While the vertical extension of culms in response to water depth rises in *Bolboschoenus* species may be seen as a plastic response to the environment that is driven by tuber reserves (Clevering and Hundscheid 1998; Blanch *et al.* 1999b;

Siebentritt and Ganf 2000; Morris and Ganf 2001), the changes to culm heights in this study resulted from salt toxicity, low water potential and the interruption of photosynthetic pathways and cannot be confused with plasticity.

A lack of plasticity was found also for *B. caldwellii* and *B. medianus* rhizomes, as their growth appeared to be pre-determined and individual ramets within each clone did not significantly adjust their rhizome lengths in response to higher salinity. The lack of plasticity found for rhizome lengths in *B. caldwellii* and *B. medianus* are suggestive of the phalanx rather than the guerilla strategy of clonal spread (sensu Lovett-Doust 1981). Although there was a higher number of rhizomes in almost all rhizome length categories under control conditions compared to saline, most length categories were represented irrespective of saline treatment, indicating that the range of phenotypic (developmental) plasticity in both species outweighs any plastic variation caused by the environment. These results agree with previous findings for *B. maritimus* grown under different water depths (Clevering and Hundscheid 1998).

The development of *B. caldwellii* and *B. medianus* also involved increasing the width of rhizomes with successive generations (Fig. 10.9). This adaptation acts to increase the storage capacity of each clone prior to entering annual dormancy cycles. While both rhizome width and length were variable they were largely predictable. The size of *B. caldwellii* and *B. medianus* rhizomes were therefore less susceptible to environmental modification than their tubers. This finding is in agreement with the review of de Kroon and Hutchings (1995) who argued that it is the localised plastic responses of roots and shoots in rhizomatous species to changing environmental conditions, that are more important than plasticity within rhizomes (i.e. the orthotropic rather than the plagiotropic growth plane).

The spatial organisation of *Bolboschoenus* clones is therefore not compromised by increasing salinity as clones do not ramify or conversely elongate their overall genet shape, but instead continue to follow a normal (non-plastic) growth pattern – albeit with reduced overall biomass and a slower RGR. Periodic salinity increases within a wetland are likely to have a negative influence on sexual reproduction for *B. caldwellii* and *B. medianus*, though the asexual growth capacity of

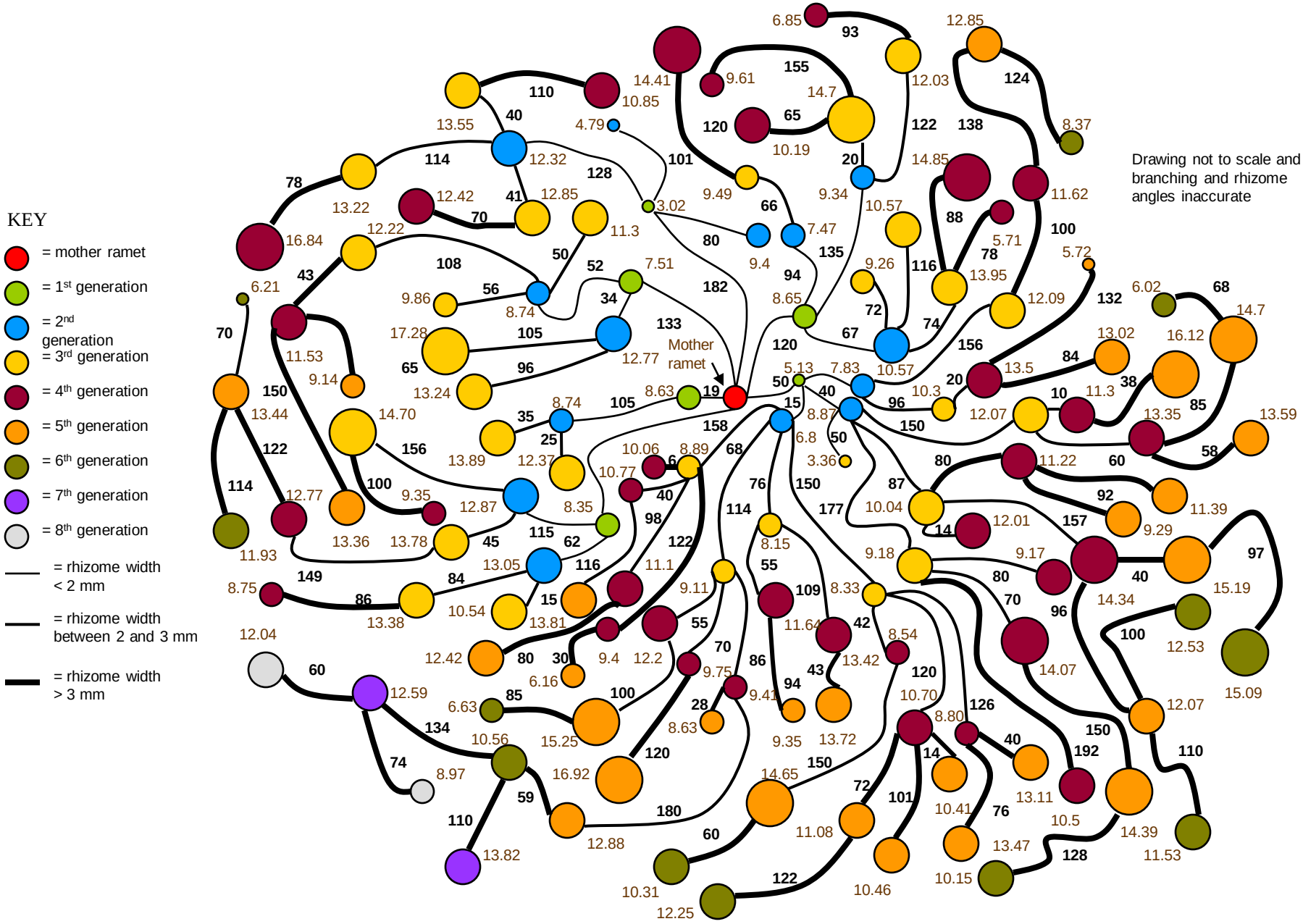


Figure 10.9. Diagrammatic representation of tubers and rhizome connections from a 4 month old *B. caldwellii* clone grown at 12 g L^{-1} salt.

both species should continue largely unimpeded at salinities as high as 12 g L^{-1}, as rhizome extension is not compromised and banks of dormant tubers are still produced to over-winter and re-emerge in spring.

10.4.4 How does the growth of B. caldwellii and B. medianus fit with trade-off models?

The following text refers to question four posed on p.145 of this chapter, which asked "how do the biomass allocation patterns of each species fit with analytical models that attempt to predict the responses of clonal plants to changing environmental conditions". Interpretations of resource trade-offs between sexual and asexual reproduction in *B. caldwellii* and *B. medianus* must be made with caution, as only *B. caldwellii* managed to flower during the experimental period, which in itself was only 3 months long, and mesocosm results appeared to contradict those observed in the field. For example, achene production results from field sites (see Chapter 3) suggested that asexual reproduction was favoured over sexual reproduction in both test species when environmental conditions were more productive (i.e. less salty); a result that fits well with the models of Sakai (1995) and Gardner and Mangel (1999). However, mesocosm results for sexual investment in *B. caldwellii* from this study were more in agreement with the alternative models of Loehle (1987) and Olejniczak (2001), as achenes were only produced under low salinity and freshwater controls. *Bolboschoenus medianus* allegedly only flowers in its second year of growth, so no conclusions could be made regarding trade-offs between sexual and asexual reproduction in this study. However, as the above-ground portion of *Bolboschoenus* plants senesce over winter and tubers become dormant, it can be concluded that *B. medianus* is totally dependent on the success of clonal growth in its first year, in order to (potentially) sexually reproduce in later years.

With regard to biomass trade-offs to specific ramet components (roots, tubers, rhizomes and culms), the responses of *B. medianus* and *B. caldwellii* differed across salinity treatments. Biomass trade-offs for *B. medianus* followed the predictions of Munns and Termaat (1986) and Munns (1993), as the ratio of above to below-ground biomass decreased with increasing salinity. Asexual growth of *B. caldwellii* in contrast, differed to *B. medianus* as the ratio of above to below-ground biomass

increased as salinity increased. Neither test species agreed with the predictions of Hutchings and de Kroon (1994) that clonal plants increase allocation to storage (tubers and rhizomes) with increased resource availability. Indeed, mesocosm experiments showed that percentage allocation to tubers was stable across treatments for *B. caldwellii* and clones produced proportionally more rhizome biomass as resource availability decreased (high salt), while *B. medianus* increased resource allocation to tubers as salinity increased.

10.5 Summary of asexual reproduction

The survival of *B. caldwellii* and *B. medianus* plants in both the mesocosm experiments conducted in this chapter and also at each of the field sites (particularly Dowd Morass and Clydebank Morass) highlight the importance of asexual growth mechanisms to the persistence of *Bolboschoenus* species growing under salinised conditions. Whereas almost all aspects of sexual reproduction were significantly inhibited at salinities > 4 g L^{-1}, asexual reproduction continued largely unaffected at a salinity of 12 g L^{-1}, which far exceeds reported limits for freshwater macrophytes.

Although this chapter focused on artificially simulated saline conditions by growing plants in man-made mesocosms, the importance of the clonal growth habit to *Bolboschoenus* survival in the field is illustrated in Figure10.10. An individual *B. medianus* genet (determined through AFLP analysis – see Chapter 11) is shown at Dowd Morass in January over three consecutive years (2006-8). The genet expanded and contracted through space and time in relation to environmental variation, through the use of tuber and rhizome storage reserves. Figure 10.10.A shows the outer margins of the clone expanding during draw-down conditions in 2006, while the internal (senescent) portion of the clone remained dormant. In 2007, the entire clone remained in dormancy due to extreme draw-down conditions (Fig. 10.10.B). In contrast, Dowd Morass remained flooded in January of 2008 and tuber sprouting was restricted to the centre of the genet, which was on higher ground (Fig. 10.10.C).

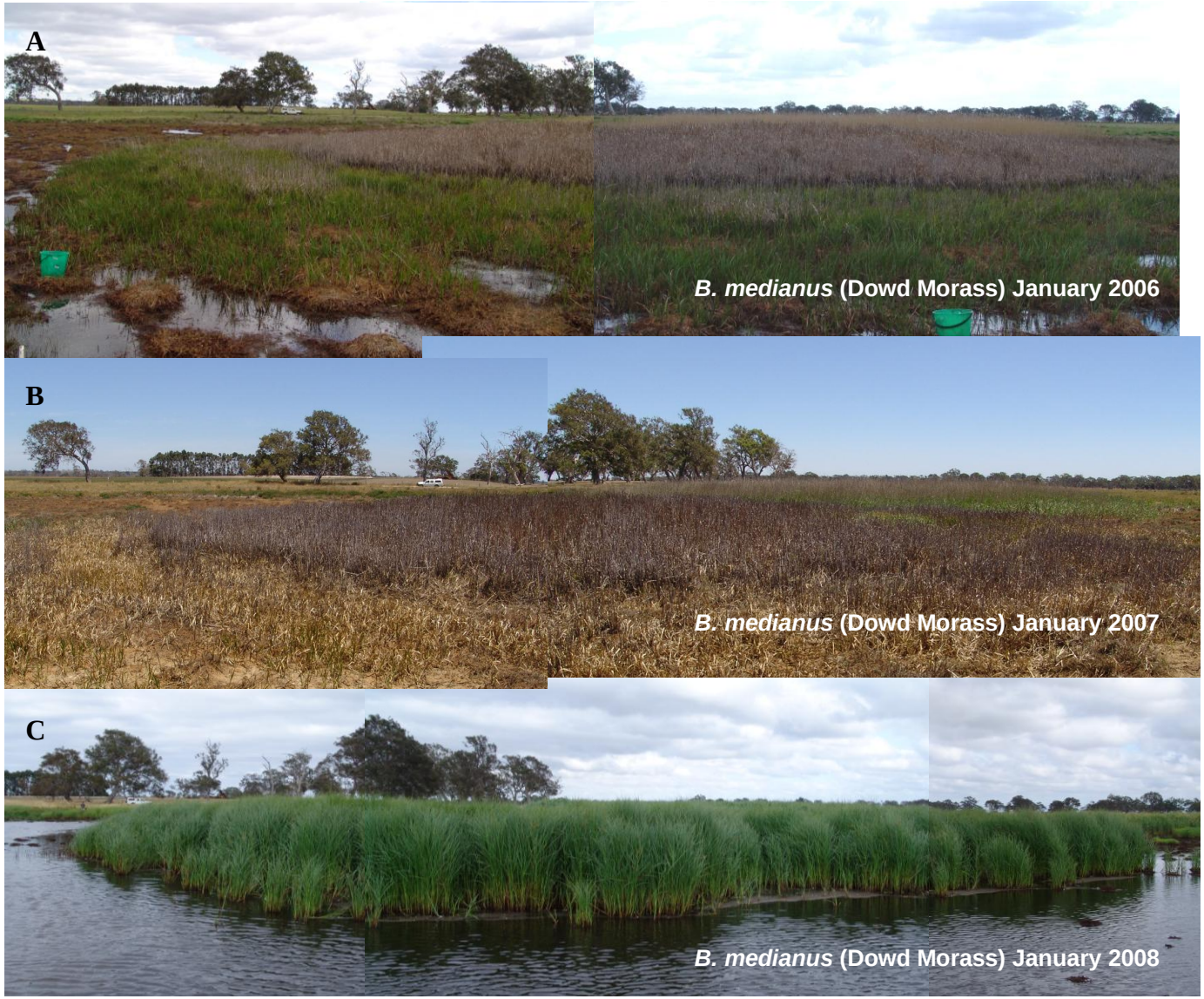


Figure 10.10. The importance of clonal growth: the same *B. medianus* genet photographed at Dowd Morass in January over three consecutive years

Section IV:

Genetics

Chapter 11.

