SEED BIOLOGY AND *EX SITU* STORAGE BEHAVIOUR OF AUSTRALIAN *NYMPHAEA* (WATER LILIES): IMPLICATIONS FOR CONSERVATION

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Cover image: *Nymphaea violacea* flower in the far north Kimberley, Western Australia. Emma Dalziell all rights reserved.

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SUMMARY

The iconic, basal angiosperm genus *Nymphaea* (Nymphaeaceae) occupy many of the world's freshwater wetlands, with 18 species occurring in northern Australia. Generally, our knowledge of wetland plant species is limited, and even more so for species occurring in the monsoonally driven wet-dry tropics of northern Australia. In order to guide the management and conservation of *Nymphaea* in Australia, this thesis seeks to improve our understanding of these charismatic plants, and presents the first detailed investigation into the seed biology of the genus.

Investigations into the seed dormancy of Australian *Nymphaea* revealed that five species underwent embryo growth prior to radicle emergence, and were thus classified as having morphophysiological dormancy (MPD). This finding adds to the increasing body of literature suggesting MPD is basal within the angiosperms. Additionally, all study species showed a requirement for light, and maximal germination occurred in a small temperature window between 30-35 °C. A phenomenon previously reported in the literature that crowding seeds under flooded conditions was observed, and produced high germination in all species of *Nymphaea* tested in this study, when incubated under optimal temperature and light conditions. This response, termed 'crowding' was attributed to the endogenous production and concentration of ethylene by the seeds, which acts as a germination stimulant.

As all species displayed specific germination requirements, I further investigated how dormancy may be naturally overcome under natural conditions, and tested treatments that can be applied to break dormancy *ex situ*. I found that dormancy loss under simulated natural conditions occurs in conjunction with increasing (over-night) temperatures at the onset of the summer wet season, when seeds are permanently inundated with water. However, when tested under laboratory conditions, temperature alone did not break dormancy. Cycles of wetting and drying, used to mimic the sporadic rainfall of northern Australia, acted to completely alleviate dormancy in seeds of one species (*N. lukei*), in conjunction with the exogenous application of ethylene. Under simulated burial conditions, seeds were found to be semi-persistent, and can remain

viable in dry soils for > 12 months. However, viability rapidly declined when under simulated wetting and drying cycles.

Given the increasing threat of habitat decline for many Australian *Nymphaea,* the investment in other conservation measures such as *ex situ* seed banking forms an essential component of biodiversity management for the continued preservation of these important species. Seeds of all Australian *Nymphaea* tested in this study were found to be desiccation tolerant (drying to an internal moisture content of 5 %), which further supports hypotheses regarding the evolution of this trait in the basal angiosperms. Upon testing their amenability to standard seed banking procedures, all species showed orthodox storage behaviour, however, some species and accessions lost viability more rapidly than others. All species of *Nymphaea* were shown to be short-lived in storage when compared with other species from global datasets. As such, many of the species of *Nymphaea* assessed in this study will not be able to survive conventional seed storage, however rigorous ultra-low temperature cryogenic storage may provide an alternative solution.

In light of current climate change predictions expected to impact on low-lying coastal wetlands and floodplains in northern Australia, I investigated the impact on seed germination and early seedling morphology to increasingly saline conditions. All species tested displayed a marked decline in germination and early seedling growth (biomass and total length) when sodium chloride concentrations exceeded that of brackish water. Upon recovery in fresh water, the majority of species showed a tolerance to salinity, however there was a significant decrease in seed germination when seeds were incubated in high salinities (up to sea-water equivalent). There was no observed correlation between ability to withstand increasing salinity and the geographical range of the species, as increasing salinity was not more detrimental to a short-range endemic species, when compared with widely distributed species.

Overall, the findings in this thesis have, for the first time, contributed to the knowledge of seed biology and *ex situ* longevity of the genus *Nymphaea*. Furthermore, this thesis

serves to highlight some of the conservation challenges Australia faces, if the current trend in the decline of freshwater wetlands continues.

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TABLE OF CONTENTS

ABBREVIATIONS

CE	Controlled environment room (15 % RH at 15 °C)
Crowd	Crowding (seed crowding technique, see Chapter 3)
CS	Cold stratification
DAR	Dry after-ripening
DI	Deionised water
Eth	Ethrel
E:S	Embryo to seed ratio
GA or GA_3	Gibberellic acid
ISTA	International Seed Testing Association
KAR or KAR_1	Karrikinolide
LC_{50}	50 % lethal dose
LN	Liquid nitrogen (vapour phase)
MC	Moisture content
MD	Morphological dormancy
MPD	Morphophysiological dormancy
PD	Physiological dormancy
P_{50}	50 % germination
RH	Relative humidity
RO	Reverse osmosis
TGP	Thermogradient plate
TZ	Tetrazolium staining
UWC	Unfrozen water content
WDC	Wet dry cycling
WS	Warm stratification

ACKNOWLEDGEMENTS

Completing my PhD degree is probably the most challenging thing I have taken on in my 27 years of life. The best and worst moments of my four-year doctoral journey have been shared with many people, and I certainly could not have completed it without their help.

Friends, Romans, countrymen, lend me your ears!

First and foremost I would like to thank my supervisors Dr. David Merritt, Prof. Kingsley Dixon and Dr. Renee Young for their ongoing support and guidance throughout my PhD. I am so grateful for all your help, and that you all took the chance on this project – notwithstanding the initial lack of funding and rather remote field sites!

Despite my excellent supervisory team, the sheer amount of travel required for this project would not have been possible without the financial support from a number of organisations. The University of Western Australia's School of Plant Biology and the Botanic Gardens and Parks Authority provided initial funding. Additionally, I received generous support from The Friends of Kings Park (FoKP Scholarship), The Graduate Women of Western Australia (Mary Walters Bursary) and the ANZ Trustees Holsworth Wildlife Research Endowment. I was supported financially by a University Postgraduate Award (UWA), an Australian Postgraduate Award (APA), a top-up scholarship from UWA and a UWA Graduate Research School Completion Scholarship. During my candidature, I was fortunate enough to travel to Hobart, Tasmania to present at the Australasia Plant Conservation Conference (ANPC) and Nouméa, New Caledonia to present at the Society for Ecological Restoration Australasia conference. This travel was supported by funding from the UWA Graduate Research School and the School of Plant Biology. Additionally, the UWA Postgraduate Student's Association (Travel Scholarship) further supported this travel, and I am genuinely humbled to be supported by my peers. Thank you to all of the individuals and organisations who assisted me financially though the last four years.

At the start of this project, I was struck by the fact that no one had attempted to collect seeds of *Nymphaea* in Australia before (at least in no great numbers). Some 50,000 + km travelled later, to some of the most remote and crocodile, leech, mosquito and sand-fly (ugh!) infested places in Australia, I'm beginning to understand why:

The road so long and weary:

- 22 x commercial flights
- 3 x flights in a 6-seat Cessna
- 5 x flights in 13-seat Cessna
- 1 x helicopter
- 3 x Toyota Landcruisers
- 1 x Mitsubishi Pajero,
- 1 x Toyota Corolla
- 4 x quad bikes
- 1 x estuary trekker (boat)
- 1 x tinny
- 1 x purple people eater (canoe)
- 2 x truck inner tyres
- Numerous pairs of tired, soggy, bruised, blistered and insect bitten feet

I've loved (nearly) every minute of it. My deepest thanks to those that gave up their time to help me in the field. To Todd Erickson and his family for their help in the Northern Territory and Kakadu National Park in 2011. Alison Ritchie for helping at Theda in 2012-for *volunteering* to help me in the stifling heat and humidity of the late Kimberley wet season. You got me through my toughest day in the field-lightning storms, flat battery, bogged bikes, no sat-phone reception, wasp sting and spider bite and all! Todd (again) and Myles Menz for the lower half of the Gibb River Road. Never again shall the three of us (well, two of you) be seen dragging a canoe through inundated grassland for hours on end. Wolfgang Lewandrowski for Kununurra and Parry's Lagoons 2012-for not giving up when I wanted to. The docs- Sean (Fuzz) Tomlinson and Ryan Phillips- I am indebted to you both- for not only for stepping in at the last minute when I desperately needed the help, but pulling off the best trip of my PhD. The Gibb River Road from start to finish without a hitch. I couldn't have asked for more. Finally, Christine Best and Fuzz for making the dream come true- far north Queensland. Daintree. Cape Tribulation. Atherton Tablelands. Cairns. Enough said.

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I'm also indebted to the kind assistance from others outside Kings Park: Dr Lynne Milne at Curtin University, for teaching me the pollen acetolysis procedures and also for encouraging my membership at the Royal Society and for always being a friendly face, Prof. Ricardo Mancera also at Curtin University for assistance with the DSC and

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For

Hugh and Esther Hanley & Jack and Dorothy Dalziell

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DECLARATION

DECLARATION FOR THESES CONTAINING PUBLISHED WORK AND/OR WORK PREPARED FOR PUBLICATION

The examination of the thesis is an examination of the work of the student. The work must have been substantially conducted by the student during enrolment in the degree.

Where the thesis includes work to which others have contributed, the thesis must include a statement that makes the student's contribution clear to the examiners. This may be in the form of a description of the precise contribution of the student to the work presented for examination and/or a statement of the percentage of the work that was done by the student.

In addition, in the case of co-authored publications included in the thesis, each author must give their signed permission for the work to be included. If signatures from all the authors cannot be obtained, the statement detailing the student's contribution to the work must be signed by the coordinating supervisor.

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2. This thesis contains **only sole-authored** work, some of which has been published and/or prepared for publication under sole authorship. The bibliographical details of the work and where it appears in the thesis are outlined below. Student Signature .....
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STATEMENT OF CANDIDATE CONTRIBUTION

This thesis contains work prepared for publication, some of which has been co-authored. The vast majority of work contained in this thesis was produced by the primary author (ELD). This included literature searches, experimental design, data collection, data analysis and manuscript write-up. However, co-authors will be included on each manuscript, acknowledging their contributions to each particular aspect of the research. The bibliographical details of intended publications arising from this thesis are listed below.

CHAPTER 3:

Contributions: ELD designed the experiments, performed the experiments, collected and analysed the data and wrote the manuscript. REY, KWD and DJM provided critique of experimental design and comments on the manuscript.

Dalziell, E. L., Young, R. E., Dixon, K. W. and Merritt, D. J. (in prep.). Seed dormancy and germination traits of Australian *Nymphaea* L. (Nymphaeaceae).

CHAPTER 4:

Contributions: ELD designed the experiments, performed the experiments, collected and analysed the data and wrote the manuscript. REY, KWD and DJM provided critique of experimental design and comments on the manuscript.

Dalziell, E. L., Young, R. E., Dixon, K. W. and Merritt, D. J. (in prep.). Breaking dormancy in seeds and longevity in the soil seed bank of tropical *Nymphaea* L. species.

CHAPTER 5:

Contributions: ELD designed the experiments, performed the experiments, collected and analysed the data and wrote the manuscript. BF and RM assisted with interpretation and data analysis of DSC data. Both will provide comments on the manuscript.

REY, KWD and DJM provided critique of experimental design and comments on the manuscript.

Dalziell, E. L., Young, R. E., Funnekotter, B., Mancera, R., Dixon, K. W. and Merritt, D. J. (in prep.) Seed storage behaviour of Australian *Nymphaea*. L.

All other work presented in this thesis is my own (ELD), with comments on the manuscripts provided by my supervisors, REY, DJM and KWD.

During the course of my candidature, I have presented results arising from my thesis at two conferences. The details of these presentations are as follows:

- Emma Dalziell. *Ex situ* seed storage of Australian *Nymphaea* (waterlilies): Implications for conservation. Presented at the 10th Annual Australasian plant Conservation Conference. Hobart, Tasmania. November 2014.

- Emma Dalziell. *Ex situ* seed storage of Australian *Nymphaea* (waterlilies): Implications for conservation and restoration. Presented at the 2nd conference of the Society for Ecological Restoration Australasia. Noumèa, New Caledonia. November 2014.

Also during the course of my candidature, I have contributed to several manuscripts in preparation for publication, that are broadly related to the themes addressed in this thesis:

- **Dalziell, E. L.,** Tomlinson, S., Stevens, J. C., Dixon, K. W., Withers, P. C. and Merritt, D. M. (in prep). Seed respiration is used as an alternative method of determining viability non-destructively in native Australian seeds.

- **Dalziell, E. L.,** Erickson, T. E. and Merritt, D. M. (in prep). Morphophysiological dormancy in *Hibbertia glabberima*: Evidence of wetter refugia in Western Australia's Pilbara bioregion.

- Tomlinson, S., **Dalziell, E. L.,** Dixon, K. W., Withers, P. C., and Merritt, D. M. (in prep). From little things big things grow: The energetic basis to seed ecology and evolution.

- Miller, B. P. *et al.* **Dalziell, E. L.** (in prep). A comprehensive framework of the science necessary for sustainable, biodiverse, ecological restoration.

- Tomlinson, S. *et al.* **Dalziell, E. L.** (in prep). Future proofing conservation decisions: Deconstructing the oxymoron to identify caveats, assumptions and knowledge gaps.

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CHAPTER 1

GENERAL INTRODUCTION & LITERATURE REVIEW

Part 1: Wetlands

Wetlands of the world

While freshwater wetlands only occupy a small proportion (4-6 %) of the Earth's surface, their value cannot be understated (Ramsar Convention Bureau 1996; Mitsch and Gosselink 2000). Not only are they reservoirs for biodiversity, wetlands also provide significant ecosystem services in terms of clean drinking water, water purification, flood control, shoreline protection, food supply, carbon sequestration, along with significant cultural, recreational and aesthetic values (Mitsch and Gosselink 2007).

Classification of wetlands

The Ramsar Convention (Ramsar Convention Bureau 1996) states that wetlands are defined as:

"Areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six metres".

Of these, five distinct types of naturally occurring wetlands are recognised:

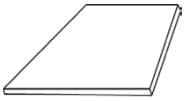
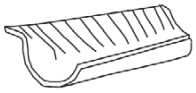
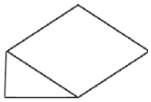
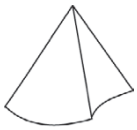
1. Marine (coastal wetlands including coastal lagoons, rocky shores, and reefs);
2. Estuarine (including deltas, tidal marshes, and mangrove swamps);
3. Lacustrine (wetlands associated with lakes);
4. Riverine (wetlands along rivers and streams);
5. Palustrine (meaning "marshy" - marshes, swamps and bogs).

For the purposes of this introduction, marine wetlands and mangrove systems are not further considered.

In order to compare the values and significance of wetland areas, a rigorous classification system is required. However, the issue of classifying wetlands in one unified system is contentious (Finlayson and van der Valk 1995). There are several different systems accepted and used throughout the world, for example; Cowardin *et al.*'s (1979) system that divides waterways into systems and subsystems, is used

extensively in the United states, while Seminiuk and Seminiuk's (1995) system is based on underlying landform and geology (Table 1.1) and is often used in Australia (Finlayson and van der Valk 1995).

Table 1.1: Modified version of Seminiuk and Seminiuk's (1995) classification system (Department of Environment and Conservation 2012) whereby wetlands are classified based on hydroperiod and landform characteristics.

	Landform				
Periodicity of inundation	 **Basin**	 **Flat**	 **Channel**	 **Slope**	 **Highland**
Permanent	Lake	-	River	-	-
Seasonal	Sumpland	Floodplain	Creek	-	-
Intermittent	Playa	Balkarra	Wadi	-	-
Seasonal waterlogging	Dampland	Palusplain	Trough	Paluslope	Palusmont

The majority of the worlds wetlands are found in tropical to subtropical regions, while the remainder are predominantly peatland swamps in boreal regions such as northern Europe and Asia (Cronk and Fennessy 2001; Mitsch and Gosselink 2007). In northern Australia, the majority of coastal wetlands are seasonally inundated floodplains, rivers and creeks, while inland systems are generally basins (lakes, sumplands or damplands) or channels (rivers creeks and wadis).

Wetland hydrology

Wetland hydrology is considered to be the most important factor influencing wetland maintenance and functioning (Mitsch and Gosselink 2007) as it influences both the physicochemical environment and biotic environment of all wetlands (Fig. 1.1). The hydrological regime, period and frequency of inundation and factors such as flow rate

and recharge rate directly impact the nutrient availability, temperature and sediment decomposition. All of these factors then influence the species composition of the biota occurring in the wetland (Mitsch and Gosselink 2007).

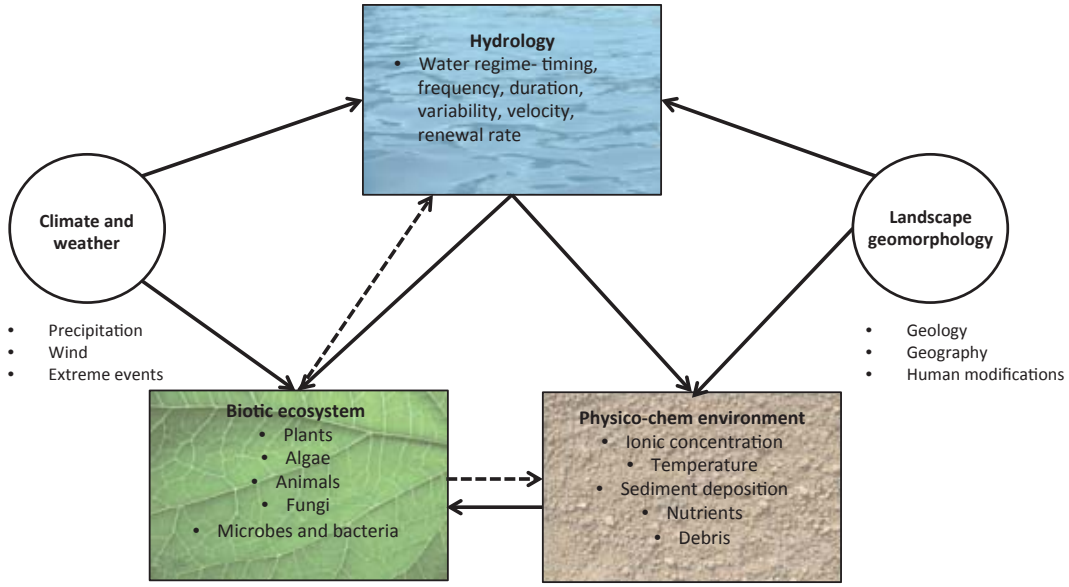


Figure 1.1: The physiochemical environment in a wetland, modified from Mitsch and Gosselink (2007). Solid lines represent direct interactions, while dashed lines represent indirect interactions.

The hydrological regime, combined with landscape morphology and climate ultimately controls the distribution, abundance and diversity of biota within a wetland. Other biogeochemcial factors that control the biotic environment include the ionic composition of the water (i.e. salinity), pH, temperature, sediment deposition, dissolved oxygen, nutrient availability, along with physical factors such as water flow and velocity (Cronk and Fennessy 2001; Mitsch and Gosselink 2007).

Wetland flora

There are some 71 angiosperm families that contain aquatic taxa (Cook *et al.* 1974; Baskin and Baskin 2014) that inhabit any number of the wetland habitats previously described. Much like the classification system for the world's wetlands, defining wetland plant taxa within an organised system has been contentious, and many authors do not differentiate between wetland plants and true aquatic plants (Cronk and Fennessy

2001). However, the following classification of (vascular) wetland plants is well accepted e.g. (Cronk and Fennessy 2001; Sainty and Jacobs 2003):

- Free-floating species are plants that freely float in the water column. If they have roots, they do not attach to substrate. Examples include *Lemma minor*, *Wolfia* sp. and *Azolla* spp.
- Floating attached species have (mature) leaves that float on the waters surface to allow gas exchange with the atmosphere, while their roots are anchored in substrate. Examples include *Nymphaea* and *Nelumbo* species.
- Submerged plants are continuously underneath the surface of the water and have roots that anchor them to the soil substrate. These plants take up dissolved oxygen and carbon dioxide directly from the water column. Examples include *Vallisneria*, *Ceratophyllum* and *Myriophyllum.*
- Emergent plants are those species that are rooted in the soil substrate, with the majority of their leaves and reproductive organs above the water. Woody species include the trees and shrubs found in riparian wetlands. Examples include many monocotyledonous grass (Poaceae), rush (Juncaceae) and sedge (Cyperaceae) species.

Many wetland plants are cosmopolitan in distribution (or at least widespread) due to movement of plant propagules via water (hyrdochory via water flow and flooding events), wind (wind dispersal), animals (zoochory via migratory waterbirds, fish, turtles etc), or human mediated dispersal (Cronk and Fennessy 2001) combined with their phenotypic plasticity and clonal reproduction strategies (Santamaria 2002). Patterns of distribution are fairly predictable, often following bird migration routes or water flow in large catchment areas (Cronk and Fennessy 2001). However, some species occupy a small range or are endemic to certain areas, usually due to the patchy nature and lack of interconnectedness between systems. Examples can be found in vernal pool systems in California, and the southwest of Western Australia along with systems in South America and South-East Asia (Cronk and Fennessy 2001; Santamaria 2002; Les *et al.* 2003).

Much like wetlands as a whole, wetland plants also provide significant ecosystem services in terms of; timber production, food (e.g. rice), medicines, stabilisation of sediments and embankments, habitat and food for numerous aquatic animals, carbon

sequestration, nutrient mobilisation and serve to decrease water velocity where it would otherwise be destructive (Cronk and Fennessy 2001; Sainty and Jacobs 2003).

Threats to wetlands

Although wetlands provide significant ecosystem services, they are among the most highly threatened ecosystems in the world (Hay *et al.* 2000). Human-induced processes such as land clearing, urbanisation, agriculture and invasive weeds and animals combined with physical and chemical alterations to freshwater ecosystems (damming, diverting, dredging, straightening and channelization combined with dumping of sewage and other pollutants) (Gibbs 2000; Hay *et al.* 2000; Levin *et al.* 2009) are contributing to a reduction in aquatic species diversity worldwide (Amezaga *et al.* 2002). In the United States alone, figures of up to 90 % loss of wetlands in inland agricultural areas are not uncommon (Cronk and Fennessy 2001). In the last 200 years, more than 50 % of all wetlands within Australia have been lost (Finlayson and Rea 1999) and this figure extends to wetlands throughout the world.

Current global climate predictions suggest that sea-level rise, increased temperatures, evaporation, the probability of extreme events, and the decreased predictability and amount of rainfall will significantly impact freshwater wetlands and their biota on a number of fronts (Parmesan 2006; Le Quesne *et al.* 2010; Church and White 2011). These combined climatic impacts will affect water quality, hydroperiod (timing or seasonality) and overall water quantity in the majority of wetlands worldwide (Le Quesne *et al.* 2010).

In order to retain functioning wetland ecosystems for the benefit of human civilisation and biodiversity, a holistic understanding of wetland processes as well as the individual species that occur in such wetlands is required.

Wetlands of northern Australia

The wet-dry tropics

The wet-dry tropics of northern Australia encompass a vast area from Derby in Western Australia to south of Townsville in Queensland (Fig. 1.2). The area was first defined by Landsberg *et al.* (1966) who categorised the region based on annual rainfall of 600-1600 mm over 4-7 months of the year (Finlayson 1999). The wetlands of the region are relatively undisturbed compared with the rest of the country, based on the disturbance index developed by Stein *et al.* (2002).

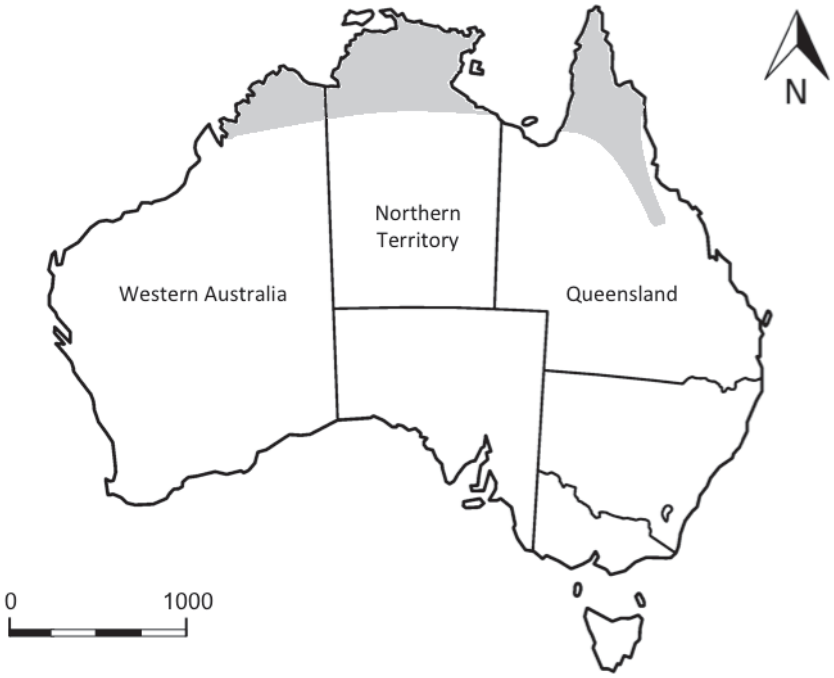


Figure 1.2: The wet dry tropics of northern Australia, shown in grey. Modified from Finlayson (1999).

Climate

The area is characterised by heavy precipitation (Fig. 1.3) from monsoonal weather systems through the summer (late November through to March/April) and a period of drought coupled with high evaporation rates over the winter (May-October) leading to the typical 'wet-dry' climate (Peel *et al.* 2007) However, precipitation is variable; the majority of rainfall falls near the coastline, usually in areas with a north-westerly aspect

and decreases significantly with increasing distance from the coast (Butler 2008). The area is also impacted heavily by tropical cyclones and thunderstorms through the months of December to March (Peel *et al.* 2007).

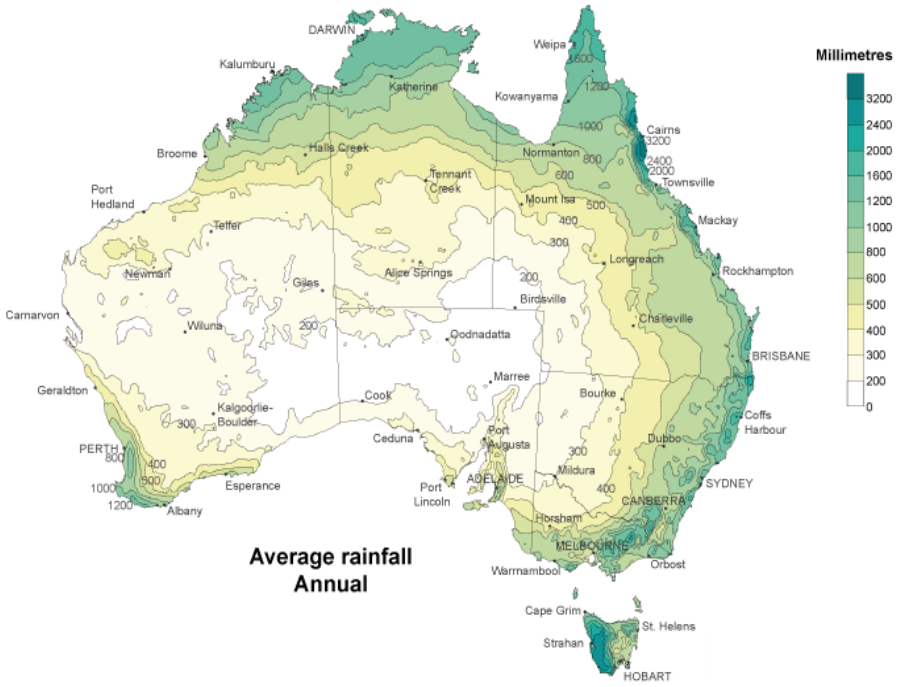


Figure 1.3: Average annual rainfall in Australia, based on 30-year climatology records from 1961-1990 (Bureau of Meteorology 2011).

Geology

The upland geology of the Northern Territory was formed approximately 2 billion years ago (East 1996), while the floodplains of the Northern Territory are comparatively young and were formed after the last interglacial maximum (approx. 20,000 years ago) (Cowie *et al.* 2000a). Once sea levels had stabilised after the melting of the Northern Hemisphere ice sheets (approx. 7,000 years ago) many of the coastal estuaries in the Northern Territory filled with sediment and were subsequently colonised by extensive estuarine mangrove ecosystems (East 1996; Cowie *et al.* 2000a). Over time, many of these wetlands became fresh water (Mulrennan and Woodroffe 1998; Cowie *et al.* 2000a).

The Kimberley region of Western Australia is estimated to be approximately two billion years old (Tyler *et al.* 2012). The area is heterogeneous with regard to landscape,

topography and soil type, all of which has contributed to the regions high levels of biotic diversity (Pepper and Keogh 2014). The dominant landform in the region is the Kimberley Plateau, which is comprised of heavily weathered and dissected sandstone (Pepper and Keogh 2014) along with areas of limestone formed by Devonian reefs (Playford 1980). Millions of years of weathering has led to heavily dissected sandstone pavements, platforms and the formation of gorges and broad flat valleys (Daniel and Mucina 2013).

The geography of the wet-dry tropics of Queensland is diverse, encompassing a range of geological features. The Cape York Ranges are a large sandstone escarpment, bounded to the east by the Coral Sea and to the west by the clay pans of the Gulf of Carpentaria (Bowman *et al.* 2010). Further to the south-east, volcanic activity, occurring up until 6000 BP, has resulted in the formation of a number of basalt ranges (Frakes 1999).

Vegetation

The wet dry tropics can be broadly described as tropical to subtropical grasslands, savanna and shrublands (Department of Sustainability Environment Water Population and Communities. Commonwealth of Australia 2012). According to Bridgewater's (1994) floristic classification system, the area falls within four major vegetation classifications (Groves 1999): Forest to open woodland forest, predominately *Eucalyptus* with a grassy understory reminiscent of the Indo-Malayan monsoonal forest (Blakella-Gardenia Division, 1); Open savanna dominated by *Melaleuca* that becomes inundated in the wet season (Melaleuca-Sorghum Division, 2); Open woodland dominated by *Acacia* shrubs and trees and *Triodia* hummock grassland (Acacia-Triodia Division, 3); and Open woodland with a dense grassy understorey with pockets of monsoon forest (Geijera-Paspalidium Division, 9). In northern, coastal Queensland, high rainfall areas are dominated by monsoon rainforest (Groves 1999). While many individual plant species are widespread through the wet-dry tropics of Australia, there are several regions of high species richness and endemism. Some 3,000 species are known from the Kimberley region of Western Australia (pers. comms. R. L. Barrett and M. D. Barrett) and the Cape York Peninsula in Queensland, of which at least 300 species are endemic (Woinarski *et al.* 2007).

Freshwater wetlands

While Seminiuk and Seminiuk's (1995) system of classifying wetlands is commonly used throughout Australia, I have chosen to use a classification system developed by Jacobs (1983) through this thesis. Unlike Seminiuk and Seminiuk's (1995) system, Jacobs' (1983) system was developed for Australian wetlands, and makes particular reference to wetland flora. As such, the freshwater wetlands of the wet-dry tropics of northern Australia can be simply classified according to their geographical and physical characteristics and is divided into six distinct wetland types:

1. Coastal wetlands
    a. Upland swamps
    b. Rivers and their tributaries
    c. Floodplain swamps and billabongs
    d. Coastal lagoons and lakes
    e. Estuaries (with further sub classification)
2. Mountain lakes and swamps
3. Inland rivers
    a. Rivers (subdivided into perennial or ephemeral)
    b. Billabongs and floodplains
    c. Swamps
4. Inland lakes
5. Mound springs
6. Constructed wetlands (dams, ditches, channels, canals bores etc.)

Between Broome (Western Australia) and Cooktown (Queensland) there are more than 60 major rivers and hundreds of smaller streams and creeks that enter the northern oceans (Woinarski *et al.* 2007). Large seasonal floodplains are common through the Northern Territory, many of which are protected within Australia's largest national park, Kakadu (Russell-Smith *et al.* 1995; Finlayson and von Oertzen 1996). Given the seasonality of rainfall, catchments within the wet-dry tropics receive significantly different amounts of summer rainfall, with five-fold difference between some catchments (Butler 2008). Additionally, these wetlands are characterised by high evaporation rates, particularly in areas inland from the coast (Butler 2008).

Vegetation

The vegetation communities of individual wetlands in northern Australia (particularly floodplains) are usually less diverse than the surrounding terrestrial vegetation (Cowie *et al.* 2000a). However the overall diversity of wetland types in northern Australia leads to a large diversity of aquatic and wetlands plants in the region as a whole. Inland swamps, such as those common through the Kimberley region of Western Australia, are dominated by sedges and rushes (Cyperaceae and Restionaceae) surrounded by *Melaleuca* (Myrtaceae) (Sainty and Jacobs 2003). Larger, deeper inland rivers may be fringed with *Melalueca, Eucalyptus* (Myrtaceae) and *Pandanus* (Pandanaceae), while in-stream vegetation may include *Nymphoides* (Menyanthaeaceae), *Blyxa* (Hydrocharitaceae) or *Eliocharis* (Cyperaceae) (Sainty and Jacobs 2003). Coastal billabongs are usually surrounded by a fringe of *Melaleuca, Pandanus* or *Barringtonia*, with a strip of grass or sedges species (*Eliocharis, Oryza, Pseudoraphis*) and aquatics such as *Najas* (Hydrocharitaceae), *Utricularia* (Lentibulariaceae) and *Nymphoides* (Cowie *et al.* 2000b).

Threats

While many wetlands of northern Australia remain in a relatively pristine state, they are not without threats to their biodiversity and functioning. The impact of widespread agriculture and associated damage caused by stock (predominantly cattle), feral animals (pigs, goats, horses, buffalo), and water diversion for irrigation, combined with inappropriate fire regimes, introduced weeds (e.g. *Salvinia molesta* and *Mimosa pigra*) and increasing urbanisation has led to the degradation or complete loss of many waterways (Skeat *et al.* 1996; Finlayson *et al.* 1997; Finlayson and Rea 1999). The anticipated impacts of climate change, including sea-level rise and subsequent salt-water intrusion are expected to become one of the major challenges to coastal wetland management this century (Eliot *et al.* 1999; Steffan *et al.* 2014).

Part 2: The genus *Nymphaea* L.

"How stainless it rises from its slimy bed! How modestly it reposes on the clear pool… Symmetrically perfect, its subtle perfume is wafted far and wide; while there it rests in spotless state, something to be regarded reverently…"

- Chinese writer Chou Tun-I in the 11th Century AD, quoted in Giles's "Gems of Chinese Literature" referring to *Nymphaea tetragona*, as written by Conard (1905).

Common throughout many freshwater wetlands in northern Australia is the iconic genus *Nymphaea* (Nymphaeaceae)- the waterlilies. While not only providing important ecosystem services such as soil stabilisation and food for migratory waterbirds, waterlilies are of high value for Indigenous Australian's, as the plants are a traditional food source. The value of the *Nymphaea* therefore extends well beyond their presence *in situ*. Given their wide distribution through northern Australia, and importance to Australia's freshwater wetlands, this thesis focuses on the seed biology of Australian members of the *Nymphaea*. Here I provide an introduction to the genus, and highlight their importance as some of the most ancient of the flowering plants.

Evolution of the angiosperms

There has been much research into defining the evolutionary pathways and subsequent radiation of the angiosperms; the largest and most successful group of plants in the Earths' history (Willis and McElwain 2002; Soltis *et al.* 2005). Molecular techniques and the use of extant lineages from the fossil record have improved our understanding in this regard (Friis *et al.* 2001; Feild *et al.* 2003; Friis *et al.* 2005) However, the accurate placement of a number of genera (both extant and extinct) within this evolutionary lineage is often hotly contested, and much current research is focussed on the correct positioning of species within the angiosperm phylogenetic tree [e.g. Hydatellaceae (Qiu *et al.* 1999; Friis and Crane 2007; Saarela *et al.* 2007)]. The placement of taxa within the tree ultimately assists with understanding how certain traits evolved within the angiosperms; for example flowering and pollination strategies and desiccation tolerance in seeds [e.g. (Rudall *et al.* 2008; Rudall *et al.* 2009a; Rudall *et al.* 2009b; Thien *et al.* 2009; Tuckett *et al.* 2010c)].

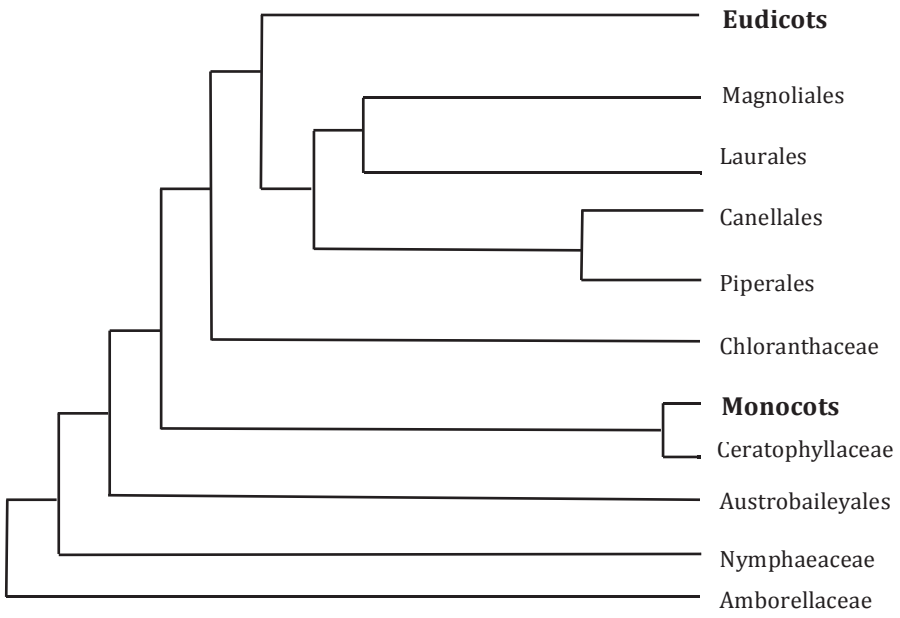


Figure 1.4: Simplified angiosperm phylogenetic supertree, showing the placement of the Nymphaeaceae in relation to the rest of the angiosperms, modified from Soltis *et al.* (2005).

The Nymphaeaceae form part of a grade of species at the base of the angiosperm phylogenetic tree (Les *et al.* 1999; Qiu *et al.* 1999; Friis *et al.* 2001; Feild *et al.* 2003; Soltis *et al.* 2005) (Fig. 1.4). These taxa are sometimes referred to as the ANITA (*Amborella*, Nymphaeaceae, Illiciales, Trimeniaceae and Austrobaileyaceae) grade angiosperms (Qiu *et al.* 1999), however due to realignments of taxa within this grade this grouping is contentious and the ANA (Amborellaceae, Nymphaeaceae and Austrobaileyaceae) grade of angiosperms has been offered as an alternative (Soltis *et al.* 2005).

Fossilised seeds of the Nymphaeaceae are frequently recognised from the Palaeocene, through the Tertiary and Quaternary (Collinson 1980), while leaves have been identified as early as the Cretaceous (Collinson 1980). The most recent fossil evidence has dated *Jaguariba wiersemana,* an extinct member of the Nymphaeaceae, to the late early Cretaceous (125-100 Ma) (Coiffard *et al.* 2013), which also suggests an earlier divergence time between the Nymphaeaceae and the Cambombaceae (e.g. from extinct species *Pluricarpellatia peltata*) in the Aptian Age of the early Cretaceous (125-113 Ma) than initially postulated by other authors (Collinson 1980; Löhne *et al.* 2008b). Differentiation of the extant genus *Nymphaea* is thought to have occurred during the late Oligocene or early Miocene (30-20 Ma), with a southward radiation of species from

Eurasia (Borsch *et al.* 2007; Löhne *et al.* 2008b). The Australasian subgenus *Anecphya* of *Nymphaea* is thought to have radiated during the Miocene (18-15 Ma), when global sea levels were low and Australia was connected to Papua New Guinea and the Indo-Malayan Archipelago (Löhne *et al.* 2008b).

Taxonomy

The Nymphaeaceae family is divided into five genera; *Barclaya, Euryale, Nuphar, Nymphaea* and *Victoria* of which *Nymphaea* is the most specious and includes the recently reclassified *N. ondinea* (previously from the monogeneric genus *Ondinea*) (Löhne *et al.* 2009).

The taxonomy of the extant members of the genus *Nymphaea* has also been debated and fraught with difficulty since the publication of Conard's (1905) monograph of the genus. Not only are plants morphologically diverse, as flowers in a single population can show different petal colouration and sepal patterning, but many species can readily hybridise [e.g. (Les 1993; Slocum 2005; Jacobs and Hellquist 2011)]. The issue is confounded further by the relative difficulty and dangers associated with accessing many field locations; soft soils and inundated areas make vehicle access almost impossible, making boats a necessity, particularly when sampling in large or deep waterways. In most of the tropical regions (the major area of concentration of *Nymphaea*), crocodilians (crocodiles, caiman and alligators) also inhabit many of the waterways making entry on foot almost impossible and highly dangerous. Due to the remote, difficult and dangerous nature of many places, it is likely that current *Nymphaea* collections are still under-representing the true species diversity.

Between Conard's (1905) publication (which described 34 species of *Nymphaea*), and the end of the 20th century, there was a static period of new species being described. However, there has been a rapid rise in the number of *Nymphaea* taxa described since the year 2000 (Borsch *et al.* 2011), including 10 newly named taxa from Australia since 2006 (Jacobs and Hellquist 2006, 2011) effectively doubling the number of *Nymphaea* species to over 60 (see distribution section of this review and Appendix 1 for full current species list). With the advancement of molecular techniques, a reasonably robust phylogeny of Australian *Nymphaea* is now available (Jacobs and Hellquist 2006;

Borsch *et al.* 2007; Jacobs 2007; Löhne *et al.* 2008a; Löhne *et al.* 2008b; 2009; 2011; Jacobs and Hellquist 2011).