Genetic variation and clonal diversity in *B. caldwellii* and *B. medianus*

11.1 Introduction

A number of findings from the examination of the sexual and asexual reproductive ecology of *B. caldwellii* and *B. medianus* have critical implications for the genetic diversity of *Bolboschoenus* populations. In Section II of this thesis, achene viability was high (~80%) in both *B. caldwellii* and *B. medianus* (Chapter 4) therefore trade-offs between sexual and asexual reproduction should not limit potential sexual recruitment and genetic diversity. The achenes of *B. caldwellii* have a high capacity for dispersal, whereas *B. medianus* achenes sank almost immediately (Chapter 5), which suggests that potential sexual recruitment into local populations could be higher in *B. medianus* than *B. caldwellii*. There was, however, wide differentiation in achene production levels between sites/populations due to environmental conditions (Chapter 3), and laboratory tests showed that germination was significantly restricted by salinity (Chapter 6). Therefore, at field sites with sediment salinity levels greater than 4 g L^{-1} (Dowd Morass and Clydebank Morass) there is likely to be highly restricted sexual recruitment in comparison to sites with low sediment salinity, such as Sale Common. Section III of this thesis, in contrast, highlighted that asexual recruitment was not inhibited by salt concentrations of up to 12 g L^{-1}, thus it was predicted that population structure would consists largely of vegetatively produced ramets and that genetic diversity would be low at brackish sites in comparison to freshwater wetlands.

Though clonality is a common factor to all *Bolboschoenus* species, the influence of asexual reproduction on the genetic diversity of populations remains unstudied. The aims of this chapter were: 1) to test the expectation that populations of *B. caldwellii* and *B. medianus* are dominated by few large clones, and 2) to compare genetic diversity within and between populations from wetlands with contrasting salinity levels. The implications of putative differences between sites are discussed with reference to population assessments (fitness), recruitment capacity, and conservation.

11.2 Methods

11.2.1 Sampling method

The sampling approach adopted in this study was dependent on population size and shape. If stands were large and wide (as per *B. caldwellii* at Dowd Morass and *B. medianus* at Sale Common) a 90 m straight-line transect was run through the centre of the population and a ramet sample was taken every 10 m (Figures 11.1 and 11.2). If the shape of a population followed the water line (*B. caldwellii* at Clydebank Morass) or was classically round in appearance (*B. medianus* at Dowd Morass), transects were run around the margins of the stand and samples were taken at 9 regular intervals along the length of each transect (Figures 11.3 and 11.4). At each sample point one complete ramet (stem, tuber and rhizome) was excavated and immediately transplanted into 6-inch garden pots containing peat. In this fashion ramets could be kept alive during transport to Victoria University and until such time as DNA extraction was conducted (up to 1 week post harvest). In total 18 samples per species were taken (9 per wetland). Only *B. medianus* was found at Sale Common and conversely only *B. caldwellii* was found at Clydebank Morass. The complete list of GPS coordinates for sample locations in each wetland is given in Appendix A.4.

Figure 11.1.A. Aerial photograph of *B. caldwellii* population at north Dowd Morass, showing 90 m transect line and the 9 points used for plant material sampling. The dotted line represents the perimeter of the population.

Figure 11.1.B. On-ground photograph of *B. caldwellii* population at north Dowd Morass, showing density of stems and approximate position of transect.

Figure 11.2.A. Aerial photograph of *B. medianus* population at Sale Common, showing 90 m transect line and the 9 points used for plant material sampling. The dotted line represents the perimeter of the population.

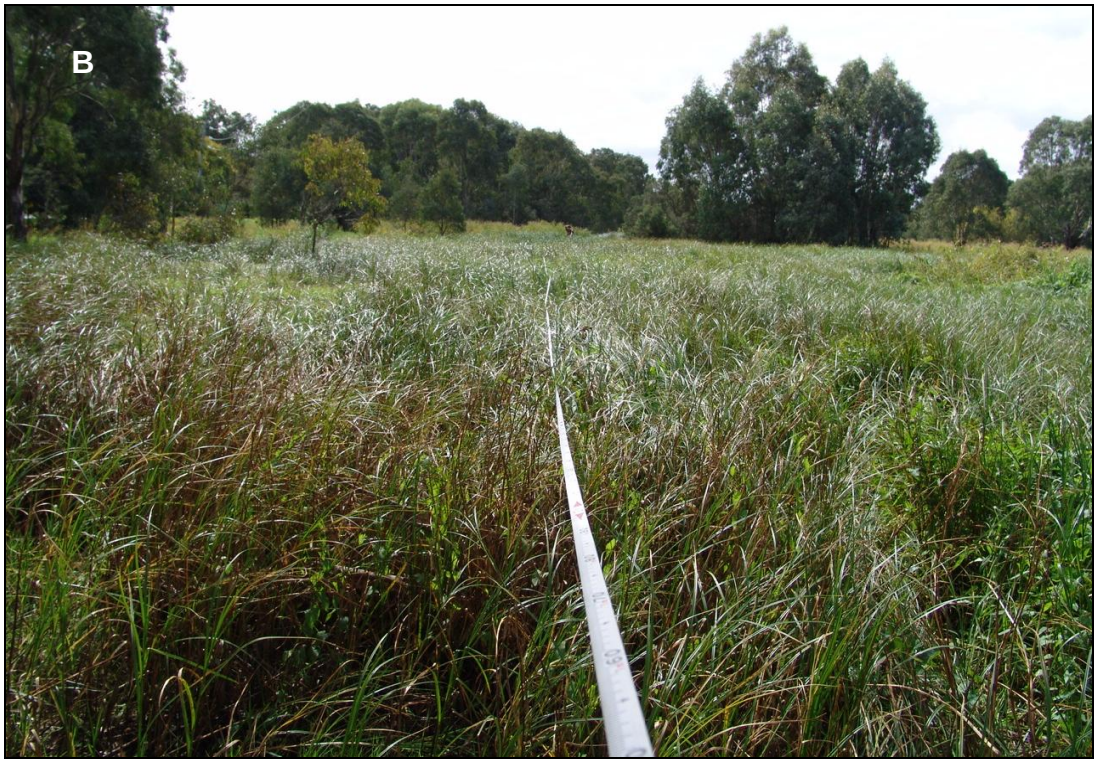


Figure 11.2.B. On-ground photograph of *B. medianus* population at Sale Common, showing density of stems and position of transect.

Figure 11.3.A. Aerial photograph of *B. caldwellii* population at Clydebank Morass, showing transect which followed the water line for approximately 420 m. The 9 samples points indicated were spaced at ~45 m intervals from each other. The dotted line represents the perimeter of the population.

Figure 11.3.B. On-ground photograph of *B. caldwellii* population at Clydebank Morass, showing part of the stand that fringed the south-eastern inlet. The pink tag in the left-hand foreground indicates a single sample point.

Figure 11.4.A. Aerial photograph of *B. medianus* population at western Dowd Morass, showing ~90 m circular transect and the 9 points used for ramet sampling. The dotted line represents the perimeter of the population.

Figure 11.4.B. On-ground photograph of *B. medianus* population at western Dowd Morass, showing one edge of the circular stand.

11.2.2 DNA extraction

Nuclear DNA was extracted from 100 mg of fresh, young leaf tissue following the protocol of the DNeasy Plant Mini Kit (QIAGEN Pty. Ltd. Doncaster, Victoria. Aust.). Leaf tissue was disrupted in 400 µl extraction buffer AP1 using five 3.2 mm chrome-steel grinding beads in a FastPrep® (FP120) tissue-lyser (Qbiogene, Montreal Canada). Samples were shaken for 40 seconds at highest frequency, put on ice for 90 seconds then disrupted again at the same frequency for a further 30 seconds. DNA was isolated according to the manufacturer's protocol, though final elution of DNA used a total of 100 µl of AE buffer instead of the recommended 200 µl, as per Ford *et al.* (2006).

Extracted DNA was quantified using a Bio-Rad SmartSpec™ Plus spectrophotometer with UV absorbance ratio of 260-280 nm. Care was taken to ensure that OD readings fell between 1.7 and 2.0, as DNA at this range is considered pure (Weising *et al.* 2005). Final DNA yields ranged from 38-105 ng μl^{-1} for *B. caldwellii* samples and 21-84 ng μl^{-1} for *B. medianus*.

11.2.3 DNA amplification

DNA fingerprinting was conducted using the AFLP® Analysis System I (Invitrogen™). Core reagents used included:

- *Eco*RI/*Mse*I restriction enzymes [1.25 units μl^{-1} each in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mg ml^{-1} BSA, 50% glycerol (v/v), 0.1% Triton X-100]
- Adapter ligation solution [*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, 50 mM K-acetate]
- 5X restriction digestion buffer [50 mM Tris-HCl (pH 7.5), 50 mM Mg-acetate, 250 mM K-acetate]
- Nuclease free distilled water
- T4 DNA ligase [1 unit μl^{-1} in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% glycerol (v/v)]
- T4 kinase [10 unit μl^{-1} in 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 1 mM 2-mercaptoethanol, 0.1 µM ATP, 50% glycerol (v/v)]
- 5X kinase buffer [350 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol]
- 10X PCR buffer plus Mg [200 mM Tris-HCl (pH 8.4), 15 mM $MgCl_2$, 500 mM KCl]
- TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA]
- *Eco*RI/*Mse*I primers at 27.8 ng μl^{-1} and 6.7 ng μl^{-1} respectively

The AFLP reaction comprised 4 main steps and followed the Invitrogen™ protocol with some minor variations. Firstly 500 ng of genomic or template DNA was digested with two different restriction enzymes to generate specific strand lengths. This is achieved as the two enzymes differ with one functioning as a frequent or four base pair cutter (*Mse*I), while the other functions as a rare or six base pair cutter (*Eco*RI). In 1.5 ml microcentrifuge tubes, 500 ng of DNA (volume dependent on sample) was added to 2 µl of *Eco*RI/*Mse*I restriction enzyme solution and 5 µl of 5X reaction buffer. The total volume of the reaction mixture was adjusted to 25 µl using distilled water. Digestion reactions were performed at 37°C for 3 hours (as per Hipp *et al.* 2006) rather than the standard 2 hours, in order to ensure the complete digestion of genomic DNA as a large starting volume was used. Mixtures were then incubated at 70°C for 15 minutes in order to inactivate the restriction enzymes.

The second step involved the ligation of adapters to each end of the liberated DNA fragments. Immediately following restriction digestion, 24 µl of adapter ligation solution and 1 µl of T4 DNA ligase were added to each mixture and incubated for 2 hours at 20°C. Upon completion, a portion of each mixture was diluted 1:10 in TE buffer in preparation for the third step known as pre-selective amplification. In 0.2 ml thin walled microcentrifuge tubes, 1 µl of diluted adapter ligation solution was added to 40 µl of preamp primer mix, 5 µl of 10X PCR buffer plus Mg and 1 µl of *Taq* DNA polymerase, then centrifuged briefly at low speed to collect the reaction. Pre-amplification was a 20 cycle event using the following steps: 30 seconds denaturation at 94°C, 1 minute annealing at 56°C, 1 minute elongation at 72°C, and a final extension of 72°C for 10 minutes (as per Scott *et al.* 1998).

The fourth step, known as selective amplification incorporates the use of specific primer sequences (see below), which bind to the adapters and allow a selective group of DNA sequences to be amplified via PCR. The reaction mixture for selective amplification was: 5 µl of 1:10 diluted pre-amplification PCR products; 5 µl of unlabeled specific *Eco*RI/*Mse*I primer mix; 2 µl of 10X PCR buffer plus Mg; 0.5 µl of *Taq* DNA polymerase (5 units/µl) and 5.9 µl of distilled water. Selective amplification was a 35 cycle event using the following parameters; 30 seconds denaturation at 94°C, 30 seconds annealing at 56°C, 1 minute elongation at 72°C and

a final extension at 72°C for 10 minutes. Enzyme digestion, pre-amplification and selective amplification events were all conducted in a Bio-Rad Mycycler™ Thermal Cycler.

11.2.4 Primer screening

PCR selective amplification used *Eco*RI and *Mse*I primers with 3 selective nucleotides. A total of 56 primer pair combinations were initially screened (see Appendix A.5) of which 5 were chosen for each species for selective amplification. Table 11.1 lists the selective nucleotide extensions used for AFLP analysis of *B. caldwellii* and *B. medianus.*

Table 11.1. Selective nucleotide extensions/combinations for EcoRI and MseI adaptors used in AFLP analysis of B. caldwellii and B. medianus.

	*Eco*RI primer extension	*Mse*I primer extension
B. caldwellii	+ AAC	+ CAA
	+ AGG	+ CAC
	+ ACG	+ CTA
	+ ACC	+ CTT
	+ AGC	+ CTT
B. medianus	+ AAC	+ CAC
	+ ACA	+ CAC
	+ ACC	+ CTC
	+ ACG	+ CTT
	+ AGC	+ CTT

11.2.5 Visualisation

AFLP products were prepared for separation by electrophoresis as follows: 2 µl of sample loading dye (50 mM Tris-HCl; 25% glycerol; 5 mM EDTA; 0.2% bromophenol blue; 0.2% xylene cyanole) were added to 5 µl of the reaction mixture. Electrophoresis used non-denaturing 5% TBE polyacrylamide ready-gels (Bio-Rad, Hercules, CA) which were pre-run for 1 hour at 100 V/cm at room temperature in a Bio-Rad, Mini-PROTEAN®II vertical electrophoresis cell. Each well contained 5 µl of AFLP product mixture and final electrophoresis was performed in TBE buffer (pH 8.0) at 40 V/cm for 125 minutes. Gels were then stained with SYBR® Green I Nucleic Acid Gel Stain (Invitrogen™) (diluted 1:10,000 in TBE buffer) for 90

minutes. Gels were photographed using a ChemiDoc™ XRS imaging system (Bio-Rad, Hercules, CA) and processed with the associated Quantity One® software package to estimate DNA fragment lengths. Specific bands were scored as present (1) or absent (0) for each sample and only unambiguous bands were scored and considered for analysis. It should be noted also that AFLP analysis produces dominant markers, therefore, homozygous dominant alleles (e.g. AA) could not be distinguished from heterozygous alleles (Aa), though homozygous recessive (aa) alleles were distinguishable.

11.2.6 Data analysis

To estimate comparative levels of clonal diversity both within and between populations, two measures of genotypic diversity were calculated after Ellstrand and Roose (1987): i) the number of clones detected (G) divided by the sample size (N); ii) Simpson's index of diversity (Parker 1979), $D = 1 - [\Sigma n_i(n_i - 1)]/[N(N - 1)]$, where N is the total number of samples, and n_i is the number of samples with the AFLP profile i present in that population. The index D ranges from 0 to 1 with 0 indicating an entirely clonal population, and 1 indicating a population composed only of unique genotypes and no clones. These statistical techniques have been employed successfully in a wide range of studies on clonal plant species using DNA fingerprinting techniques with dominant data (Escaravage *et al.* 1998; Pappert *et al.* 2000; Suyama *et al.* 2000; Dong *et al.* 2006; Kameyama and Ohara 2006).

In addition to the above calculations, each of the data sets was examined both individually and collectively by Cluster tree analysis using SYSTAT® statistical software (Cranes®, San Jose, California).

11.3 Results

Amplified fragment length polymorphism analysis proved suitable for clone identification in the genus *Bolboschoenus*. As summarised below, genetic homogeneity was apparent for stands of *B. caldwellii* at both field sites (Clydebank Morass and Dowd Morass) and also for *B. medianus* at Dowd Morass, while substantial genetic diversity was found for *B. medianus* at Sale Common.

11.3.1 Genetic diversity of B. caldwellii at Clydebank Morass

Of the five AFLP primer combinations tested on *B. caldwellii* samples from Clydebank Morass, 29 different bands were produced which ranged from 33-376 bp. All bands proved to be monomorphic within each primer pair (e.g. Fig. 11.5). Accordingly, the number of clones detected (G) divided by sample size (N) was very low (0.11), and the estimate of Simpson's diversity index was 0, which indicated an entirely clonal population. This result was supported by cluster tree analysis, which revealed no Euclidean distance between cases.

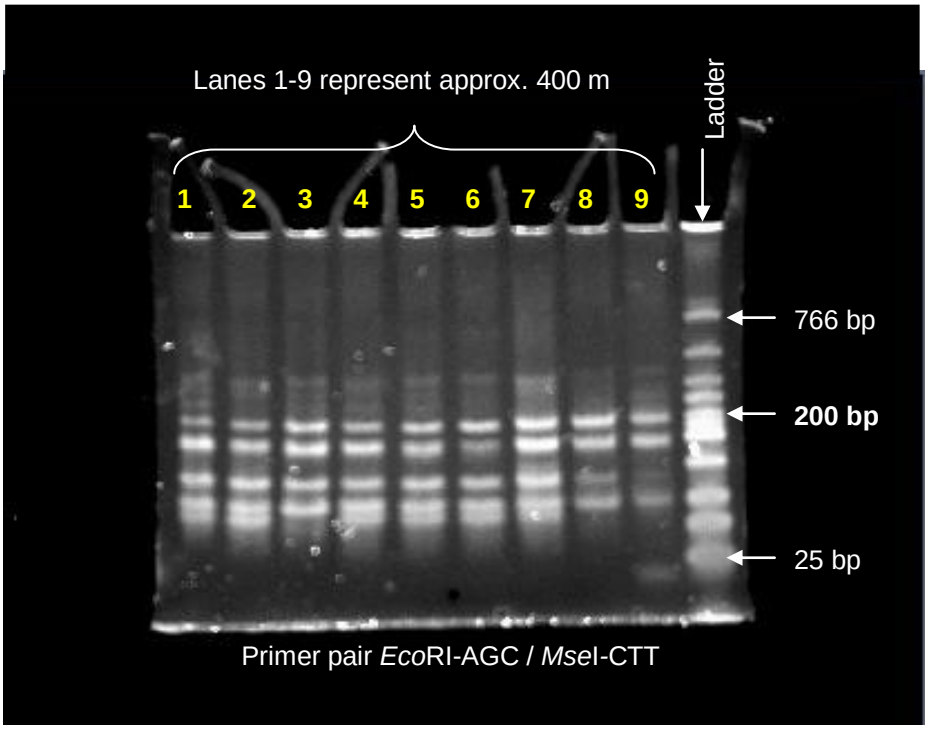


Figure 11.5 AFLP profile for nine *B. caldwellii* samples taken at Clydebank Morass using primer pair *Eco*RI-AGC and *Mse*I-CTT.

11.3.2 Genetic diversity of B. caldwellii at Dowd Morass

Of the five AFLP primer combinations tested on *B. caldwellii* samples from Dowd Morass, 31 different bands were produced, which ranged from 50-508 bp. All bands proved to be monomorphic within each primer pair (e.g. Fig. 11.6). Accordingly, the number of clones detected (G) divided by sample size (N) was very low (0.11), and the estimate of Simpson's diversity index was 0, which indicated an entirely clonal population. This result was supported by cluster tree analysis, which revealed no Euclidean distance between cases.

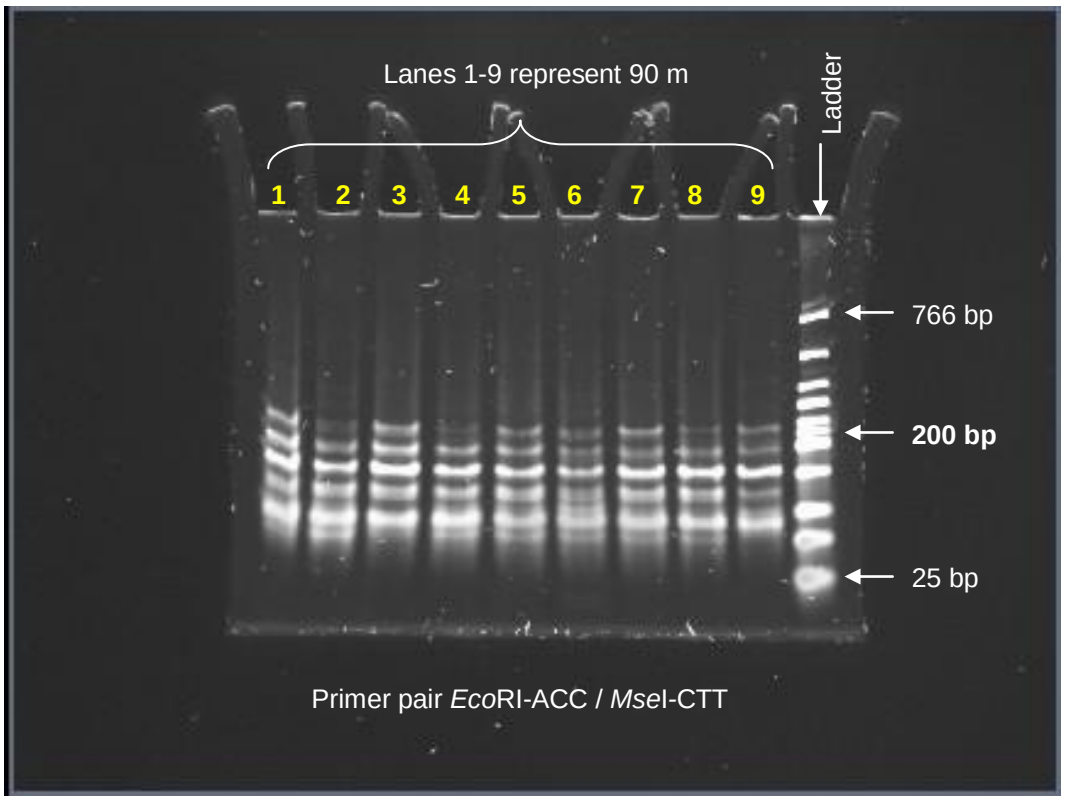


Figure 11.6 AFLP profile for nine *B. caldwellii* samples taken at Dowd Morass using primer pair *Eco*RI-ACC and *Mse*I-CTT.

11.3.3 Genetic diversity of B. medianus at Dowd Morass

Of the five AFLP primer combinations tested on *B. medianus* samples from Dowd Morass, 34 different bands were produced, which ranged from 42-489 bp. All bands proved to be monomorphic within each primer pair (e.g. Fig. 11.7). Accordingly, the number of clones detected (G) divided by sample size (N) was very low (0.11), and the estimate of Simpson's diversity index was 0, which indicated an entirely clonal population. This result was supported by cluster tree analysis, which revealed no Euclidean distance between cases.