Until recently the genus was divided into five subgenera; *Anecphya* (Australasia), *Brachyceras* (pantropical), *Hydrocallis* (tropical and sub-tropical America), *Lotos* (palaeotropical) and *Nymphaea* (northern temperate) (Conard 1905; Borsch *et al.* 2007). However, revisions by Jacobs (2007) split the subgenus *Anecphya* into two; *Anecphya* and *Confluentes*, with the former characterised by relatively large seed and the latter characterised by smaller seed (Löhne *et al.* 2008a) (Fig. 1.5).

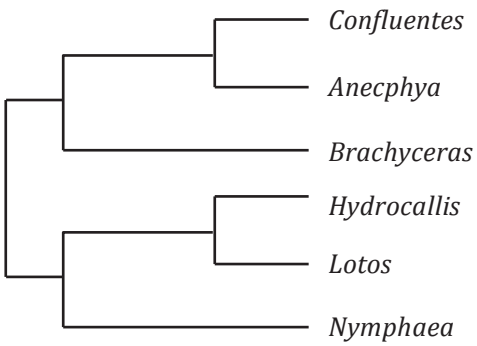


Figure 1.5: Simplified phylogeny of the *Nymphaea* subgenera. Modified from Borsch *et al.* (2011). Note that the position of subgenus *Confluentes* (Jacobs 2007) has not been confirmed via molecular data.

In Australia, the genus now includes 18 native species and 4 naturalised species (Jacobs and Hellquist 2006, 2011). Of the native species, subgenus *Anecphya* contains eight species [*Nymphaea atrans, N. carpentariae, N. georginae, N. jacobsii, N. immutabilis, N. kimberleyensis N. macrosperma, N. gigantea* and (Löhne *et al.* 2008a; 2009; Jacobs and Hellquist 2011)], subg. *Confluentes* contains eight species (*Nymphaea alexii, N. elleniae, N. hastifolia, N. lukei, N. nolea, N. ondinea, N. vaporalis* and *N. violacea*). *Nymphaea.* subg. *Lotos* is represented by the widely distributed palaeotropical species *N. pubescens* and *N.* subg. *Brachyseras* is represented by *N. nouchali* which is common in coastal areas of Asia (Löhne *et al.* 2008a).

Distribution

The extant distribution of the *Nymphaea* is cosmopolitan, with the majority of species found in low-lying tropical regions of the world. There is a distinct pattern of their distribution with regards to subgenera; *Anecphya* and *Confluentes* are restricted to Australia and Papua New Guinea (Australasia), *Brachyceras* is pantropical, *Hydrocallis* is restricted to the tropical and subtropical regions of South and Central America, *Lotos* is palaeotropical and *Nymphaea* occupies the temperate regions of the United States of America, Europe and Asia (Löhne *et al.* 2008b).

In terms of continental distribution, Africa, Australia and the Americas account for 85 % of native species distribution (Fig. 1.6), while Asia and Europe are far less specious. Australia has the greatest proportion of endemic *Nymphaea*, with a total of 13 species with ranges restricted to northern Australia (Fig. 1.6). Africa and Australia have the greatest number of native species, with both continents home to 18 natives. Australia is also home to the largest number of naturalised or invasive species in the world, with populations of *N. alba, N. mexicana, N. odorata N. tuberosa* (subg. Nymphaea), *N. caerulea and N. capensis* (subg. Brachyceras).

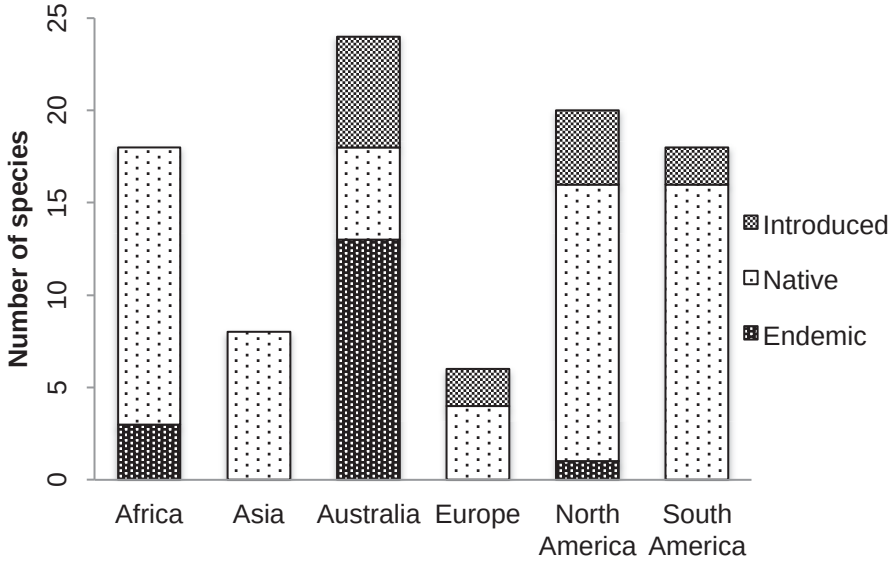


Figure 1.6: Total number of native, endemic and introduced *Nymphaea* species by continent. See Appendix 1 for full species list and their global distribution.

Within Australia, *Nymphaea* are found within a number of different wetland ecosystems, including coastal wetlands (creeks, rivers, billabongs), mountain lakes and swamps and various man-made waterways (Jacobs and Hellquist 2006; Jacobs 2007; Jacobs and Hellquist 2011). They are generally found in slow moving, fresh to brackish waters up to two metres in depth with a fine, silt-clay base, high in organic matter (Cowie *et al.* 2000b; Sainty and Jacobs 2003).

Reproduction; flowering and pollination

All sub-genera of *Nymphaea* tend to produce long pedicles, which raise flowers above the surface of the water (postulated to be an adaptation to either fluctuating water levels or to intercept pollinator flight-paths), except for members of the subgenus *Nymphaea*, which tend to have flowers floating on the water's surface (Conard 1905; Wiersema 1988). The subgenera *Hydrocallis* and *Lotos* are night flowering whereas *Anecypha*, *Brachyceras* and *Confluentes* flower during the day (Slocum 2005; Jacobs 2007; Maia *et al.* 2014). Subgenus *Hydrocallis*, including *N. rudgeana* and *N. amazonum,* are night flowering species pollinated by scarab beetles (Wiersema 1987). Members of this subgenus produce remarkably strong volatile organic compounds (Prance 1980; Maia *et al.* 2014) and heat in order to attract pollinators such as *Cyclocephala* (Ervik and Knudsen 2003; Hirthe and Porembski 2003). The day flowering genera, *Anecypha*, *Brachyceras* and *Confluentes* are brightly coloured (Wiersema 1988) and attract a range of potential pollinators from the orders Coleoptera, Diptera and Hymenoptera e.g. *N. mexicana* (Capperino and Schneider 1985). Though it should also be stated that several species of *Nymphaea* are capable of self-fertilisation (Conard 1905; Wiersema 1987, 1988). Post pollination, the production and germination of seeds is the most common method of reproduction in the *Nymphaea*. However, viviparous reproduction occurs in a few species, for example *N. micrarantha*, *N. lasiophylla* and *N. prolifera* (Slocum 2005) and propagation from detached horizontal rhizomes also occurs in other species, particularly in the subgenus *Nymphaea* e.g. *N. odorata, N. tuberosa,* and *N. alba* (Wiersema 1988).

Dispersal

Seeds of *Nymphaea* are hydrochorously dispersed via water movement (Conard 1905). At the time of release from the parent plant, seeds are surrounded by an air-filled membranous aril which provides buoyancy, allowing the seed to move downstream (Smits *et al.* 1989). However, dispersal via this method is limited within the same water body, or downstream (Smits *et al.* 1989) and does not account for movement into isolated wetland habitats (Wongsriphuek *et al.* 2008). Seed and small fragments of aquatic plants are also dispersed internally through the guts of birds, fish and turtles (endozoochory) or externally on bird feathers (epizoochory) (Figuerola and Green 2002). Seeds of *N. odorata* are consumed by a number of waterfowl including the ring-necked duck (*Aythya collaris*) (Hoppe *et al.* 1986). *Nymphaea alba* seeds are unable to survive the gut passage through a number of waterfowl and fish species, as seeds are digested prior to excretion (Smits et al. 1989), however they do survive passage through European pond turtles (*Emys orbicularis*) (Calviño-Cancela *et al.* 2007). Furthermore, *N. alba* seeds are recalcitrant (desiccation sensitive) and are therefore unlikely to survive external transport on water birds (Smits et al. 1989). However, transport within a birds crop and subsequent regurgitation has been postulated (Löhne *et al.* 2008b).

Ethnobotanical significance

There has been a long held fascination with the Nymphaeaceae that dates back through the centuries, as people use them in some fashion on every continent in which they occur. Perhaps most famous is the use of *N. caerulea* by the ancient Egyptians, as the waterlily features prominently in religion, culture and art (Conard 1905; Irvine and Trickett 1953; Emboden 1978). The use of *Nymphaea* for religious and medicinal purposes has also been known to occur within India (Conard 1905; Irvine and Trickett 1953; Roy *et al.* 2013), South America (Conard 1905) and China (Conard 1905; Irvine and Trickett 1953). In Conard's monograph (1905), aesthetic interest is well documented (and highly regarded), however the more significant and indispensible uses of *Nymphaea* are dismissed in a brief sentence stating that they are simply used for food by "a number of…races, as in Australia, Madagascar, West Africa and Central America".

Indigenous Australians predominantly use waterlilies as a year-round staple food-source, particularly in the drier months of April-December (Brockwell *et al.* 1995). All parts of the plant are utilised and their food value is high; the stalks (pedicels) have a high water content, the seeds provide carbohydrates and fat while the tubers contain a range of carbohydrates, proteins, fibre and trace elements (Issacs 1987). In the Northern Territory, the Gundjeyhmi people of Kakadu collect the large seeds of *Nymphaea macrosperma* ('mardjarkarlang') eating them raw, lightly toasted, or ground up into a flour to make small cakes (Brockwell *et al.* 1995). In the northwest Kimberley region of Western Australia (Wunambal Gaambera country) and the south-east Kimberley and western Northern Territory (Jaru country) people dig up and roast the tubers of *N. violacea* ('miiyani') and dive to the bottom of waterways to collect the ripe seed heads ('danggarri') (Deegan *et al.* 2010; Karadada *et al.* 2011). The Aboriginal women of Arnhem Land have the task of collecting tubers, which are cooked in hot coals and further ground into a paste to feed children and the elderly (Issacs 1987). Dyes can also be extracted from the seeds and flowers of *N. macrosperma* (purple) and *N. violacea* (blue), which is then used to dye cloth (Issacs 1987). As such, the conservation value of *Nymphaea* to indigenous Australians from the wet-dry tropics of northern Australia is high.

Horticulture

Horticulturally, *Nymphaea* are highly sought after, with over 3,000 registered types and varieties available (Slocum 2005). Numerous growers and hybridizers exist through America and South-East Asia and there is a well-connected international network of professionals and enthusiasts sharing information through a number of societies (e.g. the International Waterlily & Water Gardening Society).

In Australia, over-collection of wild populations has been identified as a potential threat to a number of desirable species (Sainty and Jacobs 2003). However, the *ex situ* collection of living plants is increasingly becoming a 'last ditch' option of keeping vulnerable or endangered species from extinction, for example *N. thermarum* (Fischer and Rodriguez 2010).

Conservation status

Currently, the majority of *Nymphaea* are not listed as threatened or endangered. On the IUCN red-list, only nine species have profiles, three of which are data deficient, one is extinct in the wild (*N. thermarum*) while the other species are of 'least concern' (IUCN 2014). In Australia, *N. ondinea* subsp. *petaloidea* is considered to be 'priority one' within the state of Western Australia, due to its limited distribution, small population size and overall lack of information (Western Australian Herbarium 1998). Given that the total global diversity of *Nymphaea* is poorly understood (see Taxonomy section above), it is likely that many species may in fact be short-range endemics. Additionally, given the ever-increasing threats to wetlands, the likelihood that *Nymphaea* populations will be adversely affected is high.

Part 3: Seed dormancy and germination

As outlined in part 1 of this review, the conservation significance of *Nymphaea* (and the wetlands they occupy) in northern Australia cannot be understated. While there has been a reasonable effort to conserve important wetland areas in northern Australia [e.g. Kakadu National Park covers almost 20,000 km^2 (Department of Sustainability Environment Water Population and Communities 2011)], threatening processes are complex and land management alone will not mitigate all threats (Hay *et al.* 2000). Therefore, investment in other conservation measures, such as *ex situ* seed banking, forms an essential component of biodiversity management for the continued preservation of important plant species. However, *ex situ* seed banking comes with its own set of complexities and a rigorous understanding of seed biology must be established prior to successful *ex situ* conservation.

Seed dormancy

Seed dormancy is an important ecological strategy that prevents seed germination when seedling establishment and survival is unlikely, even when conditions are otherwise favourable, contributes to the formation of a seed bank and promotes asynchronous germination to reduce competition between siblings (Forbis *et al.* 2002b; Baskin and Baskin 2014). Seed dormancy is postulated to have evolved in order to allow seeds to; 1) persist in risky environments, 2) escape direct competition with siblings or other plants 3) survive in unfavourable seasons, or 4) optimise the timing of germination (Baskin and Baskin 2014). Until recently, morphological dormancy (MD), whereby the embryo contained within the seed is small and underdeveloped (Martin 1946) was considered to be the ancestral dormancy type in the angiosperms (Forbis *et al.* 2002b). However recent evidence now suggests that morphophysiological dormancy (MPD, discussed later in this section), whereby seeds contain an underdeveloped embryo and a physiological limitation to germination, is the true ancestral state (Willis *et al.* 2014).

The size, shape and position of the embryo is inextricably linked with dormancy type and a species' position within the angiosperm phylogenetic tree (Forbis *et al.* 2002b; Finch-Savage and Leubner Metzger 2006; Baskin and Baskin 2014; Willis *et al.* 2014). For example, seeds with underdeveloped and undifferentiated (i.e. not differentiated

into a radicle and cotyledon(s)) embryos (rudimentary) are considered to be the basal state, whereas large, fully developed embryos are considered more derived (Finch-Savage and Leubner Metzger 2006; Baskin and Baskin 2014). Seeds of *Nymphaea* have been found to have a broad, fully differentiated embryo (Baskin and Baskin 2007) (Fig. 1.7).

A comprehensive, all-encompassing system for classifying seed dormancy has been widely debated in the literature [e.g. (Nikolaeva 1977; Bewley 1997; Baskin and Baskin 2004a; Finch-Savage and Leubner Metzger 2006; Finch-Savage and Footitt 2012)] and the distinction between the stimulation of germination and dormancy alleviation is contentious (Vleeshouwers *et al.* 1995; Koorneef *et al.* 2002; Thompson and Ooi 2010; Finch-Savage and Footitt 2012; Thompson and Ooi 2012). However, I choose to subscribe to the Baskin's (Baskin and Baskin 2004a) definition of seed dormancy; a dormant seed is one *"that does not have the capacity to germinate in a specified time period under any combination of normal physical environmental factors (temperature, light, moisture etc.) that are otherwise favourable for germination"*. According to Baskin and Baskin's (2004a) classification system, some seeds may simply be classified as non-dormant, while dormant seeds can be classified as having physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) or combinational dormancy (PY + PD) (Table 1.2). Seeds of *Nymphaea* that have been subjected to rigorous dormancy classification have been found to have PD (Baskin and Baskin 2007). However, there is also some evidence to suggest that seeds of *N. lotus* may have MPD (Hay *et al.* 2000). Given their placement within the basal angiosperms, this is entirely possible. As there is no evidence for PY, this dormancy type is not further discussed in this introduction.

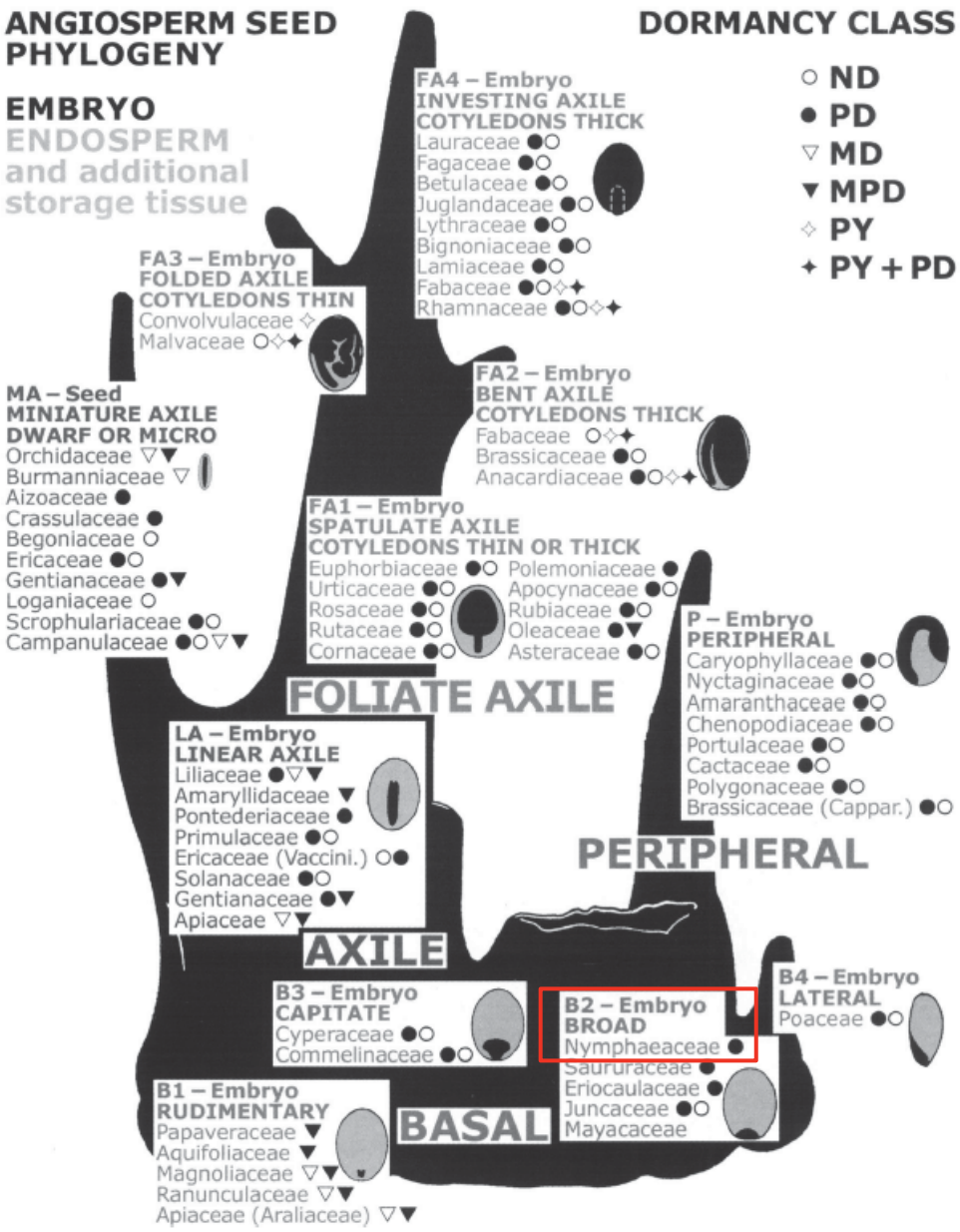


Figure 1.7: Internal seed morphology and embryo types as defined by Martin (1946), overlaid on the angiosperm phylogenetic tree, with the Baskin's dormancy classification (2004b) by Finch-Savage and Leubner Metzger (2006), with the Nymphaeaceae highlighted in the red box. Used with permission from John Wiley & Sons, Inc.

Table 1.2: The dichotomous key used to distinguish dormancy and non-dormancy modified from Baskin and Baskin (2014).

1.	Embryos differentiated and fully developed	2
2.	Seeds imbibe water	3
3.	Root emergence occurs within 4 weeks	4
4.	After root emergence, shoot emergence occurs within a few days	ND
4.	After root emergence, shoot emergence is delayed by 3-4 weeks	PD- epicotyl
3.	Root emergence requires > 4 weeks	5
5.	After root emergence, shoot emergence occurs within a few days	PD
5.	After root emergence, shoot emergence is delayed by 3-4 weeks	PD epicotyl
2.	Seeds do not imbibe water	6
6.	Scarified seed germinate within 4 weeks	PY
6.	Scarified seed do not germinate within 4 weeks	PD + PY
1.	Embryo undifferentiated or differentiated and underdeveloped	7
7.	Embryo not differentiated	8
8.	After dispersal, embryo differentiated and grows	9
9.	Germination occurs within 4 weeks	MD
9.	Germination does not occur within 4 weeks	MPD
8.	After dispersal, embryo never differentiates	10
10.	Germination occurs within 4 weeks	Specialised MD
10	Germination does not occur within 4 weeks	Specialised MPD
7.	Embryo differentiated but underdeveloped	11
11.	Germination occurs within 4 weeks	MD
11.	Germination does not occur within 4 weeks	MPD

Physiological dormancy (PD)

Physiological dormancy is the most common dormancy type in the angiosperms, in which a physiological block impedes radicle emergence i.e. the radicle doesn't have the energetic growth potential to break out of the seed coat (Nikolaeva 1977; Baskin and Baskin 2001; Baskin and Baskin 2007). Physiological dormancy can be further classified into three levels; non-deep, intermediate and deep (Nikolaeva 1977; Baskin and Baskin 2004b), with the majority of PD species falling in the non-deep category which includes many weed, herb and vegetable species (Baskin and Baskin 2004b; Baskin and Baskin 2014). Seeds with non-deep PD can lose dormancy via after-ripening in dry storage, or via cold or warm stratification, with gibberellic acid (GA) promoting germination and excised embryos producing normal seedlings (Baskin and Baskin 2004a). Intermediate PD is characterised by seeds that require dry after-ripening and/or stratification to overcome dormancy, and excised embryos produce normal seedlings, but not all seeds germinate with the addition of GA (Baskin and Baskin

2004a). Finally, deep PD is characterised by seeds that require a long (3-4 month) period of stratification, are not promoted by the addition of GA and produce abnormal seedlings from excised embryos (Baskin and Baskin 2004a). Like the angiosperms as a whole, PD is also the predominant dormancy class in seeds of aquatic species (Baskin and Baskin 2014).

For the purposes of *ex situ* conservation, a number of techniques can be used to mimic natural environmental fluctuations in order to overcome PD. Dry after-ripening, temperature stratification that mimics the shift from winter to summer or vice versa, or a series of wetting and drying cycles have been shown to overcome dormancy in a range of terrestrial and aquatic Australian species (Baskin and Baskin 2003; Baskin and Baskin 2004a; Finch-Savage and Leubner Metzger 2006; Hoyle *et al.* 2008a; Bazin *et al.* 2010; Tuckett *et al.* 2010a; Walck *et al.* 2011).

Morphophysiological dormancy (MPD)

Morphophysiologically dormant seeds not only have an underdeveloped embryo, but also require the correct set of environmental conditions to overcome PD (Baskin and Baskin 2014). MPD is less common than PD in the angiosperms, and is predominantly found in broad-leaved forests of the Northern Hemisphere (Baskin and Baskin 2014). MPD can be further subdivided into two different levels, based on the temperature at which embryo growth occurs: Simple- where embryos grow at temperatures suitable for warm stratification (≥ 15 °C), and Complex, whereby embryo growth occurs at temperatures suitable for cold stratification (< 15 °C) (Baskin and Baskin 2014). Aquatic members of the Apiaceae (e.g. *Angelica*, *Cnidium*, *Ptilimnium*) Araceae, Campanulaceae (*Lobelia*) Iridaceae (*Iris*), Lentibulariaceae (Pinguicula), Menyanthaceae (*Menyanthes, Nymphoides*), Ranunculaceae (*Thalictrum*) and Tolfieldiaceae (*Tofieldia*) have been shown to have MPD (Baskin and Baskin 2014). Additionally, the basal, aquatic genus *Trithuria* (Hydatellaceae) has been shown to have a specialized form of MPD, in which the undifferentiated embryo erupts from the seed and proceeds to differentiate outside of the seed coat (Tuckett *et al.* 2010c). The methods of overcoming MPD *ex situ* are similar to that of seeds with PD, however a

combination of treatments over a longer period may be required to allow for sufficient embryo growth and for PD to be completely overcome (Baskin and Baskin 2014).

Dormancy in the soil seed bank

PD and MPD can be naturally alleviated within the soil seed bank. In terrestrial species occurring in southern Australia, this usually relates to seeds that are dispersed in spring, are subjected to dry-hot conditions over summer and then germinate when soil moisture increases in autumn (Merritt *et al.* 2007). This period of warm, dry conditions is termed dry-after ripening (Baskin and Baskin 1986). In temperate aquatic species, a period of drying would not occur, which is reflected in the recalcitrant storage behaviour of *Nymphaea alba* (Estrelles *et al.* 2004). However, the wetlands occurring in northern Australia are often temporary and seasonal, thus potentially exposing the aquatic soil seed bank to a period of dryness, which may be conducive to dormancy loss (Cross *et al.* 2014; Cross *et al.* 2015).

Changes in soil temperature also affect seed dormancy (Baskin and Baskin 2014). For example, the herbaceous species *Gratiola viscidula* and *Scirpus lineatus* are dormant at the time of natural dispersal and lose dormancy over winter in the sediment seed bank, which allows for high germination rates in spring (Baskin *et al.* 1989). On the contrary, two species of emergent aquatics, *Cyperus oderatus* and *Penthorum sedoides* have been found to be conditionally dormant at the time of natural dispersal and only germinate to high percentages at higher temperatures (alternating 25/15-35/20 °C) (Baskin *et al.* 1989). Species that are dispersed during spring lose dormancy over the warm summer period and germination begins in autumn when temperatures are cooler. In aquatic species from temperate regions seed dispersal is likely to coincide with the cooler and wetter winters. However, seeds of aquatic species occurring in the tropics and subtropics would likely disperse through the hot, wet season. As such, germination of seeds of aquatic species inhabiting this region is likely to be optimal at temperatures ≥ 30 °C [e.g. (Cross *et al.* 2015)].

Seed germination

The term seed germination refers to a series of biochemical events, which is initiated by the uptake of water (imbibition) and is defined by radicle emergence from the seed coat or other covering structures (Bradford 1990; Bewley 1997; Baskin and Baskin 2014). The germination requirements for both terrestrial and aquatic seeds are the same, as they both need optimal temperatures, light (or lack thereof), oxygen (or lack thereof) and water to be available (Cronk and Fennessy 2001).

Temperature

Temperature is one of the most critical factors controlling seed germination and subsequent plant establishment (Walck *et al.* 2011; Rosbakh and Poschlod 2014). A seeds' requirement for optimal temperatures to promote germination may span a small or large range of temperatures. For example, the aquatic species *Myriophyllum spicatum* and *Potamogeton malaianus* have specific thresholds for germination with regards to temperature, and even a 5 °C increase from 10 °C to 15 °C significantly increased final germination of both species (Xiao *et al.* 2010). However, the temperatures optimal for germination often coincide with the season of water availability (Baskin and Baskin 2014). While there is a substantial body of literature investigating the temperature requirements of germination in many terrestrial plant species, there is a lack of information available for aquatic seeds, particularly those of submerged, free-floating or floating-leaved aquatic species. Of species that have been studied, a mean optimal temperature of 24 ± 0.3 °C has been calculated (Baskin and Baskin 2014), however this temperature is skewed in favour of species from the Northern Hemisphere. Temperate species of *Nymphaea* have shown optimal seed germination at 19/15 °C or 20 °C (*N. tuberosa, N. alba*), while tropical species germinated well under warmer temperatures of 30/10°C (*N. capensis*), 30/15 °C (*N. immutabilis*) and 35/20 °C (*N. micrantha*) (Baskin and Baskin 2006; Baskin and Baskin 2007).

Water and cycles of wetting and drying

Dormancy in seeds of aquatic species, particularly those inhabiting ephemeral wetlands, may be overcome or germination cued by fluctuating levels of soil moisture (Casanova and Brock 2000; Deil 2005). While a lack of water may be unusual for seeds of temperate aquatics, in regions where rainfall is less reliable or highly seasonal, a period of drying is common for many aquatic species (Baskin and Baskin 2014). As such, some seeds of aquatic species have been shown to overcome dormancy during a dry period and germinate readily once moisture becomes available (Leck 1989; Leck *et al.* 1989). For example, seeds of red rice (*Oryza satvia*) have been shown to overcome dormancy during the dry season and germinate in the following wet season (Vidotto and Ferrero 2000; Baskin and Baskin 2014). Additionally, the timing, duration and frequency of flooding events may influence dormancy break and subsequent germination (Casanova and Brock 2000; Brock 2011). For example, in short-lived ephemeral wetland sediment seed banks from New South Wales, Australia, short and frequent wetting events were found to promote the greatest germination and subsequent species diversity, particularly in amphibious plant species (Casanova and Brock 2000).

Light

Both the quantity (i.e day length and strength of light- photon flux density) and quality (e.g. the proportion of red and far red light received) of light a seed receives is considered important for germination (Pons 1991; Bell *et al.* 1995; Plummer and Bell 1995; Kettenring *et al.* 2006). Of 212 aquatic species surveyed from a range of authors Baskin and Baskin (2014) found that approximately 46 % had a requirement for light for successful germination, while only 3 % of species had a higher proportion of seeds germinate in complete darkness. For example, Tuckett *et al.* (2010a) found that the aquatic, Western Australian vernal pool species *Glossostigma drummondii, G. sp., G. trichodes, Myriophyllum balladoniense, M. crispatum, M. lapidicola, M. petreaum* and *Triglochin linearis* germinated significantly better when under light conditions, compared with dark. Kettenring *et al.* (2006) and Schutz (Schutz 1997; Schutz 2000) found that a number of *Carex* species required light for successful germination.

Oxygen

In seeds of aquatic species, there is evidence to suggest that there are species that require oxygen to germinate (i.e under non-flooded conditions) while others require anoxia (i.e. under flooded conditions), (Baskin and Baskin 2014). For example, time to germination and total germination was increased for the freshwater aquatic *Vallisneria americana* under oxygenated conditions (Jarvis and Moore 2008), while oxygenated conditions were found to delay germination of the seagrass *Zostera marina*. A study investigating the germination requirements of three nymphaeid species: *Nymphaea alba* (Nymphaeaceae), *Nuphar lutea* (Nymphaeaceae) and *Nymphoides peltata* (Menyanthaceae) showed different responses to the presence of oxygen. *Nymphaea alba* and *Nuphar lutea* were found to reach a higher maximum germination under anoxic, flooded conditions, while *Nymphoides peltata* only germinated under aerobic conditions (Smits *et al.* 1990). However, whether absence or presence of oxygen is to be considered a dormancy breaking or germination stimulating requirement is undecided (Baskin and Baskin 2014).

Other factors affecting seed germination of aquatic species

Seeds of aquatic species may require additional cues, such as water depth and periodicy to ensure successful germination (Cronk and Fennessy 2001). Some seeds are known to require a period of dry conditions (e.g. some vernal pool species), while others germinate readily at the onset of the wet season (Cronk and Fennessy 2001). Some authors have also suggested that the soil substrate of a wetland, along with the ionic composition of the water may also influence a seeds' ability to germinate (Titus and Hoover 1991; Badger and Ungar 1994). A range of nitrogenous compounds such as nitrates and nitrites have also been shown to have an impact on the germination response of a number of terrestrial and some aquatic species (Baskin and Baskin 2014). For example, seeds of *Hygrophila auriculata*, a marsh plant native to Asia and India have shown greater overall germination in response to the application of nitrate under light conditions (Sharma and Amritphale 1989; Baskin and Baskin 2014).

Germination stimulants

Germination may be stimulated using chemicals such as gibberellic acid (GA_3) (Metzger 1983; Adkins and Simpson 1988; Bunker 1994; Chien *et al.* 1998; Watkinson and Pill 1998; Rogis *et al.* 2004; Linkies and Metzger 2012), smoke or its chemical derivatives (Dixon *et al.* 1995; Roche *et al.* 1997, 1998; Flematti *et al.* 2004; Merritt *et al.* 2006) and ethylene (Esashi and Leopold 1969; Cornforth and Stevens 1973; Abeles 1986; Lalonde and Saini 1992; Kępczyński and Kępczyńska 1997; Matilla 2000; Bogatek and Gniazdowska 2012). While GA_3 and karrikinolide (KAR_1, chemical derived from smoke) have been shown to stimulate germination in numerous terrestrial Australian species (Plummer and Bell 1995; Bell 1999; Flematti *et al.* 2004; Merritt *et al.* 2006) and some aquatics (Tuckett *et al.* 2010a) the role that ethylene plays in stimulating the germination of native seeds (whether for conservation or restoration purposes) remains poorly studied.

Ethylene

Ethylene is one of the five major groups of plant growth regulators, which also includes abscisic acid, auxins, gibberellins and cytokinins (Arshad and Frankenberger 2002) and is one of the simplest biological hydrocarbons (C_2H_4). It is known to play a role in a number of plant activities including fruit ripening, seed germination, root initiation and formation of adventitious roots, flowering, leaf senescence and responses to biotic and abiotic disturbances (Arshad and Frankenberger 2002; Lin *et al.* 2009). While ethylene is produced by a number of plant organs, it also occurs naturally in anaerobic or waterlogged soil (Smith and Russell 1969a; Smith and Restall 1971). This process has been attributed to its biosynthetic production by soil microbia (Jackson 1985) and enzymatic activity (Smith and Russell 1969b; Smith and Restall 1971). It is suspected that ethylene may play a role in the germination of *Nymphaea* (Else and Riemer 1984; Smits *et al.* 1995; Estrelles *et al.* 2004; Sumlu *et al.* 2010)

Ethylene has been shown to stimulate germination in seeds of a number of species (Abeles 1973, 1986; Kępczyński and Kępczyńska 1997; Baskin and Baskin 2001); however, the exact role it plays in seed germination is not yet fully understood (Bogatek and Gniazdowska 2012), though it has been suggested that it contributes to cotyledon

expansion (Hays *et al.* 2000) and zygotic embryogenesis (Matilla 2000; Matilla and Matilla-Vaquez 2008). Seed dormancy is controlled and maintained by abscisic acid (ABA) and gibberellins (GA) along with a range of other signalling hormones including jasmonates and brassinosteroids (Finch-Savage and Leubner Metzger 2006; Bogatek and Gniazdowska 2012; Linkies and Metzger 2012). A number of species have been shown to produce ethylene from their seeds at the point of germination. Peanuts (*Arachis hypogaea*) produce ethylene during germination with maximal production occurring between 46-48 hours after the onset of germination in non-dormant seeds (Ketring and Morgan 1969). Furthermore, germination increased in dormant seeds that were fumigated with gaseous ethylene prior to imbibition (Ketring and Morgan 1969). *Ricinis zanzibarensis* (Castor beans) have also shown to produce ethylene at the point of germination (Spencer and Olson 1965). Whether or not the ethylene gas produced by germinating seeds is required by ungerminated seeds in the same cohort is debatable (Kahn *et al.* 2009).

There is also the possibility that ethylene provides a signalling mechanism to a seed that is in contact with the soil. Esashi and Leopold (1969) suggested that clover's (*Trifoliium subterraneum*) increased germination response to ethylene may be an ecological adaptation to being buried beneath the soil surface; either from ethylene endogenously produced by the seed being in an enclosed space, or by environmentally produced ethylene from the soil. Abeles (1973) suggested that dormancy break may be signalled by increased ethylene concentrations in the soil, and there is some evidence for this occurring in aquatic environments, including tropical rock pools (Cross *et al.* 2014).

Part 4: *Ex situ* seed storage and seed longevity

Conservation science is increasingly dependent on *ex situ* conservation measures to safeguard species diversity within threatened ecosystems, and seed banking is considered a simple yet cost effective method of storing seed (Hay *et al.* 2000). The science behind successful seed banking has received considerable attention within the last decade due to programs such as the Millennium Seed Bank Project, managed by the Royal Botanic Gardens Kew. Previous studies regarding seed banking and *ex situ* conservation have predominantly focused on storage of terrestrial species [e.g. (Merritt 2006; Crawford *et al.* 2007)]. Comparatively, the storage of seed from aquatic plants is largely in experimental infancy (Hay *et al.* 2000). Given the increasing threats to the worlds' wetlands and their plants, the need to understand how to effectively store and manage aquatic seed collections is critical to continued conservation of vulnerable species.

Classification and evolution of seed storage behaviour

Successful seed banking ultimately relies upon seeds that survive desiccation and subsequent storage. As such, seeds may be classified into one of three storage categories: orthodox, recalcitrant or intermediate. Orthodox seeds are those that are desiccation tolerant and can survive drying to 3-7 % (15 % RH) internal moisture content and storage at -20 °C (Ellis, Hong & Roberts, 1990, Roberts, 1973, Liu *et al.*, 2008, Pritchard, 2004). Recalcitrant seeds are desiccation sensitive and do not survive drying greater than 40-50 % (> 85 % RH). Intermediate seeds are so classified when they do not satisfy the requirements for classification as either orthodox or recalcitrant and can survive desiccation to < 85 % but not low-temperature storage (Ellis *et al.* 1990, 1991).

Previous authors have hypothesized that seed desiccation tolerance is the derived trait in the angiosperms, (Dickie and Pritchard 2002; Tweddle *et al.* 2003), however recent evidence now suggests that this may not be the case (Tuckett *et al.* 2010b). Additionally, it was also thought that seeds with a high internal moisture content (*c.* 40%) or those shed in wet environments would prove to be less resilient to desiccation than terrestrial species (Offord and Makinson 2009). However, in a previous study of 87

aquatic species, approximately 75 % were found to display orthodox storage behaviour (Hay *et al.* 2000). Investigations into the storage behaviour of *Nymphaea* have revealed a number of inconsistencies (Hay *et al.* 2000). *Nymphaea alba* has been shown to be desiccation sensitive (Smits *et al.* 1989; Estrelles *et al.* 2004) while *N. gigantea* a tropical species also found in Australia, was found to be desiccation tolerant (Ewart 1908; Harrington 1972).

Successful seed banking

Seeds of orthodox species can be successfully stored under standard international seed-banking conditions, i.e. 3-7 % internal moisture content at -20 °C (Crawford *et al.* 2007; FAO 2014). Storing seeds at low internal moisture content avoids potentially lethal damage caused by intracellular ice formation (Walters 2004). However, in some species over-drying can be problematic and higher moisture contents may need to be investigated to optimise storage protocols (Hong *et al.* 1998; Walters *et al.* 2001). Seeds that are particularly short-lived or difficult to store conventionally may be suited to ultra-low temperature (between -140 to -196 °C) cryogenic storage (Walters 2004; Hamilton *et al.* 2009).

Seed longevity

Due to some seeds' ability to survive desiccation and storage almost indefinitely, it is almost impossible to assess the actual amount of time a long-lived seed can maintain viability in storage. As such, models of predicted longevity have been produced based on data gathered by rapidly ageing seeds at high temperatures and relative humidities (Pritchard and Dickie 2003; Davies and Probert 2004). A large data set compiled using these methods have shown that endospermic seeds (i.e. more basal angiosperms) are shorter lived than non-endospermic seeds (i.e. more derived), particularly those from cool wet environments (Probert *et al.* 2009). However, a study on the comparative longevity of aquatic vernal pool species from the southwest of Western Australia found that two species of *Trithuria* (Hydatellaceae) and *Crassula natans* (Crassulaceae) were not short-lived in comparison with the global patterns of longevity (Tuckett *et al.* 2010b). Additionally, a recent study assessing the longevity of Australian species has

found that woody and serotinous species are significantly longer lived than herbaceous species and that seeds from warmer wetter regions were longer lived than those from cool and dry regions (Merritt *et al.* 2014b). As such, predicting the longevity of *Nymphaea* under storage conditions is difficult, given the current body of literature.

Rationale and outline of thesis

Worldwide, the knowledge of the seed biology of *Nymphaea* is very limited, and what information is available generally relates to temperate European or North American species. Tropical Australian species grow in extremely different climatic conditions to their temperate counterparts as they experience the typical tropical 'wet-dry' conditions. For the first time, a detailed ecological seed study has focussed on tropical Australian species and provides information on seed germination, embryo morphology, dormancy break, seed storage behaviour and the potential impact of climate change on seed germination. As a member of the ANA-grade angiosperms, the study of *Nymphaea* also offers a unique opportunity to investigate these traits within the context of the basal angiosperms. Overall, the outcomes of this work will assist in the conservation and preservation of these keystone wetland species by determining species germination, dispersal and storage requirements.

The aims of this research were to:

- Characterise and investigate seed dormancy in representative species of Australian *Nymphaea.*
- Examine the optimal conditions (temperature, light germination stimulant) for successful seed germination and understand natural seed persistence.
- Investigate methods of overcoming dormancy *ex situ* and under natural conditions.
- Evaluate seed storage behaviour and suggest the most effective method of storing seeds *ex situ.*
- Assess the potential implications of climate change on seed germination of a number of species.