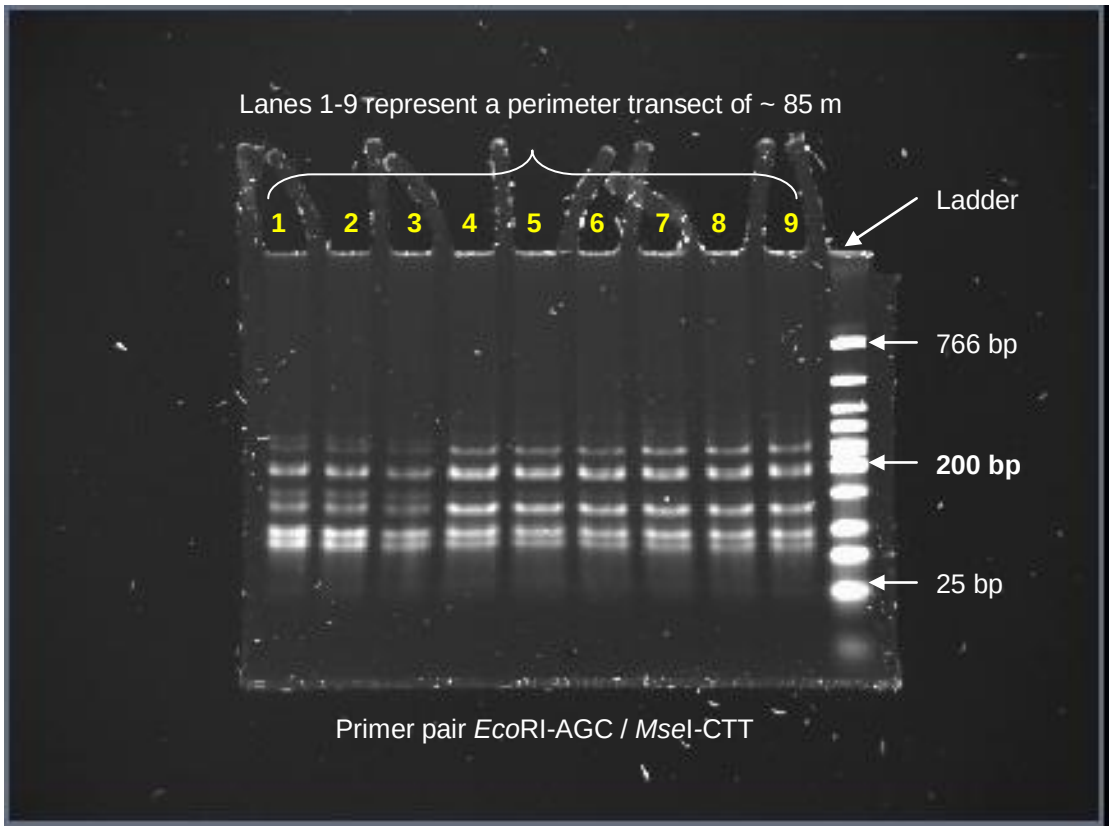


Figure 11.7 AFLP profile for nine *B. medianus* samples taken at Dowd Morass using primer pair *Eco*RI-AGC and *Mse*I-CTT.

11.3.4 Genetic diversity of B. medianus at Sale Common

The five AFLP primer combinations used to analyse *B. medianus* samples from Sale Common, revealed 159 bands (31.8 per primer pair) of which 127 were polymorphic (79.9%). Bands ranged from 42-531 bp and no bands were common (monomorphic) to all primers (e.g. Fig. 11.8). The number of clones detected (G) divided by sample size (N) was 0.66, and the estimate of Simpson's diversity index (D) was 0.86, which indicated a population of mixed genetic diversity.

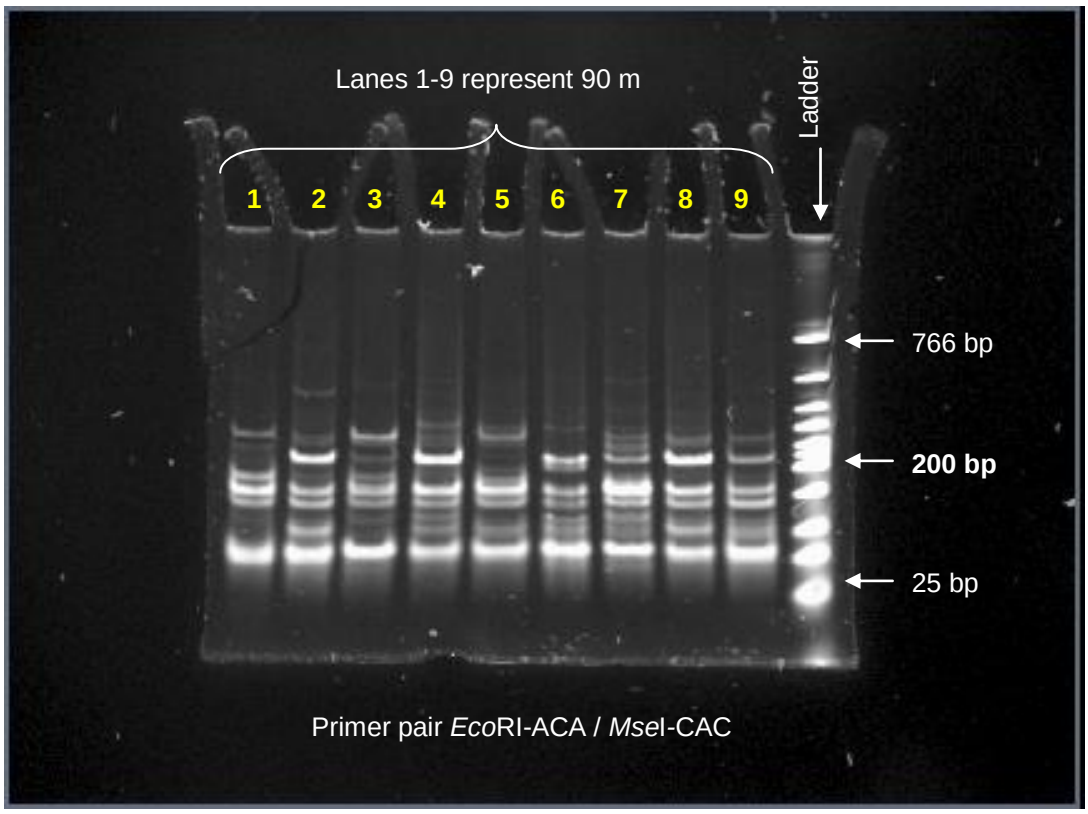
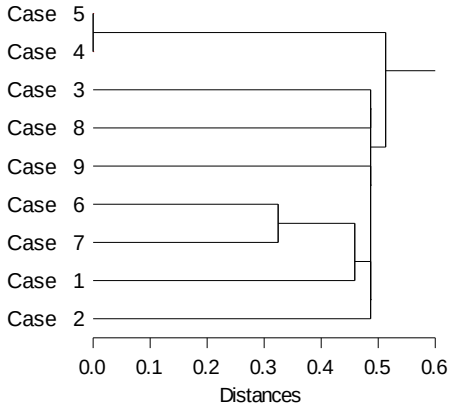


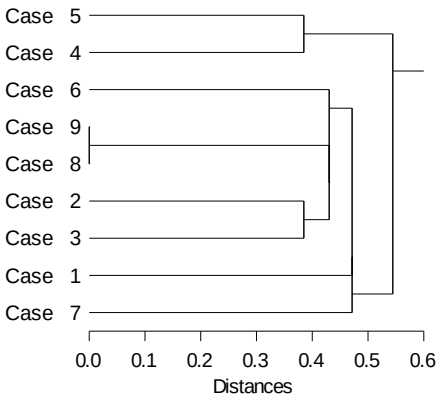
Figure 11.8 AFLP profile for nine *B. medianus* samples taken at Sale Common using primer pair *Eco*RI-ACA and *Mse*I-CAC.

Cluster tree analysis of individual primer pair combinations for *B. medianus* samples from Sale Common revealed a number of common groupings, such as cases 4 and 5, and 8 and 9, which suggested the samples were derived from the same genet (Fig. 11.9). Other samples such as cases 1 and 6 were largely independent of one another and indicative of arising from separate zygotes.

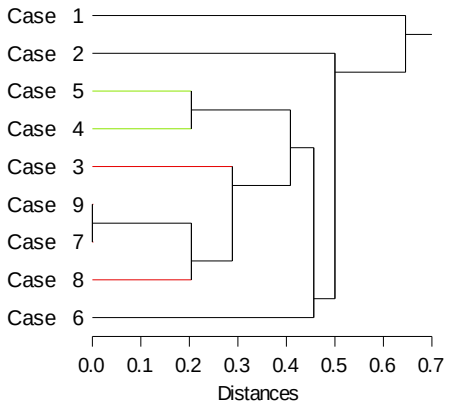
A) Primer pair *Eco*RI-AAC / *Mse*I-CAC



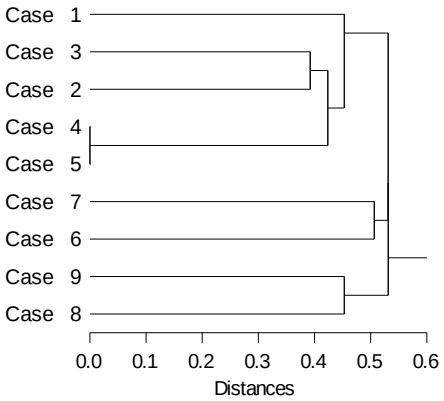
B) Primer pair *Eco*RI-ACA / *Mse*I-CAC



C) Primer pair *Eco*RI-ACC/ *Mse*I-CTC



D) Primer pair *Eco*RI-ACG / *Mse*I-CTT



E) Primer pair *Eco*RI-AGC/ *Mse*I-CTT

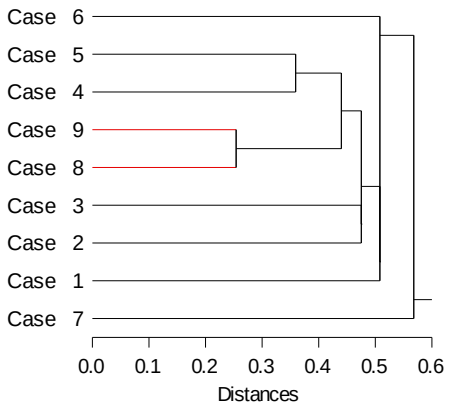


Figure 11.9.A-E Cluster tree analyses of *B. medianus* samples from Sale Common for each of five primer pair combinations.

Combined primer pair data for *B. medianus* from Sale Common produced a cluster tree of similar pattern to those in Figure 11.9, with cases 4 and 5, and 8 and 9 recognised as the same clones, though in contrast cases 3 and 7 were also indicated to share the same genotype (Fig. 11.10).

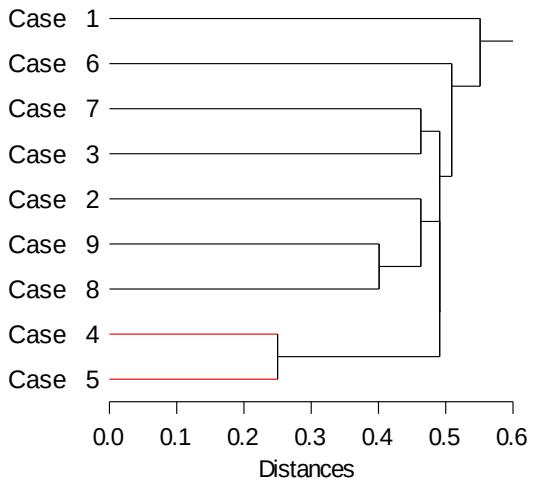


Figure 11.10 Cluster tree of combined primer pair data for *B. medianus* samples from Sale Common.

11.3.5 Combined data analysis for each species from different wetlands

Cluster tree analysis of combined primer pair data for *B. caldwellii* samples from Clydebank Morass (cases 1-9) and Dowd Morass (cases 10-18) showed no genetic overlap between wetlands (Fig. 11.11). This result indicates that there has been minimal, if any, gene flow between sites and also suggested that extant genotypes may be adaptive within each wetland. Similarly, cluster tree analysis of combined primer pair data for *B. medianus* samples from Sale Common (cases 1-9) and Dowd Morass (cases 10-18) showed no genetic overlap between wetlands (Fig. 11.12) and indicated that there was minimal, if any, gene flow between sites, as well as suggesting that genotypes may be adaptive within each wetland.

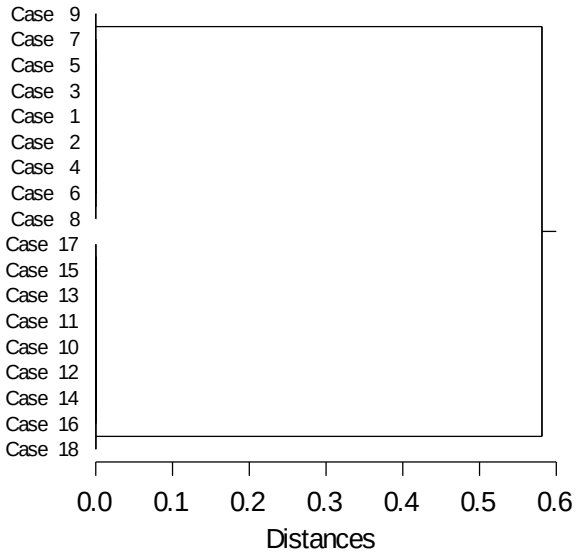


Figure 11.11 Cluster tree analysis of combined primer pair data for *B. caldwellii* samples from Clydebank Morass (cases 1-9) and Dowd Morass (cases 10-18).

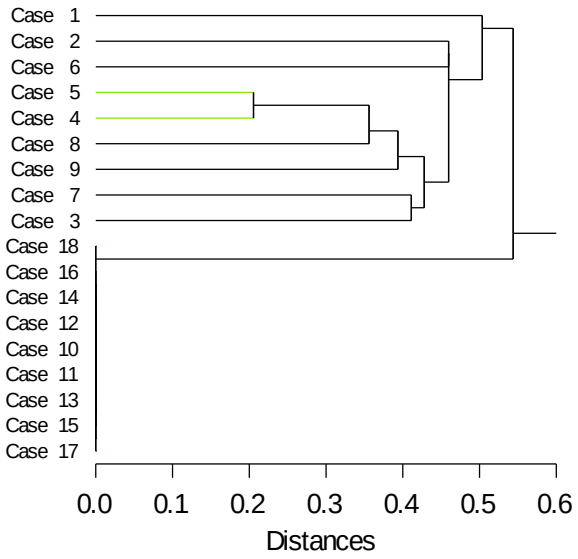


Figure 11.12 Cluster tree analysis of combined primer pair data for *B. medianus* from Sale Common (cases 1-9) and Dowd Morass (cases 10-18).

11.4 Discussion

11.4.1 Amplified fragment length polymorphisms (AFLP)

Amplifies fragment length polymorphism (AFLP) techniques were developed by Vos *et al.* (1995) and essentially incorporate former restriction fragment length polymorphism (RFLP) principles with polymerase chain reaction (PCR) technology. Amplifies fragment length polymorphism has become a widely used technique for producing DNA fingerprints due to its broad taxonomic scope, as no prior genome/sequence information is required, hence, any organism can be tested. In addition a limited set of primers can be used in multiple combinations to produce a large set of fragments/polymorphisms. The versatility of the AFLP technique has been demonstrated through application in areas such as molecular taxonomy (Kardolus *et al.* 1998; Van Droogenbroek *et al.* 2002; Deprés *et al.* 2003) and population genetics (Gaudeul *et al.* 2000; Campbell *et al.* 2003) where it has enabled the identification of clones (Pornon *et al.* 2000; Suyama *et al.* 2000), hybrids (Scotti *et al.* 2002) cultivars (Tyrka *et al.* 2002) and sports (Debener *et al.* 2000).

Amplifies fragment length polymorphisms have been shown to be more robust than the majority of other genetic marker techniques, as they are produced in stringent PCR conditions, eliminating any artifactual variation that is often found in RAPD analysis. In addition, only a small amount of starting material is required for AFLP and the analyses are relatively insensitive to variations in template concentration (e.g. Hansen *et al.* 1999; Myburg *et al.* 2001). Amplifies fragment length polymorphism products are also highly reproducible, although the genetic fingerprints generated are not always identical between two ramets from the same genet as a result of accumulating somatic mutations (Arens 1998; Cabrita *et al.* 2001; Douhovnikoff and Dodd 2003; Portis *et al.* 2004). DNA mutation rates differ greatly depending on the type of mutation, the type of organism, the genome type [nuclear, chloroplast (cp) or mitochondrial (mt)] and the type of sequence targeted. Mutation rates of silent nucleotide substitutions in plant mtDNA have been calculated at about one third of the neutral rate in cpDNA and about 1/12 of the rate of nuclear DNA (Wolfe *et al.* 1987; Yang *et al.* 1999). This study, therefore, chose to look at nuclear DNA, as the higher mutation rates in theory allow greater discrimination between clones. One

problem faced with inquiry into clonal organisms, however, is the ability to distinguish between polymorphic variation derived from meiotic recombination (sexual reproduction) and those generated by somatic mutation. For example, when plants are cloned (i.e. grown from tissue culture) regenerates may differ from the parental type. This phenomenon is known as somaclonal variation and is often expressed as a difference in phenotype (Weising *et al.* 2005). Somaclonal variation has been demonstrated in species such as *Oryza sativa* (rice) (Godwin *et al.* 1997), though conventional methodologies for DNA fingerprinting are generally not able to detect somatic mutations, which may mean that phenotypic variation is also due to epigenetic factors. However, while AFLP analysis of normal and abnormal tissue cultured clones of *Elaeis guineensis* (Oil palm), using traditional restriction enzyme combinations such as *Eco*RI-*Mse*I, were unable to discern polymorphisms, some variation was noted when methylation sensitive digestion enzymes such as *Hpa*II, *Pst*I and *Msp*I were utilised instead (Matthes *et al.* 2001). This variation of AFLP analysis is known as methylation sensitive amplified polymorphism (MSAP) (Xiong *et al.* 1999). Only the conventional coupling of *Eco*RI and *Mse*I restriction enzymes, were used in this study, hence there was very little probability that genetic differences detected resulted from somatic mutation rather than meiotic recombination. Polymorphic differences between samples were therefore thought to derive from sexual reproduction.

11.4.2 Bolboschoenus genetics and environmental variation

The use of AFLP analysis confirmed suspicions that the inherent genetic diversity of populations of *Bolboschoenus* species can be highly cryptic. While populations may appear genetically diverse with high fitness due to the presence of many thousands of separate stems, they may in fact be genetically homogenous (excepting somatic mutation). The results of this study indicate that caution must be exercised when population assessments are based purely on ramet numbers or morphological differences: primer pair combinations for both species produced distinct multilocus fingerprints that clearly indicated mono-clonal patches at Dowd Morass and Clydebank Morass, while at Sale Common several discrete clones were detected. As both Clydebank Morass and Dowd Morass are brackish to highly saline sites in comparison to Sale Common (see Chapter 3), the genetic diversity patterns

found within each wetland strongly suggest a relationship between site quality and clonal structure. Similar conclusions were made for *Sasa senanensis* (Dwarf bamboo) in relation to site quality, such as the slope of terrain and depth of the soil profile (Suyama *et al.* 2000). While sediment salinity differences between sites were significant, other factors such as pH may also be influential in producing the resultant genetic patterns and should be considered when assessing population status.

The lack of genetic similarity between like species from different wetlands supported the notion that genotypes were derived from completely unrelated parentages and that they may be locally adapted. A clonal variant with a superior genotype is well suited for survival in salinised wetlands as asexual reproduction can preserve adaptive gene complexes, whereas sexual reproduction constantly reshuffles co-adapted genomes (Vrijenhoek 1979; McLellan *et al.* 1997). Even if clonal species such as *Bolboschoenus* lack the morphological plasticity (Section III of this thesis) required to track suitable microsites, they may overcome environmental heterogeneity through ramet integration and the capacity to share resources (Silander 1985; Hutchings and de Kroon 1994).

Ultimately, interpretations of the AFLP profiles obtained in this study are open to question. The uniformity of DNA fingerprints among samples at Dowd Morass and Clydebank Morass could be due to explicit clonal reproduction, or they may represent assay insensitivity as it is possible that identical profiles could arise from sexual reproduction, whereby technically different genetic individuals do not exhibit segregational heterozygosity at the amplified loci (Hollingsworth and Bailey 2000). As *Bolboschoenus* species may self-pollinate, a shift to inbreeding could, in theory, also result in the sexual reproduction of offspring that are as genetically uniform as those produced by asexual means (Philbrick and Les 1996). Given the successful delineation of clones within Sale Common, as well as the fact that five different primer pair combinations were utilised for each inquiry, the most likely explanation is that identical AFLP fingerprints represent ramets arising from one genet.

11.4.3 Recruitment patterns in B. caldwellii and B. medianus

It is very difficult to determine the recruitment patterns of plant populations *post-hoc*, though in freshwater wetlands such as Sale Common, which has sediment salinity typically <1-2 g L^{-1} (Chapters 2 & 3) sexual recruitment is likely to have occurred repeatedly over time as conditions are within the limits for seed germination. This was reflected in the higher levels of genetic diversity found within the examined *B. medianus* population at Sale Common in comparison to the other field sites. Alternatively, in salinised sites such as the *B. caldwellii* population at northern Dowd Morass, where sediment salinity exceeded 40 g L^{-1} (Chapter 3), it is highly unlikely that repeated sexual recruitment into existing populations has occurred. In such situations, seed recruitment for clonal plants is expected to follow the 'strawberry coral' model (Williams 1975), whereby an initial mass seed colonisation event occurs, then as ramet densities increase via vegetative growth, less fit genets are progressively thinned out until a single genet reaches fixation (Hartnett and Bazzaz 1985; Ellstrand and Roose 1987; McLellan *et al.* 1997). Alternatively, it is conceivable that a 'population' may begin via a single seed germinating within a suitable microsite, before progressing to a large genet size through vegetative growth as proposed for species such as *Lomatia tasmanica* (Lynch and Balmer 2004). The genetic uniformity of *B. caldwellii* and *B. medianus* at Dowd Morass and Clydebank Morass support both scenarios and it is not possible to discriminate between the two alternatives.

The extent of clonality in a given population is likely to reflect the relative opportunity for sexual recruitment and will vary widely between locations. For example, Ellstrand and Roose (1987) and Widen *et al.* (1994) reviewed 17 and 35 clonal species respectively, and both studies reported Simpson's diversity indexes (D) to range from 0.13 to 1.0 depending on the species and location. In addition, Widen *et al.* (1994) reported that 91% of the surveyed species displayed multi-clonal populations, though uni-clonal populations were also found for 22 of the 35 species surveyed (63%).

Changes to environmental conditions over time will also be of great influence to the genetic make-up of populations. For example, major landscape-scale alterations in the Gippsland Lakes region such as the formation of the Macalister

irrigation district and the permanent opening of Lakes Entrance to Bass Strait, have altered the historical hydrological and salinity regimes of the wetlands in this region and shifted vegetation communities from a dominance of freshwater macrophytes to predominantly halophytic (salt tolerant) species (Boon *et al.* 2007; Hatton *et al.* 2008). In many instances, the conditions required for germination are no longer available and plant species are forced to rely on asexual reproduction. With reference to *B. caldwellii* and *B. medianus*, these species were likely present prior to the salinisation of the Gippsland Lakes region and able to recruit sexually, though the environmental changes now appear to have restricted their recruitment to exclusively clonal reproduction.

A number of recent studies have revealed similar dynamics. For example, prolonged flooding has shifted the vegetation composition of a Florida swamp towards clonal species (Ernst and Brooks 2003). Conversely, heavily restricted water flows into floodplain and riparian zones have left species such as *Muehlenbeckia florulenta* entirely reliant on asexual reproduction (Chong and Walker 2005). Similarly, habitat fragmentation and associated pollen flow reductions or pollen incompatibility can reduce fecundity and lead to asexual growth dependence, as shown for *Scirpus maritimus* (Charpentier *et al.* 2000), *Santalum lanceolatum* (Warburton *et al.* 2000) and *Elaeocarpus williamsianus* (Rossetto *et al.* 2004). Clonal dependence is often also the norm at the present-day distributional range limits of many species, such as *Decodon verticillatus* (Eckert 2002) and *Juniperus sabina* (Wesche *et al.* 2005). Seed germination is now extremely rare or non-existent in these populations, as the initial establishment of seedlings is proposed to have taken place during periods defined by more favourable climatic conditions (Wesche *et al.* 2005).