This thesis is in agreement with the Postgraduate and Research Scholarship Regulation 1.3.1.33 (1) of the University of Western Australia and is presented as a series of four experimental chapters, three of which are currently in preparation for submission and publication. There are a total of seven chapters in this thesis: A general introduction and literature review, a description of the study species and seed collection and handling, four experimental chapters and a general discussion. The four experimental chapters are presented in the format of scientific papers that can be read individually, or as part of the whole thesis. Each experimental chapter includes the following sections: Abstract, introduction, materials and methods, results, discussion and references. This "thesis-as-a-series-of-papers" format inevitably results in some unavoidable repetition, especially in the materials and methods. While I have tried to keep repetition to a minimum, I have included Chapter 2 in order to ensure the materials and methods are thoroughly clarified.

Chapter 1 (this chapter): summarises the overall aims of the thesis and provides a contextual review of the literature to provide essential background information. The literature review is separated in to four parts; part one gives a general overview of wetlands and their ecological significance, particularly in relation to northern Australia, (the study area for this research) part two is a detailed account of the study genus, *Nymphaea*. Part three specifically reviews the literature in relation to seed biology, germination and dormancy, with a focus on aquatic species including *Nymphaea*. Finally, part four reviews the literature with regard to seed banking, storage and longevity.

Chapter 2: provides a detailed summary of the species of *Nymphaea* used in this dissertation along with some more detailed site-specific information. Additionally, this chapter provides a summary of the common methods and procedures used throughout this dissertation, in relation to seed collection and handling.

Chapter 3: Examines the seed biology and dormancy of six native, tropical Australian *Nymphaea*. In this chapter I show evidence of morphophysiological dormancy within these species- the first conclusive record of this dormancy type within the Nymphaeaceae. This chapter is in preparation for publication.

Chapter 4: further examines the physiological dormancy of *Nymphaea* and discusses the impact of seasonal variability on seed germination. Seed persistence is also explored through an *ex situ* burial trial. This chapter is in preparation for publication.

Chapter 5: is focussed on the seed longevity and storage behaviour of some selected *Nymphaea* species. This chapter also examines the potential for cryogenic preservation as a long-term storage technique. This chapter is in preparation for publication.

Chapter 6: investigates the potential impact of climate change on Australian *Nymphaea*. The salt-tolerance of a number of species of *Nymphaea* is investigated by determining the germination response and changes to early seedling vigour when incubated under increasingly saline conditions.

Chapter 7: Serves to summarise the information presented and discusses the implications of my findings in the context of species conservation and management, particularly in Australia.

There are also several appendices to this dissertation which have not been included in the main text:

1. A total species list of the genus *Nymphaea* and their global distribution
2. Images of Australian wetland habitats
3. SEM and other microscopy of Australian *Nymphaea* seeds
4. Supplementary information for Chapter 3
5. Supplementary information for Chapter 4
6. Supplementary information for Chapter 5
7. Supplementary information for Chapter 6

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CHAPTER 2

DESCRIPTION OF STUDY SPECIES & SEED COLLECTION AND HANDLING

This thesis represents an aggregate of a number of smaller research studies, each of which has been designed for separate publication. However, there are some methodologies that are common throughout the thesis and have been summarised here to ensure that all elements of seed collection and handling procedures have been fully explained.

Species selection

Six species, of a total 18 native species in Australia, were selected for study; *Nymphaea lukei* S. W .L. Jacobs & Hellq., *N. immutabilis* S. W. L. Jacobs , *N. macrosperma* Merr. & L. M. Perry, *N. ondinea* Lohne, Wiersema & Borsch, *N. pubescens* Willd. and *N. violacea* Lehm (Table 2.1).

Nymphaea immutabilis S. W. L. Jacobs Subg. *Anecphya*

Nymphaea immutabilis is native to the northern tropical regions of the Northern Territory and Queensland in Australia, parts of northern New South Wales along with parts of Papua New Guinea (Jacobs and Pickard 1981; Jacobs and Porter 2007) (Fig. 2.1). It is distinguished by its toothed acute leaf margins and blue petals (Jacobs and Pickard 1981; Cowie *et al.* 2000b) (Plate 2.1). Seeds are red when immature, maturing to a brownish-grey with rows of white hairs (Jacobs and Pickard 1981; Cowie *et al.* 2000b).

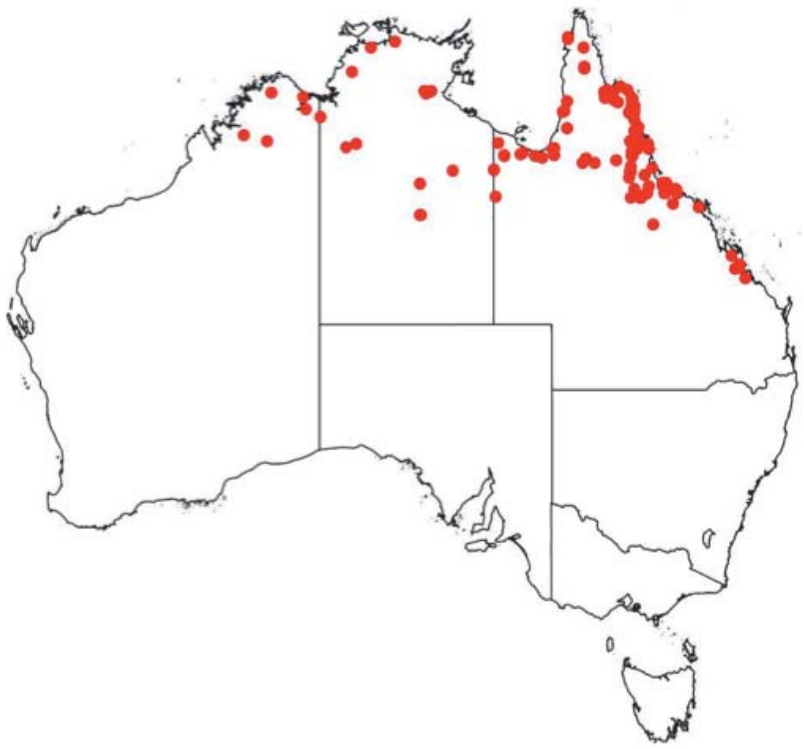

Figure 2.1: Map of Australia, showing known locations of *Nymphaea immutabilis* (red dots). Image from the Atlas of Living Australia (2015).

Plate 2.1: *Nymphaea immutabilis* collected from Barrett's Lagoon, Cooktown, QLD. A) flower B) fruit C) Barrett's Lagoon, typical of northern QLD billabongs.

Nymphaea lukei S. W. L. Jacobs & Hellq subg. *Confluentes*

Nymphaea lukei was separated from *N. violacea* and raised as a new species in 2011 (Jacobs and Hellquist 2011). It is distinct from *N. violacea* morphologically in that it has distinctive two-toned bluish/white petals and strongly sinuate margins of mature leaves (Jacobs and Hellquist 2011) (Plate 2.2). It grows exclusively in ephemeral creek lines originating from the King Leopold Ranges in the Kimberley Region of Western Australia (Fig. 2.2) and is notably absent from larger water bodies such as billabongs and rivers (Jacobs and Hellquist 2011).

Figure 2.2: Map of Western Australia, showing known locations of *Nymphaea lukei* (red dots). Image from the Atlas of Living Australia (2015).

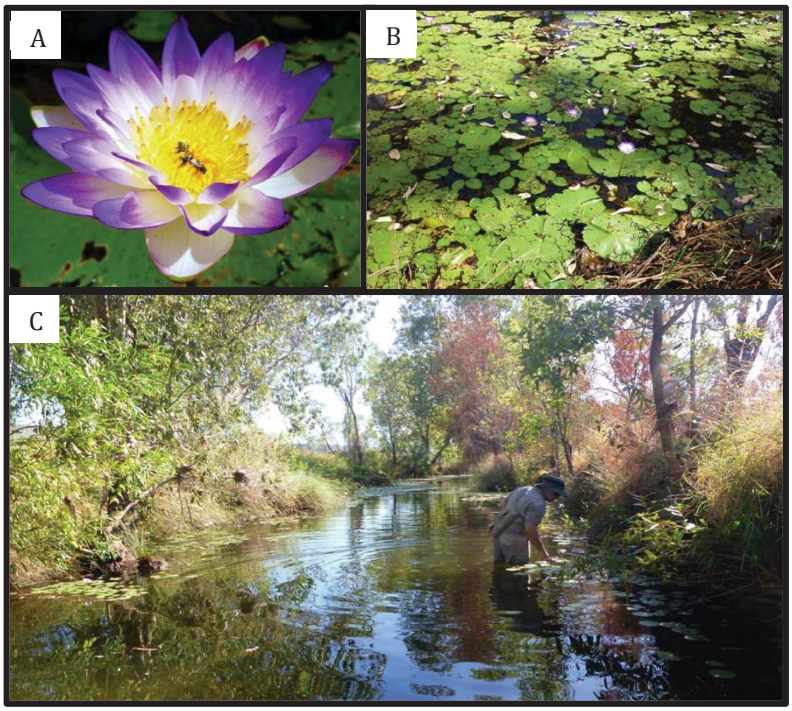

Plate 2.2: *Nymphaea lukei* collected from the Kimberley region of Western Australia. A) flower of *N. lukei,* B) flowers and leaves at March Fly Glen, Western Australia, C) typical habitat of *N. lukei* at Mornington Wildlife Sanctuary.

Nymphaea macrosperma Merr. & L.M.Perry subg. *Anecphya*

Known from northern parts of Western Australia, Northern Territory and Queensland (Fig. 2.3), in Australia and parts of New Guinea and Papua New Guinea *Nymphaea macrosperma* is characterised by large, rounded to globuose seeds with longitudinal rows of hairs (Cowie *et al.* 2000a). Found in a range of water bodies, in water up to 2.5 m deep; usually found fringing the edge of permanent billabongs or semi-permanent drainage channels and backwater swamps (Cowie *et al.* 2000a). Petals are white tinged, pale purple or blue through to dark purple (Cowie *et al.* 2000b) (Plate 2.3).

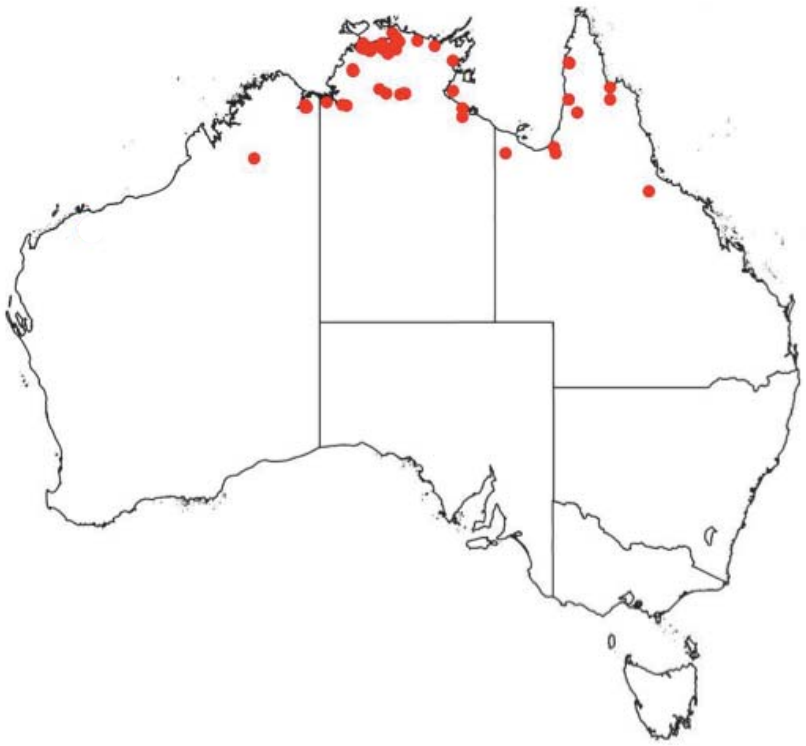

Figure 2.3: Map of Australia, showing known locations of *Nymphaea macrosperma* (red dots). Image from the Atlas of Living Australia (2015).

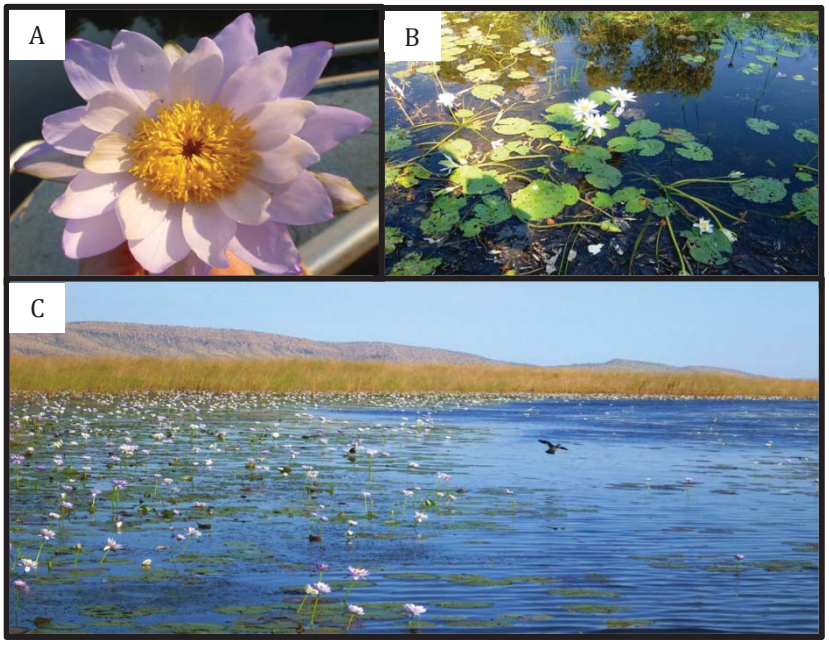

Plate 2.3: *Nymphaea macrosperma* collected from Parry's Lagoons Nature Reserve, WA. A) flower B) whole plant C) Parry's Lagoon, a floodplain billabong typical of northern Australia.

Nymphaea ondinea* Lohne, Wiersema & Borsch subg. *Confluentes

Originally considered to be a monospecific genus within the Nymphaeaceae, *Ondinea purpurea* den Hartog (Hartog 1970) was recently revealed to sit within the *Nymphaea* and has been reclassified as *Nymphaea ondinea,* within the subgenus *Anecypha* (Löhne *et al.* 2009). Based upon morphological characteristics alone, it is easy to understand how this confusion arose- it's mainly submersed mature leaves with the occasional floating saggitate leaves and extremely small, apetalous flowers are extremely unusual within the family (Plate 2.4). It grows exclusively in ephemeral, sandstone based creeks in the far north Kimberley region of Western Australia (Fig. 2.4).

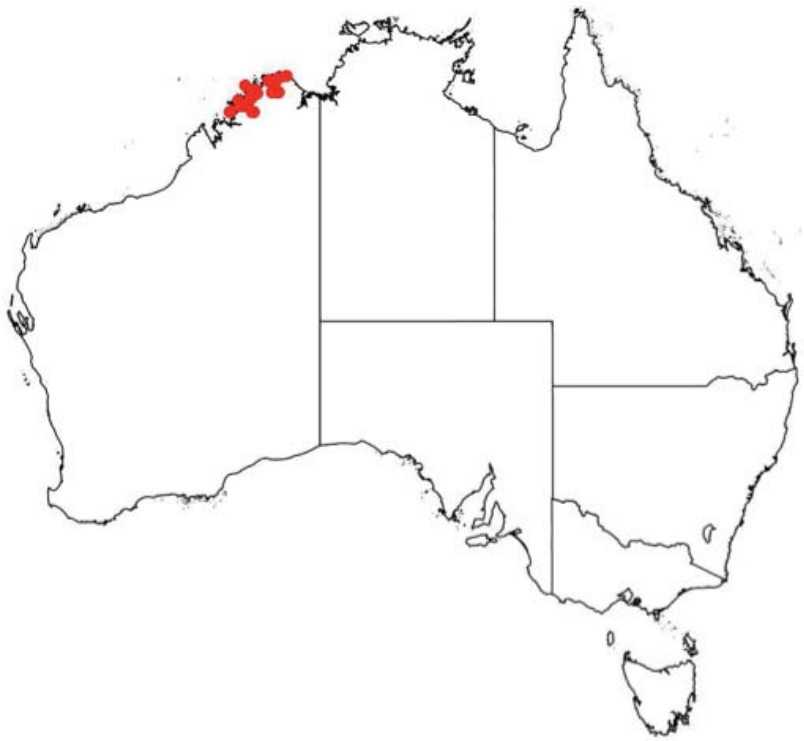

Figure 2.4: Map of Australia, showing known locations of *Nymphaea ondinea* (red dots). Image from the Atlas of Living Australia (2015).

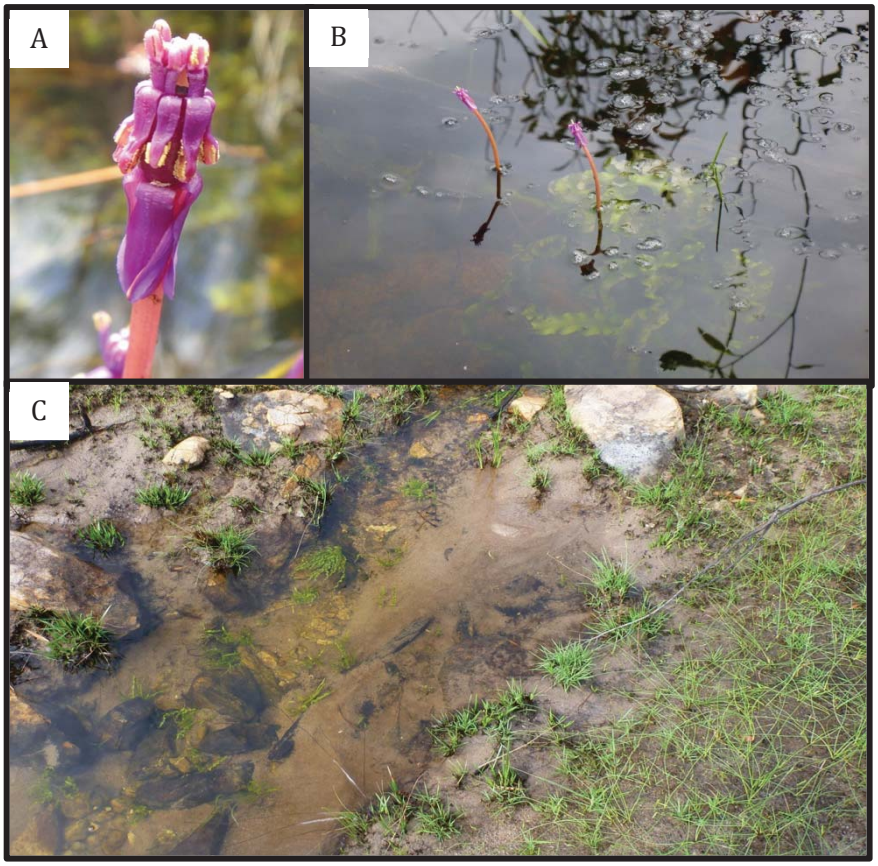

Plate 2.4: *Nymphaea ondinea* collected from the far northern Kimberley region of Western Australia. A) unusual apetalous flower B) apetalous flower above the waterline with submerged leaves C) seedlings growing in a small creek two weeks after the commencement of the 'wet season' in the Kimberley.

Nymphaea pubescens Willd. Subg. *Lotos*

Nymphaea pubescens is common throughout the Northern Territory and Queensland in Australia and is also known from various parts of SE Asia and India (Fig. 2.5). It is readily distinguished from other *Nymphaea* by thick floral filaments and dentate leaf margins (Jacobs and Hellquist 2011). I readily distinguished this species from others by its exceptionally dark-green leaves and flowers that almost sit on the water's surface (Plate 2.5). Seeds are ellipsoidal and covered in longitudinal rows of fine hairs (Cowie *et al.* 2000a).

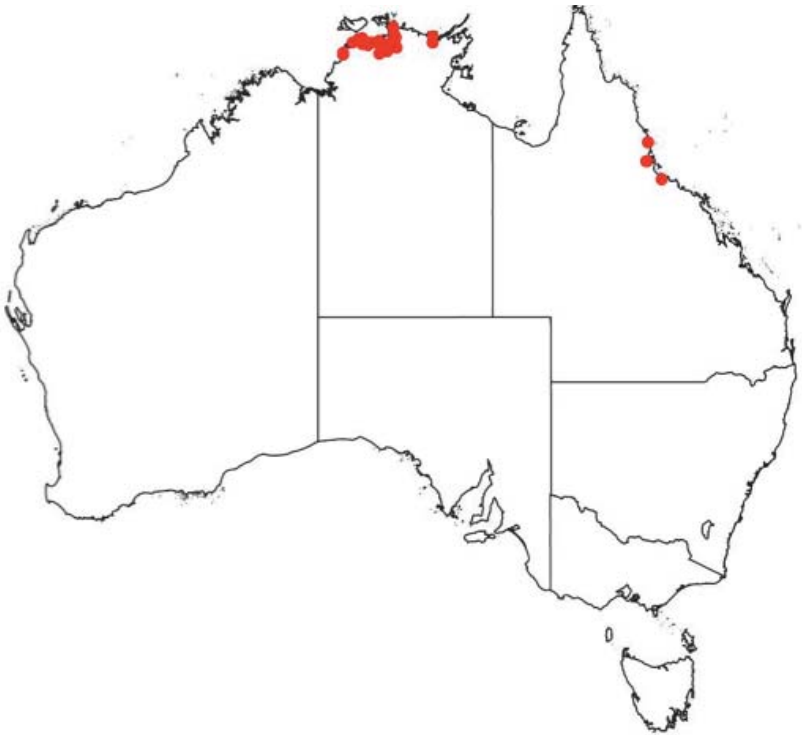

Figure 2.5: Map of Australia, showing known locations of *Nymphaea pubescens* (red dots). Image from the Atlas of Living Australia (2015).

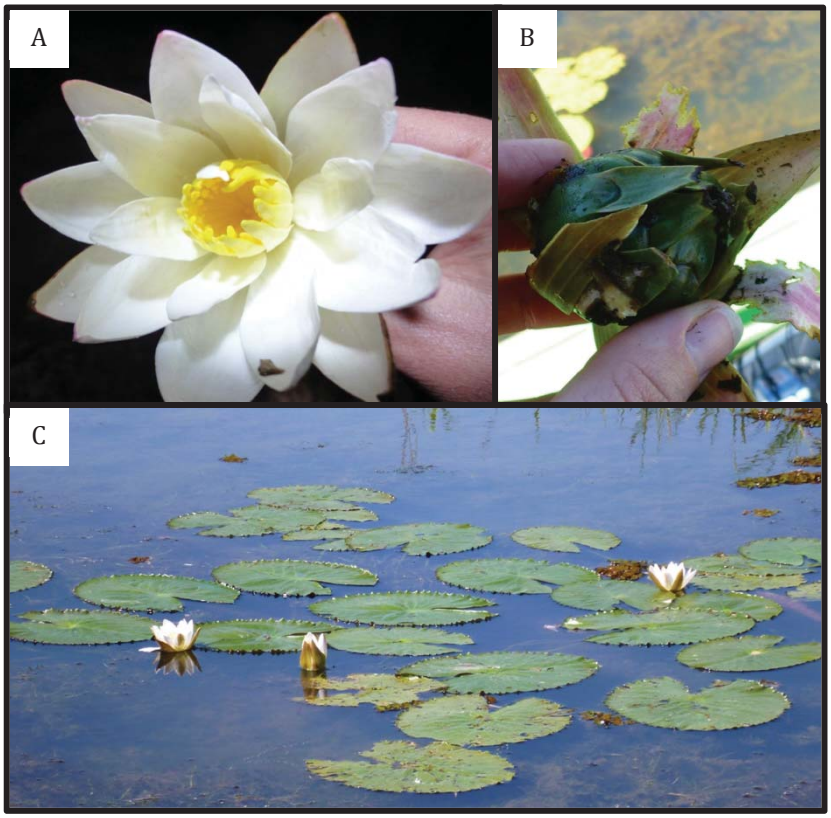

Plate 2.5: *Nymphaea pubescens* collected from the Kakadu National Park, Northern Territory. A) flower B) mature fruit C) whole plant showing position of flowers in relation to water level.

Nymphaea violacea Lehm subg. *Confluentes*

Nymphaea violacea is the most common of the *Nymphaea* in Australia, and is found throughout the tropical regions of Western Australia, Northern Territory and Queensland (Cowie *et al.* 2000a) (Fig. 2.6). Flowers can range between blue, white, mauve or pink and vary within populations (Plate 2.6). Seeds are small and glaborous, ranging between 1.25- 2.2 mm in length (Jacobs and Hellquist 2011). Throughout this study, *N. violacea* was the most often collected- sometimes from the same population in a different season or year. A considerable amount of morphological variation between populations of *N. violacea* has been observed throughout the progression of this thesis- some of which may warrant further investigation to determine whether this is true within-species variation or simply that these are new species which are yet to be classified. As such, each separate seed collection was given a unique accession number and detailed notes about the population were taken. For ease of reading within this thesis, I have included the accession number of each collection in order to differentiate between species if needed.

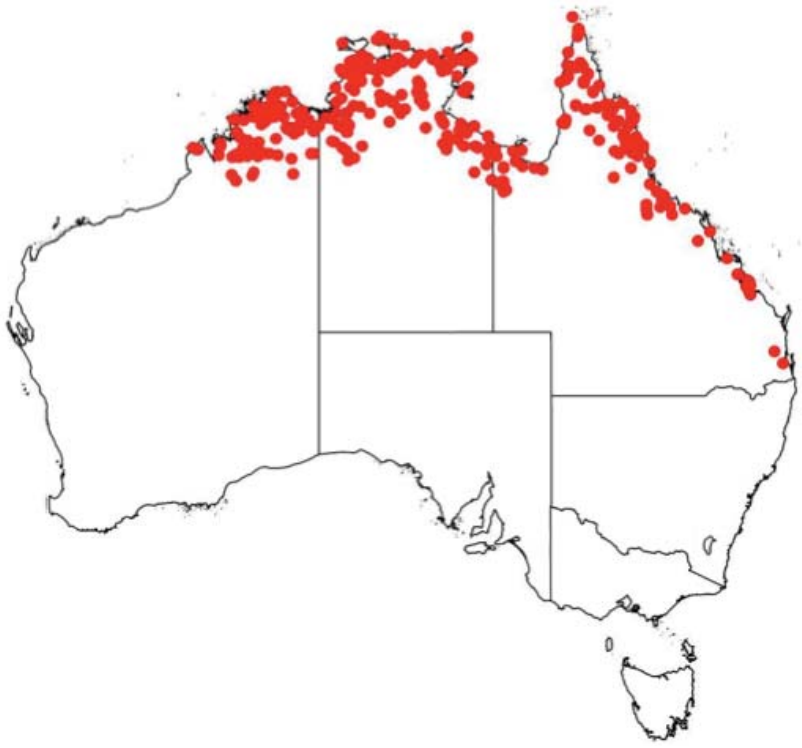

Figure 2.6: Map of Australia, showing known locations of *Nymphaea violacea* (red dots). Image from the Atlas of Living Australia (2015).

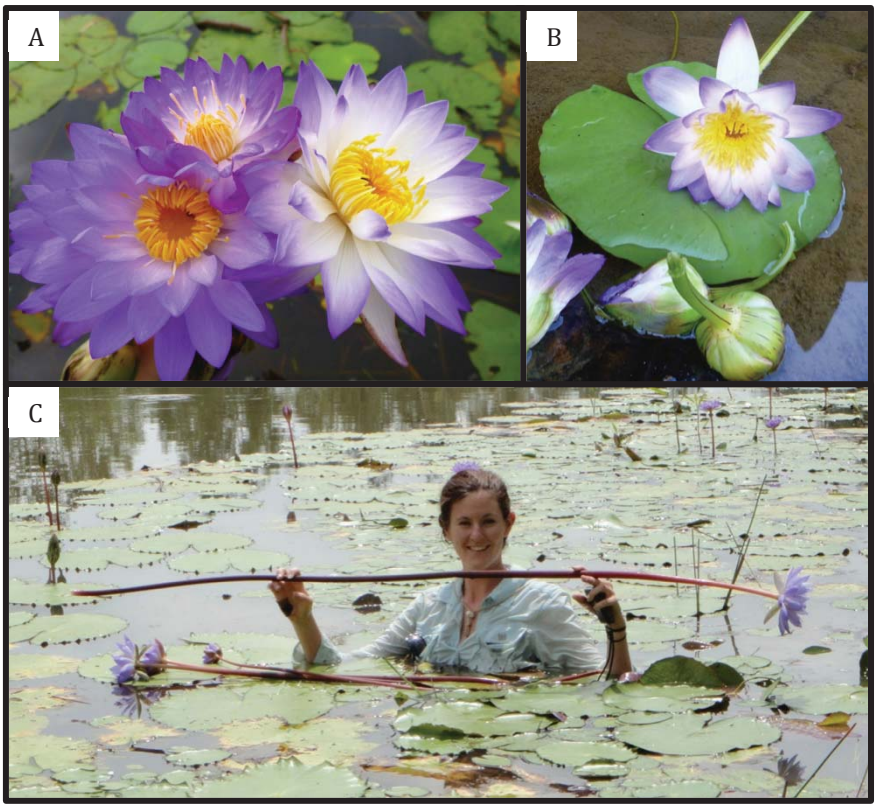

Plate 2.6: *Nymphaea violacea* collected from the Kimberley region of Western Australia A) group of three flowers from the same billabong showing some petaloid colour differentiation B) flower, leaf and mature fruit C) the author displaying the length of the peduncle- image by David Merritt.

Seed collection, processing and initial viability testing

All *Nymphaea* used in this study produce a dehiscent syncarpous berry (fruit), which contains tens to thousands of arrilate seeds (Cowie *et al.* 2000a). Mature seed heads were selected for collection when the peduncle had coiled under water, the ovary wall was in the process of disintegrating, and the berry contained dark-coloured seeds with fully developed arils (i.e seeds were at the point of natural dispersal). All seed used in this study were collected in this state, unless otherwise specified. Multiple collections were made of *N. lukei, N. macrosperma* and *N. violacea,* either from the same location in a different month or year, or from completely separate populations (Table 2.1). Unless otherwise stated, whole fruits of *Nymphaea* were collected in the field and transported in Glad® Snap Lock® bags (approximately 30 mL of water was placed in the bottom of each bag) to Kings Park and Botanic Garden and were left to naturally

dehisce at room temperature (*c.* 24 °C). Collections were made between March and August in 2011, 2012 and 2013.

Table 2.1: Seed collection details for all seeds used experimentally in this study. Initial seed fill was determined via digital x-ray upon arrival at the laboratory, before seed batches were cleaned.

Species	Habitat	Voucher No	Accession No.	State	Collection location (GDA 94)	Date	Initial fill (%)
Nymphaea immutabilis	Freshwater pools, billabongs, swamps, streams and rivers	ELD085	NI1	QLD	S15°25'35.7", E145°9'14.5"	Jul-13	99
Nympahea lukei	Freshwater streams	ELD046	NL1	WA	S17°34'47.6" E126°04'59.6"	May-12	97
		ELD076	NL2	WA	S16°43'0.2", E125°27'35.8"	Jun-13	89
Nymphaea macrosperma	Freshwater pools, billabongs, swamps, streams and rivers	ELD018	NM1	NT	S12°42'47.9" E131°38'17.7"	Jun-11	92
		ELD047	NM2	WA	S15°35'35.7" E128°16'47.9"	May-12	95
		ELD079	NM3	WA	S15°35'35.7" E128°16'47.9"	May-13	98
Nymphaea ondinea	Small, ephemeral, sandstone based creeks	ELD002	NO1	WA	S14°48'30.1" E126°33'01.1"	Mar-11	64
Nymphaea pubescens	Freshwater pools, billabongs, swamps, streams and rivers	ELD013	NP1	NT	S12°53'27.8" E132°30'59.7"	Jun-11	99
Nymphaea violacea	Freshwater pools, billabongs, swamps, streams and rivers	ELD005	NV1	WA	S14°46'33.1" E126°30'52.6"	May-11	74
		ELD010	NV2	WA	S14°47'18.8" E126°31'47.5"	May-11	98
		ELD011	NV3	WA	S14°48'23.9" E126°31'47.5"	May-11	100
		ELD015	NV4	NT	S12°53'27.8" E132°30'59.7"	Jun-11	99
		ELD020	NV5	WA	S15°56'46.5" E128°56'56.6"	Jun-11	98
		ELD044	NV6	WA	S14°46'33.1" E126°30'52.6"	Mar-12	88
		ELD048	NV7	WA	S15°35'35.7" E128°16'47.9"	May-12	68
		ELD077	NV8	WA	S15°51'39.8" E127°14'20.8"	May-13	99
		ELD078	NV9	WA	S15°51'39.8" E127°14'20.8"	May-13	99
		ELD081	NV10	QLD	S16°54'55.3" E145°22'20.6	May-13	98

Upon arrival at the laboratory, each individual seed collection was assigned a unique accession number (e.g. ELD002). The number of days the seed spent in transit, the presence of foreign material (e.g. soil, plant material) and the state of the seeds (i.e. loose seed, whole fruit etc) was also recorded. In order to calculate initial purity, five replicates of 50 seeds were randomly selected from each accession. Seeds were x-rayed (Faxitron MX-20 x-ray cabinet, Tucson, Arizona, USA) at 20 kVa for a total exposure time of 10 seconds. Seeds were determined to be filled upon visual inspection of a digital x-ray image showing uniformly grey/white perispermic tissue and an intact embryo (Figure 2.7). All seed accessions used experimentally were additionally assigned an experimental seed batch number (accession number), consisting of the initials of the species and a number e.g. *Nymphaea violacea* ELD005 is NV1 (Table 2.1).

Seeds were rinsed through brass sieves (Endecotts 710 μm – 2.36 mm) under running water to remove any remaining plant material. Repeated vacuum separation using a seed aspirator ('Zig Zag' Selecta, Machinefabriek BV, Enkhuizen, The Netherlands) and x-raying ensured all experimental batches were ≥ 99 % filled before storage in a controlled environment facility (CE, 15 % RH**,** 15 °C). Seed viability was also confirmed via a cut test, whereby imbibed seeds were cut in half longitudinally and inspected for healthy embryonic tissue. Firm, white embryos were considered viable. Five replicates of 50 filled and intact seeds were also weighed in order to estimate individual seed weight and the number of seeds in the entire lot.

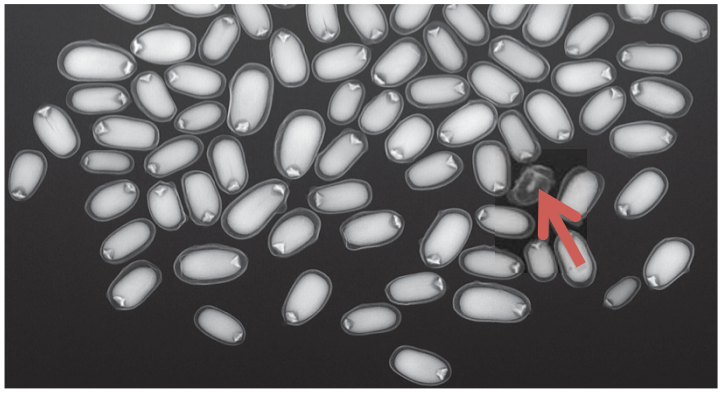

Figure 2.7: Digital x-ray image of *Nymphaea immutabilis.* Arrow indicates an empty seed that would be removed prior to experimental work.

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CHAPTER 3

SEED DORMANCY AND GERMINATION TRAITS OF AUSTRALIAN *NYMPHAEA* L. (NYMPHAEACEAE)

In preparation for publication as:

Dalziell, E. L., Young, R. E., Dixon, K. W. and Merritt, D. J. (in prep.). Seed dormancy and germination traits of Australian *Nymphaea* L. (Nymphaeaceae).

ABSTRACT

Background and aims: Despite their importance as members of the basal angiosperms, the seed biology of Australian *Nymphaea* is under represented in the literature. The aims of this study were to classify dormancy and understand the germination requirements of six *Nymphaea* species occurring in Australia's wet-dry tropics.

Methods: Seeds of *N. immutabilis, N. lukei, N. macrosperma, N. ondinea, N. pubescens* and *N. violacea* were chosen for study. Dormancy was classified via imbibition, precision nicking, embryo excisions and measurements of embryo growth. In order to determine the optimal conditions for germination, seeds were incubated under a range of temperatures (10, 15, 20, 25, 30, 35, 40 °C and alternating 20/35 °C), with and without light, and in conjunction with a range of germination stimulating treatments (gibberellic acid, karrikinolide, Ethrel® and crowding- whereby 50 seeds are clustered together under water). Additionally, multiple seed collections of *N. lukei*, *N. macrosperma* and *N. violacea* were tested in order to determine whether germination requirements differed between accessions.

Key results: Embryo growth occurred prior to radicle emergence in seeds of *N. immutabilis, N. lukei, N. macrosperma, N. ondinea* and *N. violacea*. Seeds of *N. pubescens* did not germinate under any conditions. Germination was greatest in seeds of all species when incubated in the light, at temperatures between 30- 35 °C and in the crowding treatment. Germination in complete darkness was < 19 % for all species under all treatments and was significantly reduced or absent at temperatures ≤ 25 °C. The addition of germination stimulants gibberellic acid and karrikinolide did not significantly increase germination, while the application of Ethrel® did increase germination of some species, when compared with the control.

Conclusions: Seeds of Australian *Nymphaea* (that germinated in this study) display morphophysiological dormancy, which is consistent with the hypothesis that MPD is basal among the angiosperms. Furthermore, the temperatures for optimal germination reflect the natural conditions experienced in the wet-dry tropics of northern Australia. The success of the crowding treatment was attributed to the endogenous production of ethylene, which acts as a germination stimulant.

Key words: basal angiosperm, embryo growth, morphophysiological dormancy, aquatic plants, accessional variation, wet-dry tropics.

INTRODUCTION

The placement of taxa within the angiosperm phylogenetic tree aids our understanding of how plants may have adapted and evolved in response to changing environmental conditions over time. Investigations of the key features of angiosperm seeds, such as embryo size and shape, along with patterns of dormancy and germination inform us of the basal states and evolutionary pathways undertaken by the angiosperms to become the most diverse and dominant group of plants on earth (for example see Rudall *et al.* 2008; Rudall *et al.* 2009a; Rudall *et al* 2009b; Tuckett *et al*. 2010b).

The Nymphaeaceae or waterlily family, are aquatic members of the ANA-grade (Amborella, Nymphaeales, and the Austrobaileyales) angiosperms (Les *et al.* 1999; Qiu *et al.* 1999; Friis *et al.* 2001; Feild *et al.* 2003; Soltis *et al.* 2005). Extant members of the Nymphaeaceae are cosmopolitan in distribution, with approximately 65 species occurring on every continent except Antarctica (Löhne *et al.* 2008b). In Australia, the genus *Nymphaea* is the only representative of the family and 18 species (Jacobs and Hellquist 2006; Borsch *et al.* 2007; Jacobs 2007; Löhne *et al.* 2008a; Löhne *et al.* 2009; Jacobs and Hellquist 2011) are found through the wet-dry tropics; the northern area of the continent that is characterised by hot wet summers (receiving approximately 600-1600 mm of rainfall per annum) and warm, dry winters (Finlayson 1999). Despite exhibiting the greatest species endemism of *Nymphaea* in the world (Chapter 1), Australian *Nymphaea* are under represented in the scientific literature, with the majority of studies focussing on temperate species occurring in perennial wetlands in the northern hemisphere. Given the diverse range of habitats *Nymphaea* occupy in northern Australia (Chapter 1 and Appendix 2), including ephemeral wetlands, it may be predicted that individual species or populations may have evolved different dormancy and germination responses as a result of habitat variability.

Embryo morphology and development is ultimately linked to the evolution of different types of seed dormancy (Forbis *et al.* 2002b; Finch-Savage and Leubner Metzger 2006; Willis *et al.* 2014). For example, characteristics of seeds with morphological (MD) and morphophysiological dormancy (MPD), are related to the size, shape, position and level of development of the embryo at the time of natural dispersal (Baskin and Baskin

2004b). Seeds with MD contain underdeveloped embryos, which under the right environmental conditions must grow prior to radicle emergence. Equally seeds with MPD also contain underdeveloped embryos, but dormancy is additionally regulated by environmental cues and the lack of embryonic growth potential to break through the seed coat (Baskin and Baskin 2004b).

It is generally accepted that small rudimentary embryos are basal, larger more developed embryos are more recent, and embryo size has increased over time (Martin 1946; Nikolaeva 1977; Baskin and Baskin 2001; Forbis *et al.* 2002b). As such, the majority of the extant members of the ANA grade angiosperms display MD or MPD, including the specialised type of MPD in *Trithura* (Hydatellaceae) (Tuckett *et al.* 2010c). As a basal angiosperm, it was always concluded that embryos of *Nymphaea* were similarly underdeveloped (Forbis *et al.* 2002b). However, *N. immutabilis, N. lotus, N. capensis* var. *zanzibariensis*, and the hybrid *N.* 'Albert Greenburg' were found to possess differentiated and fully developed embryos that did not undergo embryo growth prior to germination (Baskin and Baskin 2007). Thus it was initially concluded that the seeds of *Nymphaea* are physiologically dormant (PD). Nonetheless, given the capacity for multiple species within a genus to display different dormancy types, rigorous testing is required before an individual species can be ascribed a dormancy classification (Baskin and Baskin 2014). The phylogenetic placement of *Nymphaea* as basal angiosperms, combined with the relatively recent description of a number of new species of *Nymphaea* in Australia (Jacobs and Hellquist 2006; Löhne *et al.* 2008a; Jacobs and Hellquist 2011) warrants further investigation.