11.4.4 Genet sizes and growth behaviour of Bolboschoenus clones

Amplified fragment length polymorphism results clearly provided evidence that *Bolboschoenus* genets are capable of reaching far greater sizes than the 25 m diameter noted for *Scirpus maritimus* by Kantrud (1996). The exact size and nature of growth of *Bolboschoenus* genets could not be determined at each wetland due to the simplicity of the sampling designs used in this study, though the length of a single

B. caldwellii clone at Dowd Morass was demonstrated to cover at least 90 m and at Clydebank Morass, a single clone was shown to stretch over ~ 400 m. Further testing would be necessary to confirm the genetic extent of these clones, though it was suspected that the entire *B. caldwellii* populations at both Dowd Morass and Clydebank Morass were composed of a single genet each and that these clones covered approximately 13,000 m^2 and 16,600 m^2 respectively (see Figures 11.1.A and 11.3.A). Similar large-scale clones, which ranged from 1,174-3,274 m^2 were recently documented for *Melaleuca ericifolia* at Dowd Morass (Robinson *et al.* In review).

The lack of genetic variation found across the different sampling scales used to investigate *B. caldwellii* (10 m intervals at Dowd Morass and 40-45 m intervals at Clydebank Morass) suggested that *B. caldwellii* adopts a phalanx clonal growth form (*sensu* Lovett-Doust 1981). The phalanx growth form was also largely confirmed for *B. medianus* at Dowd Morass through the adoption of a circular sampling design. Even though testing was not conducted within the centre of the patch (see Fig. 11.4.A) the genetic uniformity of all samples strongly indicated a single circular genet (typical of the phalanx growth form) with a surface area of ~530 m^2. In contrast, the 10 m interval sampling strategy used to examine *B. medianus* ramets at Sale Common (Fig. 11.2.A.) suggested that genet sizes in a fresh water environment may on average be smaller than those found in brackish sites, as only two distinct genets were located in consecutive sampling points. The genetic diversity value (G = 0.66) and Simpson's diversity index (D = 0.86) indicated a mixed genet population at Sale Common and further sampling on a grid basis would be required to discover the exact size of clones and their growth behaviour. Nonetheless, genet sizes estimated in this study were far larger than previous reports for Cyperaceae species in harsh environments, such as <20 m^2 for the heath-land species *Carex bigelowii* (Jonsson 1995) and <2 m^2 for the alpine species *Carex curvula* (Steinger *et al.* 1996).

11.4.5 Conclusion

While the amount of genetic diversity found for *B. medianus* under freshwater conditions at Sale Common suggest a high level of fitness in terms of evolutionary potential, the same conclusions cannot be made for *B. caldwellii* and *B. medianus* under salinised conditions at Clydebank Morass and Dowd Morass. In the short term,

the successful spread of a single genotype may temporarily outweigh the benefits of sexual reproduction, but there may be associated costs with asexual dependence such as the 'loss of sex' itself, which can occur through the accumulation of neutral sterility mutations that are unable to be removed by meiosis, a theory known as Müller's Ratchet (Müller 1964; Eckert 2002).

The findings of this chapter are of importance to the management of wetlands in the Gippsland Lakes for a number of reasons. Firstly, the discovery of single large clones at the salinised sites of Dowd Morass and Clydebank Morass, indicates that current conservation approaches are preserving very little genetic diversity. Secondly, seed collection at these field sites is likely to occur from just one or few clones, limiting the genetic contribution of future revegetation trials. Thirdly, revegetation practices in which large numbers of individuals are planted at close densities (<1-2 m), are likely to reduce the competitive advantages of clonal growth mechanisms such as resource sharing, resource foraging and division of labour, which allow clonal plants to compensate for environmental heterogeneity.

Section V:

Thesis summary and conclusions

Chapter 12.

Summary of the reproductive ecology of *B. caldwellii* and *B. medianus*

The fundamental aim of this thesis was to investigate the reproductive ecology of *B. caldwellii* and *B. medianus* populations within the Gippsland Lakes region. The region has undergone major landscape-scale changes within the past 100-150 years particularly as a result of the permanent opening of Lakes Entrance to Bass Strait in 1889. Increasing salinity has proven one of the most pervasive influences of these changes. For those wetlands with hydrological links to Lake Wellington, increasing salinity has altered wetland vegetation communities from a dominance of freshwater species to dominance by salt tolerant species. *Bolboschoenus caldwellii* and *B. medianus* are two of the few non-salt-specialised taxa remaining in the region. Salinity increases have restricted the ability of *Bolboschoenus* species to reproduce sexually and subsequently imposed a reliance on asexual recruitment for long-term survival. Thus, the major prediction made in this study was that *Bolboschoenus* populations would be genetically sparse or indeed homogeneous, despite the fact that stands may contain many thousands of stems.

A conceptual model of *Bolboschoenus* recruitment (Fig. 1.10) was used to illustrate each of the life-history phases examined in this thesis. Eight phases/steps were included in the model to assess the sexual and asexual reproduction of *B. caldwellii* and *B. medianus*, as well as current population genetics. Each of the phases are summarised below.

12.1 Sexual reproduction

12.1.1 Achene production and sediment seed banks

The analysis of achene production in Chapter three indicated that achenes of both *B. caldwellii* and *B. medianus* are produced in very low numbers (<2,300 m^2), which is a common trait of most Cyperaceae species. The low number of achenes recovered from sediment seed banks from each field site (<11,200 m^2) was consistent with poor achene production. The findings suggested that poor achene production and low numbers of achenes stored in the sediment bank are likely to contribute to rare sexual recruitment events for *B. caldwellii* and *B. medianus*. Low achene production is also likely to reduce the probability of achene dispersal to suitable germination niches. Interestingly, achene production was low in both fresh and brackish water wetlands, suggesting that production levels are unlikely to be influenced by management intervention through changes to hydrological and salinity regimes. The successful germination of achenes recovered from sediment cores from all field sites indicated that *B. caldwellii* and *B. medianus* are capable of forming persistent seed banks. Annual achene production, therefore, is not integral to future sexual reproduction of *Bolboschoenus* species.

12.1.2 Achene viability

Both fresh and 1-year-old achenes of *B. caldwellii* and *B. medianus* had consistently high viability (~80%) irrespective of the source of plants. These results strongly indicate that poor viability was unlikely to be a contributing factor to the lack of sexual recruitment observed for the two *Bolboschoenus* species in the wetlands of the Gippsland Lakes region.

12.1.3 Dispersal

Contrasting dispersal mechanisms were displayed by *B. medianus* and *B. caldwellii*. Achenes of the former species contained little aeriferous tissue in their pericarp layer and sank almost immediately. Thus, at each field site achenes probably join the local sediment seed bank only and do not disperse far from the parental population. In contrast, *B. caldwellii* achenes contained substantial aeriferous tissue,

which enabled them to remain afloat for up to 3 months. This ability likely offers *B. caldwellii* achenes a higher probability of long-distance dispersal and allows them to find recruitment windows in both time and space. The differences in buoyancy between the two species could account for the typical differentiation reported in their landscape positions by Siebentritt and Ganf (2000). Endozoochory via waterbirds is likely to increase the scope of achene dispersal in both species to a regional or even global scale.

12.1.4 Light, temperature and salinity effects on germination

Germination of achenes for both species was light dependent and required wide diurnal temperature variations of at least 20-25°C. Salinity significantly affected germinability, though achenes of both species were able to recover from salinity as high as 32 g L^{-1} when transferred to freshwater. As little germination was recorded above a salinity of about 4 g L^{-1}, salt could be a major factor in affecting achene germination and thus sexual recruitment. Unless salt is leached from wetlands (such as Clydebank Morass and Dowd Morass) by periodic flushing events, sexual recruitment is highly unlikely. Large-scale irrigation, levee banks and periodic drought restrict freshwater input to the majority of wetlands fringing the Gippsland Lakes, thus, salinity is likely a significant environmental sieve for the sexual reproduction of *B. caldwellii* and *B. medianus*.

12.1.5 Additional requirements for germination

Cold-wet stratification of 4 weeks or greater and scarification of achenes, either for several days in weak acid or by manual scarification with a razor blade, significantly improved germination for both species. Stratification and scarification pre-treatments were especially effective at improving germination of *B. medianus* achenes. Most importantly, stratification and scarification trials widened the temperature range at which germination could take place for both species.

12.1.6 Hypocotyl hairs

Hypocotyl hairs were found in a range of Cyperaceae species, including *B. caldwellii* and *B. medianus*. The production of hypocotyl hairs was independent of

the true root system and critical to early germination phases and the establishment of young seedlings. Fine-scale water variation of just 1-2 mm and low salinity (2-4 g L^{-1}) was enough to impede the development of hypocotyl hairs, and in all cases where hypocotyl hairs were absent or did not form completely, radicle formation was compromised and achenes failed to complete germination. Hypocotyl hair formation differed between species with *B. caldwellii* producing hypocotyl hairs under drier conditions than *B. medianus*, which in turn produced hypocotyl hairs under wetter conditions than *B. caldwellii*. The species-specific differences in hypocotyl hair formation may define the typical gradient differentiation noted for each species (i.e. *B. caldwellii* at higher and drier elevations than *B. medianus*, which is more often found at the waterline). Hypocotyl hairs have never been reported for Cyperaceae species in the scientific literature and may be an overlooked element that is influential to the early stages of seedling development and establishment.

12.1.7 Summary

Table 12.1 summarises the sexual reproductive (achene) differences and similarities found between the two sympatric species examined in this thesis.

Table 12.1. Comparative achene characteristics of *B. caldwellii* and *B. medianus*

Achene characteristic	*B. caldwellii*	*B. medianus*
Anatomy	Complex pericarp with substantial aeriferous tissue	Complex pericarp with minimal aeriferous tissue
Viability	High	High
Dispersal	High capacity for hydrochory and endozoochory	Low capacity for hydrochory (though high potential for endozoochory)
Polymorphic	Size and rare shape	Size and shape
# Produced / m^2	Low (< 2,300)	Low (< 2,000)
Seed bank	Persistent	Persistent
Dormancy	High though easily broken	High and difficult to break
Stratification	Slight positive influence	Highly positive influence
Scarification	Slight positive influence	Highly positive influence
Hypocotyl hairs	Present	Present

12.2 Asexual reproduction

In contrast to almost all aspects of sexual reproduction, which were significantly inhibited at salinities >4 g L^{-1}, asexual reproduction in both *B. caldwellii* and *B. medianus* continued largely unaffected at a salinity of 12 g L^{-1}. The difference highlighted the importance of clonal growth to population dynamics in salinised wetlands. Although total biomass was reduced by ~50% at 12 g L^{-1} in both species, trade-off responses in biomass allocation were species-specific. The most prominent response of *B. medianus* to increasing salinity was to increase biomass allocation to tubers. This pattern of biomass accumulation suggested an ecological strategy in *B. medianus* to trade-off exploration for storage and dormancy as environmental conditions deteriorated. In contrast, *B. caldwellii* allocated greater biomass to rhizomes under increasing salinity. Rhizome lengths did not differ between salinity treatments, indicating a lack of genet plasticity in the plagiotropic (horizontal) plane. In contrast, tuber sizes were highly variable within each clone and many tubers did not produce culms. This pattern suggested that plasticity in the orthotropic (vertical) plane was of greater value to a genet in terms of future survival and spatial organisation.

12.3 Genetics

Molecular AFLP analysis confirmed predictions that the genetic diversity of populations of *Bolboschoenus* can be highly cryptic. Despite the presence of many thousands of individual stems at each field site, genetic homogeneity was found within three of the four sampled populations. The findings suggested a strong relationship between site quality and clonal structure, as molecular fingerprints were polymorphic from the population growing in fresh water conditions only (Sale Common). Thus, it appears that salinity is highly influential to the genetic diversity of *B. caldwellii* and *B. medianus*. The AFLP results indicated that caution must be exercised when population assessments of status and fitness (in terms of genetic diversity) are based purely on ramet numbers or morphological differences.

The discovery of large individual clones of both species at Dowd Morass and *B. caldwellii* at Clydebank Morass, indicates that current conservation approaches at

these wetlands are preserving very little genetic diversity. Further research may investigate more appropriate hydrological and salinity regimes for the promotion of sexual recruitment at these sites. The collection of *B. caldwellii* and *B. medianus* achenes at Dowd Morass and Clydebank Morass is also likely to occur from few, if not a single clone, which will limit the genetic contribution of potential future revegetation trials, as achenes are likely produced by self pollination.