Once dormancy has been overcome, like those of terrestrial species, seeds of aquatic plants require the optimal combination of temperature and light (or lack thereof), along with water availability in order for germination to proceed (Cronk and Fennessy 2001). Temperature is one of the most critical factors controlling seed germination and subsequent plant establishment (Walck *et al.* 2011; Rosbakh and Poschlod 2014). While a temperature optima of 24 ^{o}C has been suggested for successful germination in seeds of most aquatic plants (Baskin and Baskin 2004a), the majority of species presented in this dataset are from the northern hemisphere. Due to the hot climatic conditions experienced in northern Australia, it is reasonable to hypothesise that the optimal temperature for seed germination occurring in this region would be higher than the

global average. The presence of light has also been shown to be a requirement for germination for the vast majority of aquatic seeds (Baskin and Baskin 2014) and seeds of *N. odorata* and *N. alba* have both been shown to germinate to higher percentages in the presence of light, compared to complete darkness (Else and Riemer 1984; Sumlu *et al.* 2010).

Interestingly, germination of *N. odorata* has been shown to increase significantly from < 50 % to 100 % after five months when a large number of seeds are crowded together in vials of water (Else and Riemer 1984). These results were assumed to be caused by the stimulatory action of endogenously produced ethylene, but was never conclusively confirmed. However, the addition of 2-chloroethylphosphonic acid (Ethephon, which is metabolised to ethylene; a commonly available agricultural plant growth regulator) to dormant *N. odorata* seeds also significantly increased germination (Else and Riemer 1984). A number of other studies have confirmed the stimulatory effect of ethylene on seed germination, both in terrestrial and aquatic species (Smith and Russell 1969a; Ketring 1977; Osborne 1984; Kępczyński and Kępczyńska 1997; Bogatek and Gniazdowska 2012; Baskin and Baskin 2014). In Australia, a recent study investigating the germination response of ephemeral rock pool aquatics from the monsoonal Kimberley region of Western Australia has also confirmed that ethylene serves to stimulate germination in aquatic species of *Portulaca* and *Myriophyllum* (Cross *et al.* 2014).

For *ex situ* germination requirements of *Nymphaea*, in addition to the use of ethylene, the application of chemical stimulants found to be effective for terrestrial species may also prove successful. For example, the application of gibberellic acid (GA_3) (Bell *et al.* 1995; Plummer and Bell 1995) or smoke and its chemical derivatives (Dixon *et al.* 1995; Roche *et al.* 1997, 1998; Flematti *et al.* 2004; Baker *et al.* 2005; Merritt *et al.* 2006) have both been found to increase the germination response of a range of terrestrial and aquatic (Tuckett *et al.* 2010a) species.

Given their significance as basal angiosperms, popularity among horticulturalists (Sainty and Jacobs 2003) and value to indigenous Australians as a traditional food source (Issacs 1987; Press *et al.* 1995b), the conservation significance of *Nymphaea* in Australia cannot be overstated. As such, we addressed three aims in order to improve *ex situ* conservation of Australian *Nymphaea*:

1. Classify seed dormancy,
2. Determine the optimal conditions (light, temperature, germination stimulant) to promote germination, and
3. Assess whether different seed collections (accessions) of the same species exhibit the same dormancy and germination responses *ex situ*.

MATERIALS AND METHODS

Species selection and seed collection

Seeds of six species of *Nymphaea* were used experimentally in this study; *N. immutabilis* Hook., *N. lukei* S.W.L. Jacobs & Hellq., *N. macrosperma* Merr. & L.M.Perry, *N. ondinea* Löhne, *N. pubescens* Willd. and *N. violacea* Lehm. were collected over three years between March 2011 and August 2013 from multiple locations across northern Australia (Fig 3.1). As collections took place over a number of years and seasons, mean monthly climate data (minimum and maximum temperatures and mean monthly rainfall) for each collection was obtained from the nearest Australian Bureau of Meteorology weather station (Table 3.1). Mature seeds were collected at the point of natural dispersal. Due to a number of small seed collections, multiple collections (accessions) were made for a number of species (Table 3.1). Unless otherwise stated (Table 3.1), whole seed heads were collected, kept moist (approximately 30 mL of water was placed in the bottom of each bag) and transported to Kings Park, Perth in Glad® Snap Lock® bags. Upon arrival, seed heads were left in shallow pans of water to naturally dehisce at room temperature (*c.* 24 °C). All seeds were separated from chaff using an aspirator (SELECTA BV Gravity Seed Seperator "Zig Zag", Netherlands) or by hand. Initial seed fill was determined by x-ray analysis using a Faxitron Specimen Radiography System MX-20 Cabinet.

Seed quality assessment and seed characteristics

To determine initial viability, a seed cut test was used (ISTA 1985) whereby three replicates of 20 seeds were cut in half and visually inspected for damage. Seeds with a fully formed, turgid, white embryos and perisperm were considered viable. Seed morphometrics (length and width) were assessed on 50 randomly chosen seeds using an

Olympus SZX16 dissecting microscope equipped with a Nikon DSFi1 camera and digital micrometer. Five replicates of 50 filled and intact seeds were also weighed in order to estimate individual seed weight.

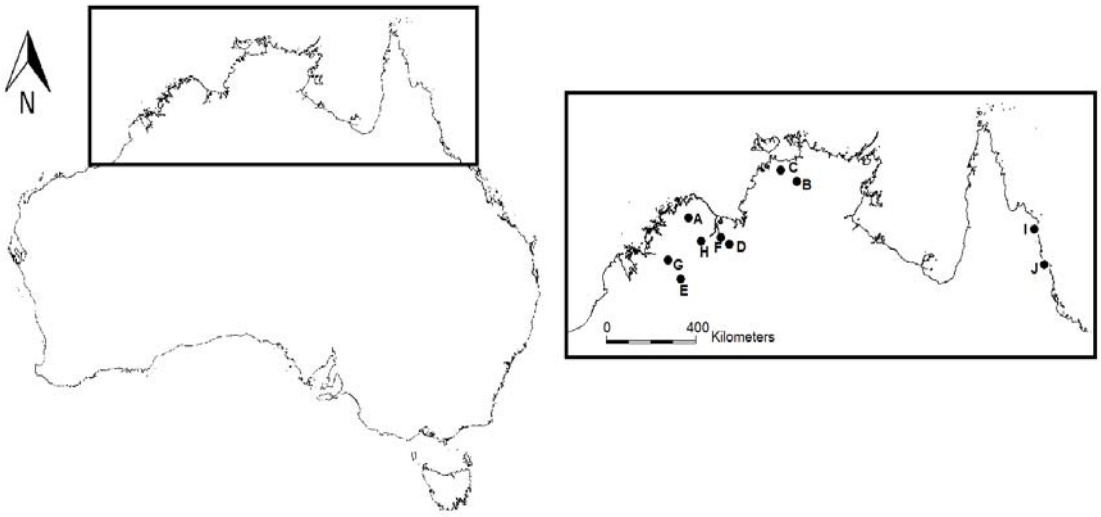

Figure 3.1: Collection locations for *Nymphaea* seeds used in this study: (A) *N. ondinea* (NO1), *N. violacea* (NV1, NV2, NV3, NV6) (B) *N. pubescens* (NP1), *N. violacea* (NV4) (C) *N. macrosperma* (NM1) (D) *N. violacea* (NV5) (E) *N. lukei* (NL1, NL2) (F) *N. macrosperma* (NM2, NM3), *N. violacea* (NV7) (G) *N. lukei* (NL3) (H) *N. violacea* (NV8, ELD78 NV9) (I) *N. immutabilis* (NI1) (J) *N. violacea* (NV10).

Dormancy classification

In order to classify dormancy according to Baskin and Baskin (2004b; 2014), we conducted a series of experiments in order to systematically rule out dormancy types. Water uptake (imbibition) was used to rule out physical dormancy, while precision nicking, embryo growth measurements and embryo excisions were used to determine the presence of PD, MD, or MPD.

Water uptake (imbibition)

Seeds underwent imbibition tests whereby, three replicates of ≥ 0.05 g of seed (or 15-25 individual seeds for larger seeds) of each species were weighed and placed on a circle of nylon mesh on glass filter paper (Advantec, 84 mm), inside a Petri dish under

standard laboratory conditions (*c*. 22.5 °C). Initial seed weight (t_0) was recorded before the filter paper was irrigated with RO filtered water. At intervals of 1, 2, 5 and 30 minutes and 1, 2, 4, 6, 8, 24, 48, 72 and 96 hours, the mesh and seeds were removed from the Petri dish and seeds were blotted dry with paper towel to absorb surface moisture and reweighed. Percent moisture uptake was determined gravimetrically using the following equation:

$$\%\ increase\ in\ mass = \frac{W_i - W_d}{W_d} \times 100$$

Where W_i is the weight of imbibed seeds at any given time (t_x) and W_d is the dry weight at t_0. In order to assess whether seeds possessed physical dormancy, three replicates of ≥ 0.05 g of seed from each were nicked with a scalpel through the seed coat along the longitudinal axis and subjected to the same imbibition procedure.

Precision nicking

Seeds of *N. lukei, N. violacea and N. macrosperma* were scarified to determine whether this treatment would alleviate dormancy. Four replicates of 25 seeds had a small portion of the seed coat removed with a scalpel along the longitudinal axis before being plated on either water or GA_3 agar (described below in 'Germination requirements') and incubated 25, 30, 35 and 40 °C under 12h/ 2h light/dark. Treatments were scored daily for germination for the first two weeks and weekly for a further 8 weeks. Seeds were considered to have germinated upon radical emergence ≥ 1 mm.

Embryo classification and growth measurements

In order to confirm the classification of *Nymphaea* embryos as broad according to Martin's (1946) system, and that embryos were differentiated, seeds were left to imbibe for 24 hours on moist filter paper before being dissected and visually inspected using a microscope. To determine whether embryo growth occurred prior to radicle emergence, two separate experiments were performed:

Experiment 1

Two hundred seeds of *N. lukei, N. macrosperma* and two accessions of *N. violacea* were placed in 10 ml plastic tubes filled with 10 ml of sterile RO water and placed in a 30 °C

incubator under a 12h/12h light regime. Every two days (or every six to eight days for *N. macrosperma*), 20 seeds were randomly selected and removed, cut in half longitudinally with a scalpel blade and total seed length, embryo length and the perisperm length were measured using a digital micrometer. In order to determine when embryo growth occurred in each species, embryo length from each sampling period was assigned to a size class (Adams *et al.* 2011). Six size classes were assigned to *N. violacea* (ELD48) [(1) < 0.20 mm; (2) 0.20- 0.29 mm; (3) 0.30- 0.39 mm; (4) 0.40- 0.49 mm; (5) 0.50- 0.59 mm and (6) 0.60- 0.69 mm] and *N. macrosperma* [0.4- 0.49 mm; 0.5- 0.59 mm; 0.6- 0.69 mm; 0.7-0.79 mm; 0.8- 0.8 9 mm and 0.9- 0.99 mm. Seven classes were assigned to *N. lukei* [(1) 0.3- 0.39 mm; (2) 0.4- 0.49 mm; (3) 0.5- 0.59 mm; (4) 0.6- 0.69 mm; (5) 0.7- 0.79 mm; (6) 0.8- 0.89 mm and (7) 0.9- 0.99 mm].

Experiment 2

In the second experiment, 100 seeds of *N. immutabilis* (NI1) *N. lukei* (NL1 and NL2), *N. macrosperma* (NM3), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV7-NV10) were placed in 10 ml plastic tubes filled with 10 ml of sterile RO water and placed in a 30 °C incubator under a 12h/12h light regime. Seeds were inspected daily and any seeds showing a split in the seed coat (indicating imminent germination) were removed and embryos were excised and measured (Baskin and Baskin 2007).

Embryo excisions

Two hundred individual embryos of *N. immutabilis, N. lukei, N. macrosperma, N. pubescens* and *N. violacea* and 100 embryos of *N. ondinea* were excised from seeds and cultured *ex situ.* Seeds were surface sterilized for 30 minutes in a 2 % (w/v) solution of calcium hypochlorite ($Ca(OCl)_2$) and rinsed three times in sterile RO water, and embryos excised using a binocular microscope under aseptic conditions in a laminar flow. Excised embryos were plated on 90 mm Petri dishes containing either ½ MS basal salts medium (Sigma Australia) (Murashige and Skoog 1962) and 0.7 % w/v agar or ½ MS, 0.7 % w/v agar and 3 μM of GA_3 (Sigma Australia). Petri dishes were sealed and wrapped in aluminum foil and incubated at 30 °C for seven days. After seven days, the aluminum foil was removed and seeds were exposed to a 12h/12 h light regime for a further 49 days.

Table 3.1: Seed collection details for all seeds used experimentally in this study. Conservation status 'NT': not threatened in the state from which they were collected (information from the Department of Parks and Wildlife-Western Australian Herbarium, Department of Land Resource Management- Northern Territory Herbarium, Queensland Department of Environment and Heritage Protection). Endemism refers to species' endemism within Australia, as such 'cosmopolitan' species are common throughout Australia. Seeds that were dried prior to transport to Kings Park in Western Australia (due to quarantine regulations) are denoted with a Y in the "dried for transport" column. Accessions from Queensland (NI1 and NV10) were spread in a thin layer and dried in the sun for 10 days, while accessions from the Northern Territory (NM1, NP1 and NV4) were dried over silica gel for 14 days prior to transport. Accessions denoted with a 'N' were kept moist during transport. Rainfall, mean minimum and maximum temperatures (average for the month of collection) were taken from the Australian Bureau of Meteorology Station nearest to the collection site: *N. immutabilis* = Cooktown Aero; *N. lukei* (NL1) = Mornington (rainfall) and Fitzroy Crossing (temperatures); (NL2) = Charnley River (rainfall) and Mt Elizabeth (temperatures); *N. macrosperma* (NM1) = Cooinda aero (rainfall) and Jabiru (temperatures); (NM2 and NM3) = Parry Creek Farm (rainfall) and Wyndham (Temperatures); *N. ondinea* (NO1) = Theda (rainfall) and Doongan (temperatures); *N. violacea* (NV1, NV2 and NV3) = Theda (rainfall) and Doongan (temperatures); (NV4) = Kununurra border (rainfall) and Kununurra (temperatures); (NV5) = Cooinda aero (rainfall) and Jabiru (temperatures); (NV6) = Theda (rainfall) and Doongan (temperatures); (NV7) = Parry Creek Farm (rainfall) and Wyndham (Temperatures); (NV8 and NV9) = Mt Elizabeth (rainfall and temperatures); (NV10) = Mareeba Airport (rainfall and temperatures).

Species	Subgenus	Cons. Status	Habitat	Endemism	Seedlot No.	State	Collection location (GDA 94)	Date	Dried for Transport?	Rainfall	Mean min (°C)	Mean max (°C)
Nymphaea immutabilis	*Anecphya*	NT	Freshwater pools, billabongs, swamps, streams and rivers	Cosmopolitan	NI1	QLD	S15°25'35.7", E145°9'14.5"	July 2013	Y	15.2	17.2	26.4
Nymphaea lukei	*Confluentes*	NT	Small, ephemeral, sandstone based creeks	Endemic WA	NL1	WA	S17°34'47.6" E126°04'59.6"	May 2012	N	0.0	12.1	35.1
					NL2	WA	S16°43'0.2", E125°27'35.8"	June 2013	N	1.8	12.8	29.6
Nymphaea macrosperma	*Anecphya*	NT	Freshwater pools, billabongs, swamps, streams and rivers	Cosmopolitan	NM1	NT	S12°42'47.9" E131°38'17.7"	June 2011	Y	0.0	16.6	30.3
					NM2	WA	S15°35'35.7" E128°16'47.9"	May 2012	N	0.0	17.3	32.4
					NM3	WA	S15°35'35.7" E128°16'47.9"	May 2013	N	1.8	21.8	34.9
Nymphaea ondinea	*Confluentes*	NT	Small, ephemeral, sandstone based creeks	Endemic WA	NO1	WA	S14°48'30.1" E126°33'01.1"	March 2011	N	257.4	22.3	30.4
Nymphaea pubescens	*Lotos*	NT	Freshwater pools, billabongs, swamps, streams and rivers	NT, QLD	NP1	WA	S12°53'27.8" E132°30'59.7"	June 2011	Y	0.0	16.6	30.3
Nymphaea violacea	*Confluentes*	NT	Freshwater pools, billabongs, swamps, streams and rivers	Cosmopolitan	NV1	WA	S14°46'33.1" E126°30'52.6"	May 2011	N	0.0	12.4	28.9
					NV2	WA	S14°47'18.8" E126°31'47.5"	May 2011	N	0.0	12.4	28.9
					NV3	WA	S14°48'23.9" E126°31'47.5"	May 2011	N	0.0	12.4	28.9
					NV4	NT	S12°53'27.8" E132°30'59.7"	June 2011	Y	0.0	16.6	30.3
					NV5	WA	S15°56'46.5" E128°56'56.6"	June 2011	N	0.0	11.3	27.7
					NV6	WA	S14°46'33.1" E126°30'52.6"	Mar 2012	N	482.5	22.2	31.5
					NV7	WA	S15°35'35.7" E128°16'47.9"	May 2012	N	0.0	17.3	32.4
					NV8	WA	S15°51'39.8" E127°14'20.8"	June 2013	N	0.0	12.8	29.6
					NV9	WA	S15°51'39.8" E127°14'20.8"	June 2013	N	0.0	12.8	29.6
					NV10	QLD	S16°54'55.3" E145°22'20.6"	July 2013	Y	9.4	15.1	24.7

Germination requirements

Factors investigated included light, temperature, germination stimulants (GA_3, KAR_1 and Ethrel®) and crowding (Else and Riemer 1984). All seeds were used in germination experiments within 28 days of collection. Prior to plating, fresh seeds were sterilised in a 2 % (w/v) solution of calcium hypochlorite ($Ca(OCl)_2$) with two drops of surfactant (Tween 80) for 30 minutes under alternating vacuum (10 minutes on/off/on) and then rinsed three times in sterile RO water. Each treatment consisted of four replicates of 25 seeds. Seeds were sown in 90 mm round, sterile Petri dishes containing 0.7% (w/v) water agar, or water agar and gibberellic acid (GA_3, Sigma® Australia, 0.29 mM) or water agar and karrikinolide [KAR_1, 0.67 µm, the butanolide 3-methyl-2*H*-furo[2,3*c*]pyran-2-one, provided by G. Flematti, School of Biomedical, Biomolecular and Chemical Science, University of Western Australia, (Flematti *et al.* 2004)]. The Ethrel® (Bayer Crop Science) treatment was administered by placing seeds inside a nylon mesh bag and soaking in a 20 mM solution of Ethrel® for 24 h prior to plating on 0.7% (w/v) water agar. Petri dishes were wrapped four times in GLAD® Wrap to prevent excess evaporation. To test the effect of crowding on germination, 50 seeds of each species were placed in a 10ml plastic tube (TechnoPlas, Australia) and filled with 10 ml of sterile RO water.

Dark treatments (both Petri dishes and 10 ml tubes) were immediately wrapped twice in aluminum foil following plating and placed in a light-tight box for the duration of the experiment. Light treatments were not wrapped in aluminum foil. Petri dishes and 10ml tubes were incubated in Contherm incubators at 10, 15, 20, 25, 30, 35 and 40 °C and alternating 20/35 °C. Light treatments received a 12 hour photoperiod of 30 µmol m^{-2} s^{-1}, 400-700 nm, cool-white fluorescent light. Light treatments were scored daily for germination for the first two weeks and weekly for a further 8 weeks. Dark treatments were only scored on the last day.

At the conclusion of each experiment, all ungerminated seeds were subjected to a cut test to assess viability (ISTA 1985). Seeds with a fully formed, turgid, white embryos and perisperm were considered viable. Empty, necrotic or dead seeds (characterised by a blackening or browning of the embryo and/or perisperm) were considered non-viable and excluded from further analysis.

Statistical analyses

Germination data were analysed using the '*glm'* function in R (R Core Team 2012) using a generalised linear model with an inbuilt 'logit' link function with binomial errors. The initial model compared all main factors (temperature, light, germination stimulants) and their interactions. To simplify the main model, a stepwise reduction was used for simplification based on the Akaike Information Criterion (AIC) index (Akaike, 1974). When significant differences were detected, *post-hoc* analyses using Holm's pairwise comparisons were used to determine factorial differences. Dark treatments were later omitted from the analysis, due to low germination response in the dark. A Tukey's HSD test ($P < 0.05$) was used to determine differences between all levels of all factors. All other data (imbibition, embryo growth measurements) were analysed using ANOVA in R, and *post hoc* analysis conducted using Tukey's HSD ($P < 0.05$) to determine significant differences.

RESULTS

Quality assessment and seed characteristics

Initial seed fill as determined via x-ray analysis of each species ranged from 64 to 100 % (Table 3.2). In terms of seed size (length and width), *N. immutabilis* and *N. macrosperma* (subg. *Anecphya*) were the larger of the species and *N. violacea* and *N. ondinea* (subg. *Confluentes*) were the smallest, however some variation within species (between accessions) was noted (Table 3.2). This finding supports the taxonomic differentiation between subgenera whereby *Anecphya* is characterised by relatively large seed and *Confluentes* is characterised by smaller seed (Jacobs 2007; Löhne *et al.* 2008a). Seed mass (1000 seed weight) was also variable within species, particularly for accession of *N. macrosperma*, which varied between 7.96 g^{-1} and 18.07 g^{-1} per 1000 seeds.

Dormancy classification

Water uptake (imbibition)

Imbibition experiments demonstrated that seeds of all six species readily took up water over the 72 hour testing period. Seeds that were nicked took up water at a faster rate, however final increase in seed mass after 72 hours was not significantly different (P= 0.9) for both nicked and un-nicked seeds (Fig. 3.2). Therefore, seeds of the tested species of *Nymphaea* are not physically dormant.

Precision nicking

Precision nicking of *N. lukei, N. macrosperma* or *N. violacea* did not result in significantly greater germination than the un-nicked controls (P = 0.9, data not shown).

Embryo classification and growth measurements

Embryos were found to be broad, in accordance with Martin's (1946) classification system, whereby embryos are wider than they are tall and surrounded by abundant endosperm or perisperm (Fig 3.6, Table 3.3). The embryos of all species of *Nymphaea* were also found to be fully differentiated, whereby some development of the radicle and cotyledons was present.

Experiment 1

Embryos of *N. lukei* were found to significantly increase in size over the 10-day incubation time ($P < 0.007$) and the proportion of embryos in larger size classes also increased (Fig. 3.3). Embryo growth initiated after two days of incubation, with the majority of embryos starting to grow after 8-10 days in incubation at 30 °C under crowded conditions. Similarly, embryos of *N. macrosperma* showed a slight increase in size, as illustrated by the number of embryos moving in to higher embryo length categories over the 34-day incubation period (P = 0.06) (Fig. 3.4).

Table 3.2: Average length, width, 1000 seed weight and initial seed fill of all accessions of *Nymphaea immutabilis, N. lukei, N pubescens, N. macrosperma* and *N. violacea.*

Species	Accession	State collected	Ave length (mm)	Ave width mm	1000 seed weight (g)	Seed fill (%, x-ray)
Nymphaea immutabilis	NI1	QLD	4.64 ± 1.30	2.74 ± 0.07	18.89	99
Nymphaea lukei	NL1	WA	2.54 ± 0.05	1.96 ± 0.03	3.84	97
	NL2	WA	2.35 ± 0.06	1.67 ± 0.05	3.47	98
Nymphaea macrosperma	NM1	NT	4.05 ± 0.07	3.35 ± 0.06	18.07	92
	NM2	WA	3.00 ± 0.10	2.61 ± 0.07	7.96	95
	NM3	WA	3.38 ± 0.07	2.85 ± 0.07	11.16	98
Nymphaea ondinea	NO1	WA	1.81 ± 0.04	1.46 ± 0.03	0.81	64
Nymphaea pubescens	NP1	NT	1.93 ± 0.06	1.61 ± 0.06	2.49	99
Nymphaea violacea	NV1	WA	1.90 ± 0.06	1.58 ± 0.05	1.89	74
	NV2	WA	1.73 ± 0.06	1.21 ± 0.05	1.45	98
	NV3	WA	1.68 ± 0.03	1.23 ± 0.03	1.60	100
	NV4	NT	1.52 ± 0.13	0.91 ± 0.08	3.19	80
	NV5	WA	1.58 ± 0.07	1.15 ± 0.06	1.11	95
	NV6	WA	1.19 ± 0.01	0.80 ± 0.01	3.80	88
	NV7	WA	1.60 ± 0.05	1.13 ± 0.04	0.75	68
	NV8	WA	1.62 ± 0.06	1.19 ± 0.05	1.14	99
	NV9	WA	1.57 ± 0.04	1.10 ± 0.04	1.10	99
	NV10	QLD	1.56 ± 0.05	1.08 ± 0.03	0.94	98

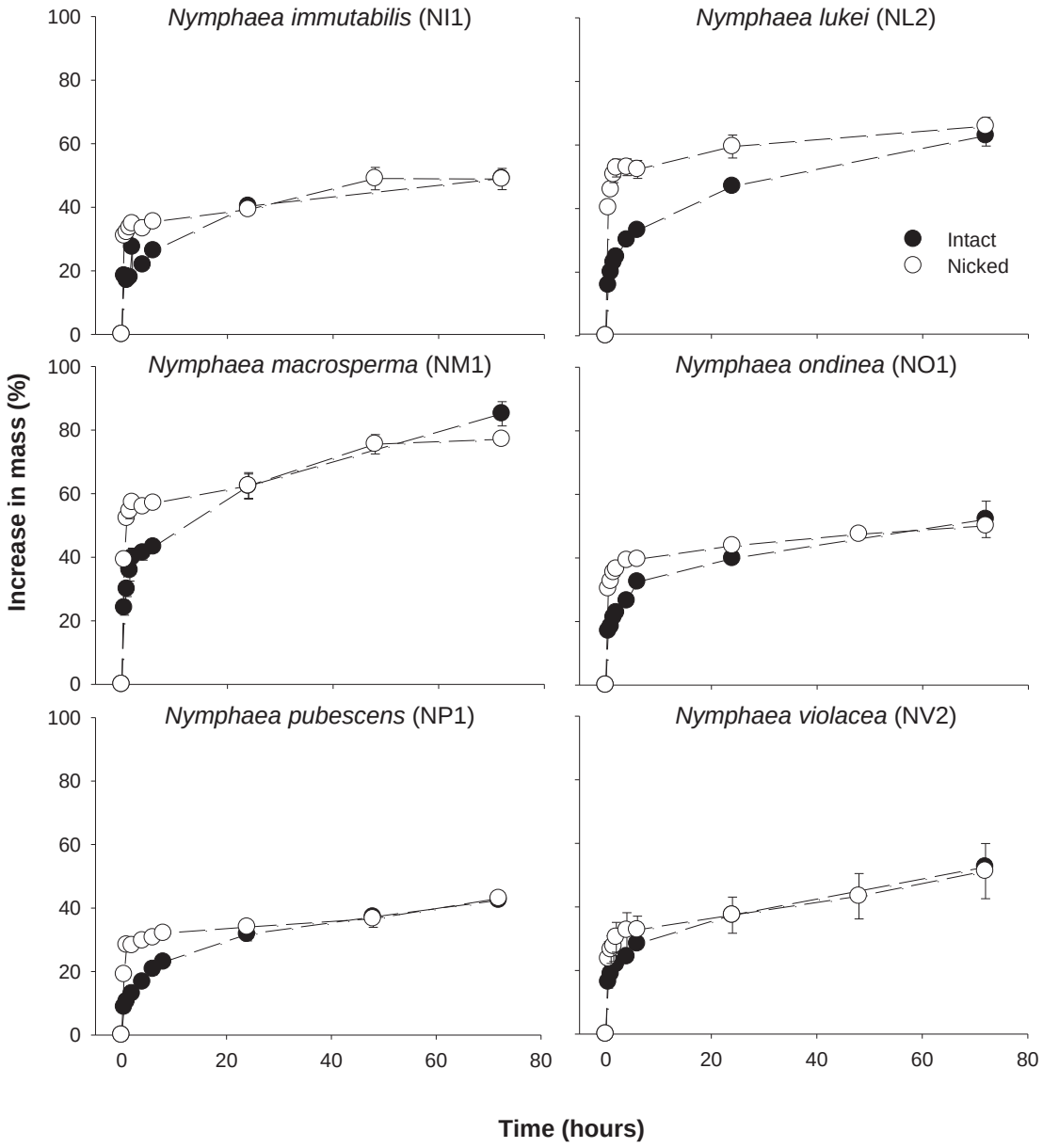

Figure 3.2: Imbibition (% water uptake ± S.E.) of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM1), *N pubescens* (NP1) and *N. violacea* (NV2) seeds left intact or precision nicked. Seeds were kept at room temperature (*c*. 22°C).

Embryos of *N. macrosperma* did not appear to start growing until at least day 14 (Fig. 3.4). Approximately 10 % of embryos in seeds of *N. violacea* transitioned from the 0.20- 0.49 mm to size classes ≥ 0.5 mm over the 10-day incubation period, however this was found to not be statistically significant (P = 0.3; Fig. 3.5).

Experiment 2

During the second embryo growth experiment, all species showed a significant increase in both embryo length (P < 0.001) and E:S ratio (P < 0.001) (Table 3.3, Fig. 3.6). Only one accession of *N. lukei* (NL2) did not show significant embryo growth prior to radicle emergence (P = 0.14). In all accessions time to first germinant took less than 10 days (Table 3.3).

Embryo excisions

Excised whole embryos of *N. immutabilis, N. lukei, N. macrosperma, N. pubescens, N. ondinea* and *N. violacea* failed to germinate under all conditions (data not shown).

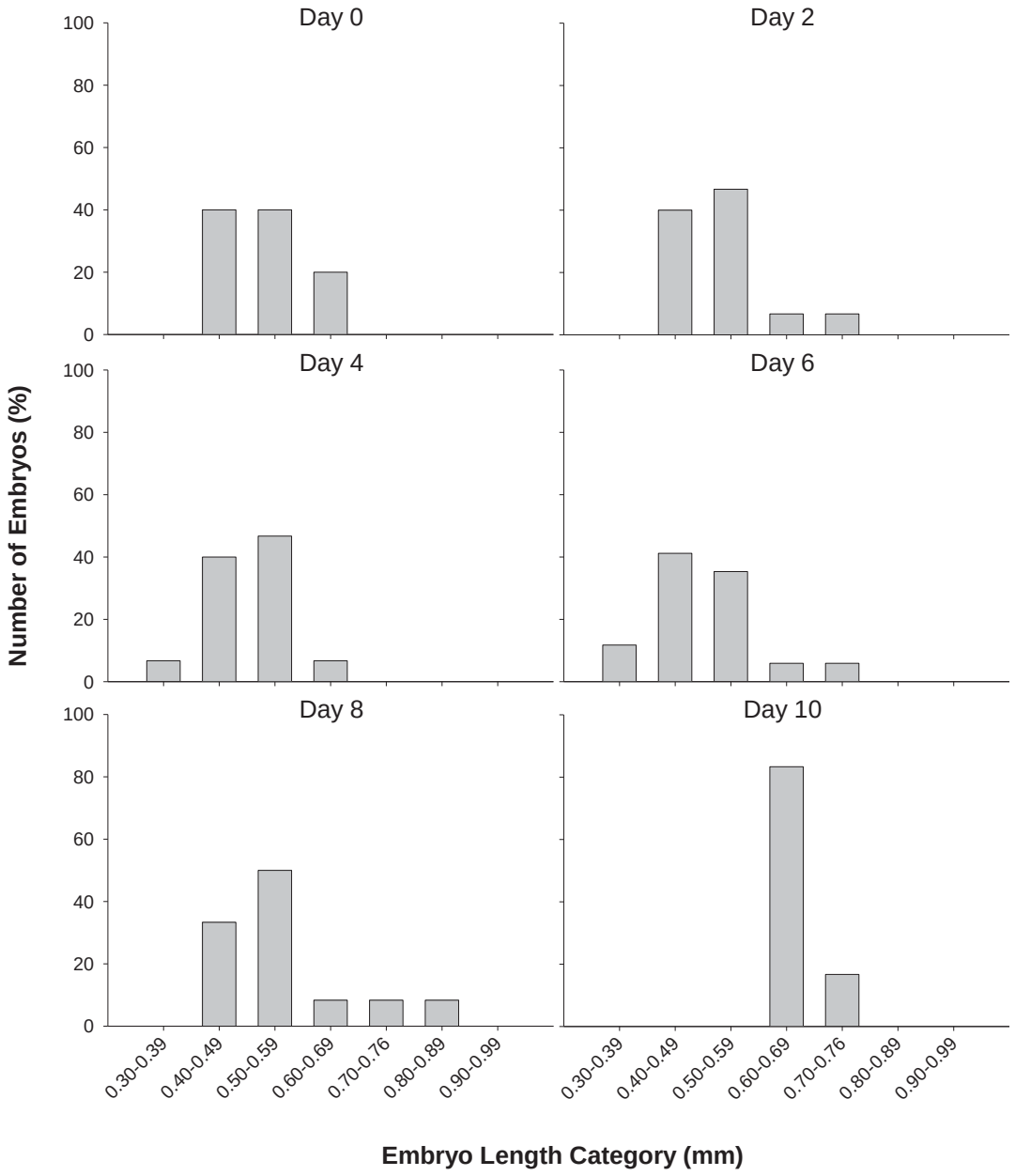

Figure 3.3: Embryo length in seeds of *Nymphaea lukei* after incubation at 30°C for 10 days. A random sample of 20 seeds were removed and embryos measured every second day, with embryos being placed in one of seven size categories. The proportion of seeds in each size class is represented here. Day 0 represents the size class structure in dry seeds imbibed for two hours prior to measurement.

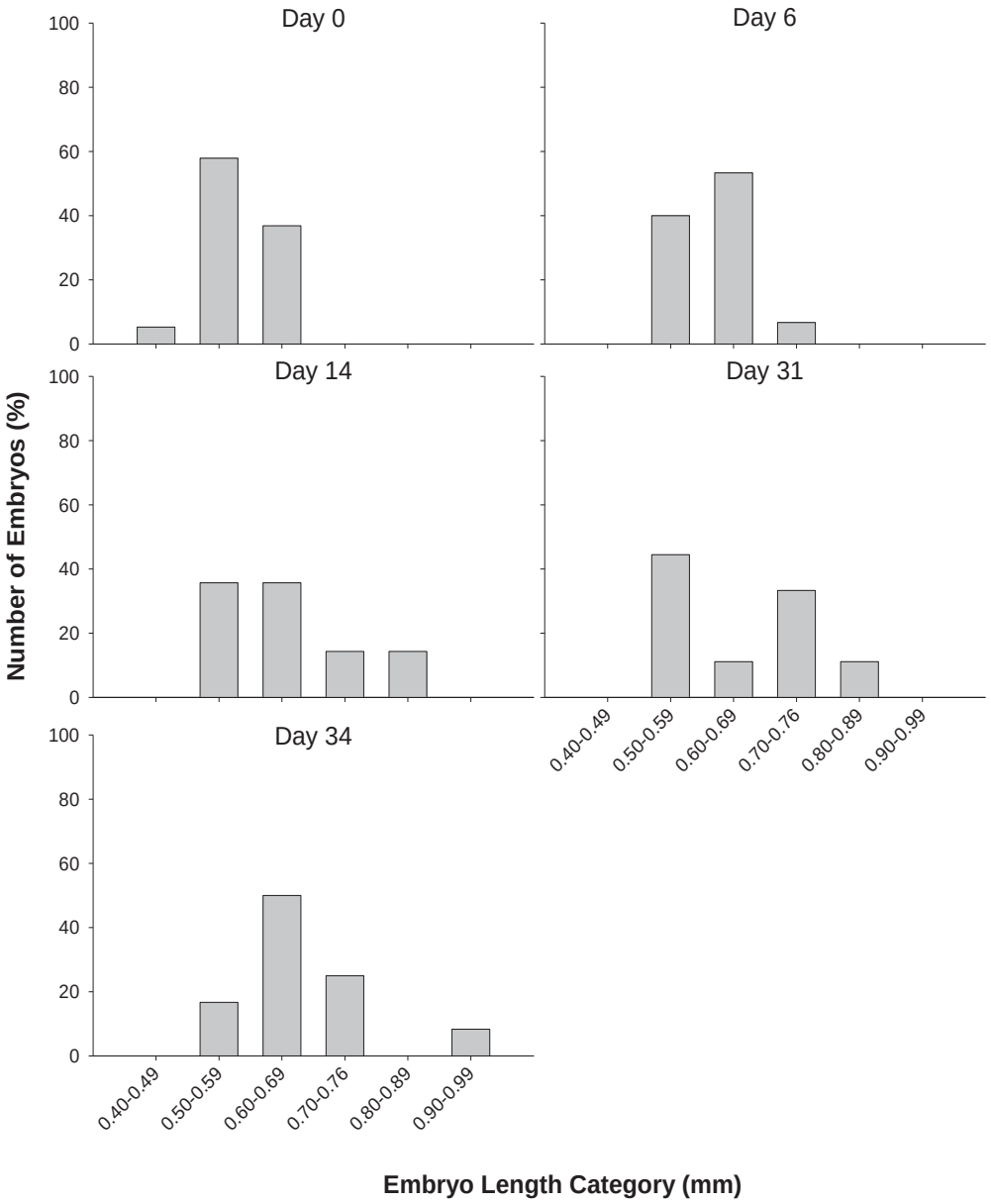

Figure 3.4*:* Embryo length in seeds of *Nymphaea macrosperma* after incubation at 30°C for 35 days. A random sample of 20 seeds were removed and embryos measured at 6, 14, 31 and 34 days, with embryos being placed in one of six size categories. The proportion of seeds in each size class is represented here. Day 0 represents the size class structure in dry seeds imbibed for two hours prior to measurement.

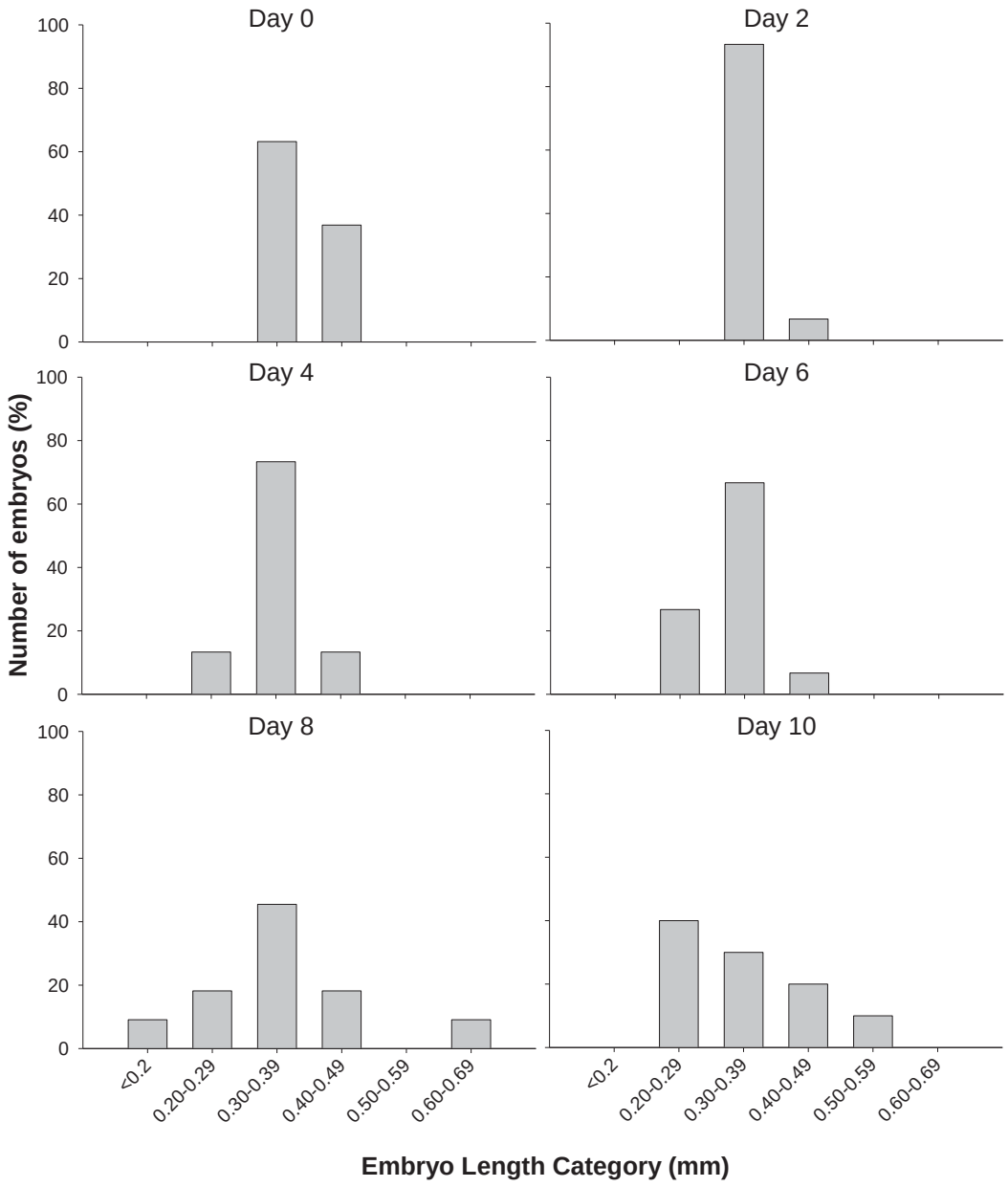

Figure 3.5: Embryo length in seeds of *Nymphaea violacea* after incubation at 30°C for 10 days. A random sample of 20 seeds were removed and embryos measured every second day, with embryos being placed in one of seven size categories. The proportion of seeds in each size class is represented here. Day 0 represents the size class structure in dry seeds imbibed for two hours prior to measurement.

Table 3.3: Embryo type (according to Martin's 1946 embryo classification), embryo differentiation, seed coat thickness, mean fresh seed embryo length, mean embryo length at the point of radicle emergence, mean fresh seed embryo to seed ratio (E:S), mean E:S ratio at the point of radicle emergence and mean time to first germinant in seeds of *Nymphaea immutabilis, N. lukei, N. macrosperma, N. ondinea, N. pubescens* and *N. violacea.* Different superscript letters denote significant differences within each species for mean embryo lengths and E:S ratios, respectively. ($P < 0.001$).

Species	Seed lot No.	Embryo type	Differen-tiated?	Mean fresh seed embryo length (mm ± SE)	Mean embryo length at radicle emergence (mm ± SE)	Mean fresh seed E:S ratio ± SE	Mean E:S ratio at radicle emergence ± SE	Mean time to first germinant (days)
Nymphaea immutabilis	NI1	Broad	Y	0.78 ± 0.02^{a}	0.85 ± 0.01^{b}	0.19 ± 0.006^{a}	0.25 ± 0.003^{b}	< 10
Nymphaea lukei	NL1	Broad	Y	0.53 ± 0.01^{a}	0.61 ± 0.01^{b}	0.22 ± 0.005^{a}	0.28 ± 0.003^{b}	< 10
	NL2	Broad	Y	0.58 ± 0.01^{a}	0.59 ± 0.01^{a}	0.24 ± 0.005^{a}	0.26 ± 0.001^{a}	< 10
Nymphaea macrosperma	NM1	Broad	Y	0.71 ± 0.02^{a}	0.80 ± 0.01^{b}	0.22 ± 0.004^{a}	0.28 ± 0.004^{b}	< 10
Nymphaea ondinea	NO1	Broad	Y	0.49 ± 0.02^{a}	0.67 ± 0.02^{b}	0.29 ± 0.008^{a}	0.37 ± 0.01^{b}	< 10
Nymphaea pubescens	NP1	Broad	Y	0.35 ± 0.01	NA	0.20 ± 0.005	NA	< 10
Nymphaea violacea	NV3	Broad	Y	0.43 ± 0.01^{a}	0.55 ± 0.01^{b}	0.25 ± 0.007^{a}	0.30 ± 0.006^{b}	< 10
	NV8	Broad	Y	0.38 ± 0.01^{a}	0.48 ± 0.01^{b}	0.25 ± 0.008^{a}	0.32 ± 0.005^{b}	< 10
	NV9	Broad	Y	0.38 ± 0.01^{a}	0.47 ± 0.01^{b}	0.23 ± 0.006^{a}	0.31 ± 0.004^{b}	< 10
	NV10	Broad	Y	0.33 ± 0.01^{a}	0.50 ± 0.01^{b}	0.22 ± 0.004^{a}	0.32 ± 0.005^{b}	< 10

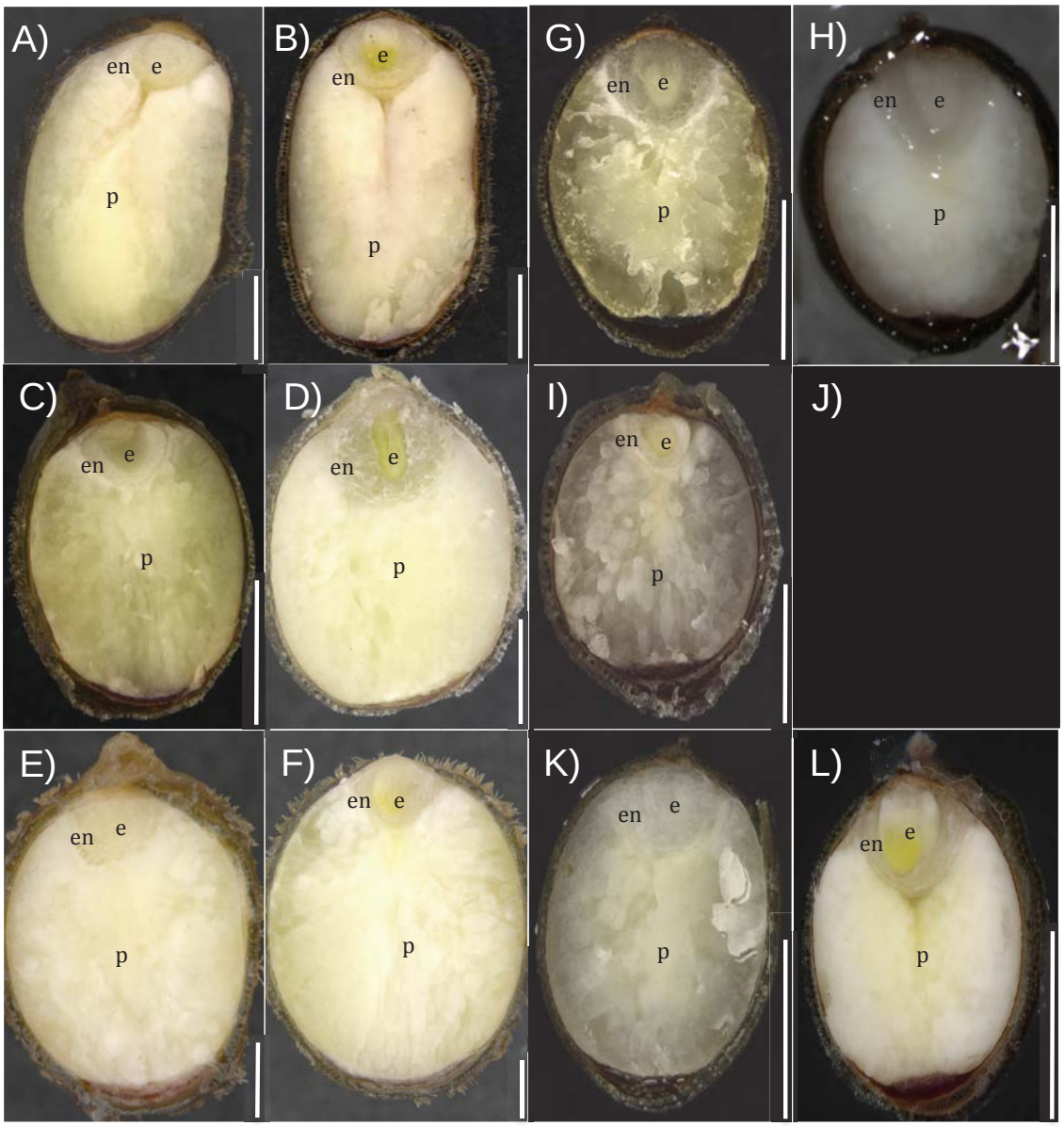

Figure 3.6: Longitudinal section of seeds of *Nymphaea* prior to and after a period of incubation, showing evidence of embryo growth. A) *N. immutabilis* (NI1) control; B) *N. immutabilis* (NI1) after incubation (10d); C) *N. lukei* (NL2) control; D) *N. lukei* (NL2) after incubation (10d); E) *N. macrosperma* (NM1) control; F) *N. macrosperma* (NM1) after incubation (34d); G) *N. ondinea* (NO1) control; H) *N. ondinea* (NO1) after incubation (10d); I) *N. pubescens* (NP1) control; J) seeds of *N. pubescens* (NP1) did not germinate during the embryo growth experiment; K) *N. violacea* (NV10) control; L) *N. violacea* (NV10) after incubation (10d). e = embryo, en = endosperm, p = perisperm. Scale bar = 1mm.

Germination requirements

The germination response of *N. immutabilis, N. lukei, N. macrosperma, N. ondinea* and *N. violacea* was negligible when seeds were incubated in the dark ($P < 0.001$; Table 3.4). Maximum germination reached in the dark was 19 % in *N. ondinea* at 30 °C (Fig. 3.7), which was 5-fold less than seeds incubated in light/dark conditions. *Nymphaea pubescens* did not germinate under any temperature and stimulant combinations tested. All species showed 0 % germinating at alternating 20/35 °C (data not shown).

Under light conditions, germination in all species did not occur at temperatures < 25 °C and was confined to a narrow temperature window between 30- 35 °C (all $P < 0.001$; Fig 3.7). Some species, for example *N. ondinea* and *N. macrosperma* were also capable of germinating at temperatures up to 40 °C (< 60 %), however they showed an overall decrease in germination when compared with 35 °C. Germination did not exceed 80 % in any species or treatment, except *N. ondinea* which showed 80- 100 % germination when crowded at 30-35 °C.

Maximum germination was generally observed at 35 °C (46 %- 100 %) under crowded conditions ($P = 0.01$; Fig. 3.7), while seeds that were not treated with a germination stimulant (i.e. controls), showed limited germination (< 20 %), except for seeds of *N. macrosperma.* Seeds of *N. macrosperma* generally showed more variation in response to germination stimulant, for example control treatments at 35°C showed germination > 65 %, while at other temperatures crowding or GA_3 were significantly higher ($P = 0.01$). In all other species, the ethrel treatment produced significantly higher germination when compared with the control, GA_3 and KAR_1 treatments as temperatures increased from 30- 35 °C (Fig. 3.7).

At optimal temperatures for germination (30- 35 °C), all species except *N. ondinea* germinated to < 75 % after 28 days of incubation (Fig. 3.8). Only seeds of *N. ondinea* in the crowding treatment showed high (80- 100 %) germination at 28 days.

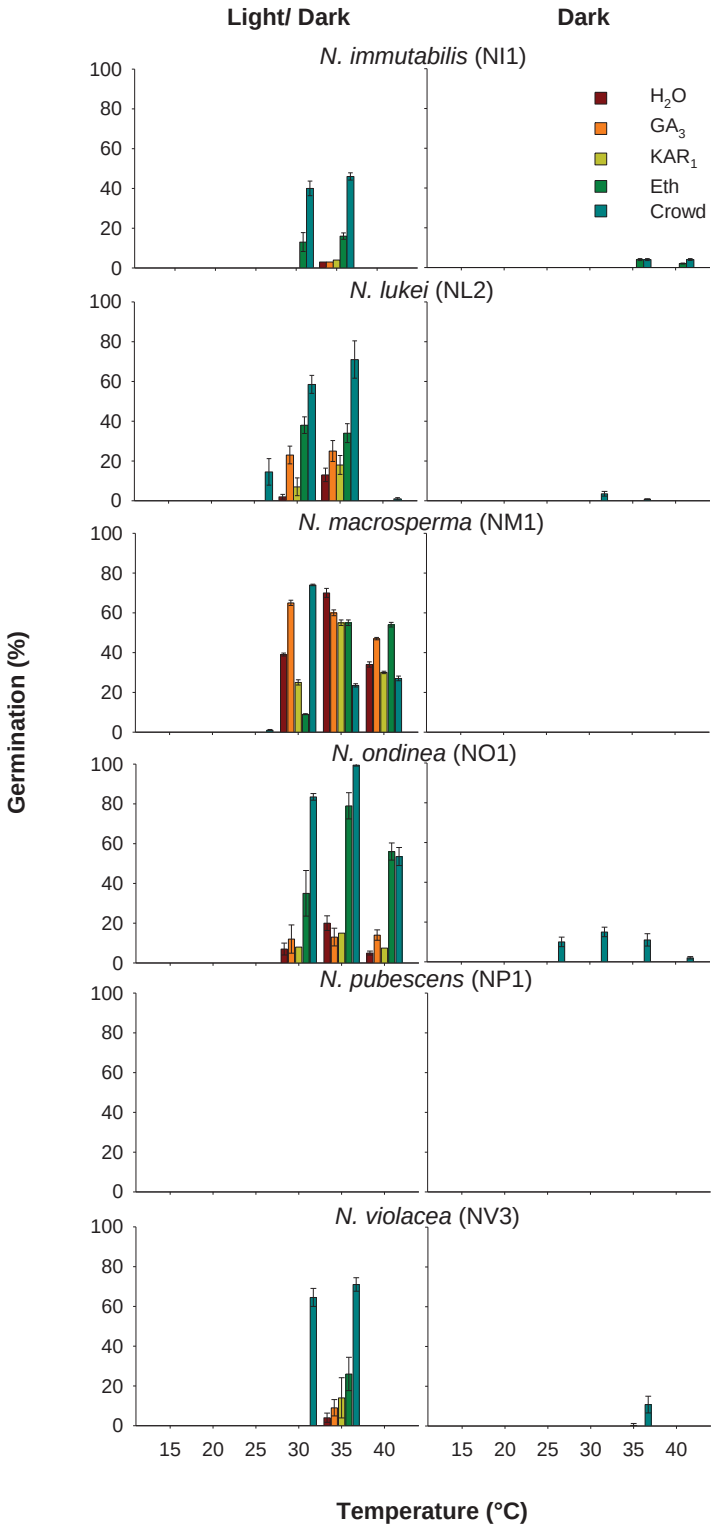

Figure 3.7: Total percent germination of seeds (± S.E) of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM1), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV3) after 8 weeks of incubation at 15, 20, 25, 30, 35 or 40°C. Seeds were either sown on to plain water agar (H_2O, 0.7% w/v), agar containing gibberellic acid (GA_3 0.29 µm) or karrikinolide (KAR_1 0.67 µm), soaked in a solution of ethrel (20 mM) for 24 h prior to plating on H_2O agar or placed in a 10 ml plastic tube and filled with 10 ml of sterile RO water (crowd). Petri dishes and tubes were incubated under 12 h light d^{-1} (light/dark) or in complete darkness (dark).

Table 3.4: Results of a generalised linear model with an inbuilt 'logit' link function with binomial errors on fresh seed germination data. The initial model compared all main factors (temperature [T], germination stimulants [S] light [L] and their interactions. Interactions that were not included in the model simplification are marked with a -). Seeds were incubated at a range of temperatures (15, 20, 25, 30, 35 and 40°C), with or without the addition of germination stimulating treatments (gibberellic acid, karrikinolide, ethrel or seed crowding) and incubated either with (12 h d^{-1}) or without light (complete darkness) for a total period of 8 weeks. *N* = 100.

Species	T	S	L	T x S	T x L	S x L	T x S x L
N. immutabilis	<0.001	<0.001	<0.001	-	-	-	NS
N. lukei	NS	NS	<0.001	-	-	NS	NS
N. macrosperma	NS	NS	<0.001	0.01	-	-	NS
N. ondinea	<0.001	NS	<0.001	-	NS	NS	NA
N. pubescens	-	-	-	-	-	-	NS
N. violacea	<0.001	<0.001	<0.001	NS	NS	0.007	NS

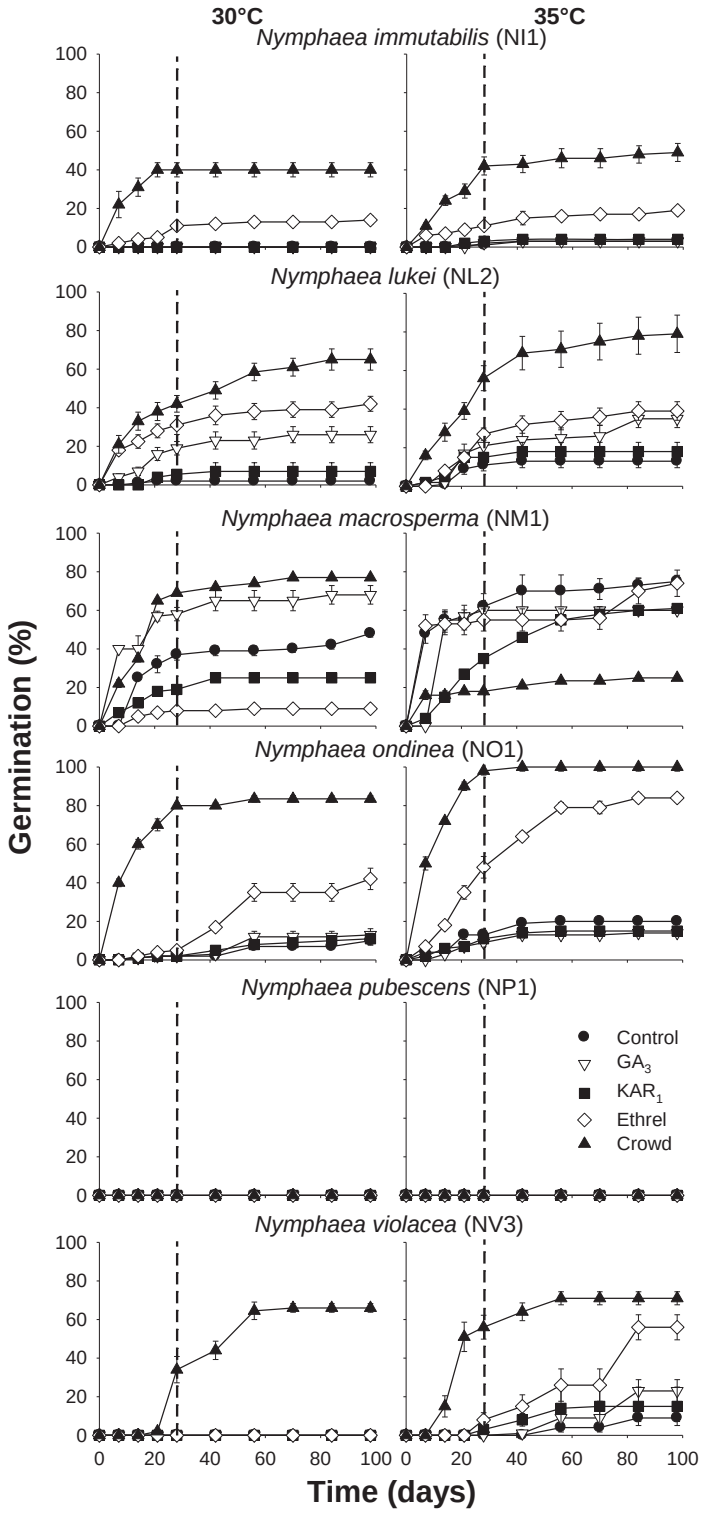

Figure 3.8: Cumulative germination (± S.E.) of *Nymphaea immutabilis, N. lukei, N. macrosperma, N. ondinea, N. pubescens* and *N. violacea* after 100 days of incubation at 30 or 35°C. Seeds were either sown on to plain water agar (H_2O, 0.7% w/v), agar containing gibberellic acid (GA_3 0.29 µm) or karrikinolide (KAR_1 0.67 µm), soaked in a solution of Ethrel (20 mM) for 24 h prior to plating on H_2O agar or placed in a 10 ml plastic tube and filled with 10ml of sterile RO water (crowd). Petri dishes and tubes were incubated under 12 hour light d^{-1} (light/dark). Dashed vertical line indicates 28 days of incubation, in order to classify dormancy type.

Accessional differences were evident within all species tested (all $P < 0.001$; Figs 3.9-3.11). Although the overall pattern of response was similar between accessions of *N. lukei,* total germination was greater in NL1 than NL2. Germination was greatest in both accessions when crowded seeds were incubated at 35 °C, with NL1 showing germination of up to 70 % compared with < 40 % in NL2. For *N. macrosperma*, there was considerably more variation among the treatments (Fig. 3.10). For example, germination in *N. macrosperma* NM1 was similar across temperatures and germination stimulants, while NM2 showed < 15 % germination. For NM3, crowding produced highest germination of up to 60 % at temperatures between 30- 35 °C ($P < 0.001$; Table 3.5). In *N. violacea*, NV1 did not germinate across any temperature or germination stimulant, while seeds of NV4 readily germinated across all temperatures and stimulants (Fig. 3.11). Although total germination was variable between accessions, the overall germination patterns were generally similar, with all species showing an increased response from 30- 35 °C and highest germination occurring within crowded treatments. In accessions NV5, NV8 and NV9 germination in crowded treatments incubated at 35 °C was 100 %. Within the same accessions, germination in the control treatments never exceeded 40 %.

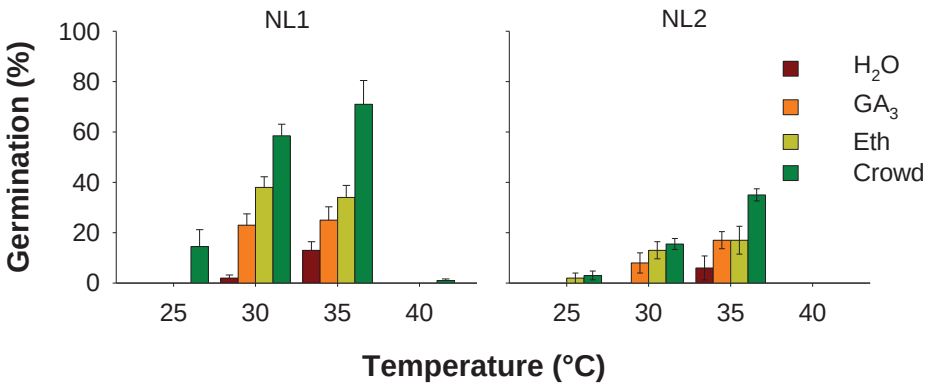

Figure 3.9: Total percent germination of seeds (± S.E.) of multiple accessions of *Nymphaea lukei* taken from different collection locations, after 8 weeks of incubation at 25, 30, 35 or 40°C. Seeds were either sown on to plain water agar (H_2O, 0.7% w/v), agar containing gibberellic acid (GA_3 0.29 µm) or karrikinolide (KAR_1 0.67 µm), soaked in a solution of Ethrel (20mM) for 24h prior to plating on H_2O agar or placed in a 10ml plastic tube and filled with 10ml of sterile RO water (crowd). Seeds were incubated in 12h/12h alternating light/dark.

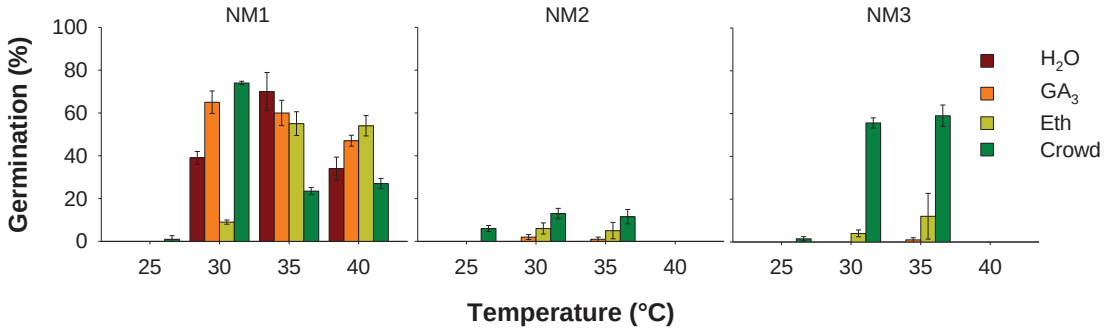

Figure 3.10: Total percent germination of seeds (± S.E.) of multiple accessions of *Nymphaea macrosperma* taken from different collection locations, after 8 weeks of incubation at 25, 30, 35 or 40°C. Seeds were either sown on to plain water agar (H_2O, 0.7% w/v), agar containing gibberellic acid (GA_3 0.29 µm) or karrikinolide (KAR_1 0.67 µm), soaked in a solution of ethrel (20mM) for 24h prior to plating on H_2O agar or placed in a 10ml plastic tube and filled with 10ml of sterile RO water (crowd). Seeds were incubated in 12h/12h alternating light/dark.

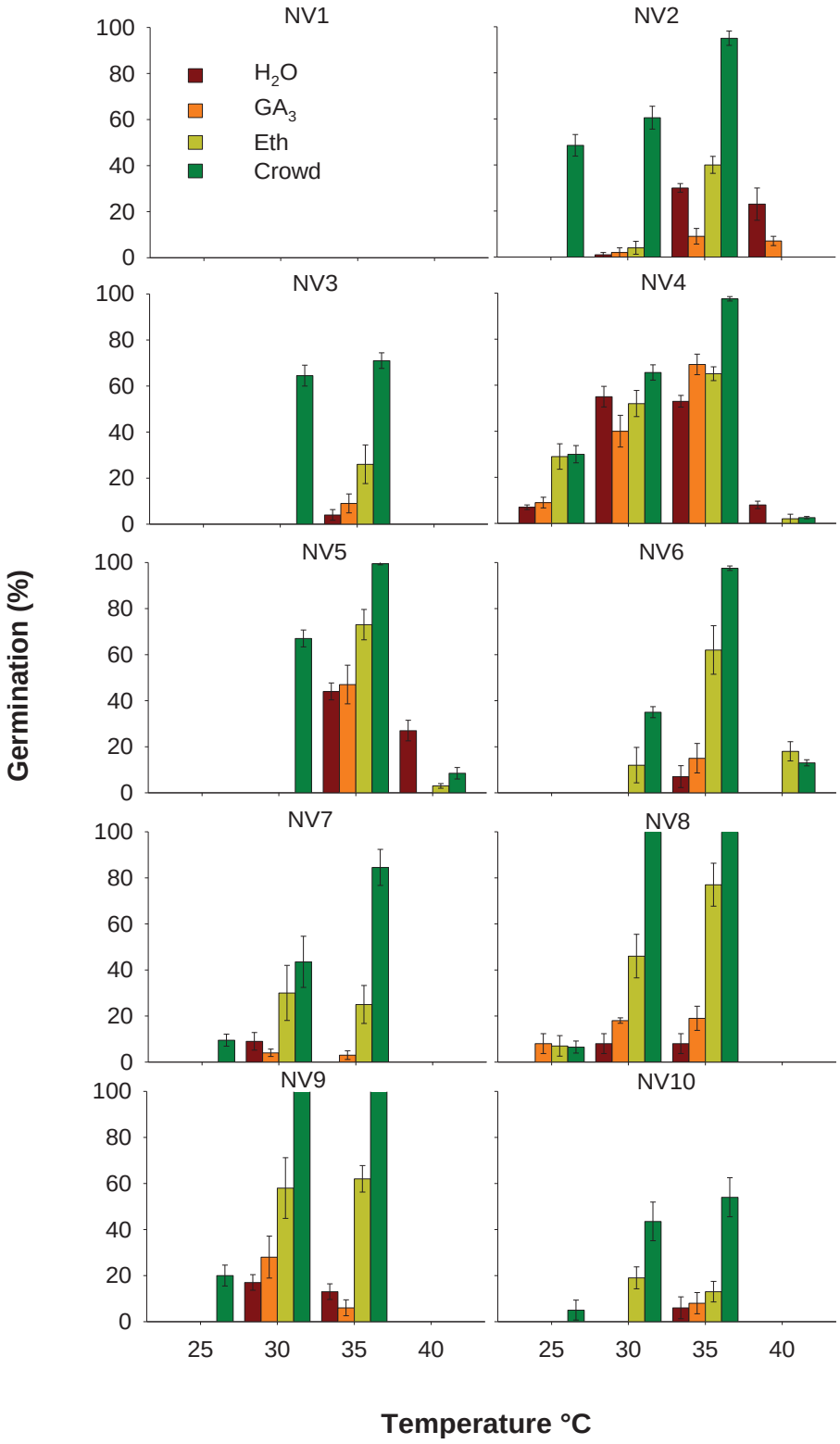

Figure 3.11: Total percent germination of seeds (± S.E.) of multiple accessions of *Nymphaea violacea* taken from different collection locations, after 8 weeks of incubation at 25, 30, 35 or 40°C. Seeds were either sown on to plain water agar (H_2O, 0.7% w/v), agar containing gibberellic acid (GA_3 0.29 µm) or karrikinolide (KAR_1 0.67 µm), soaked in a solution of Ethrel (20mM) for 24h prior to plating on H_2O agar or placed in a 10ml plastic tube and filled with 10ml of sterile RO water (crowd). Seeds were incubated in 12h/12h alternating light/dark.

Table 3.5: Final summary statistics showing differences across accessions of *N. lukei*, *N. macrosperma* and *N. violacea* for the factors of temperature (T), germination stimulant (S) and their interaction (T x S). Accessional differences were determined at $P < 0.001$ for each species.

Species	Accession no	T	S	T x S
Nymphaea lukei	NL1	<0.001	NS	-
	NL2	<0.001	<0.001	<0.001
Nymphaea macrosperma	NM1	NS	NS	0.02
	NM2	<0.001	<0.001	-
	NM3	<0.001	<0.001	-
Nymphaea violacea	NV1	-	-	-
	NV2	<0.001	<0.001	NS
	NV3	<0.001	<0.001	-
	NV4	<0.001	NS	NS
	NV5	NS	NS	NS
	NV6	<0.001	<0.001	NS
	NV7	<0.001	<0.001	NS
	NV8	<0.001	<0.001	NS
	NV9	<0.001	<0.001	NS
	NV10	<0.001	<0.001	NS

DISCUSSION

Dormancy classification

Each of the species of *Nymphaea* we examined in this study had a fully differentiated embryo (i.e. cotyledons and radicle were visible), and embryos were broad according to Martin's classification system (1946). All species readily imbibed water (Fig. 3.2), and thus the presence of physical dormancy was excluded. While in incubation at optimal temperatures (30- 35 °C), embryos of seeds of *N. immutabilis*, *N. lukei, N. macrosperma, N. ondinea, and N. violacea* increased significantly in length, on average by 22 % (9, 15, 13, 37 and 33 % for *N. immutabilis, N. lukei, N macrosperma, N. ondinea* and *N. violacea,* respectively), indicating the presence of morphological dormancy. Furthermore, when seeds were incubated on water agar alone (i.e. in the absence of a germination stimulant) at optimal temperatures, germination after 28 days was less than 20 % in all species except *N. macrosperma* (which showed germination of approximately 60 %), confirming that seeds were also physiologically dormant (Fig. 3.8). Therefore, as embryo growth occurred and significant germination did not occur without the addition of a germination stimulant (such as the crowding treatment) we concluded that seeds of *N. immutabilis*, *N. lukei, N. macrosperma, N. ondinea, and N. violacea* possess morphophysiological dormancy (MPD), according to the classification system described by Baskin and Baskin (2004; 2014). As such, this study provides the first evidence for embryo growth occurring within seeds of *Nymphaea,* the Nymphaeaceae and within seeds with broad embryos.

Further classification of the type of MPD is not possible with the present data as the length of time between root and shoot emergence is not known. However, the fact that temperatures in northern Australian rarely fall below 10 °C (Bureau of Meteorology 2005) would suggest that the type of MPD is likely to be 'Nondeep, Type 1', 'Deep Simple Epicotyl Type 1' or 'Nondeep Simple Epicotyl Type 2 or 3' (Baskin and Baskin 2014). In order to elucidate this classification, further warm or cold stratification experiments need to be performed, and the timing of root and shoot emergence noted (Baskin and Baskin 2014). As seeds of *N. pubescens* did not germinate, embryo growth was not observed during this study. Given *N. pubescens* readily imbibed water (Fig. 3.2), we can only confirm the presence of physiological dormancy (PD) in this species.

Small, underdeveloped embryos are considered to be basal within the angiosperms (Forbis *et al.* 2002a; Finch-Savage and Leubner Metzger 2006) and recent evidence supports the placement of MPD as the ancestral trait (Willis *et al.* 2014). Considering the placement of *Nymphaea* within the ANA-grade angiosperms, our results further support both hypotheses.

While we observed that the embryos of *Nymphaea* grew prior to germination, their total percentage increase in size (22 %) is small compared to other species with underdeveloped embryos. For example, underdeveloped linear embryos in *Corydalis ambigua* (Papaveraceae) and members of the Campanulaceae may increase in size by as much as 1000 % prior to radicle emergence (Baskin *et al.* 2005; Kondo *et al.* 2005). However, at present there is no system for classifying morphological dormancy that explicitly states the definition of 'embryo growth'. Given the small, but consistent increase in embryo length we have observed in this study, we recommend that the definition of embryo growth constitute any observable increase in embryo size that cannot be attributed solely to cell elongation. Given this definition, the finding by Baskin and Baskin (2007) that embryos of *N. capensis* subsp. *zanzibarensis* increased by 12 % prior to radicle emergence may be evidence of embryo growth, however further research is needed in order to confirm this.

Germination requirements

Seeds of *N. immutabilis*, *N. lukei, N. macrosperma, N. ondinea, and N. violacea* germinated to significantly higher percentages when exposed to light, compared with < 19 % germination under completely dark conditions. This result is congruent with the findings of a range of studies showing that the majority of aquatics have a requirement for light (Baskin and Baskin 2014), including aquatics from temporary pools in the Mediterranean (Carta *et al.* 2013) south-west Western Australia (Tuckett *et al.* 2010a) and the monsoon tropical Kimberley region in Australia (Cross *et al.* 2015). This requirement for light may ensure that seed germination occurs at shallow enough depths to allow sufficient light penetration to support subsequent seedling establishment or allow for germination of seeds in the soil seed bank in response to disturbance (Grime 1981; Pons 1991).

All species of *Nymphaea* in this study germinated to highest percentages at temperatures between 30- 35 °C. *Nymphaea macrosperma* and *N. ondinea* also showed > 50 % germination in some treatments when incubated at 40 °C. Outside of the optimal range, particularly at lower temperatures, germination was absent or significantly reduced in all species. In northern Australia, summer temperatures frequently exceed 35 °C, which coincide with the summer monsoonal rainfall (McQuade *et al.* 1996; Bureau of Meteorology 2011). As seeds of many aquatic species are cued to germinate in conjunction with water availability, particularly those from ephemeral wetlands (Casanova and Brock 2000; Deil 2005) this preference for higher temperatures would allow Australian *Nymphaea* to germinate and establish when water is available. These temperatures are significantly higher than the global average optimal temperature of 24 °C for seed germination in freshwater aquatic plants (Baskin and Baskin 2014). However, this mean temperature is dominated by species from the northern hemisphere, and our understanding of seeds from the southern hemisphere, the tropics and from particularly floating-attached plants, is somewhat limited (Baskin and Baskin 2014). A recent study investigating the germination responses of other aquatic species occurring in the wet-dry tropics of northern Australia also found a similar preference for temperatures ≥ 30 °C (Cross *et al.* 2015).

Interestingly, when *Nymphaea* were subjected to an alternating temperature regime of 20/35 °C, all species tested in this study did not germinate. Seeds of some aquatic species use daily temperature fluctuations as a means of detecting water depth (Baskin and Baskin 2014). For example, seeds of the sedge *Fimbristylis littoralis*, were found to have an absolute requirement for alternating temperatures to enable successful germination (Pons and Schröder 1986). When large containers of water were heated from above, the water temperature became stratified, with the surface water fluctuating with diurnal temperature to a much greater extent that the water at the bottom of the container. Seeds of *F. littoralis* placed on the waters surface germinated readily to 100 % when the surface temperature fluctuation exceeded 11.9 °C daily, while those at the bottom of the container only germinated to < 20 % (Pons and Schröder 1986). As such, a requirement for stable daily temperatures in Australian *Nymphaea* may act to prevent germination occurring at the waters' surface where seedlings are unable to establish in the absence of sediment.

The crowding treatment, whereby a number of seeds (50) were clustered in a 10 ml vial of water, elicited the greatest germination response in all species (and the vast majority of accessions) at optimal temperatures. These results are similar to those found by Else and Riemer (1984), who also found that crowding seeds (60- 100) of *N. odorata* in a small volume of water increased germination from 0 to up to 60 %. While the mechanism of dormancy break or germination stimulation under crowded conditions is not yet fully understood, a number of other studies have reported similar results. For example, germination of the aquatic Duck-Lettuce (*Ottelia alismoides*) was significantly increased when the number of seeds in a volume of water was increased from 20 to 200 (Yin *et al.* 2009). Additionally in this study, the crowded treatments were the only treatments to produce significant germination outside the optimal temperature range of 30- 35 °C, with the exception of one accession of *N. macrosperma* in which the control and GA_3 treatments produced higher germination. This response may be attributed to the endogenous production of ethylene, as postulated by Else and Riemer (1984). Although, when seeds of Australian *Nymphaea* were treated with 20 mM ethaphon, seeds did not germinate to the same degree as the crowded treatment in most species. However, in all species, the ethaphon treatment showed significantly ($P < 0.01$) higher percentage germination when compared to the control (water), GA_3 or KAR_1 treatments. Ecologically, ethylene is produced in soils rich in organic matter by bacteria or fungi when waterlogged or under anaerobic conditions (Esashi and Leopold 1969; Smith and Russell 1969a; Smith and Restall 1971; Abeles 1973) and has been shown to occur in permanently (Smith and Russell 1969a; Smith and Restall 1971; Zeikus and Winfrey 1976) and ephemerally (Cross *et al.* 2014) inundated wetland soils. While ethylene's mechanism of action remains unresolved, it has been suggested that the gas may act as a 'flood detection' mechanism that signals to aquatic seeds that there is enough water to support subsequent seedling development and establishment (Baskin and Baskin 2014). As such, it may be expected that a number of aquatic plant species, including previously unstudied members of the *Nymphaea,* may also show a similar stimulatory germination response to the presence of ethylene.

The addition of another germination stimulant, the smoke derivative KAR_1 did not significantly increase germination beyond that of the controls for the majority of species and accessions of *Nymphaea* tested in this study. The wet-dry tropics of northern Australia are dominated by savanna grassland, which are extremely fire-prone during

the winter dry-season (Woinarski *et al.* 2007; Bowman *et al.* 2010). In other fire-prone regions of Australia, the flora have adapted to this disturbance by becoming fire responsive, and subsequently responsive to KAR_1 (Dixon *et al.* 1995; Flematti *et al.* 2004). However *Nymphaea*, like the basal genus *Trithuria* from south-west Western Australia, have apparently not developed a positive response to the impact of fire (Tuckett *et al.* 2010c).

While ethylene certainly appears to contribute to the success of the crowding phenomenon, other factors including flooding, anoxia (either an increase in CO_2 or decrease in O_2), and the stimulatory effect of ethanol may also serve to break dormancy and stimulate germination in seeds of *Nymphaea* (Else and Riemer 1984; Smits *et al.* 1995; Estrelles *et al.* 2004). In a number of seasonally inundated wetland species, flooding delays or inhibits germination (Poschlod 1996) while serving to stimulate germination in others such as *N. odorata* (Baskin and Baskin 2014). Under anaerobic, flooded conditions, seeds of *N. alba* also produce ethanol, which promotes germination in the species (Smits *et al.* 1995). Oxygen, or lack thereof may also determine whether seeds of *Carex comosa, C. stricta,* a number of *Cyperus* species, *Heteranthera limosa* and rice-field weeds remain dormant (Pons and Schröder 1986; Baskin *et al.* 1996; Baskin *et al.* 2003). Therefore, it is likely that a combination of these factors may contribute to the dormancy release and germination stimulation in Australian *Nymphaea*.

Accessional differences in germination response

Different accessions of the same species of *Nymphaea* (*N. lukei, N. macrosperma* and *N. violacea*) showed significantly different depth of dormancy and subsequent germination responses. Seeds of *N. violacea* NV1 did not germinate under any conditions, while *N. violacea* NV4 germinated over a broad range of temperatures (25-40 °C), with and without the addition of germination stimulants. Likewise, seeds of *N. macrosperma* NM1 germinated to > 70 % in some treatments, while *N. macrosperma* NM2 only showed germination of < 15 %. These results suggest a difference in the depth of dormancy between collection locations, both spatially or temporally. Variation in germination response between accessions of the same species is not uncommon, and may be attributed to a number of factors; the most common of which is the influence

from the maternal environment (Roach and Wulff 1987; Fenner 1991; Donohue 2009; Baskin and Baskin 2014). The physical variation between sites, with respect to latitude, longitude, season and precipitation have all been shown to contribute to differences in the depth of seed dormancy (Baskin and Baskin 2014). As such, multiple accessions of single species should be incorporated into any *ex situ* germination study to account for dormancy variability. Therefore, any future work focussing on the *ex situ* dormancy and germination response of *Nymphaea* should consider collections from multiple locations in order to fully capture this natural variation.

Conclusions

Seeds of *N. immutabilis, N. lukei, N. macrosperma, N. ondinea* and *N. violacea* underwent embryo growth prior to radicle emergence, and therefore display MPD. This finding adds further support to recent hypotheses that MPD is basal within the angiosperms. Seeds of *N. pubescens* did not germinate, therefore embryo growth was not observed. As such, seeds could only be classified as being PD. The conditions that produce maximal germination (light, 30-35 °C, crowded) may reflect a series of adaptations in order to ensure that dormancy break and subsequent germination occur in conjunction with summer rainfall.

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CHAPTER 4

BREAKING DORMANCY IN SEEDS AND LONGEVITY IN THE SOIL SEED BANK OF TROPICAL *NYMPHAEA* L. SPECIES

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ABSTRACT

Background and aims: The wet-dry tropics of northern Australia are characterised by extremes in climate, with hot, erratically wet summers (often associated with intense cyclonic events) and warm dry winters. As such, the wetland plants occurring through the region are uniquely adapted to this ephemeral environment, and must be resilient to periods of drought. *Nymphaea* are a genus of basal, aquatic plants that are common through the wetlands of tropical northern Australia. In this study, we aimed to understand how physiological dormancy in Australian *Nymphaea* might be alleviated by fluctuations in air temperature and moisture- both under simulated natural conditions, and under laboratory conditions. Additionally, we also aimed to understand seed persistence and dormancy alleviation in a simulated natural environment.

Methods: In order to inform further experimental design and to understand the optimal daily temperature fluctuations required for germination, seeds of *N. violacea* were placed on a two-way, alternating temperature thermogradient table (running bi-directionally from 10- 40 °C). A seed burial trial was used to simulate natural wetland soil conditions (wet, dry or wet/dry) in order to assess seed persistence and dormancy loss of *N. violacea*. Exhumed seeds were assessed for persistence and germination tested under laboratory conditions. Additionally, laboratory-based treatments (dry after-ripening and wet/dry cycling) were used to break dormancy in seeds of *N. lukei, N. macrosperma* and *N. violacea.* Furthermore, warm and cold stratification was trialled in conjunction with a move-along experiment to determine the temperature changes necessary for dormancy break.