12.4 Overall conclusions

There are many conflicting theories on the benefits and potential costs of reliance on asexual growth in botanical literature. The primary argument in favour of clonality for plant survival is that there is a two-fold advantage for asexual reproduction over sexual reproduction, as 100% rather than 50% of the genome is passed on to the next generation (Williams 1975). This ability has lead some authors to suggest that asexual reproduction offers clonal organisms theoretical genetic immortality (Kneitel and Chase 2004). Others conclude that a reliance on clonal growth is essentially a pathway to extinction due to the loss of sex and the accumulation of deleterious mutations (Eckert 2002; Honnay and Bossuyt 2005). Another argument suggests that clonal growth increases the number of networked ramets, thereby raising the collective fitness of genets and increasing lifetime fecundity (Snow and Whigham 1989; Oborny and Bartha 1995).

All of the above theories are potentially applicable to *B. caldwellii and B. medianus*, though it seems that the mechanisms and responses used by each species are population specific and largely dependent on site quality alone. The mesocosm experiments conducted under different salinity conditions in Section III of this thesis, demonstrated that plants do not always invest in achenes, nor exhibit similar above-ground to below-ground biomass ratios, but instead trade-off resources to different ramet components depending on the environment. In saline sites, there is little doubt that asexual reproduction in *B. caldwellii* and *B. medianus* is far more effective at consolidating and exploring space than relying on achene germination. Achene production is energy intensive, dispersal is highly randomized and germination is uncertain. In contrast, vegetative growth offers clonal plants the ability to actively place new stems into a local environment, where conditions are likely to be

favourable for growth given that the parental plant is already established *in situ* (Williams 1975). *Bolboschoenus* tubers and rhizomes act as an effective storage mechanism during unfavourable conditions, while having the added advantage of being ready for immediate reconsolidation and expansion of space under a much wider set of environmental parameters than is offered by recruitment from seed.

The molecular AFLP analysis in Section IV, highlighted that the status change of wetlands immediately surrounding the Gippsland Lakes from freshwater to brackish conditions, has shifted the recruitment dynamics of *B. caldwellii* and *B. medianus* to an exclusive reliance on clonal reproduction. Prevailing salinity concentrations are now beyond the tolerance levels for achene germination in wetlands such as Dowd Morass and Clydebank Morass. Because of this shift, should anything happen to the remaining plants, local populations will become extinct without appropriate windows of opportunity for sexual recruitment from the sediment seed bank. The clonal growth habit enables *B. caldwellii* and *B. medianus* to cope with year-to-year changes in wetting and drying conditions, however, further increases to salinity throughout the Gippsland Lakes system may also eventually prevent asexual or clonal growth mechanisms and thereby all forms of recruitment for these species.

APPENDIX A

Appendix A.1. GPS locations of soil core samples taken from each wetland

Sample	Population	Site	Latitude	Longitude
1	*B. caldwellii*	Clydebank 1	38°02'54.96"	147°13'53.37"
2	*B. caldwellii*	Clydebank 1	38°02'54.68"	147°13'53.78"
3	*B. caldwellii*	Clydebank 1	38°02'55.33"	147°13'53.82"
4	*B. caldwellii*	Clydebank 1	38°02'55.31"	147°13'53.02"
5	*B. caldwellii*	Clydebank 1	38°02'54.69"	147°13'53.01"
6	*B. caldwellii*	Clydebank 1	38°02'54.29"	147°13'54.29"
7	*B. caldwellii*	Clydebank 1	38°02'55.97"	147°13'54.25"
8	*B. caldwellii*	Clydebank 1	38°02'53.76"	147°13'57.50"
9	*B. caldwellii*	Clydebank 1	38°02'56.62"	147°13'56.33"
10	*B. caldwellii*	Clydebank 1	38°02'52.09"	147°13'59.26"
1	*B. caldwellii*	Clydebank 2	38°03'34.25"	147°14'43.33"
2	*B. caldwellii*	Clydebank 2	38°03'34.33"	147°14'43.51"
3	*B. caldwellii*	Clydebank 2	38°03'34.34"	147°14'43.70"
4	*B. caldwellii*	Clydebank 2	38°03'34.21"	147°14'43.14"
5	*B. caldwellii*	Clydebank 2	38°03'34.14"	147°14'42.94"
6	*B. caldwellii*	Clydebank 2	38°03'34.31"	147°14'44.25"
7	*B. caldwellii*	Clydebank 2	38°03'34.20"	147°14'44.69"
8	*B. caldwellii*	Clydebank 2	38°03'33.99"	147°14'42.43"
9	*B. caldwellii*	Clydebank 2	38°03'33.91"	147°14'41.82"
10	*B. caldwellii*	Clydebank 2	38°03'33.67"	147°14'40.89"
1	*B. caldwellii*	Dowd Morass 1	38°08'17.60"	147°11'42.34"
2	*B. caldwellii*	Dowd Morass 1	38°08'17.67"	147°11'41.52"
3	*B. caldwellii*	Dowd Morass 1	38°08'17.22"	147°11'43.05"
4	*B. caldwellii*	Dowd Morass 1	38°08'17.80"	147°11'43.57"
5	*B. caldwellii*	Dowd Morass 1	38°08'18.38"	147°11'41.44"
6	*B. caldwellii*	Dowd Morass 1	38°08'17.16"	147°11'43.80"
7	*B. caldwellii*	Dowd Morass 1	38°08'16.68"	147°11'45.22"
8	*B. caldwellii*	Dowd Morass 1	38°08'18.28"	147°11'40.54"
9	*B. caldwellii*	Dowd Morass 1	38°08'18.55"	147°11'38.56"
10	*B. caldwellii*	Dowd Morass 1	38°08'17.94"	147°11'37.44"
1	*B. caldwellii*	Dowd Morass 2	38°08'17.60"	147°11'49.21"
2	*B. caldwellii*	Dowd Morass 2	38°08'17.34"	147°11'48.91"
3	*B. caldwellii*	Dowd Morass 2	38°08'17.41"	147°11'49.44"
4	*B. caldwellii*	Dowd Morass 2	38°08'17.79"	147°11'49.25"
5	*B. caldwellii*	Dowd Morass 2	38°08'17.80"	147°11'48.61"
6	*B. caldwellii*	Dowd Morass 2	38°08'17.57"	147°11'50.16"
7	*B. caldwellii*	Dowd Morass 2	38°08'17.38"	147°11'51.04"
8	*B. caldwellii*	Dowd Morass 2	38°08'17.34"	147°11'48.26"
9	*B. caldwellii*	Dowd Morass 2	38°08'16.92"	147°11'47.61"
10	*B. caldwellii*	Dowd Morass 2	38°08'16.49"	147°11'47.20"
1	*B. medianus*	Dowd Morass 3	38°08'57.26"	147°08'51.35"
2	*B. medianus*	Dowd Morass 3	38°08'57.18"	147°08'51.42"
3	*B. medianus*	Dowd Morass 3	38°08'57.24"	147°08'51.22"
4	*B. medianus*	Dowd Morass 3	38°08'57.44"	147°08'51.23"
5	*B. medianus*	Dowd Morass 3	38°08'57.48"	147°08'51.55"
6	*B. medianus*	Dowd Morass 3	38°08'57.67"	147°08'51.86"
7	*B. medianus*	Dowd Morass 3	38°08'57.11"	147°08'51.80"
8	*B. medianus*	Dowd Morass 3	38°08'57.05"	147°08'51.05"

Sample	Population	Site	Latitude	Longitude
9	*B. medianus*	Dowd Morass 3	38°08’57.58”	147°08’51.07”
10	*B. medianus*	Dowd Morass 3	38°08’56.76”	147°08’52.15”
1	*B. medianus*	Dowd Morass 4	38°08’43.40”	147°13’58.79”
2	*B. medianus*	Dowd Morass 4	38°08’43.36”	147°13’58.70”
3	*B. medianus*	Dowd Morass 4	38°08’43.36”	147°13’58.80”
4	*B. medianus*	Dowd Morass 4	38°08’43.40”	147°13’58.80”
5	*B. medianus*	Dowd Morass 4	38°08’43.41”	147°13’58.70”
6	*B. medianus*	Dowd Morass 4	38°08’43.41”	147°13’58.89”
7	*B. medianus*	Dowd Morass 4	38°08’43.41”	147°13’58.96”
8	*B. medianus*	Dowd Morass 4	38°08’43.45”	147°13’58.73”
9	*B. medianus*	Dowd Morass 4	38°08’43.45”	147°13’58.67”
10	*B. medianus*	Dowd Morass 4	38°08’43.48’	147°13’58.40”
1	*B. medianus*	Sale Common 1	38°07’50.04”	147°04’18.07”
2	*B. medianus*	Sale Common 1	38°07’49.75”	147°04’18.52”
3	*B. medianus*	Sale Common 1	38°07’50.35”	147°04’18.56”
4	*B. medianus*	Sale Common 1	38°07’50.30”	147°04’17.86”
5	*B. medianus*	Sale Common 1	38°07’49.73”	147°04’17.98”
6	*B. medianus*	Sale Common 1	38°07’49.29”	147°04’18.48”
7	*B. medianus*	Sale Common 1	38°07’49.91”	147°04’19.87”
8	*B. medianus*	Sale Common 1	38°07’50.63”	147°04’18.87”
9	*B. medianus*	Sale Common 1	38°07’50.16”	147°04’17.41”
10	*B. medianus*	Sale Common 1	38°07’49.12”	147°04’18.02”
1	*B. medianus*	Sale Common 2	38°08’39.97”	147°05’16.51”
2	*B. medianus*	Sale Common 2	38°08’39.88”	147°05’16.71”
3	*B. medianus*	Sale Common 2	38°08’40.09”	147°05’16.71”
4	*B. medianus*	Sale Common 2	38°08’40.08”	147°05’16.32”
5	*B. medianus*	Sale Common 2	38°08’39.84”	147°05’16.31”
6	*B. medianus*	Sale Common 2	38°08’40.09”	147°05’17.61”
7	*B. medianus*	Sale Common 2	38°08’40.45”	147°05’16.57”
8	*B. medianus*	Sale Common 2	38°08’39.94”	147°05’15.52”
9	*B. medianus*	Sale Common 2	38°08’39.53”	147°05’16.51”
10	*B. medianus*	Sale Common 2	38°08’39.32”	147°05’15.30”

Appendix A.2. Mean salinity (g L^{-1}) taken weekly between 10 am and 2 pm over 3 months, for *B. caldwellii* replicates from individual mesocosms. Salt was added to the appropriate level in each treatment on the week beginning Monday 30th October 2006 (grey shading).