Key results: Some dormancy loss in the soil seed bank (under simulated natural conditions) appears to occur over the dry season, until germination is stimulated in conjunction with increasing overnight temperatures after the onset of the wet season. Seeds of *N. violacea* were found to form a semi-persistent seed bank, which may remain viable for > 12 months under dry conditions. Dry after-ripening did not break dormancy or increase germination, however wet/dry cycling significantly increased germination of *N. lukei.* The mimicked changes in temperature between the dry season and summer (and *vice versa*) (cold and warm stratification and move-along) produced germination of < 5 %, even when tested in conjunction with a germination stimulant.

Conclusions: Controls of dormancy loss and germination in Australian *Nymphaea* are complex, and are postulated to rely on a series of interconnected environmental events such as wetting, drying, temperature changes and the presence of ethylene.

Key words: Dry after-ripening, wet/dry cycling, warm and cold stratification, move-along, seed burial, persistence, wet-dry tropics, Australia.

INTRODUCTION

The *Nymphaea* (Nymphaeaceae) are a cosmopolitan genus of approximately 65 species of aquatic, floating leaved macrophytes (Löhne *et al.* 2008b). While a number of species occur through the temperate regions of Europe, Asia and North America, the greatest species diversity is found through the tropics of Africa, South America, South-East Asia and Australia (Löhne *et al.* 2008; Appendix 1 of this thesis). In Australia, 18 native species of *Nymphaea* occupy the freshwater wetlands of northern Western Australia, the Northern Territory and Queensland (Jacobs and Hellquist 2011). Their distribution spans the extent of the wet-dry tropics of Australia, which is characterised climatically by extremes in temperature and rainfall (Finlayson 1999).

Rainfall in the region predominantly occurs through November to March, and is often associated with severe cyclonic events (Cowie *et al.* 2000b; Woinarski *et al.* 2007). Rainfall is sporadic and total annual precipitation may vary considerably between years (Cowie *et al.* 2000b). For example in the Northern Territory, Darwin receives rainfall between 900 mm and 2400 mm per annum (Bureau of Meteorology 2011). Outside of the 'wet' season, rainfall during the 'dry' season is scarce, which leads to high rates of evapotranspiration and drought (Finlayson 1999; Woinarski *et al.* 2007). As such, wetlands in the wet-dry tropics are often transient and ephemeral, while perennial wetlands exhibit large inter-seasonal shifts in water level. These seasonal oscillations in rainfall and drought have a significant impact on the local flora, particularly those species occupying wetlands (Cowie *et al.* 2000b; Bowman *et al.* 2010). Furthermore, reproduction in aquatic species is often limited to the wet season when subsequent seedling establishment and growth is supported by high water availability (Cowie *et al.* 2000b; Bowman *et al.* 2010). Consequently, it is the resilience of aquatic species to episodic drying events which allows a community to re-establish to the diversity and abundance seen during wetting events (Brock 2011).

Many species of *Nymphaea* in Australia inhabit large permanent or semi-permanent coastal floodplains and billabongs and a number of species also persist in ephemeral creeks and wetlands further inland (Cowie *et al.* 2000a; Sainty and Jacobs 2003). For example, *N. violacea* is widespread, and occupies both perennial and ephemeral wetlands across the northern expanse of the continent, while in Western Australia, *N. lukei* is restricted to small pools and sandstone-based creeks to the west of the King Leopold Range in the Kimberley region (Jacobs and Porter 2007; Jacobs and Hellquist 2011). However, given the transient nature of annual rainfall and variability in water availability as influenced by monsoonal rainfall events, even perennial waterways experience large fluctuations in water level. As such, *Nymphaea*, like many other wetland plant species in the wet-dry tropics, must rely upon a persistent sediment seed bank or underground storage organs or in order to survive the dry period (Bonis *et al.* 1995; Brock 2011).

Seed dormancy is a property of the seed to time germination to when conditions are most favourable for seedling establishment. In seeds of aquatic species, like that of the angiosperms as a whole, physiological dormancy (PD) is most prevalent (Baskin and Baskin 2014). Physiological dormancy may be overcome in the sediment seed bank by a series of environmental fluctuations in temperature or moisture (Schütz and Rave 1999; Baskin and Baskin 2003; Baskin and Baskin 2004a; Baskin and Baskin 2014). Seasonal changes in temperature and the progression through seasons can act to overcome dormancy in both terrestrial and aquatic species (Baskin *et al.* 1989; Schutz 1997), and inundation and drying events can alleviate dormancy and stimulate germination in a range of aquatic plants from ephemeral wetlands (Casanova and Brock 2000; Brock *et al.* 2003; Brock *et al.* 2005; Brock 2011).

Furthermore, the conditions that overcome dormancy naturally in the soil seed can be co-opted for use *ex situ,* in the laboratory. Previous studies investigating the dormancy of *Nymphaea* initially found seeds to possess PD (Baskin and Baskin 2007) and laboratory-based dormancy breaking treatments are species-specific; cold stratification was required for temperate species *N. alba* (4 °C for at least 2 months), *N. odorata* (4 °C for 5- 9 months) and *N. tuberosa* (1- 3 °C for 7 months), while warm stratification overcame dormancy in tropical species; *N. capensis* (30/10 °C for 56 days), *N. immutabilis* (30/15 °C for 168 days), *N. micarantha* (35/20 °C for 42 days),

(Muenscher 1936; Else and Riemer 1984; Smits *et al.* 1990; Smits *et al.* 1995; Estrelles *et al.* 2004; Baskin and Baskin 2006, 2014). Recently though, a number of tropical Australian *Nymphaea* have been shown to possess morphophysiological dormancy (MPD), whereby seeds require a period of time at high temperatures (30- 35 °C) to allow for embryo growth within the seed prior to germination (Chapter 3 of this thesis). Thus, these warm conditions alleviate the morphological component of seed dormancy, but the temperature requirements to overcome PD in Australian *Nymphaea* are currently unknown.

The process of after-ripening, whereby seeds are subjected to a warm, dry period, can be mimicked under laboratory conditions and has become a widely used method for overcoming dormancy in PD and MPD species (Baskin and Baskin 2004a; Cochrane and Probert 2006; Finch-Savage and Leubner Metzger 2006; Turner *et al.* 2009; Bazin *et al.* 2010). Germination of a number of aquatic species occurring in temporary wetlands has also been shown to increase in response to a period of after ripening (Tuckett *et al.* 2010a; Carta *et al.* 2013; Cross *et al.* 2015). Furthermore several temperate *Carex* species occurring in perennial wetlands have been shown to increase after a period of 4- 8 weeks of dry after-ripening (Schutz 1997). Therefore, as many wetlands occupied by *Nymphaea* in the wet-dry tropics experience a period of drought (Osland *et al.* 2011) Australian *Nymphaea* may respond positively to a period of dry after- ripening.

Wet-dry cycling is an *ex situ* technique that mimics natural environmental fluctuations in temperature and moisture availability commonly experienced by semi- tropical and tropical species (Hoyle *et al.* 2008a). Seeds are subjected to a series of wetting and drying cycles at temperatures optimal for dormancy break. Unlike dry after-ripening, which is based on the climatic conditions in temperate regions, wet-dry cycling more closely represents the conditions experienced by seeds in the wet-dry tropics (Hoyle *et al.* 2008a). While some success has been reported using this technique on Mediterranean climate temporary pool systems in Western Australia (Tuckett *et al.* 2010a), it has yet to be tested on aquatic species from northern Australia.

Though the manipulation of temperature and moisture availability may assist with dormancy alleviation, the application of germination stimulating treatments (such as ethylene, gibberellic acid or crowding) may further enhance the *ex situ* germination response (Chapter 3 of this thesis).

While the genus *Nymphaea* has received much attention for its position among the basal angiosperms (Qiu *et al.* 1999) and for its horticultural importance, the plants also play a vital ecological role in the wetlands they inhabit and to the people who use them. *Nymphaea* are culturally important to indigenous Australians, as they are traditionally used as a food source through the dry season (Issacs 1987; Press *et al.* 1995b) As such, the genus is of high conservation value, particularly in Australia, and conservation measures must be considered in order to future-proof important populations, particularly in the face of global climate change. The *ex situ* storage of seeds provides one of the most effective and reliable methods of aquatic plant conservation (Hay *et al.* 2000) However, the issue of seed dormancy must first be overcome before any successful *ex situ* storage program is initiated (Hoyle *et al.* 2008b). In order to better inform future conservation of *Nymphaea* in Australia, we aimed to:

1) Understand how physiological dormancy in Australian *Nymphaea* might be alleviated by fluctuations in air temperature and moisture- both under simulated natural conditions, and under laboratory conditions
2) Understand seed persistence and dormancy alleviation in a simulated natural environment.

MATERIALS AND METHODS

Species selection, seed collection and seed quality

Whole mature fruits of *Nymphaea* accessions of the following subgenera were collected over three years between March 2011 and August 2013. Subgenus Confluentes included *Nymphaea lukei* S. W. L. Jacobs & Hellq., (accession numbers: NL1 and NL2) and *N. violacea* Lehm. (accession numbers: NV1, NV3, NV4, NV6, NV7, NV8, NV9 and NV10), subgenus Anecphya included *N. immutabilis* Hook. (accession number: NI1)

and *N. macrosperma* Merr & L. M. Perry (accession number NM1, NM2 and NM3) and subgenus Lotos consisted of *N. pubescens* Willd. (accession number: NP1) (Table 4.1). Seeds were rinsed through brass sieves (Endecotts 710 μm – 2.36 mm) under running water to remove any remaining plant material. All seed accessions were separated from chaff using an aspirator (SELECTA BV Gravity Seed Seperator "Zig Zag", Netherlands) or by hand before being stored in a controlled environment facility (CE 15 % RH**,** 15 °C). Prior to all experimental work, all seeds (except NV6) were x-rayed (Faxitron Specimen Radiography System MX-20 Cabinet) to ensure all seeds were filled with embryonic tissue and thus likely to be viable. All seed lots were experimentally within 28 days of collection, unless otherwise stated.

Temperatures for germination testing

While there are extremes of climate in any biome, daytime temperatures in Australia's tropics are relatively stable, while overnight temperatures are more variable with season (Fig. 4.1). Average mean minimum and maximum temperatures for the last 30 years (Australian Bureau of Meteorology) were averaged across the range of *Nymphaea* collection sites (Table 4.2) in order to choose suitable germination temperatures for the laboratory-based components of the following experiments: Alternating day and night temperatures, seed bank persistence and dormancy loss, dry after ripening, wet-dry cycling, warm/cold stratification and the fixed day-temperature move-along.

Germination testing

For all experiments investigating germination response, the following germination testing procedures were used. Prior to plating, seeds were sterilised in a 2 % (w/v) solution of calcium hypochlorite ($Ca(OCl)_2$, Poolbrite pool chlorine) with two drops of surfactant (Tween 80, Hurst Scientific) for 30 minutes under alternating vacuum (10 minutes on/off/on) and then rinsed three times in sterile RO water. Seeds (four replicates of 25 seeds, unless otherwise stated) were sown in 90 mm round, sterile Petri dishes containing 0.7 % (w/v) water agar, or water agar and gibberellic acid (GA_3, Sigma® Australia, 0.29 mM). The Ethrel® (ethephon, Bayer Crop Science) treatment was administered by placing seeds inside a nylon mesh bag and soaking in a 20 mM solution of Ethrel® for 24 hours prior to plating on 0.7 % (w/v) water agar. Petri dishes

were wrapped four times in GLAD® Wrap to prevent excess evaporation. To test the effect of crowding on germination, 50 seeds of each species were placed in a 10 ml plastic tube (TechnoPlas, Australia) and filled with 10 ml of sterile RO water.

Table 4.1: Collection details of *Nymphaea immutabilis, N. lukei, N. macrosperma, N. pubescens* and *N. violacea,* including the nearest Bureau of Meteorology weather station that provided at least 10 years of rainfall and temperature data (Australian Government Bureau of Meteorology 2013).

Species	Accession No.	Nearest BoM station	State	Collection location (GDA 94)	Date
Nymphaea immutabilis	NI1	Cooktown	QLD	S15°25'35.7", E145°9'14.5"	July 2013
Nymphaea lukei	NL1	Fitzroy Crossing	WA	S17°34'47.6" E126°04'59.6"	May 2012
	NL2	Mount Elizabeth	WA	S16°43'0.2", E125°27'35.8"	June 2013
Nymphaea macrosperma	NM1	Darwin	NT	S12°42'47.9" E131°38'17.7"	June 2011
	NM2	Wyndham	WA	S15°35'35.7" E128°16'47.9"	May 2012
	NM3	Wyndham	WA	S15°35'35.7" E128°16'47.9"	May 2013
Nymphaea pubescens	NP1	Jabiru	NT	S12°53'27.8" E132°30'59.7"	June 2011
Nymphaea violacea	NV1	Doongan Station	WA	S14°46'33.1" E126°30'52.6"	May 2011
	NV3	Doongan Station	WA	S14°48'23.9" E126°31'47.5"	May 2011
	NV4	Jabiru	NT	S12°53'27.8" E132°30'59.7"	June 2011
	NV6	Theda Station	WA	S14°46'33.1" E126°30'52.6"	March 2012
	NV7	Wyndham	WA	S15°35'35.7" E128°16'47.9"	May 2012
	NV8	Wyndham	WA	S15°51'39.8" E127°14'20.8"	May 2013
	NV9	Wyndham	WA	S15°51'39.8" E127°14'20.8"	May 2013
	NV10	Cairns	QLD	S16°54'55.3" E145°22'20.6"	May 2013

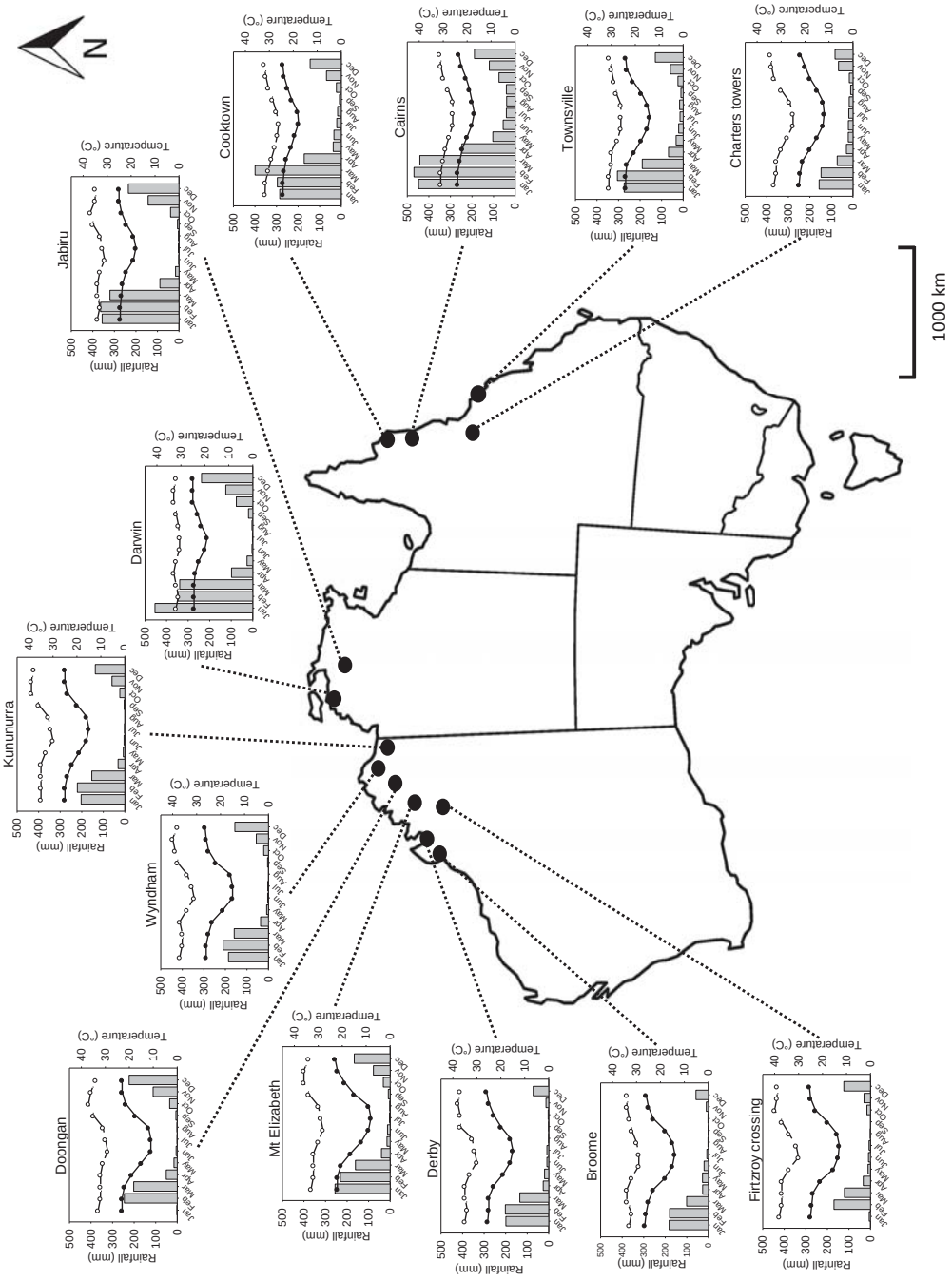

Figure 4.1: Mean monthly maximum (empty circles) and minimum (filled circles) temperature and rainfall from a number of collection locations of *Nymphaea* through northern Australia (Australian Government Bureau of Meteorology 2013).

Table 4.2: Mean maximum and minimum temperatures (± S.E) averaged across the following Bureau of Meteorology weather stations: Broome (WA), Derby (WA), Doongan (WA), Fitzroy Crossing (WA), Kununurra (WA), Mt Elizabeth (WA), Wyndham (WA), Darwin (NT), Jabiru (NT), Cairns (QLD), Charters Towers (QLD) and Townsville (QLD) and Cooktown (QLD) (Australian Government Bureau of Meteorology 2013).

Month	Mean maximum temperature (°C ± S.E)	Mean minimum temperature (°C ± S.E)	Mean daily temperature fluctuation (°C)
January	33.2 ± 2.2	24.2 ± 1.4	9.0
February	32.4 ± 2.0	24.1 ± 1.3	8.3
March	32.7 ± 2.3	23.5 ± 1.5	9.2
April	32.6 ± 2.7	21.7 ± 2.4	10.9
May	30.8 ± 2.5	18.8 ± 3.1	12.0
June	28.7 ± 2.1	16.1 ± 3.2	12.6
July	29.1 ± 2.4	15.3 ± 3.1	13.8
August	30.0 ± 2.9	16.5 ± 3.3	13.5
September	32.4 ± 3.5	19.4 ± 2.6	13.0
October	34.3 ± 3.6	22.3 ± 2.3	12.0
November	34.3 ± 3.1	23.8 ± 1.7	10.5
December	34.0 ± 2.7	24.0 ± 1.4	10.0

Alternating day and night temperatures

In order to assess the impact of alternating temperatures on dormancy and germination in *N. violacea*, two bidirectional temperature gradient plates (TGP) (Grant Instruments, Cambridge, UK) were used to provide a range of diurnally (12h/12h) alternating temperatures between 10 and 40 °C. Fifty seeds were sterilised as above and plated on to water agar (control) or placed in sterile 10 mL plastic tubes topped up with sterile water (crowding). Sixty-four Petri dishes (or tubes) were placed across the thermogradient plates in an 8 x 8 grid. Both thermogradient plates were run synchronously in order to obtain two replicates of each temperature. Temperature was logged at five points on the plate using a Grant Instruments Squirrel 1000 Series data

logger. Surface temperature of agar in Petri dishes was checked weekly using a thermocouple to ensure diurnal temperature fluctuations were consistent. Germination was scored as above, for a total period of 8 weeks.

Seed bank persistence, germination and dormancy loss

In order to assess dormancy break under simulated natural conditions, seeds of *N. violacea* (NV6) were collected in March and were transported to Broome, Western Australia (Fig 4.1). Prior to burial, seeds were assessed for viability via a cut test, whereby 5 replicates of 20 (imbibed) seeds were cut in half longitudinally and visually inspected under a microscope. Seeds that had a fully intact, turgid and white embryo and endosperm/ perisperm were considered viable. Total seed fill prior to experimentation exceeded 98 %. Thirty-six replicates of 300 seeds were placed in nylon mesh bags with 50 g of sterilised white sand. Each individual nylon bag was then sealed and placed inside a secondary, stainless steel mesh bag for burial. The stainless steel mesh bags were used to ensure that seeds remained within the allocated replicates, in the event that the nylon mesh broke down during the course of the experiment. Three plastic containers (500 x 600 x 100 mm) were filled with sterilised white sand, so as to remove soil-produced ethylene as a factor in the experiment, and placed in a ventilated glasshouse. Twelve burial bags (i.e a nylon mesh bag containing seeds and sand, enclosed in the stainless steel bag) were then randomly positioned within each container and buried to a depth of 10 mm. The three containers were either kept constantly dry (dry), constantly wet (wet- water level held at 50 mm above soil surface) or kept wet or dry based on mean monthly rainfall at the collection site (Theda Station, mean climate statistics shown in Table 4.2) (wet/dry). The wet/dry treatment was kept wet for March and April, allowed to dry back over the dry period and then gradually wetted up again in November until completely inundated by the end of December 2011 and then kept constantly wet. Temperature data loggers (Tinytag- Gemini Data Loggers, UK) were installed 10 mm below the soil surface in each container and logged temperature every minute for the duration of the experiment. Burial bags were exhumed monthly, sealed in Glad® Snap Lock® bags and freighted to Kings Park within 24 hours of extraction. Upon arrival, the contents of the bag were sieved and all seeds removed by hand under a microscope. Seeds were then x-rayed and determined to be a) filled (intact embryo and endosperm/perisperm) b) decayed (obvious signs of predation and decay resulting in a

non-intact embryo, endosperm or perisperm) or c) germinated (radicle intact or seed coat ruptured in such a way as to suggest germination). Filled seeds were then surface sterilised and plated on to water agar or treated with ethrel as describe previously to determine level of dormancy alleviation within the soil seed bank.

Dry after ripening (DAR)

Seeds of *N. violacea* (NV2 and NV4), *N. macrosperma* (NM1) and *N. lukei* (NL1) were bagged in nylon mesh and placed in DAR conditions within 4 weeks of collection, for a total period of 8 months. Seeds were suspended above a non-saturated solution of lithium chloride (LiCl, 370 g L^{-1}; 50 % RH) enclosed in a polycarbonate electrical enclosure box (NHP, Fibox, Australia) according to the methods of Hay *et al.* (2008), and incubated at 35 °C. Two hundred seeds were removed from the box after 0 (control), 1, 2, 3, 4, 5 and 8 months of storage. Seeds incubated prior to storage at 35 °C served as the control. Seeds were surface sterilised and plated on water agar or treated with ethrel as described previously. Plates were placed in 30 °C incubator on a 12h/12 h light/dark regime (30 µmol m^{-2} s^{-1}, 400- 700 nm, cool-white fluorescent light). Germination was deemed to have occurred upon radicle emergence ≥ 1 mm. Germination was scored daily for two weeks and weekly for a further four weeks.

Wet dry cycling (WDC)

Wet dry cycling was conducted in accordance with the method described by Hoyle *et al.* (2008a), with some modifications. Seeds of *N. violacea* (NV2 and NV4), *N. macrosperma* (NM1) and *N. lukei* (NL1) were bagged in nylon mesh and placed in DAR conditions as described above. Every 12 days, seeds were removed from the electrical enclosure box and placed on to 0.6 % w/v water agar for 48 hours at 30 °C. After the 48-hour wet period, seeds were removed from the Petri dishes, patted dry with absorbent towel and placed back in the electrical enclosure boxes under DAR conditions. This process was repeated for 8 months. Two hundred seeds were removed from the box after 0 (control), 1, 2, 3, 4, 5 and 8 months and surface sterilized and subjected to the germination procedure as described above (DAR).

Warm/cold stratification (WS/CS)

In order to determine the temperature changes required to break dormancy in *Nymphaea*, seeds were subjected to a period of warm or cold stratification. Four replicates of 25 seeds were used for each germination temperature treatment and germination stimulant (i.e. control, GA_3, and ethrel). *Nymphaea immutabilis* (NI1), *N. lukei* (NL1, NL2) *N. macrosperma* (NM2, NM3), and *N. violacea* (NV7, NV8, NV9, NV10) seeds were surface sterilized and sown in Petri dishes containing water agar, GA_3, or ethrel as described above ('Germination testing'). Seeds were then placed in one of four temperature control treatments (germination temperature treatments = GT): a) 30 → 10 °C (GT1), b) 10 → 30 °C (GT2), c) 32/25 °C → 32/10 °C (GT3), d) 32/10 °C → 32/25 °C (GT4), e) 30 °C, (GT5) f) 10 °C (GT6), g) 32/25 °C (GT7) or h) 32/10 °C (GT8). Warm or cold stratification treatments consisted of 12 weeks and the first temperature, followed by 12 weeks at the second temperature. Seeds incubated at fixed temperatures remaining at that temperature for the entire experimental period of 24 weeks. Germination was scored weekly. It should also be noted that seeds of NL1 and NM2 were not freshly collected, and remained under C.E. room conditions (15°C and 15 % RH) prior to experimental use.

Fixed day temperature move-along

Five hundred seeds (four replicates of 25 seeds for each temperature treatment and germination stimulant) each of *Nymphaea immutabilis* (NI1), *N. lukei* (NL1, NL2) *N. macrosperma* (NM2, NM3), and *N. violacea* (NV7, NV8, NV9, NV10) were surface sterilized and sown in Petri dishes containing water agar, GA_3, or ethrel. A modified version of the move-along experiment described by Baskin and Baskin (2003) was used. Two fixed day temperature move-alongs, similar to that of Hidayati *et al.* (2012) were designed to simulate the natural temperature sequence experienced by seeds dispersed in the summer wet season and winter dry season.

The summer cycle consisted of: 12 weeks of 1st summer (32/25 °C) → 8 weeks of autumn (32/20 °C) → 12 weeks of winter (32/10 °C) → 8 weeks of spring (32/20 °C) → 12 weeks of 2nd summer (32/25 °C).

The winter cycle was the exact opposite and consisted of 12 weeks of 1st winter (32/10 °C) → 8 weeks of spring (32/20 °C) → 12 weeks of summer (32/25 °C) → 8 weeks of

autumn (32/20 °C) → 12 weeks of 2nd winter (32/10 °C). It should also be noted that seeds of NL1 and NM2 were not freshly collected, and remained under C.E. room conditions (15°C and 15 % RH) prior to experimental use

Statistical analysis

Germination data were analysed using a generalised linear model with an inbuilt 'logit' link function binomial errors in R (R Core Team 2012). The model compared all main factors (light or germination stimulants or treatment or time) in each separate experiment (alternating day and night temperatures, germination after retrieval from burial trial, DAR, WDC, stratification, move along.) and their interactions. Where significant differences were detected, *post-hoc* analyses were conducted via a Holm's pairwise comparison. For intact, filled seeds exhumed from the burial trial, longevity data were analysed via probit analysis in Genstat (version 12, VSN International Ltd. UK) to estimate the time for viability to fall to 50 % (p_{50}) and to fit the following seed viability equation (Ellis and Roberts 1980):

$$v = K_i - p/\sigma$$

where v is the viability in normal equivalent deviates (NED) of the seeds after p days in burial: K_i is the initial viability (NED) and σ is time (in days) for viability to fall by 1 NED.

RESULTS

Alternating day and night temperatures

Germination of seeds plated on water agar alone was < 15 % (P = 0.4) (Fig. 4.2). However, seeds in the crowded treatment germinated to high percentages over a wide range of temperatures (Fig. 4.2). In the model describing the overall effect of alternating temperatures on germination of *Nymphaea violacea* (NV6), the day temperature (P < 0.001), night temperature (P < 0.001) and the interaction of temperatures (P < 0.001) were all significant terms in the regression. Germination occurred at all day temperatures (10- 40 °C), and a subsequent Holm's pairwise comparison revealed that

the day temperature alone did not influence total germination. However, high germination (> 75 %) was restricted to regions of the thermogradient table that received overnight temperatures greater than 25 °C ($P < 0.001$).

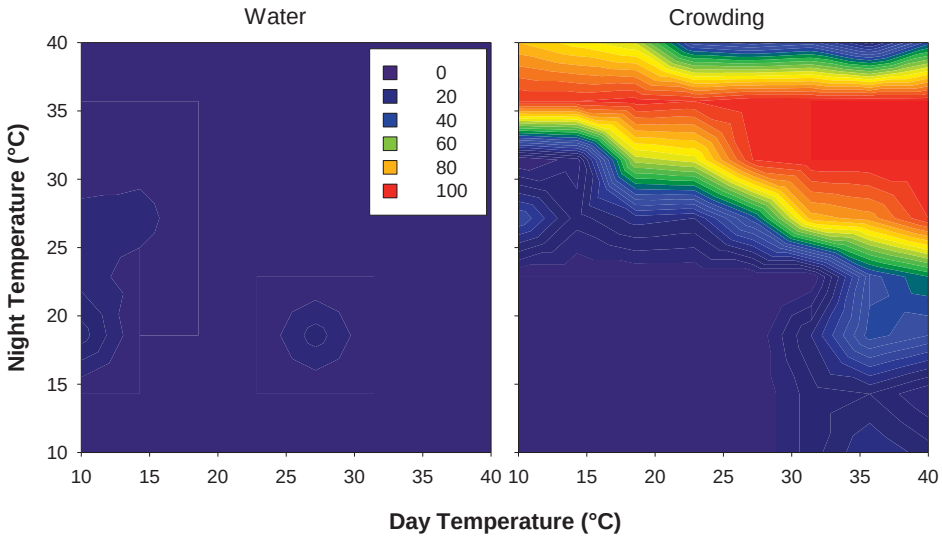

Figure 4.2: Contour plot displaying percentage germination (shown as different colours for increments of 20%) of *Nymphaea violacea* (NV6) seeds incubated on bi-directional temperature thermogradient plates running diurnally (12 h/12 h) alternating temperatures between 10 and 40 °C. Seeds were either plated on to Petri dishes containing water agar (water), or placed in 10 mL plastic vial filled with 10 mL of sterilized water (crowding).

Seed bank persistence, germination and dormancy loss

The seeds of *N. violacea* (NV6) that were buried in soil and kept constantly wet germinated to significantly higher percentages (i.e. four months of germination greater than 30 %, $P < 0.001$) over the 12- month burial period when compared with those seeds kept constantly dry, or under the wet-dry treatment (Fig. 4.3). The highest germination recorded for this treatment was 61 % for seeds exhumed in February ($P < 0.001$). Germination in this treatment first increased in November and remained constant through until March 2013 (all months significant, $P < 0.01$). Interestingly, this increase in germination also appears to be correlated with the increasing overnight (mean minimum) temperature (Fig. 4.3 and Fig. 4.4). The wet/dry treatment showed negligible (< 5 %) germination for all months until March 2013, when germination

increased to 12.5 % and 27 % in April ($P < 0.001$). There was no germination recorded in seeds from the dry treatment (Fig. 4.3). It should be noted however, that many seeds in the 'wet' treatment had decayed by the end of the experiment- hence some seeds that may have germinated prior to the March/April extractions may have been misidentified as 'decayed' rather than 'germinated'.

The number of seeds still intact and completely filled after 12- months decreased significantly ($P < 0.001$) in all treatments. In the wet treatment, the number of decayed seeds reached a maximum after 12-months burial, with 78 % decay and only 6 % remaining fully intact and viable. Seeds in the wet treatment had an estimate P_{50} value of 263 days (Table 4.3). In the dry treatment, the number of intact seeds declined by 45 % over the 12- month burial period (Fig. 4.3), with an estimated P_{50} value of 367 days, which exceeded the 12- month burial period (Table 4.3). In the wet/dry treatment, the number of decayed seeds steadily increased from 0 % in March 2012 to 73 % in April 2013, leaving 0 % intact and filled at the end of the burial period ($P < 0.001$). This treatment showed the lowest P_{50} value of 222 days. In the overall model describing seed persistence under all water regimes (wet, dry and wet/dry), the months of November and December in 2012 and January, February, March and April in 2013, were all significantly different ($P < 0.001$) to the non-buried controls.

Seed bank germination and dormancy loss under laboratory conditions

In the overall model describing germination response of exhumed seeds, the month of November ($P = 0.04$), the wet treatment ($P < 0.01$) and the germination stimulant ethrel ($P < 0.001$) were significant factors in the logistical regression. Within the wet treatment, germination was generally low (< 20 %), however maximum germination of 31.7 % occurred in seeds retrieved in February 2013 that were treated with ethrel ($P = 0.01$; Fig. 4.5). Within the dry treatment, ethrel significantly increased germination ($P < 0.001$) when compared with the water control, and maximum germination occurred in seeds retrieved in June (29 %, $P = 0.02$), August (30 %, $P < 0.01$) and October (36 %, $P < 0.01$) (Fig. 4.5). A similar pattern of germination was observed in seeds in the wet-dry treatment. Ethrel significantly increased germination ($P < 0.001$), and seeds exhumed in months 3, 4, 5 and 8 (June, July, August and November) displayed germination ≥ 24 % ($P < 0.001$) (Fig. 4.5).

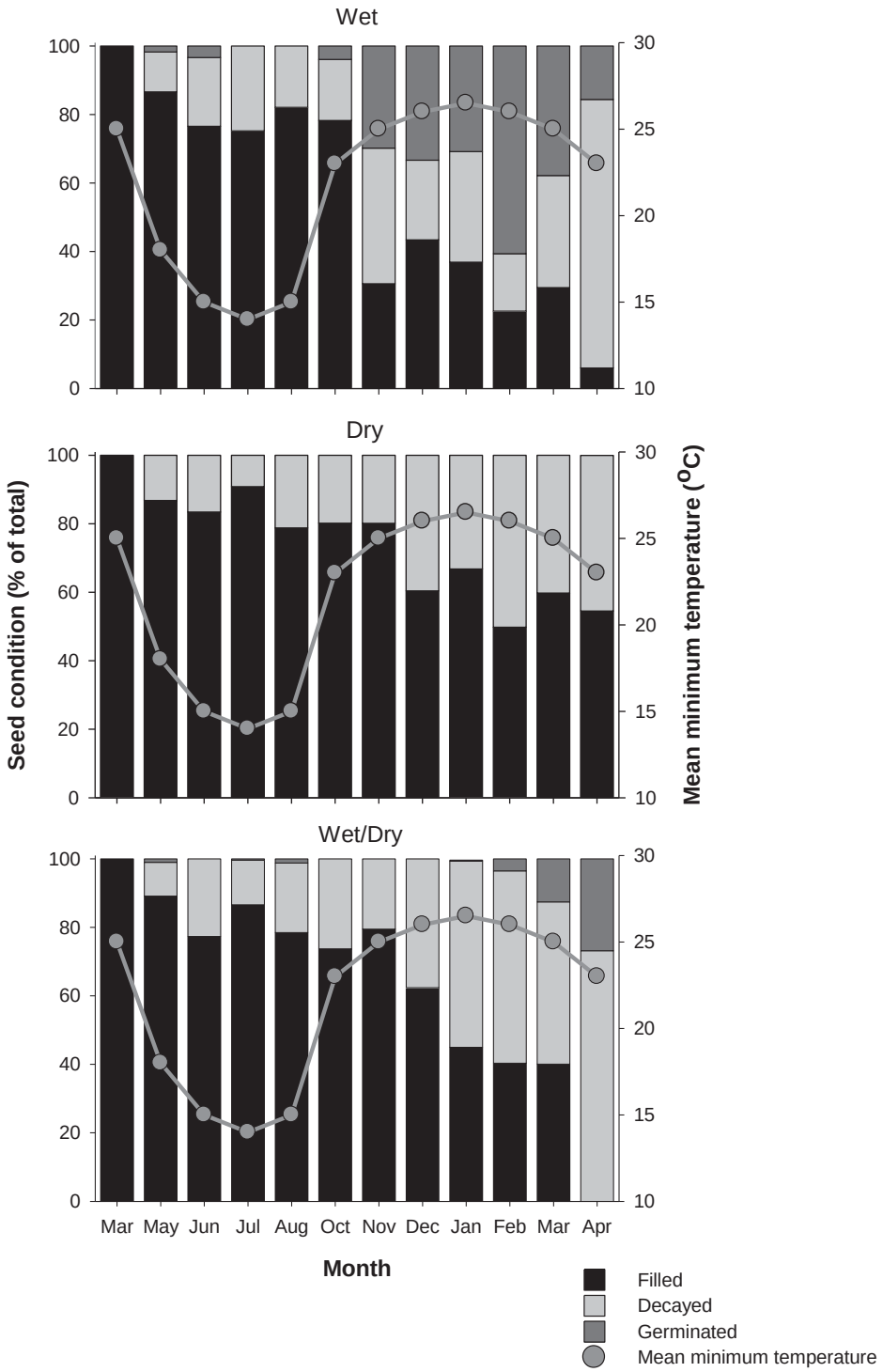

Figure 4.3: Percentage of *Nymphaea violacea* seeds that have decayed, germinated or remained filled after 12 months burial under simulated natural conditions. The 'wet' treatment was kept wet for the entire burial period; the 'dry' treatment was kept dry and the 'wet-dry' treatment was kept wet until the end of April 2013, whereby it was allowed to dry back until it was wetted-up at the end of November. Burial data has been overlayed with mean minimum temperature from Broome (Bureau of Meteorology, 2015).

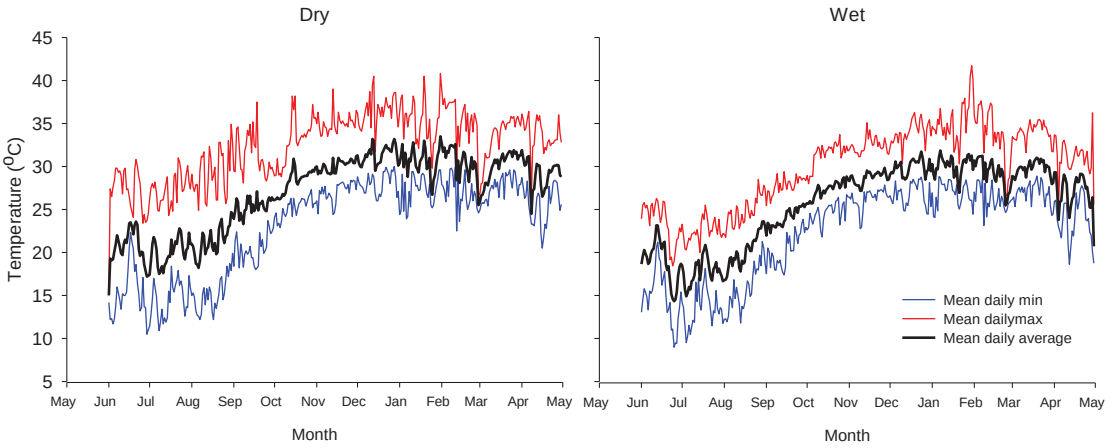

Figure 4.4: Mean daily minimum, maximum and average temperature recorded from data loggers (Tinytag, Gemini Data Loggers, UK) installed in the wet and dry burial treatments.

Table 4.3: Estimates of initial viability (K_i), $1/\sigma$ (σ is time in days for viability to fall by 1 normal equivalent deviate) and p_{50} (time in days for viability to fall to 50 %).

Treatment	K_i	$1/\sigma$	p_{50}
Dry	1.57	0.004	367.5
Wet	1.87	0.007	263.5
Wet/Dry	1.70	0.008	222.3

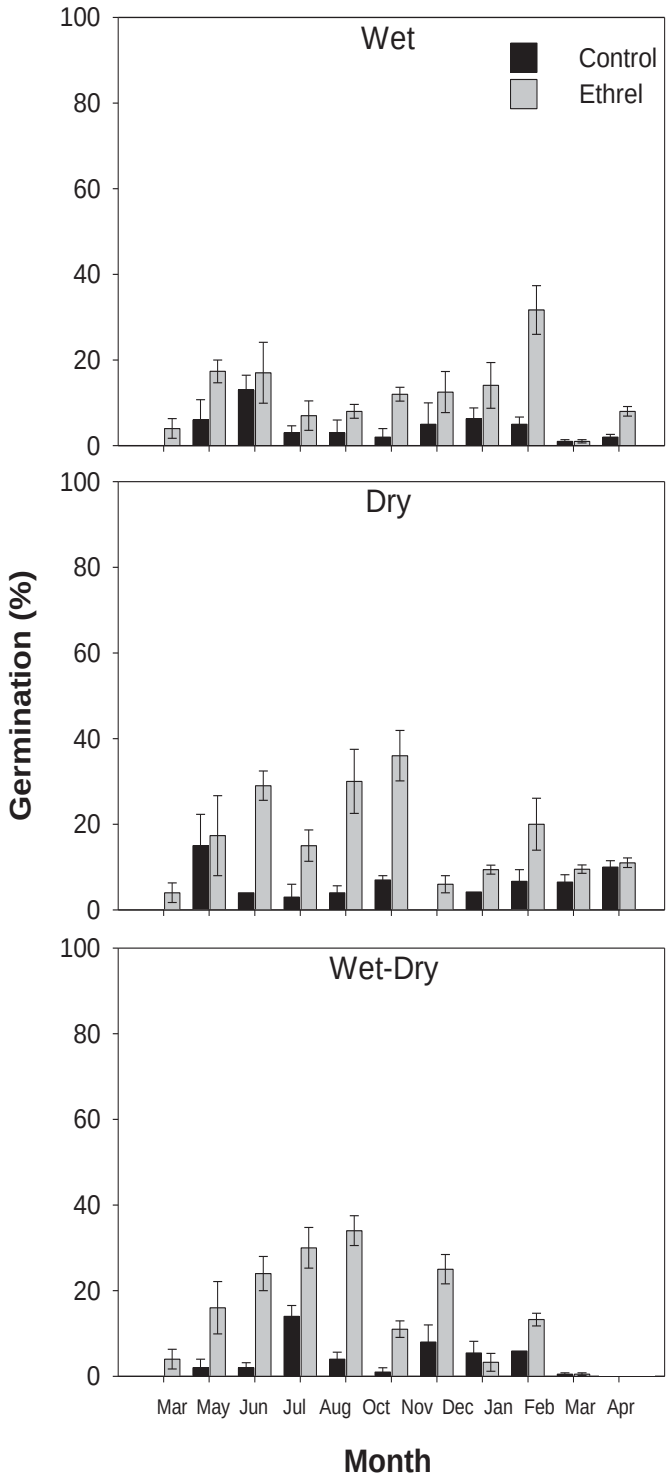

Figure 4.5: Germination of viable *Nymphaea violacea* seeds after retrieval from simulated burial conditions. The 'wet' treatment was kept wet for the entire burial period; the 'dry' treatment was kept dry and the 'wet-dry' treatment was kept wet until the end of April 2013, whereby it was allowed to dry back until it was wetted-up at the end of November. Seeds were sown on plain water agar or pre-treated with Ethrel (20 mM) prior to being plated on plain water agar incubated with 12 h light d^{-1} at 30 ^{o}C and scored for germination for 8 weeks.

Dry after ripening (DAR)

Seeds of *N. lukei* showed a significant ($P < 0.001$) increase in germination from 0 % in the non-after ripened control to 30 % germination after eight months in DAR conditions, when seeds were plated on water agar (Fig. 4.6). Seeds that were treated with ethrel prior to plating also showed a small, but significant ($P < 0.001$) increase in germination after 3 (16 %), 4 (12 %) and 8 (18 %) months in DAR conditions, compared to the non- DAR control (Fig. 4.6). Germination in *N. macrosperma* did not increase with DAR ($P = 0.3$). Germination was reduced after 8 months under DAR conditions ($P < 0.001$), decreasing from 38 % to 0 % when plated on water and from 12 % to 4 % when treated with ethrel in the non-DAR control (Fig. 4.6). There was no effect of ethrel on germination of *N. macrosperma*, as seeds treated with ethrel did not show higher germination when compared with seeds plated on water alone ($P = 0.6$). Seeds of *N. violacea* (NV1) did not germinate under any conditions. Germination of *N. violacea* (NV4) did not change significantly with DAR ($P = 0.42$) (Fig. 4.6). The non-DAR control of *N. violacea* (NV4) germinated to 46 % while the ethrel treatment germinated to 60 %.

Wet dry cycling (WDC)

Overall, WDC did not have a significant positive effect on the germination response of *N. macrosperma* or *N. violacea,* however did significantly increase the germination response of *N. lukei* ($P < 0.001$) (Fig. 4.7). Seeds of *N. lukei* not exposed to WDC did not germinate (0 %), however exposure to ≤ 5 months WDC significantly increased germination ($P < 0.001$; Fig. 4.7). Seeds plated on water agar alone germinated to high percentages (≥ 75 %) after two (78 %; 4 cycles) and three (90 %; 6 cycles) months in WDC. Ethrel increased germination in combination with WDC, and seeds germinated to high percentages after two (100 %), three (100 %) and five (94 %; 10 cycles) months ($P = 0.02$). Eight months of WDC resulted in a decline in germination to 0 % in both the water and ethrel treatments ($P < 0.001$). Seeds of *N. macrosperma* treated with ethrel were negatively affected by WDC (Fig. 4.7). Seeds treated with ethrel and WDC decreased from 30 % in the non-WDC control to 4 % after eight months in WDC ($P < 0.001$). Seeds plated on water agar alone showed a decrease in germination (14 %) after four months of WDC when compared with the control (38 %).

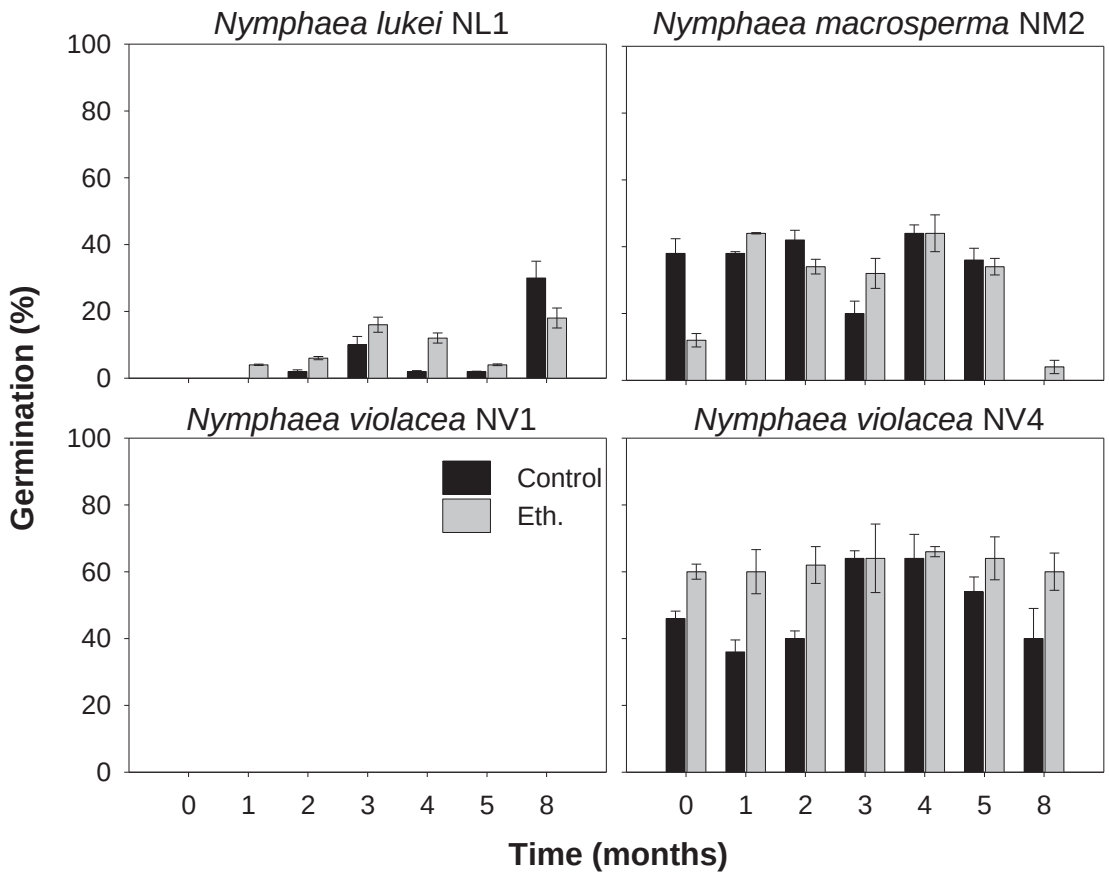

Figure 4.6: Germination of seeds of *Nymphaea lukei* (NL1), *N. macrosperma* (NM2) and two accessions of *N. violacea* (NV1 and NV4) after dry after-ripening. Seeds were placed in a FiBox electrical box containing a 50 % RH solution of LiCl at 35 ^{o}C and extracted after 1, 2, 3, 4, 5 and 8 months. Seeds were sown on plain water agar or pre-treated with Ethrel (20 mM) prior to being plated on plain water agar incubated with 12 hour light d^{-1} at 30^{o}C and scored for germination for 8 weeks.

Seeds of *N. violacea* (NV2) only germinated after one and two months of WDC, to a maximum of 20%, when treated with ethrel ($P < 0.001$; Fig 4.7). Conversely, seeds of *N. violacea* NV4 that were exposed to WDC showed a significant decrease in germination ($P = 0.01$) in both the water and ethrel treatments. This decrease was most apparent in seeds plated on water agar alone as germination decreased from 46 % in the control, to 0 % after 8 months in WDC.

Warm/cold stratification (WS/CS)

Germination was very low (< 5 %) and not significantly different to 0 % in all treatments and species tested (Fig. 4.8). Seeds of *N. macrosperma* (NM2) did not germinate under any conditions, even with the addition of GA_3 or ethrel. Maximum germination of 2.5 % (± 1.4) was observed in seeds of *N. lukei* (NL1) incubated at constant 30 °C for the duration of the experiment.

Fixed day temperature move-along

No significant germination was detected and total germination was very low (< 5 %) in all treatments and species tested (Fig. 4.4). No germination was observed in the treatment mimicking winter dispersal [12 weeks of 1st winter (32/10 °C) → 8 weeks of spring (32/20 °C) → 12 weeks of summer (32/25 °C) → 8 weeks of autumn (32/20 °C) → 12 weeks of 2nd winter (32/10 °C)], or the control treatment at 32/10 °C. While not statistically significant, maximum germination of 2.3 % (± 0.9) was observed in *N. violacea* (NV10) incubated under the summer [12 weeks of 1st summer (32/25 °C) → 8 weeks of autumn (32/20 °C) → 12 weeks of winter (32/10 °C) → 8 weeks of spring (32/20 °C) → 12 weeks of 2nd summer (32/25 °C)] move along conditions

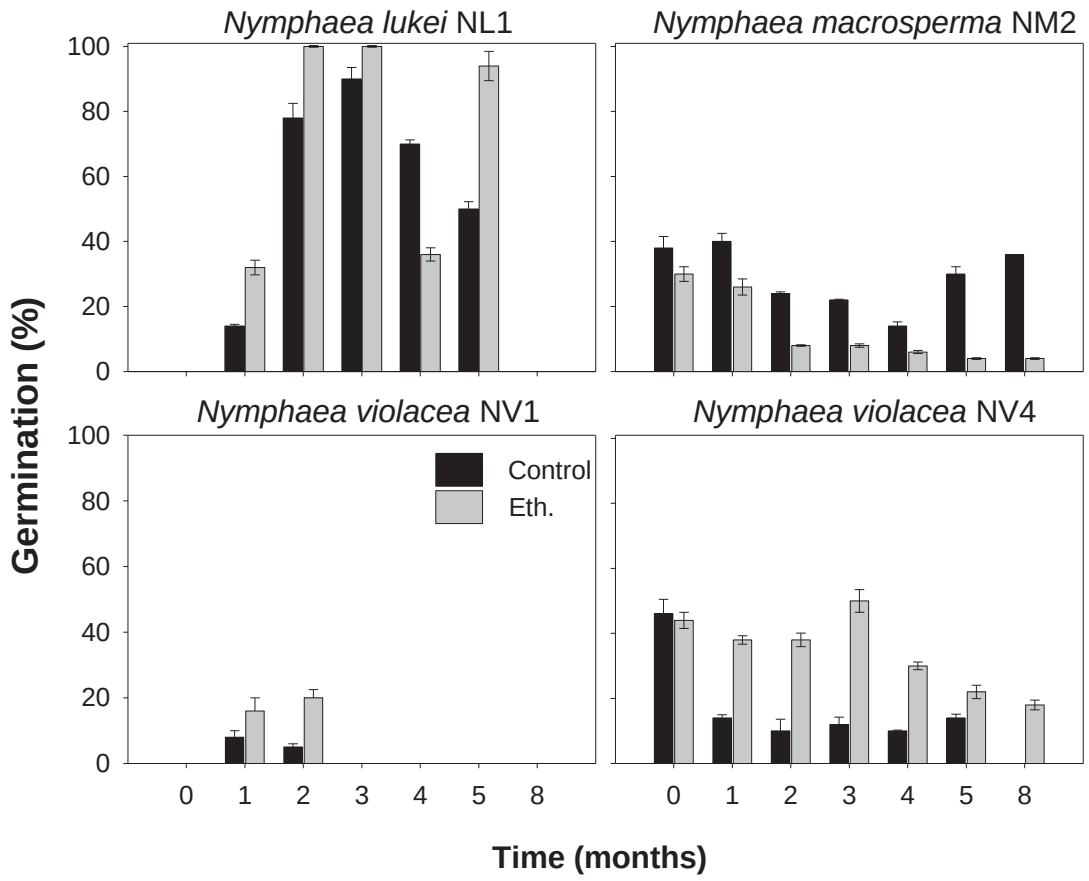

Figure 4.7: Germination of seeds of *Nymphaea lukei* (NL1), *N. macrosperma* (NM2) and two accessions of *N. violacea* (NV1 and NV4) after wet dry cycling. Seeds were paced in a FiBox electrical box containing a 50 % RH solution of LiCl at 35^{o}C for 12 days, then removed and placed on 0.7 % water agar for 2 days. This was repeated twice a month for 8 months. Seeds were extracted at 1, 2, 3, 4, 5 and 8-month intervals. Seeds were sown on plain water agar or pre-treated with Ethrel (20 mM) prior to being plated on plain water agar incubated with 12 h light d^{-1} at 30^{o}C and scored for germination for 8 weeks.

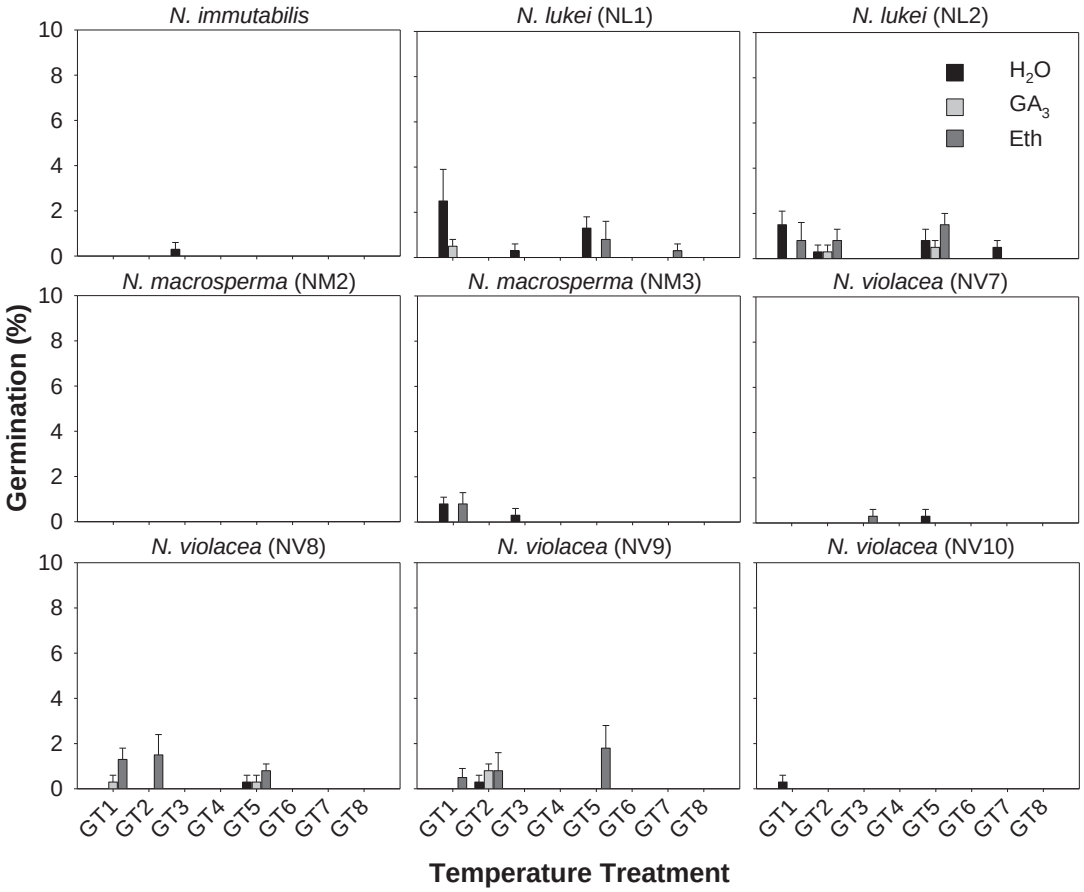

Figure 4.8: Germination of *Nymphaea immutabilis, N. lukei* (NL1 and NL2), *N. macrosperma* (NM2 and NM3) and *N. violacea* (NV7, NV8, NV9 and NV10) after 24 weeks of temperature stratification. Seeds were plated on to water agar (H_2O), water agar containing 0.67 µM GA_3 or pre-treated with ethrel and then incubated under a range of temperature treatments (germination treatments): GT1; 30 °C (12 weeks) → 10 °C (12 weeks), GT2; 10 °C (12 weeks) → 30 °C (12 weeks), GT3; 32/25 °C (12 weeks) → 32/10 °C (12 weeks), GT4; 32/10 °C (12 weeks) → 32/25 °C (12 weeks), GT5; 30 °C (24 weeks), GT6; 10 °C (24 weeks), GT7; 32/25 °C (24 weeks) and GT8; 32/10 °C (24 weeks).

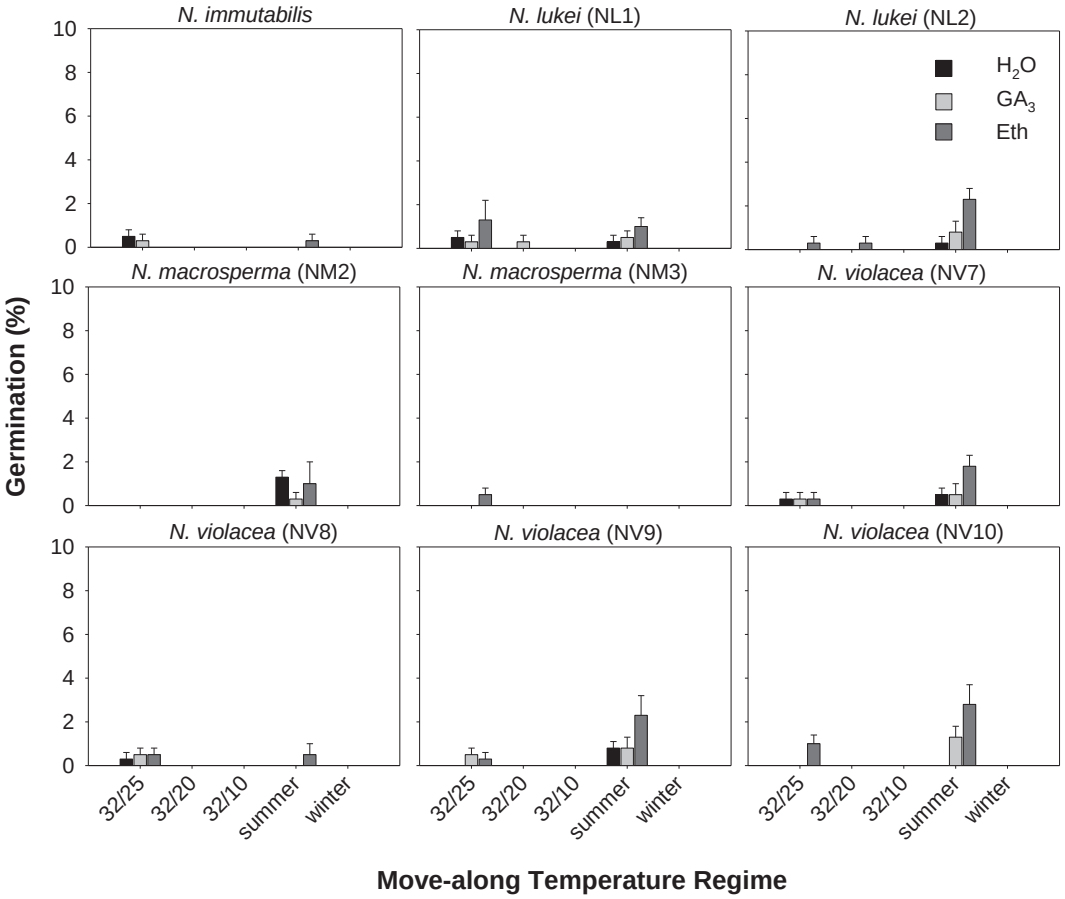

Figure 4.9: Germination of *Nymphaea immutabilis, N. lukei* (NL1 and NL2), *N. macrosperma* (NM2 and NM3) and *N. violacea* (NV7, NV8, NV9 and NV10) under experimental temperature regimes. Seeds were plated on to water agar (H_2O), water agar containing 0.67 µM GA_3 or pre-treated with ethrel. Seeds in the 32/25, 32/20 and 32/10 treatments were kept at these temperatures for the duration of the experiment. The summer cycle consisted of: 12 weeks of 1st summer (32/25 °C) → 8 weeks of autumn (32/20 °C) → 12 weeks of winter (32/10 °C) → 8 weeks of spring (32/20 °C) → 12 weeks of 2nd summer (32/25 °C). The winter cycle was the exact opposite and consisted of 12 weeks of 1st winter (32/10 °C) → 8 weeks of spring (32/20 °C) → 12 weeks of summer (32/25 °C) → 8 weeks of autumn (32/20 °C) → 12 weeks of 2nd winter (32/10 °C). Total incubation time was 365 days.

DISCUSSION

Through manipulations of seed moisture content and temperature, we found that dormancy loss in tropical Australian *Nymphaea* to be complex, and species specific. There is a significant amount of variation within and between species, and multiple cues are needed in order to overcome dormancy successfully.

Dormancy break and germination under simulated natural conditions

By investigating the germination response of *N. violacea* seeds incubated under a range of alternating day and night temperatures, we found that seeds germinated preferentially in conjunction with warm (≥ 25 °C) over-night temperatures, when under crowded conditions (Fig. 4.2). One hundred percent germination occurred predominantly where warm/hot day temperatures (≥ 25 °C) occurred in conjunction with hot night temperatures (≥ 28 °C). However, when seeds were subjected to alternating day and night temperatures and not treated with a dormancy breaking treatment (i.e. plated on water), germination was < 14 % (Fig. 4.2). Temperature stratification (both warm and cold) and the move-along experiment did not overcome MPD in any of the *Nymphaea* species we tested (Figs. 4.8 and 4.9). While temperature obviously plays a critical role in successful seed germination (Fig. 4.2), temperature alone is not the critical driver influencing dormancy break in Australian *Nymphaea.*

When comparing results from the seed burial trial, the highest germination of *N. violacea* was observed in the treatment under complete inundation (wet, Fig. 4.3). While minimal germination (≤ 3.9 %) occurred through the burial period until November 2012 (Fig. 4.3), increased germination (≥ 30 %) occurred from November to March, reaching a maximum in February (60 %) before declining in April 2013 (15.6 %). Interestingly, the observed increase in germination appears to coincide with increased mean minimum temperatures (Figs. 4.3 and 4.4) associated with the onset of the wet season in the arid tropics (Broome, Western Australia) (i.e. overnight temperatures ≥ 25 °C), which supports our findings from the alternating day/night temperature experiment. Given that dormancy was not overcome by alternating temperatures (either under warm stratification or move-along, Figs. 4.8 and 4.9), this increase in germination may be a response to a reduction in diurnal temperature

fluctuation. Additionally, filled seeds exhumed from the wet treatment in February 2013 were the least dormant and germinated to 30 % when treated with ethrel prior to incubation at 30 °C (Fig. 4.5). The fact that dormancy was lost in conjunction with decreasing diurnal temperature fluctuations and germination was stimulated in the presence of ethrel (ethylene), may suggest an ecological response that cues germination to occur after the onset of the summer wet season. As wetland sediments become increasingly inundated and anoxic, ethylene production increases (Smith and Restall 1971; Zeikus and Winfrey 1976), acting to stimulate germination (Baskin and Baskin 2014).

Under simulated natural conditions (wet- dry treatment), germination was very low (≤ 3.5 %) until March and April 2013 where germination increased to 12.5 % and 26.8 %, respectively. Seed dormancy of exhumed seeds tested *ex situ,* decreased over the dry winter period in both the dry and wet-dry treatments (Fig. 4.5), suggesting that the seeds may have after-ripened. A loss of dormancy over winter is common in many aquatic species, including a number of *Nymphaea* from the northern hemisphere (Else and Riemer 1984; Smits *et al.* 1995; Schütz and Rave 1999; Hay *et al.* 2000; Baskin and Baskin 2014). However, this would rarely occur in conjunction with a period of after-ripening, as most temperate regions receive rainfall over the colder winter period. Few aquatic species (< 20 % with PD) have been shown to require warm stratification or dry storage (Baskin and Baskin 2014). However, given the extent of the wet-dry tropics (or tropical savanna (Peel *et al.* 2007)) through northern Australia, south-east Asia, India, South and Central America and central Africa, it would seem likely that many aquatic species occurring in these regions would display similar dormancy breaking requirements to those presented here, however are under represented in the literature.

Seed persistence under simulated natural conditions

Seed viability of *N. violacea* declined under all three water regimes tested, and was most pronounced in the wet-dry treatment whereby p_{50} was equal to 222 days (Table 4.3), compared to 368 days (dry) and 264 days (wet). While no seeds remained intact under wet-dry conditions, over 50 % of seeds in the dry treatment remained filled and intact after more than 12 months burial (Fig. 4.8). Given the vast number of seeds produced by individual plants during the season, it would seem likely that *N. violacea* may be described as 'short-term persistent' (Garwood 1989; Csontos and Tamás 2003)

or 'Type III, wetland', species which have a transient seed bank component and a smaller persistent component (Leck 1989). Seeds of *N. violacea* are transient in the seed bank under wet conditions, but semi-persistent under dry conditions. Our results clearly show that seeds of *Nymphaea* are unlikely to survive multiple wetting and drying events, as seeds will likely germinate or decay, especially when compared with other temperate aquatic species that can persist for > 12 cycles (Brock 2011). These results however do not take into account other species occurring in more transient environments (e.g. *N. lukei*), which may be expected to show increased persistence under dry conditions. Additionally, seeds that are artificially buried tend to live longer than those dispersed and buried naturally (Pons 1989; Bekker *et al.* 1998) and persistence in the sediment seed bank is determined by a number of factors including inundation patterns (timing, duration, amount) and the physical and chemical properties of the substrate (or water) (Leck 1989). It is therefore likely that many tropical *Nymphaea* species invest in both underground storage organs (for mature plants), and large numbers of dormant seed, of which a fraction will survive multiple years of drying in order to compensate for inconsistent and unreliable water availability.

Dormancy break and germination under laboratory conditions

The response of *Nymphaea* seeds to DAR and WDC was variable, however *N. lukei* was the only species to show a significant increase in germination in response to either treatment (Figs. 4.6 and 4.7). The process of WDC in combination with ethrel increased germination from 0 % to 100 % germination after four wetting and drying cycles (Fig. 4.7). Of the species tested *N. lukei* has the most restricted geographical range and occurs in small, ephemeral creek lines that are persistent only for the wet summer months (December- April). As such, dormant seeds that remain in the sediment seed bank will be exposed to a period of up to seven months of drought, followed by a period of gradual, intermittent wetting prior to complete inundation. Given that wet/dry cycling only overcame dormancy completely after four wetting and drying cycles, MPD in *N. lukei* may represent an ecological adaptation that has evolved to ensure that seeds do not germinate at the onset of the wet season, postponing germination until a number of rainfall events have occurred and regular, reliable and sufficient rainfall would be expected over the coming months. While *N. macrosperma* and *N. violacea* can inhabit seasonal waterways, they are more commonly found in more perennial waterways, or at

least those that retain some water through the majority of the dry season (e.g. large billabongs and floodplains). However as water levels recede during the dry season, seeds distributed at the margins may only be exposed to a short period of drought, which may explain why WDC and DAR was not effective in overcoming dormancy in these species. However, this study represents the first to demonstrate that WDC can effectively overcome dormancy in some aquatic species with PD or MPD.

It should also be noted that there is some discrepancy between the germination responses of two accessions used in this study- *N. lukei* (NL1) and *N. macrosperma* (NM2). Both of these accessions showed low to no germination under any conditions in experiments performed in 2013 (the warm/cold stratification experiment, and the fixed day temperature move-along), while displaying significantly higher germination in the experiments performed in 2012 (dry after ripening and wet-dry cycling). Despite seeds of both accessions appearing to be viable prior to experimental use, viability may have declined, or seeds became more dormant during the one-year storage period (see Chapter 5 of this thesis).

Conclusions

In this study, we have shown that the processes governing dormancy loss and germination of Australian *Nymphaea* are complex, and likely governed by a range of environmental interplays including temperature and the availability of water. This study also serves to highlight our limited understanding of seed dormancy and seed bank dynamics in tropical aquatic species (Leck 1989; Cronk and Fennessy 2001; Baskin and Baskin 2014). Current climate change predictions for northern Australia show a change in the seasonality, periodicy and amount of rainfall received in the region (Steffan *et al.* 2013; Steffan *et al.* 2014). As a consequence of these changes, wetlands and wetland plant communities in Australia's north are likely to change in terms of distribution, composition and abundance (Casanova and Brock 2000; Brock *et al.* 2005; Bowman 2010; Brock 2011). Therefore, an understanding of the ecological drivers currently controlling germination will enable us to make more informed predictions in relation to potential ecosystem changes that may occur from a changing climate.

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CHAPTER 5

SEED STORAGE BEHAVIOUR OF AUSTRALIAN *NYMPHAEA* L.

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ABSTRACT

Background and aims: The Nymphaeaceae are aquatic plants that form part of the basal ANA-grade angiosperms. Seeds of these ancient plants have previously been used to identify the evolutionary origins of traits such as seed desiccation tolerance. Conflicting evidence suggests that seeds of *Nymphaea* may or may not be tolerant to drying. Additionally, some authors suggest that basal angiosperms are short-lived in storage as they evolved in warm, moist environments. Given these hypotheses, our aim was to determine whether seeds of tropical Australian *Nymphaea* are desiccation tolerant and display orthodox storage behaviour and to investigate the effects of water content and storage temperature on seed viability and comparative longevity in order to assist future seed bank storage of these species.

Methods: Four species (*N. immutabilis, N. lukei, N. macrosperma* and *N. violacea*) were placed in experimental storage conditions at a range of relative humidities (15, 30, 50, 70, 95 %) and temperatures (25, 5, -20, -160 °C). Seeds and lipids were investigated via differential scanning calorimetry to determine whether phase changes in water or fatty acids were contributing to the observed storage behaviour. Finally, seeds were experimentally aged in order to assess their comparative longevity.

Key results: Seeds of four *Nymphaea* species (*N. immutabilis, N. lukei, N. macrosperma* and *N. violacea*) displayed orthodox storage behaviour. However, seeds of *N. immutabilis, N. lukei* and *N. macrosperma* lost viability quickly under the majority of storage conditions over the 12- month testing period. Furthermore, upon testing their comparative longevity, the majority of accessions were found to be short-lived (values between 0 and 24.8 days). Unusual lipid crystallisation and melting events were detected in fatty acids of all species, despite presenting orthodox storage behaviour.

Conclusions: Seeds of *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea* are desiccation tolerant, providing further empirical support for hypotheses that desiccation tolerance is basal within the angiosperms. However, despite displaying orthodox storage behaviour, seeds are relatively short-lived under conventional storage conditions. Furthermore, storage behaviour is quite complex, and further investigations are required to determine the optimal methods for storing these seeds *ex situ.*

Key words: basal angiosperms, desiccation tolerance, water content, storage temperature, comparative longevity, conservation, cryogenic storage.

INTRODUCTION

The Nymphaeaceae (Nymphaeales) is a family of aquatic angiosperms comprised of *c.* 80 species, with *Nymphaea* being the most specious genus of approximately 65 species (Löhne *et al.* 2008a). Along with the Amborellaceae and Austrobaileyales, they form the ANA grade angiosperms; early divergent flowering plants that form the base of the angiosperm phylogenetic tree (Qiu *et al.* 1999). Studies regarding these basal plants' morphology, phenology and development have often been used to inform hypotheses of the evolution of flowering plants (Qiu *et al.* 1999; Rudall *et al.* 2008; 2009a; 2009b; Tuckett *et al.* 2010a) including the evolution of seed desiccation tolerance (Dickie and Pritchard 2002). While there is considerable debate in the literature as to whether desiccation tolerance is the basal (Dickie and Pritchard 2002; Tweddle *et al.* 2003) or derived state (Pammenter and Berjak 2000), the majority of basal angiosperms are tolerant of drying (Tweddle *et al.* 2003; Tuckett *et al.* 2010b). However, information regarding this trait in the *Nymphaea* is sparse and conflicting; *N. alba* and *N. odorata* are temperate species from the northern hemisphere and were found to be desiccation sensitive (Guppy 1897; Else and Riemer 1984; Smits *et al.* 1989; Hay *et al.* 2000; Estrelles *et al.* 2004) while *N. gigantea* a tropical species occurring in Australia, was found to be desiccation tolerant (Ewart 1908).

Worldwide, wetlands are among the most highly threatened ecosystems from processes such as water diversion, draining, eutrophication, competition with weeds and climate change (Gibbs 2000; Hay *et al.* 2000; Levin *et al*. 2009) which is contributing to a reduction in aquatic species diversity worldwide (Amezaga *et al*. 2002). In Australia, the genus *Nymphaea* occupies a range of wetland habitats in Australia's monsoonal north (Jacobs and Hellquist 2006, 2011). These wetland ecosystems remain relatively pristine and intact (Woinarski *et al.* 2007), however increasing development, introduced weeds and the potential impact of climate change, through altered hydrologic regimes and saltwater intrusion may prove to be detrimental (Knighton *et al*. 1991; Bowman 2010; WBM 2010). Within Australia there has been a reasonable effort to conserve such areas in reserves [e.g. Kakadu National Park covers almost 20,000 km^2 (Department of Sustainability 2011)], including a vast expanse of freshwater wetlands and floodplains, however threatening processes are complex and implementation of management strategies alone will not mitigate all threats (Hay *et al*. 2000). Therefore, investment in

other conservation measures, such as *ex situ* seed banking, forms an integral component of biodiversity management for the continued preservation of important species. *Nymphaea* play an important role in the functional ecology of the northern wetlands by providing food and habitat to a range of animal species, including migratory water birds such as Green Pygmy Geese (*Nettapus pulchellus*) (Press *et al.* 1995a). Additionally they are culturally important to Indigenous Australians occupying the region as the plants are traditionally used as a food source (Issacs 1987; Brockwell *et al.* 1995; Deegan *et al.* 2010; Karadada *et al.* 2011). The conservation of Australian *Nymphaea* is important given their botanical, ecological and cultural importance, combined with increasing threats to their habitat.

Seed banking is considered a simple and cost effective method of storing plant germplasm (Hay *et al.* 2000). However, the success of *ex situ* seed storage is entirely dependent on comprehensive scientific research; historically heavily focused on agricultural species, but increasingly on terrestrial native species e.g. (Merritt 2006; Crawford *et al.* 2007). Species are broadly classified into one of three storage categories: orthodox, intermediate or recalcitrant, based on their amenability to desiccation and subsequent storage (Baskin and Baskin 2001). Orthodox seeds are those that are desiccation tolerant and can survive drying to 3-7 % (*c.* 20 % RH) internal moisture content and storage at -20 °C and is displayed by vast majority of the angiosperms (> 90 %) (Hong *et al.* 1998; Tweddle *et al.* 2003; Baskin and Baskin 2014). Conversely, recalcitrant seeds are desiccation sensitive and do not survive drying below *c.* 40- 50 % (< 85 % RH) moisture content (Hong and Ellis 1996; Hong *et al.* 1998), and are most commonly found in woody species from tropical rainforests (Roberts and King 1980; Pammenter and Berjak 2000; Daws *et al.* 2006; Walters *et al.* 2008). Seeds that do not satisfy the requirements for the aforementioned categories are termed intermediate and generally tolerate the removal of water to a moisture content equivalent to 20- 50 % RH, but generally do not tolerate storage below 0 °C (Ellis, Hong & Roberts, 1990, Roberts, 1973, Liu *et al.*, 2008, Pritchard, 2004). However, there is some variability within this category; for example, seeds of some orchids can survive desiccation and storage below 0 °C, however lose viability more rapidly when compared with orthodox species (Pritchard and Seaton 1993; Pritchard *et al.* 1999). Furthermore, in a number of orchid species, the optimal temperature for seed storage may vary depending on their natural environment, i.e. tropical species may be more

amenable to storage at warmer temperatures (15 °C), while temperate species may store optimally at cooler temperatures (0- 5 °C) (Pritchard and Seaton 1993; Pritchard *et al.* 1999).

In broad terms, the longevity of seeds in *ex-situ* storage is dependent on seed water content and storage temperature (Probert *et al.* 2009) and decreasing these parameters in orthodox seeds can extend their longevity (Roberts and Ellis 1989; Hong *et al.* 1998; Walters 2004). Ultimately, reducing internal seed moisture content to 3-7 % has proven most effective at increasing longevity in the majority of species, and drying beyond this point (ultradrying) may also be detrimental (Roberts and Ellis 1989; Vertucci and Roos 1993a). However, the thermodynamic interaction between water content and temperature must also be taken into consideration (Ellis *et al.* 1989; Vertucci and Roos 1993b; Pritchard and Dickie 2003). For example, viability in some seeds may be maintained for longer at lower temperatures when seed water content is higher than the recommended 5 % (Vertucci and Roos 1993a).

Increasing the storage longevity of intermediate seeds is further constrained by desiccation sensitivity at the lower limit of internal seed water content, and the formation of lethal intracellular ice formation at the upper limit when stored at sub-zero temperatures (Vertucci 1989; Walters-Vertucci *et al.* 1996; Dussert *et al.* 2001). As such, storage in ultra-low temperature cryogenic storage (-130 to -196 °C), provides a viable alternative to the standard seed banking temperature of -20 °C, and has proven relatively successful for seeds of a number of intermediate species including *Coffea* (Ellis *et al.* 1990; Dussert *et al.* 2001). However, storage at sub-zero temperatures can be further confounded by the internal damaged caused by lipid phase changes and triacylglycerol crystallisation in some species, particularly those with high oil content such as *Cuphea* (Vertucci 1989; Dussert *et al.* 2001; Crane *et al.* 2003; Crane *et al.* 2006). Such damage may be ameliorated by heating seeds to 45 °C after removal from storage, thus melting the solidified lipids and avoiding subsequent imbibitional injury.

While much research has focussed on finding optimal seed storage conditions to maximise longevity, seed viability may still deteriorate over time due to a range of other factors including seed maturity, the collection environment and other intrinsic genetic

factors (Walters 1998; Probert *et al.* 2009; Merritt *et al.* 2014b). While determining seed longevity in real-time is difficult, rapid seed ageing protocols have been established in order to approximate long-term longevity in many species (Probert *et al.* 2009). Of 195 species tested, Probert *et al.* (2009) found variation in seed longevity of up to four orders of magnitude under the same storage conditions. Environmental correlates between the environment and seed longevity have also been demonstrated, with seeds originating in hot, dry climates are longer lived than seeds originating from moist, temperate regions (Walters *et al.* 2005; Probert *et al.* 2009).

In contrast with terrestrial species, the storage and longevity of seed from aquatic plants is largely unexplored (Hay *et al.* 2000), and often contradictory. In a study of 87 aquatic species, predominantly from temperate environments, approximately 75 % were classified as orthodox (Hay *et al.* 2000). However, more recent hypotheses suggest that seeds with a high internal moisture content (*c.* 40%) at maturity or those shed in wet environments, including many basal angiosperms, would prove to be less resilient to desiccation and subsequent storage than terrestrial species (Offord and Makinson 2009; Probert *et al.* 2009). Furthermore, hypotheses regarding the longevity of seeds from basal angiosperms have been mixed. Some authors have suggested that basal angiosperms may be short-lived as they had evolved in moist habitats (Probert *et al.* 2009). However more recent evidence from Western Australia has shown that seeds of *Trithuria* (Hydatellaceae) were not short lived compared to other temporary wetland species, however, were short-lived compared with a number of Australian terrestrial species.

Given the importance of *Nymphaea* as basal angiosperms, conservation significance in northern Australia, the apparent lack of information available about seed storage behaviour and seed longevity in aquatic angiosperms the aims of this study were to:

1. Investigate desiccation tolerance of a number of Australia *Nymphaea* species
2. Classify seed storage behaviour as orthodox, recalcitrant or intermediate
3. Examine the effects of storage temperature and seed water content on the viability of seeds over a 12 month storage period
4. Model the comparative longevity of seeds under controlled ageing conditions.

MATERIALS AND METHODS

Species selection, seed collection and seed quality

Seeds of *Nymphaea lukei* (NL2) S.W.L.Jacobs & Hellq, *N. violacea* (NV8, NV9 and NV10) Lehm, *N. macrosperma* (NV3) Merr. & L.M.Perry and *N. immutabilis* S.W.L.Jacobs (NI1) were collected in June, July and August 2013 from northern Western Australia and Queensland (Fig 5.1). Whole fruits of *Nymphaea* were collected in the field and transported in Glad® Snap Lock® bags, with approximately 30 mL of water placed at the bottom of each bag, to Kings Park and Botanic Garden, Perth, Western Australia, and were left to naturally dehisce at room temperature (*c.* 24 °C). Seeds were rinsed through brass sieves (Endecotts 710µm – 2.36mm) under running water to remove any remaining plant material. All seed accessions were separated from chaff using an aspirator (SELECTA BV Gravity Seed Seperator "Zig Zag", Netherlands) or by hand. Initial seed fill was determined by x-ray analysis using a Faxitron Specimen Radiography System MX-20 Cabinet. Prior to experimental work, subsamples of seed were further cleaned to remove empty or decayed seeds, until seed fill was determined to be > 98%. Seeds were used within a month of collection for all experiments, except differential scanning calorimetry.