Replicate	4-Oct-06	17-Oct-06	30-Oct-06	6-Nov-06	13-Nov-06	21-Nov-06	27-Nov-06	4-Dec-06	11-Dec-06	21-Dec-06	29-Dec-06	4-Jan-07	11-Jan-07	Average	Std dev
1	0.04	0.06	11.9	13.5	13.4	11.9	11.4	12.8	14.4	11.4	15	11.2	12.6	12.68	1.27
2	0.05	0.07	12.4	13.2	12.9	11.5	12	11.3	14.1	11.7	14.6	12.7	12.9	12.66	1.04
3	0.05	0.07	11.8	12.8	13.6	12.1	12.3	14.4	13.6	12.3	14.6	11.1	13	12.87	1.09
4	0.06	0.07	12.3	12.7	13.0	11.9	13.5	13.4	14.2	11.6	14.8	11.4	12.8	12.87	1.06
5	0.06	0.06	12.1	12.8	13.4	12.1	12.2	12.1	13.4	12.5	14.1	12.3	12.7	12.70	0.67
6	0.07	0.09	11.9	13.5	12.6	11.6	13.5	13.1	13.2	12.3	13.7	11.7	12.7	12.71	0.76
7	0.05	0.06	12.0	13.4	13.0	11.6	13.2	14	13.8	11.9	14.9	11.5	13.1	12.95	1.09
8	0.04	0.07	12.0	13.4	13.4	11.7	13.1	12.2	13.8	12.1	14.1	12.3	13.1	12.84	0.81
9	0.05	0.07	12.0	13.0	12.9	11.8	12.7	12	13.3	11.7	14.1	12.4	13.4	12.66	0.76
10	0.06	0.07	12.3	13.2	12.8	11.6	12.2	11.4	13.8	12.2	14.5	12.6	13.3	12.72	0.93
Avg	**0.05**	**0.07**	**12.1**	**13.2**	**13.1**	**11.8**	**12.6**	**12.7**	**13.8**	**12.0**	**14.4**	**11.9**	**13.0**	**12.77**	**0.95**
11	0.05	0.07	3.8	4.2	4.0	3.9	3.7	3.9	4.5	3.8	4.4	3.5	4.1	3.98	0.30
12	0.06	0.09	3.8	4	4.0	3.9	3.8	3.6	5	3.7	4.7	3.6	4.2	4.03	0.45
13	0.06	0.08	3.8	4.1	4.1	3.9	3.5	4.4	5	3.5	4.7	3.6	4.1	4.06	0.48
14	0.07	0.14	3.8	4.2	4.0	4	3.6	3.7	4.2	3.9	4.7	3.6	3.6	3.94	0.34
15	0.12	0.14	3.9	4.2	4.0	3.9	3.7	3.5	4.6	3.5	4.5	3.7	3.7	3.93	0.37
16	0.05	0.10	3.8	4.6	3.9	4	3.6	5	3.8	3.8	4.5	3.3	3.8	4.01	0.49
17	0.09	0.17	3.8	4.4	4.0	3.9	4.3	4.2	4.7	3.9	4.5	3.8	4	4.14	0.30
18	0.05	0.13	3.9	4.4	4.1	3.8	3.7	4.5	5	3.7	4.4	3.5	4.2	4.11	0.44
19	0.07	0.11	3.9	4.1	3.9	3.9	3.5	4.8	4.1	3.6	4.3	3.6	3.8	3.95	0.37
20	0.11	0.14	3.8	4	3.9	3.9	3.4	3.4	4.4	3.5	4.8	3.4	3.5	3.82	0.46
Avg	**0.07**	**0.12**	**3.8**	**4.2**	**4.0**	**3.9**	**3.7**	**4.1**	**4.5**	**3.7**	**4.6**	**3.6**	**3.9**	**4.00**	**0.40**
21	0.04	0.09	0.10	0.14	0.15	0.22	0.24	0.16	0.12	0.13	0.16	0.10	0.08	0.13	0.05
22	0.05	0.11	0.12	0.16	0.19	0.21	0.27	0.17	0.19	0.19	0.19	0.17	0.11	0.16	0.04
23	0.04	0.15	0.18	0.17	0.17	0.19	0.20	0.16	0.11	0.15	0.15	0.12	0.09	0.14	0.03
24	0.06	0.14	0.15	0.16	0.19	0.20	0.28	0.24	0.23	0.21	0.18	0.19	0.12	0.18	0.04
25	0.07	0.12	0.11	0.13	0.15	0.17	0.35	0.22	0.17	0.19	0.26	0.21	0.11	0.17	0.07
26	0.05	0.14	0.14	0.19	0.13	0.16	0.20	0.13	0.08	0.12	0.11	0.08	0.07	0.12	0.04
27	0.07	0.11	0.17	0.10	0.13	0.17	0.23	0.16	0.10	0.11	0.15	0.09	0.07	0.13	0.05
28	0.09	0.12	0.13	0.13	0.18	0.21	0.27	0.22	0.14	0.17	0.21	0.14	0.10	0.16	0.05
29	0.07	0.14	0.18	0.19	0.17	0.20	0.21	0.17	0.12	0.17	0.15	0.08	0.10	0.15	0.04
30	0.05	0.17	0.16	0.17	0.18	0.18	0.27	0.17	0.14	0.18	0.18	0.08	0.11	0.16	0.05
Avg	**0.06**	**0.13**	**0.14**	**0.15**	**0.16**	**0.19**	**0.25**	**0.18**	**0.14**	**0.16**	**0.17**	**0.13**	**0.10**	**0.15**	**0.05**

Appendix A.3. Mean salinity (g L^{-1}) taken weekly between 10 am and 2 pm over 3 months, for *B. medianus* replicates from individual mesocosms. Salt was added to the appropriate level in each treatment on the week beginning Monday 30th October 2006 (grey shading).

Replicate	4-Oct-06	17-Oct-06	30-Oct-06	6-Nov-06	13-Nov-06	21-Nov-06	27-Nov-06	4-Dec-06	11-Dec-06	21-Dec-06	29-Dec-06	4-Jan-07	11-Jan-07	Average	Std dev
1	0.05	0.07	11.7	13.1	12.7	11.8	14.4	12.4	13.6						
2	0.07	0.11	12	13.9	12.6	11.7	13	11.8	14.1	12.1	14.4	11.5	12.7	12.71	1.02
3	0.07	0.13	12.5	12.7	12.5	11.6	14.2	13.6	13.7	12.1	14.4	12.9	13.6	13.07	0.89
4	0.09	0.13	12.4	13.5	13	12.1	12.3	12.4							
5	0.05	0.12	12.6	13.5	13	12.5	12.6	12.3	13.6	11.6	14.5	12.4	12.8	12.85	0.78
6	0.05	0.11	12.5	13.4	12.6	12.1	14								
7	0.07	0.18	11.7	13.2	12.9	11.9	12	13	13.4						
8	0.06	0.13	12.6	13.1	12.9	11.9	12.6	12.7	13.6	11.7	14.3	12.4	13.2	12.82	0.74
9	0.04	0.11	12.6	13.2	12.4	12.3	13.3	13.4	14.3	12.2	15	11.9	13.5	13.10	0.95
10	0.05	0.10	12.5	13.6	13.3	11.7	13								
Avg	**0.06**	**0.12**	**12.3**	**13.3**	**12.8**	**12.0**	**13.1**	**12.7**	**13.8**	**11.9**	**14.5**	**12.2**	**13.2**	**12.91**	**0.88**
11	0.07	0.13	3.8	4.3	3.9	3.6	3.6	3.8	4.1	3.6	4.9	3.3	3.7	3.87	0.43
12	0.04	0.12	3.8	4.3	3.8	3.6	4	4.1	4.6	3.8	4.3	3.5	3.7	3.95	0.34
13	0.05	0.14	3.8	4.1	3.8	4.2	4.6	4.1	4.8	3.9	4.8	3.9	4.1	4.19	0.38
14	0.12	0.10	3.9	4.3	3.9	3.5	3.8	4.2	4.7	3.7	4.4	4.2	4.7	4.12	0.39
15	0.09	0.12	3.9	4.3	4	3.5	3.7	4.5	5	4	4.6	3.6	4	4.10	0.46
16	0.05	0.10	3.9	4.2	4	4.1	4.2	3.8	4.8	3.8	4.3	3.8	3.8	4.06	0.31
17	0.07	0.11	3.8	4.1	4	3.7	3.9	3.9	4.3	3.6	4.4	3.5	3.7	3.90	0.28
18	0.07	0.11	4	4.7	3.9	3.6	4.2	4.1	4.2	3.6	4.5	3.4	3.7	3.99	0.40
19	0.04	0.07	3.8	4.2											
20	0.05	0.07	3.8	4.2	4	4.1	4.3	3.7	4.6	3.8	4.6	3.5	3.8	4.04	0.36
Avg	**0.06**	**0.11**	**3.9**	**4.3**	**3.9**	**3.8**	**4.0**	**4.0**	**4.6**	**3.8**	**4.5**	**3.6**	**3.9**	**4.03**	**0.37**
21	0.05	0.07	0.09	0.14											
22	0.13	0.18	0.19	0.11	0.11	0.14	0.24	0.23	0.3	0.29	0.17	0.13	0.17	0.19	0.08
23	0.06	0.12	0.13	0.17	0.16	0.17	0.22	0.23	0.2	0.17	0.19	0.14	0.10	0.17	0.04
24	0.04	0.09	0.12	0.16	0.13	0.16	0.19	0.19	0.1	0.13	0.15	0.14	0.08	0.14	0.03
25	0.05	0.09	0.08	0.14	0.12	0.13	0.28	0.32	0.1	0.12	0.21	0.22	0.07	0.16	0.08
26	0.05	0.08	0.07	0.13											
27	0.04	0.06	0.09	0.11	0.19	0.19	0.24	0.13	0.1	0.19	0.15	0.09	0.11	0.15	0.05
28	0.05	0.06	0.05	0.13	0.18	0.17	0.07	0.39	0.4	0.31	0.41	0.39	0.19	0.25	0.14
29	0.06	0.07	0.11	0.13	0.21	0.22	0.31	0.25	0.2	0.22	0.21	0.14	0.13	0.19	0.06
30	0.12	0.12	0.13	0.19	0.21	0.21	0.34	0.25	0.2	0.21	0.21	0.19	0.13	0.20	0.06
Avg	**0.06**	**0.09**	**0.11**	**0.14**	**0.16**	**0.17**	**0.24**	**0.25**	**0.21**	**0.21**	**0.21**	**0.18**	**0.12**	**0.18**	**0.07**

Appendix A.4. GPS coordinates of sample locations from each wetland used in collecting plant material for genetics enquiries.

	B. caldwellii		*B. caldwellii*		*B. medianus*		*B. medianus*	
	Clydebank Morass		Dowd Morass		Dowd Morass		Sale Common	
Sample #	Latitude	Longitude	Latitude	Longitude	Latitude	Longitude	Latitude	Longitude
1	38° 2'59.91"S	147°14'6.46"E	38° 8'18.23"S	147°11'41.22"E	38° 8'57.37"S	147° 8'50.18"E	38° 7'49.33"S	147° 4'16.42"E
2	38° 2'58.68"S	147°14'4.65"E	38° 8'18.08"S	147°11'41.57"E	38° 8'57.62"S	147° 8'49.97"E	38° 7'48.99"S	147° 4'16.37"E
3	38° 2'57.46"S	147°14'2.91"E	38° 8'17.96"S	147°11'42.04"E	38° 8'57.92"S	147° 8'50.01"E	38° 7'48.60"S	147° 4'16.36"E
4	38° 2'57.02"S	147°14'0.17"E	38° 8'17.83"S	147°11'42.45"E	38° 8'58.11"S	147° 8'50.27"E	38° 7'48.22"S	147° 4'16.37"E
5	38° 2'56.62"S	147°13'57.32"E	38° 8'17.74"S	147°11'42.86"E	38° 8'58.18"S	147° 8'50.63"E	38° 7'47.89"S	147° 4'16.37"E
6	38° 2'55.51"S	147°13'55.36"E	38° 8'17.59"S	147°11'43.27"E	38° 8'58.03"S	147° 8'51.01"E	38° 7'47.58"S	147° 4'16.35"E
7	38° 2'53.79"S	147°13'55.21"E	38° 8'17.44"S	147°11'43.68"E	38° 8'57.71"S	147° 8'51.16"E	38° 7'47.26"S	147° 4'16.35"E
8	38° 2'53.65"S	147°13'57.87"E	38° 8'17.29"S	147°11'44.19"E	38° 8'57.42"S	147° 8'50.98"E	38° 7'46.91"S	147° 4'16.35"E
9	38° 2'52.42"S	147°13'59.65"E	38° 8'17.19"S	147°11'44.63"E	38° 8'57.32"S	147° 8'50.57"E	38° 7'46.52"S	147° 4'16.36"E

Appendix A.5.

Primer pair screening results for *B. caldwellii*

		***Mse*I Primer**							
		CAA	**CAC**	**CAG**	**CAT**	**CTA**	**CTC**	**CTG**	**CTT**
***Eco*RI Primer**	**AAC**	√	√	√		√	√	?	√
	AAG	√	√	√			√	?	
	ACA	√	√	√	√	√	√	?	√
	ACC	√	√			√	√	?	√
	ACG	√	√	√	√	√	√	?	√
	ACT	√	√	√		√	√	?	√
	AGC	√	√	√	√	√	√	?	√
	AGG	√	√	√	√	√	√	?	√

√ = Primer pair recommended (polymorphisms found)
Blank = Primer pair not recommended (no polymorphisms found)
? = Untested

Bands / loci were found for 49 of 56 primer pair combinations tested for *B. caldwellii*.

Primer pair screening results for *B. medianus*

		***Mse*I Primer**							
		CAA	**CAC**	**CAG**	**CAT**	**CTA**	**CTC**	**CTG**	**CTT**
***Eco*RI Primer**	**AAC**	√	√	√		√	√	?	√
	AAG	√	√	√			√	?	√
	ACA	√	√	√	√	√	√	?	√
	ACC	√	√			√	√	?	√
	ACG		√	√	√	√	√	?	√
	ACT	√	√	√	√	√	√	?	√
	AGC	√	√	√	√		√	?	√
	AGG	√	√	√	√	√	√	?	

√ = Primer pair recommended (polymorphisms found)
Blank = Primer pair not recommended (no polymorphisms found)
? = Untested

Bands / loci were found for 48 of 56 primer pair combinations tested for *B. medianus.*

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