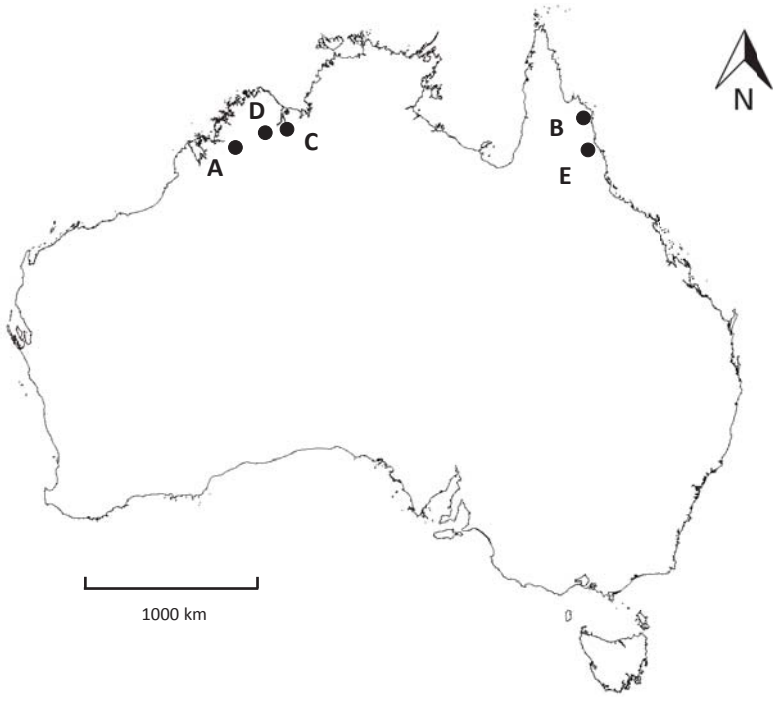

Figure 5.1: Collection locations of *Nymphaea* species used in this study: (A) *N. lukei* NL2 (B) *N. immutabilis* NI1 (C) *N. macrosperma* NM3 (D) *Nymphaea violacea* NV8 and NV9 and (E) *N. violacea* NV10.

Fresh seed moisture content and desiccation tolerance

Seeds collected freshly from the plant were cleaned from the fruit and sealed inside a glass vial and tested with the portable hygrometer (Rotronic HygroPalm - HP AW1) in the field, within a few minutes of collection. Upon return to the laboratory, following seed cleaning and quality assessments, seeds were dried in a controlled environment facility (15 % RH**,** 15 °C) at Kings Park and Botanic Garden, Perth, Western Australia. After 28 days under these conditions, seeds were removed and seed moisture content after drying was determined gravimetrically, whereby three replicates of 0.05 g or 25 seeds (whichever was the greater) were weighed before and after drying in an oven at 103 °C for 17 hours (ISTA 1999). Moisture content was then calculated as a percentage of water on a fresh weight basis.

Seed germination

Seed germination tests are used in a number of the experiments presented below, and all germination tests were conducted in the same manner, as follows. Seeds were surface sterilized in 2 % (w/v) calcium hypochlorite ($Ca(OCl)_2$, solution with two drops of Tween 80 LR surfactant (Hurst Scientific) for 30 minutes then rinsed three times in sterile water. Unless otherwise stated, 100 seeds (replicated four times) were placed in 10 ml plastic tubes (TechnoPlas, Australia) with 10 ml of sterile water and incubated at 35 °C under a daily photoperiod of 30 μmol m^{-2} s^{-1}, 400-700 nm cool white fluorescent light (Chapter 3 of this thesis). Seeds were regularly scored for germination, which was defined as radicle emergence ≥ 1 mm.

In order to assess total viability, after an eight-week germination period, all ungerminated seeds were subjected to a cut-test; whereby seeds were cut in half and visually assessed (i.e firm, white turgid embryos were considered viable) and stained with tetrazolium chloride (ISTA 1985). For the tetrazolium chloride staining (TZ), seeds previously cut in half (from the cut tests) were placed cut-side down on glass filter paper irrigated with 1 % tetrazolium chloride buffered to pH 7 with a phosphate buffer (KH_2PO_4 and Na_2HPO_4) for 24- 36 hours until viable embryos stained red.

Effects of storage temperature and moisture content on seed germination and viability

Firstly, in order to assess seeds' ability to germinate, germination testing was undertaken on freshly collected seeds prior to drying, and after seeds were dried for 28 days at 15 % RH at 15 °C. Secondly, an experiment was conducted to assess the impact of variable storage temperature and moisture content on seeds of *Nymphaea.* To adjust seed moisture content prior to storage, seeds of *N. immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM3) and *N. violacea* (NV8, NV9 and NV10) were sealed inside porous nylon mesh (0.2 µm) bags and stored at 15, 30, 50, 70 and 95 % RH by placing inside polycarbonate electrical enclosure boxes (NHP, Fibox, Australia) containing saturated and non-saturated solutions of lithium chloride (LiCl) at 20 °C for two weeks (Hay *et al.* 2008). To ensure seeds had equilibrated to experimental RHs, seeds of each species were tested with a hygrometer (Rotronic HygroPalm - HP AW1) and discarded. Seeds stored at -20 °C, 5 °C and 25 °C were first hermetically sealed inside two laminated aluminium foil bags, while seeds stored in vapour-phase liquid nitrogen (LN) (-160 °C) were first sealed inside 1 ml Nunc CryoTube™ vials (Thermo Fisher Scientific, USA) and then plunged directly into the LN vapour. Four replicates of 50 seeds of *N. lukei* and *N. violacea* and one replicate of *N. macrosperma* and *N. immutabilis* were extracted from all storage temperatures at 1, 6 and 12-month intervals and tested for germination as described above. Fresh seeds acted as a control.

Moisture sorption isotherms

Seeds of all accessions were stored in polycarbonate electrical enclosure boxes at 15, 30, 50, 70, 85, 90, 95 and 100 % RH (as described above) at 20 °C for six weeks. To ensure seeds had equilibrated to the correct RH after six weeks, a small subsample of seeds of each species were tested with a hygrometer (Rotronic HygroPalm - HP AW1) and discarded. In order to assess moisture sorption in each species, three replicates ≥ 0.05g for small seeds or 30 individual seeds for large seeds (whichever was the larger amount) were allowed to imbibe on irrigated glass filter paper for 48 hours prior to drying. Seeds were then weighed and dry weights calculated after drying at 103 °C for 17 hours (ISTA, 1999).

Seed lipid content

Seed lipid content was determined following the methods of Senaratna *et al.* (1985). Three replicates of 1 g seed (at air dry water contents) were homogenized in methanol: chloroform (1:2 by volume) using a mortar and pestle. The homogenate was transferred to clean 10 mL tubes and centrifuged at 12,000 rpm for 15 minutes. The supernatant was washed with 0.7 % NaCl to remove non-lipid material and centrifuged again at 10, 000 rpm for 10 minutes. The organic layer was separated and dried under a constant flow of N_2 for one hour and residual lipids determined gravimetrically (g lipid g^{-1} FW).

Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) (Perkin-Elmer DSC 8000, calibrated with indium, equipped with a Perkin-Elmer CLN_2 controlled liquid nitrogen cooling system) was used to determine the unfrozen water content (UWC) of all species. Prior to DSC analysis, seeds were equilibrated at 20 °C for 14 days at 15, 30, 50, 70, 85, 90, 95 and 100 % RH (described above). A sample of each species was also allowed to imbibe on irrigated glass filter paper for 48 hours prior to DSC analysis. Empty, hermetically sealed (Perkin-Elmer crucible sealing press) aluminium crucibles (Perkin-Elmer) were used to obtain the baseline curvature. Three replicates of each species at each RH were individually sealed within an aluminium crucible and cooled to -50 °C at 10 °C/min and rewarmed to 30 °C at 50 °C/min. Following DSC analysis, crucibles were punctured and dry weights calculated after drying at 103 ^{o}C for 17 hours (ISTA 1999). Lipids extracted from *Nymphaea* seeds (see previous) were also analysed using DSC. Extracted lipids were sealed inside individual aluminium crucibles and warmed to 50°C, cooled to -40 °C at 10 °C/min and rewarmed to 50 °C at the same rate in reverse. Thermal transitions were measured as endothermic (melting) peaks in the baseline and enthalpy of the transitions measured in J g^{-1} d.wt. from the area above the baseline using Pyris software (Perkin-Elmer, version 10.1.0.0412).

Comparative longevity

Seeds were placed inside sealed nylon mesh bags and enclosed inside an air-tight polycarbonate electrical enclosure box (NHP, Fibox, Australia) and hydrated above a

non-saturated solution of LiCl (370 g L^{-1}) creating an RH of 47 % (Hay *et al.* 2008) at 20 °C for 14 days. To ensure seeds had equilibrated to 47 % RH, seeds of each species were tested with a hygrometer (Rotronic HygroPalm - HP AW1) and discarded. Remaining seeds were then transferred to a second polycarbonate electrical enclosure box above a non-saturated solution of LiCl (300g L^{-1}) creating an RH of 60 % at 45 °C. Four replicates of 50 seeds were removed from the experimental enclosure box at the following intervals: 0, 1, 2, 5, 9, 20, 30, 50, 75, 100 and 125 days and tested for germination, as previously described. A T-tec data logger (Temperature Technology, Adelaide) was used to determine RH and temperature inside the electrical enclosure box to ensure a constant RH and temperature was maintained during the experiment.

Statistical analyses

Germination data for seeds stored at -150 °C, -20 °C, 5 °C and 25 °C were analysed using logistic regression using the inbuilt 'logit' link function, with binomial error distribution in R (R Core Team 2012). Germination response was analysed across the full model inclusive of all factors (storage temperature, RH and storage time), which was then stepwise reduced to simplify the main model. Pairwise comparisons of means were made using a Holm's test.

Comparative longevity data were analysed in Genstat (version 12, VSN International Ltd. UK). Survival data from the comparative longevity experiments were analysed via probit analysis to estimate P_{50} (the time for viability to fall to 50 %) and to fit the seed viability equation (Ellis and Roberts 1980):

$$v = K_i - p/\sigma$$

where v is the viability in normal equivalent deviates (NED) of the seeds after p days in storage: K_i is the initial viability (NED) and σ is time (in days) for viability to fall by 1 NED. Where germination was found to increase over the storage period (potentially as a result of after-ripening), only data showing a decline in viability were included in the analysis. Seed survival curves were plotted in OriginPro 8 (version 8, 2010, OriginLab Corporation).

Enthalpy data determined using the DSC were plotted against seed sample water content (H_2O g^{-1} d.wt.) at the various RHs. Two linear regression lines were fitted using

Sigma Plot (Systat Software, San Jose, CA), one based on water contents at which no change in enthalpy was detected, the other where there was an increase in enthalpy with increasing moisture content. The unfrozen water content (UWC) was determined to be at the intersection of these two lines.

RESULTS

Fresh seed moisture content and desiccation tolerance

Initial seed water content of freshly collected seeds of *Nymphaea* was high, with eRH as measured by the hygrometer ranging between 90- 99 % RH. After two weeks of drying at 15% RH, internal seed moisture content reached 5.1 % in all species. The drying of *Nymphaea* seeds to internal moisture content of 5 % did not significantly affect the germination of the majority of species and accessions tested (Fig. 5.2) although there was some variation between species. Seeds of *N. violacea* (accessions NV8 and NV9) germinated to 100 % prior to and after drying, and seeds *N. macrosperma* (NM3) and *N. violacea* (NV10) did not show a significant reduction in germination ($P > 0.05$). However, seeds *N. immutabilis* (NI1) and *N. lukei* (NL2) did show a small, but significant reduction in germination following drying ($P < 0.01$, 0.04 and 0.01 respectively), although subsequent tetrazolium staining revealed that all ungerminated seeds were still viable ($P > 0.05$).

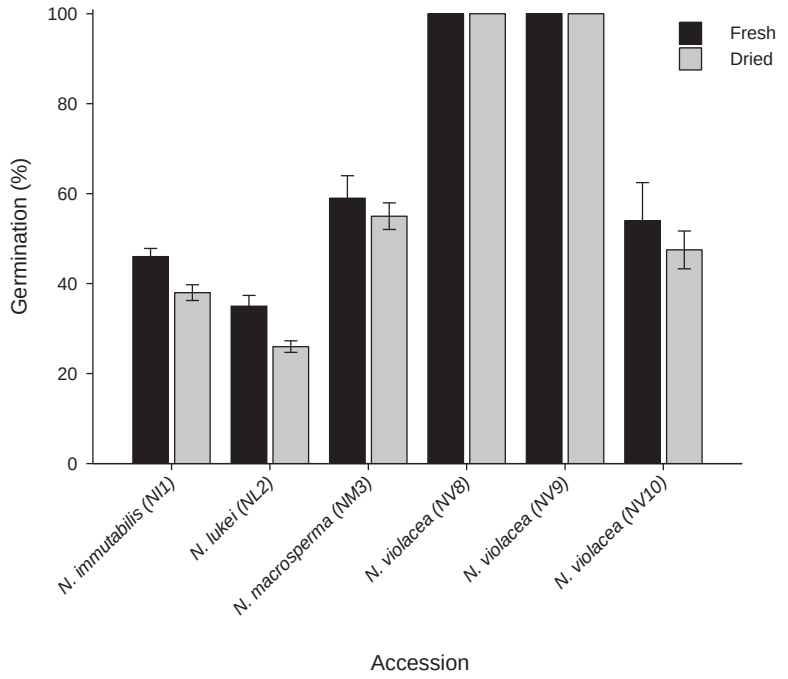

Figure 5.2: Fresh seed and dried (in a CE room at 15 % RH and 15 °C) seed germination of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM3) and three accessions of *N. violacea* (NV8, NV9 and NV10).

Effects of moisture content and storage temperature on seed viability

Under all experimental storage conditions, the ability for seeds of *N. immutabilis* to germinate declined significantly when compared with fresh seed germination (46 %; $P < 0.01$; Figs. 5.2 and 5.3, Table 5.1). The greatest germination in *N. immutabilis* was observed when seeds were stored at 25 °C and equilibrated at 30 % or 50 % RH (37 %; Fig 5.3). When non-germinated seeds were assessed for viability (via cut-tests and TZ), all treatments (time, storage, RH) showed a significant reduction viability when compared with fresh seeds ($P < 0.01$; Fig. 5.4). The highest viability in seeds of *N. immutabilis* was 77 % when stored for one month after equilibration at 30 % RH (Fig. 5.4). Maximum viability (55 %) after 12 months was observed in seeds equilibrated at 15 % RH at -160 °C. No seeds of *N. immutabilis* remained alive after 6 month storage equilibrated at 95 % RH at all temperatures (Fig. 5.4).

The ability of seeds of *N. lukei* to germinate was < 20 % under all experimental storage conditions tested compared with > 60 % in fresh seeds ($P < 0.001$; Table 5.1; Fig. 5.3) and germination decreased with increasing time in storage and RH ($P < 0.01$). Viability decreased over all temperatures and RHs with increasing time in storage ($P < 0.001$; Fig. 5.5). This decline was particularly pronounced when seeds were stored at -160 °C, and at 25 °C. Less than 20 % of seeds remained viable after 12 months storage at -160 °C, 5 °C, or 25 °C (Fig. 5.5). Maximum viability of 55 % after 12 months was recorded when seeds were stored at 15 % RH at -20 °C (Fig. 5.5). *Nymphaea macrosperma* also showed a significant decrease in germination under all temperatures and RHs when compared with fresh seed germination (59 %; $P < 0.01$; Fig. 5.2, Table 5.1, Fig. 5.3). Germination decreased with increasing RH and time in storage ($P = 0.01$; Fig. 5.3). Germination of seeds stored for one month at warmer temperatures (5 °C and 25 °C) was significantly greater than seeds stored below 0 °C ($P < 0.01$; Fig. 5.3, Table 5.1), however was still less than germination of freshly collected seeds. Seed viability (assessed via cut-tests and TZ) after one month in storage was greater (> 60 %) in all RHs when stored at -160 °C or -20 °C when compared with warmer temperatures ($P < 0.01$; Fig. 5.6). Viability decreased with increasing RH and time in storage ($P < 0.01$; Fig. 5.6). Maximum viability (46 %) after 12 months occurred when seed were equilibrated at 15 % RH and stored at -20 °C (Fig. 5.6).

The three accessions of *N. violacea* showed different responses to storage conditions (Fig. 5.3). Germination was maintained at 100 % in two accessions (NV8 and NV9) equilibrated at RHs between 15 % and 75 % at all temperatures, but fell significantly when equilibrated at 95 % RH and stored at -160 °C (Fig. 5.3). In the third accession (NV10), germination decreased when stored at temperatures below 0 °C in the first month and also when equilibrated at low RHs (15 %- 30 %) and high RHs (70 %- 95 %; Fig. 5.3). However, when stored for 6 months or longer, viability (as tested by cut-tests and TZ) was highest in those seeds equilibrated at low RH below 0 °C (Figs. 5.8 and 5.9). Overall for all three accessions, the contribution of temperature (25 °C, 5 °C, -20 °C and -160 °C) was found to be a significant ($P \leq 0.01$) factor in the logistic regression (Table 5.1), as germination decreased significantly when seeds were stored at warmer temperatures for six months or longer. Additionally, the interaction between RH and temperature ($P \leq 0.05$) and RH and time ($P < 0.01$) were significant, with viability decreasing with increased RH and temperature and length of time in storage (Table 5.1; Figs 5.7, 5.8 and 5.9).

Table 5.1: Significance of regression terms from logistic regression (binomial) analysis seeds of *Nymphaea* stored for up to 12 months (Ti =time) following equilibration at 20 °C and RH of 15, 30, 50, 70 or 90 %) and two storage temperatures (Te = -20 °C and -160 °C). Factors were fitted in the order shown, however changing the order of the factors did not affect the results of the model. Significance codes: P= *** < 0.001, ** < 0.01, * < 0.05.

Species and Accession	RH	Te	Ti	RH x Te	RH x Ti	Te x Ti	RH x Te x Ti
N. immutabilis (NI1)	NS	NS	***	*	***	NS	NS
N. lukei (NL2)	NS	NS	NS	NS	NS	NS	NS
N. macrosperma (NM3)	NS	***	NS	NS	***	**	NS
N. violacea (NV8)	NS	**	***	***	***	*	***
N. violacea (NV9)	NS	***	***	***	***	*	***
N. violacea (NV10)	**	***	NS	*	***	NS	NS

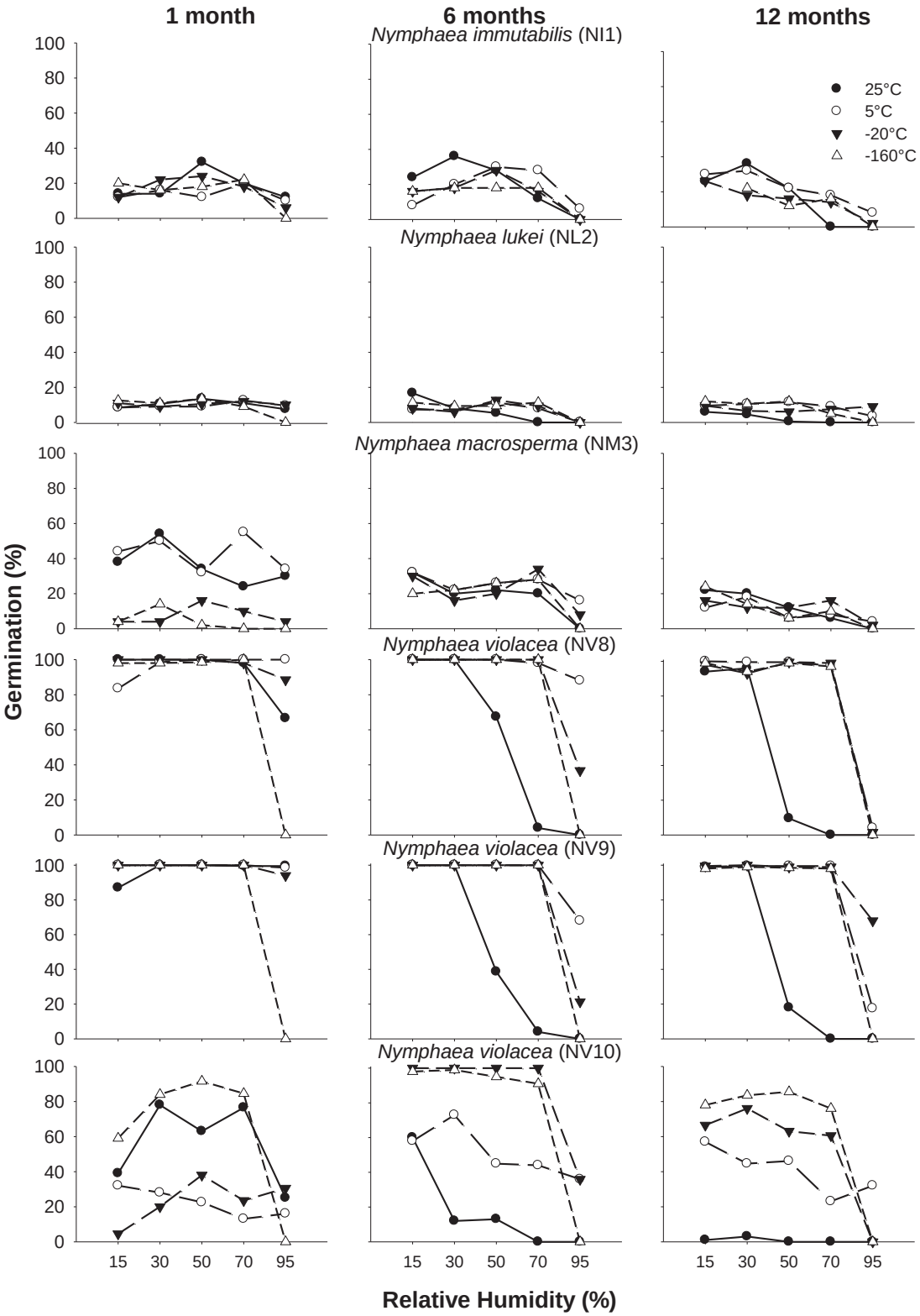

Figure 5.3: Germination of *Nymphaea immutabilis, N. lukei*, *N. macrosperma* and *N. violacea* after 1, 6 and 12 months storage at 25 °C (black circles), 5 °C (white circles), -18 °C (black triangles) or -150 °C (white triangles) after being equilibrated at 20 °C and indicated RH.

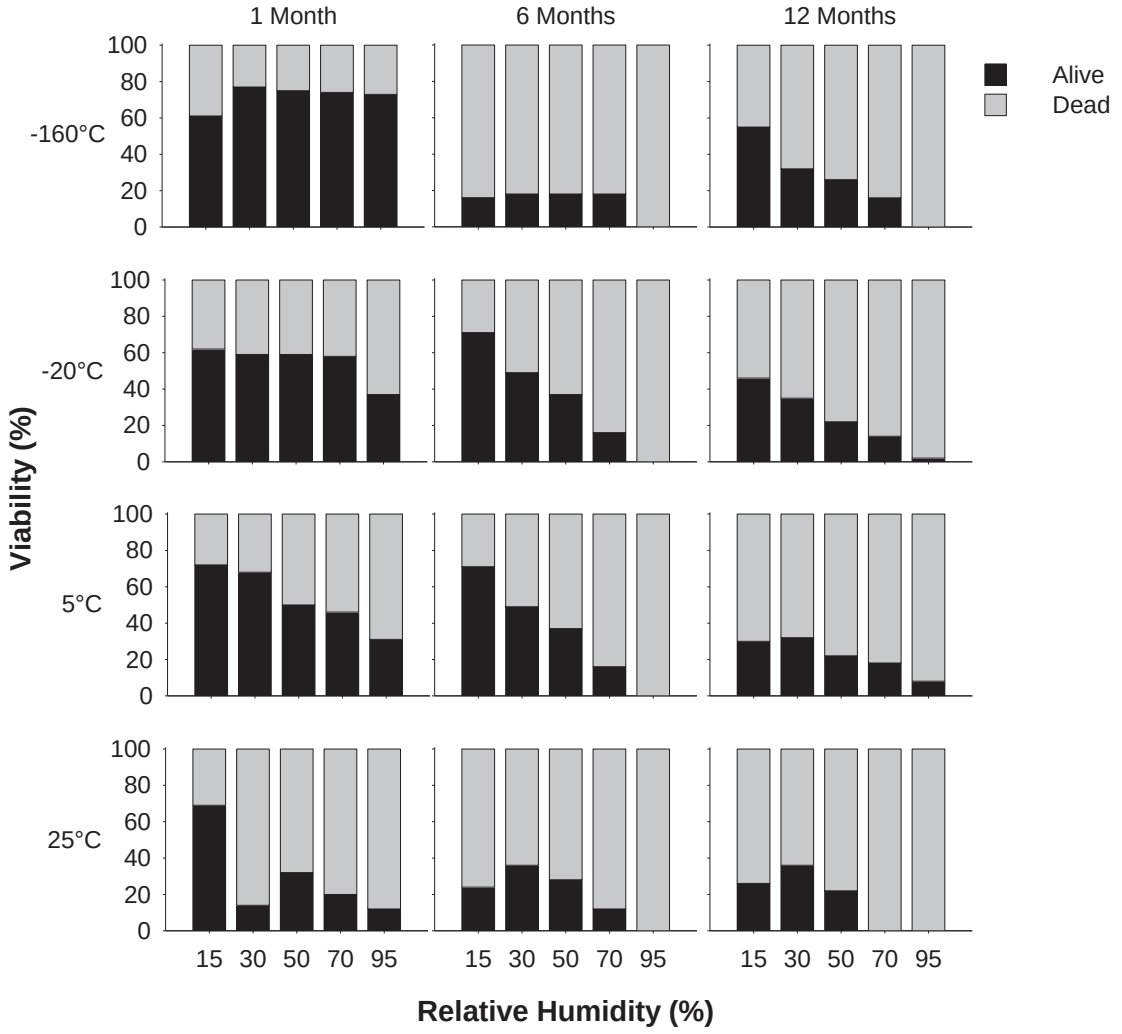

Figure 5.4: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea immutabilis* (NI1) seeds stored under experimental storage conditions. Seeds that had germinated, embryos were turgid, white and fully intact upon cut-testing or positively stained were considered to be alive.

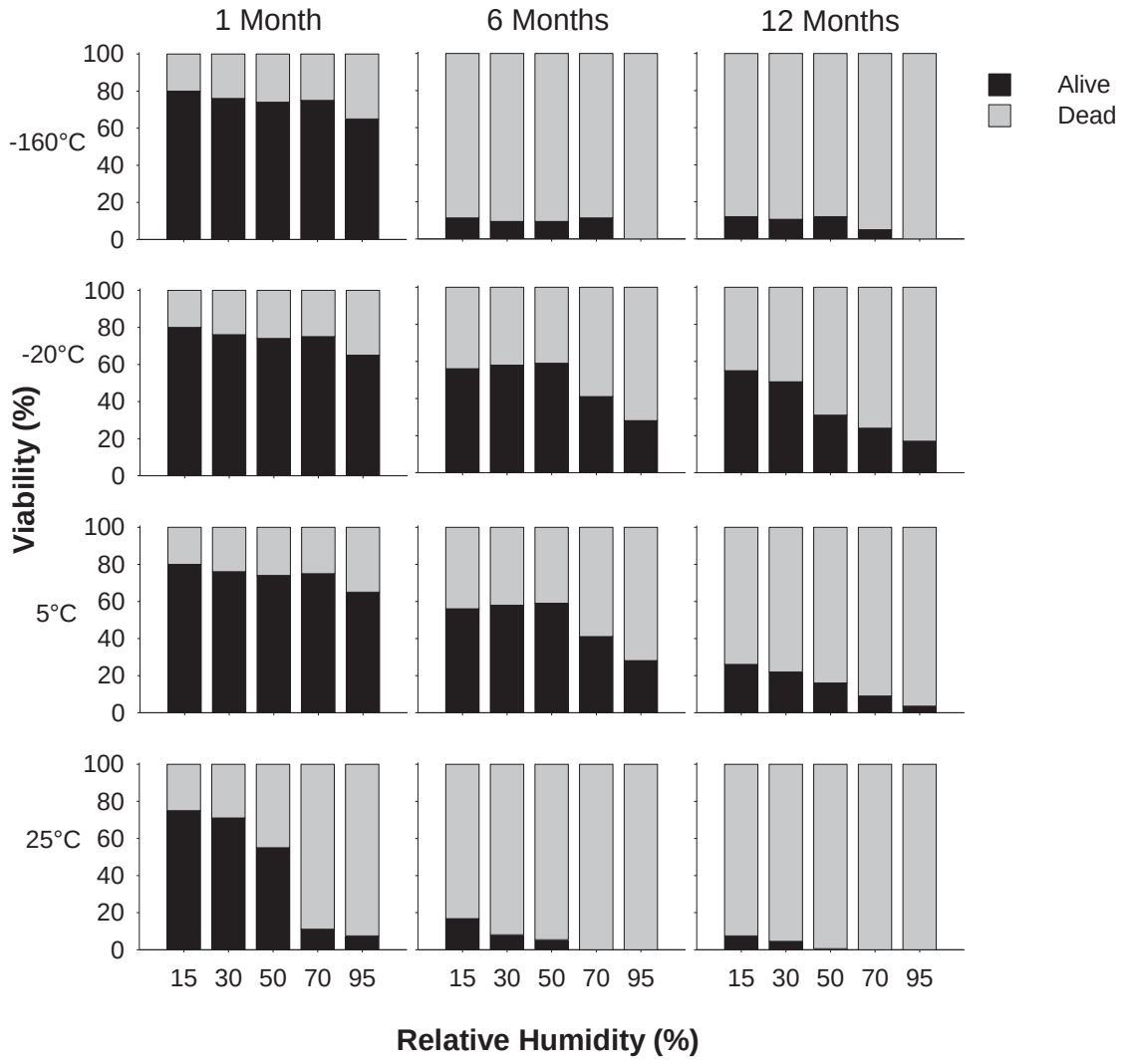

Figure 5.5: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea lukei* (NL2) seeds stored under experimental storage conditions. Seeds that had germinated, embryos were turgid, white and fully intact upon cut-testing or positively stained were considered to be alive.

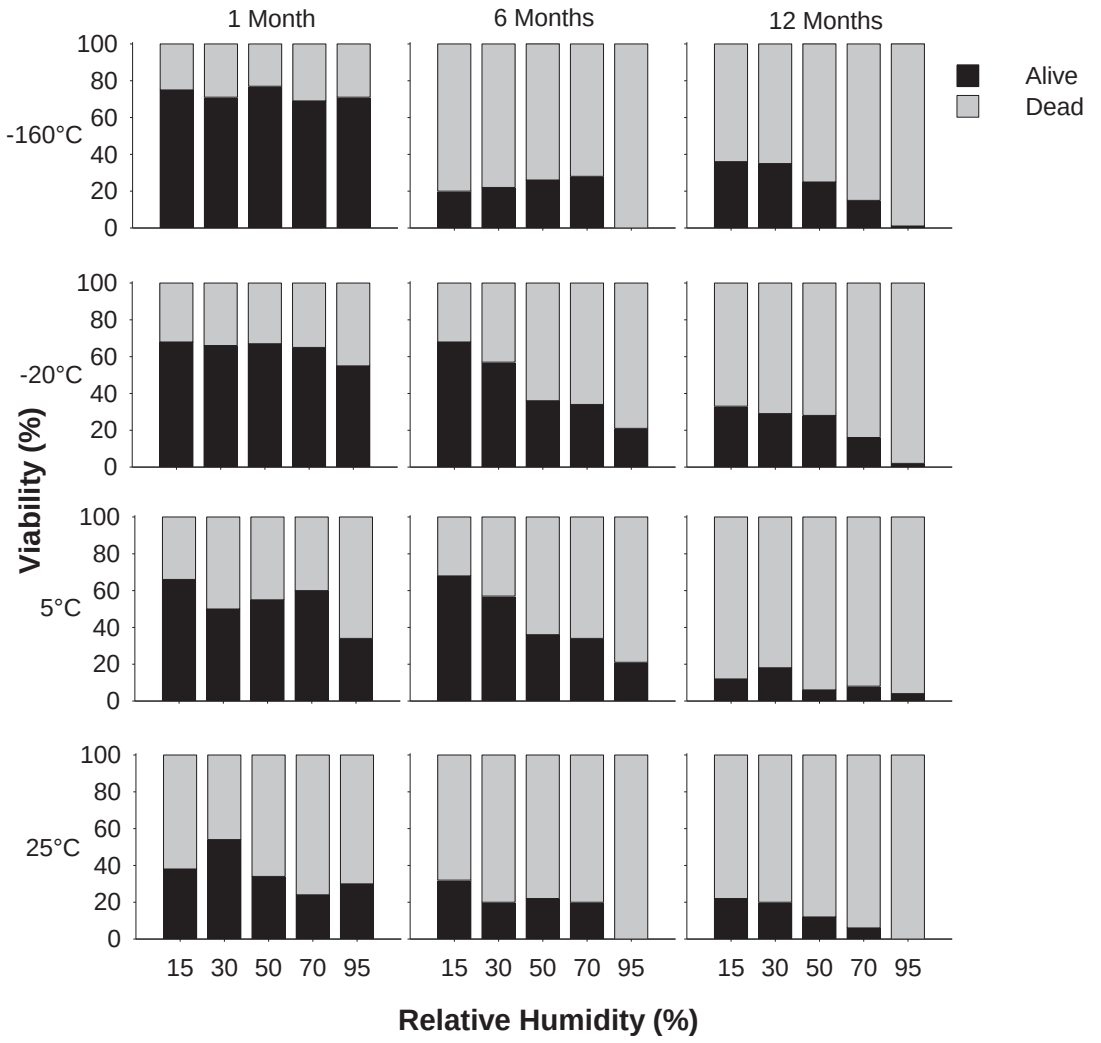

Figure 5.6: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea macrosperma* (NM3) seeds stored under experimental storage conditions. Seeds that had germinated, embryos were turgid, white and fully intact upon cut-testing or positively stained were considered to be alive.

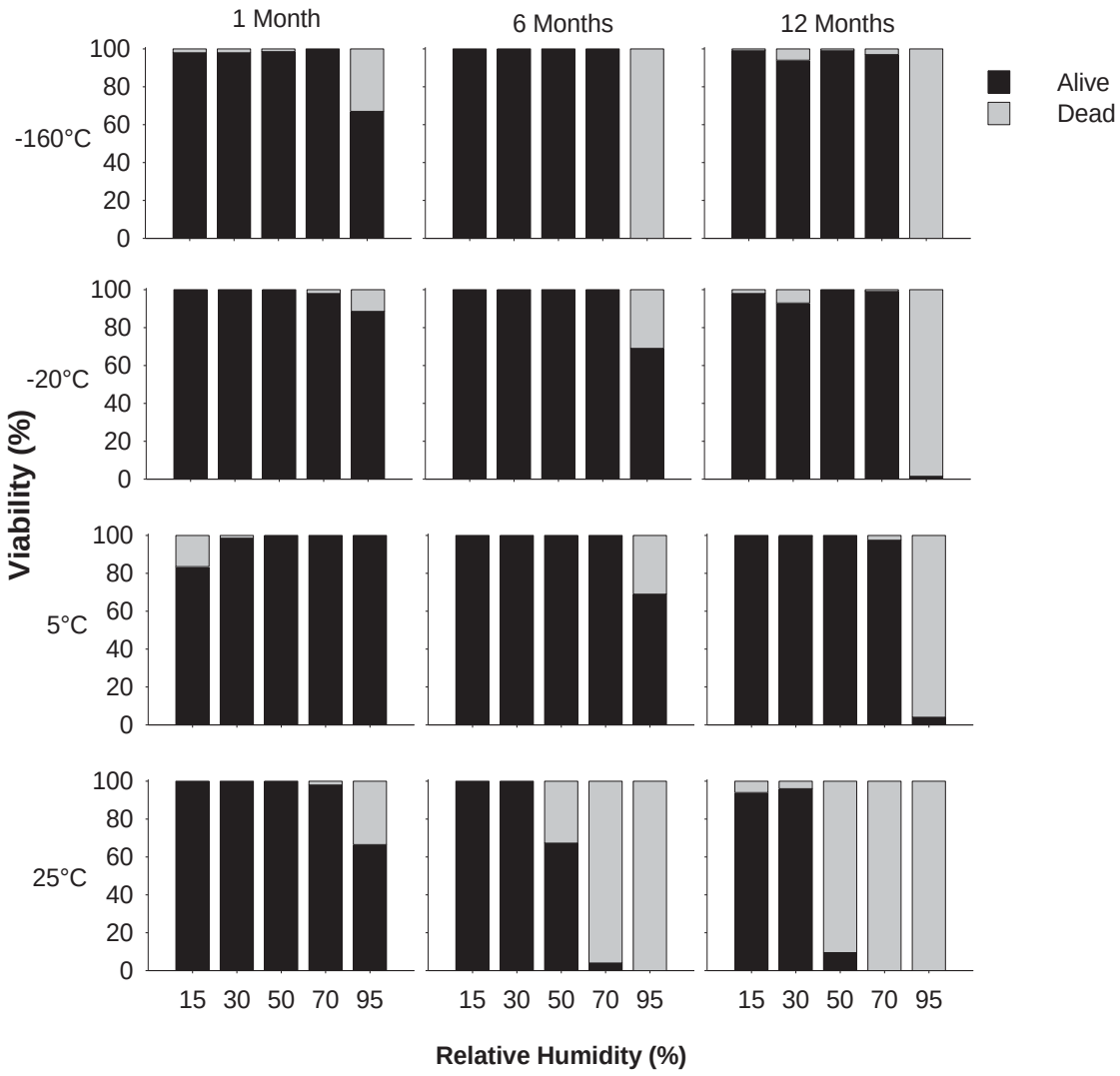

Figure 5.7: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea violacea* (NV8) seeds stored under experimental storage conditions (storage experiment two). Seeds that had germinated, embryos were turgid, white and fully intact upon cut-testing or positively stained were considered to be alive.

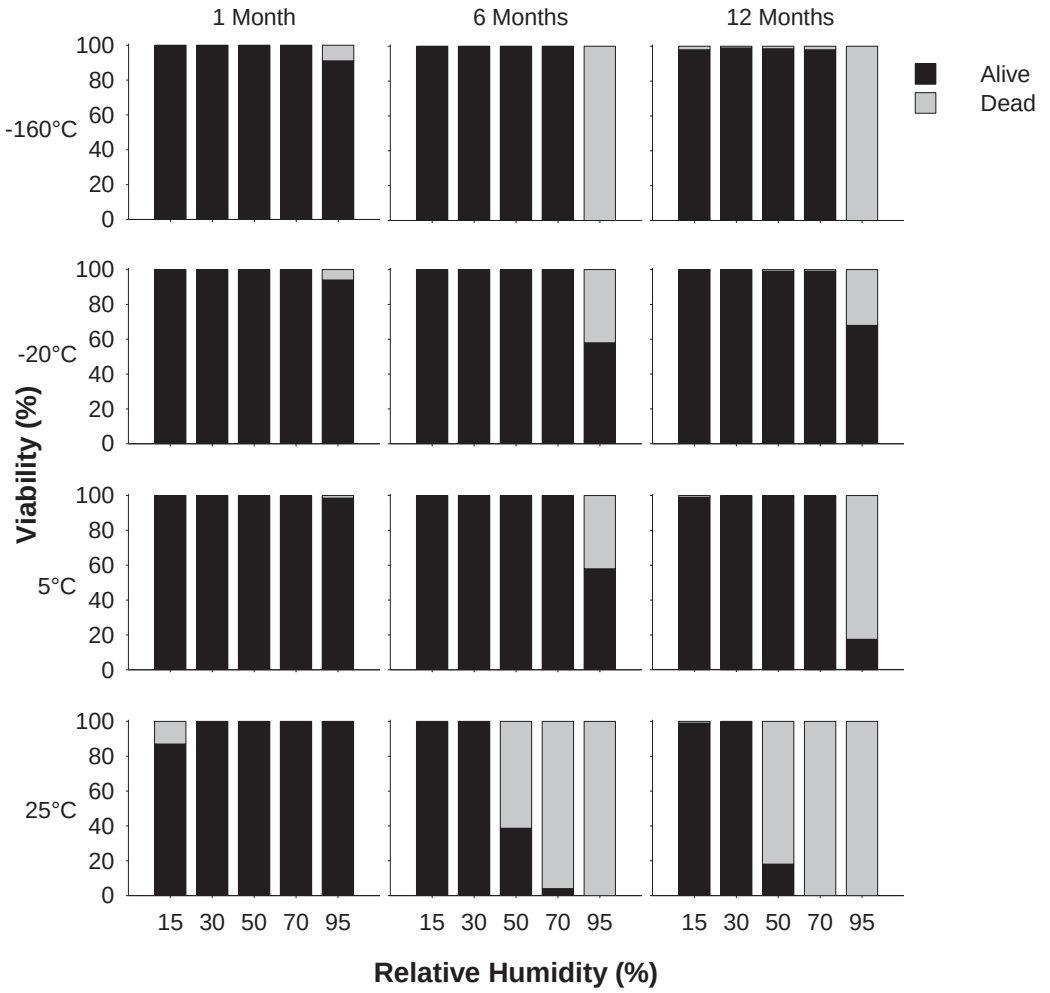

Figure 5.8: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea violacea* (NV9) seeds stored under experimental storage conditions. Seeds that had germinated, embryos were turgid, white and fully intact upon cut-testing or positively stained were considered to be alive.

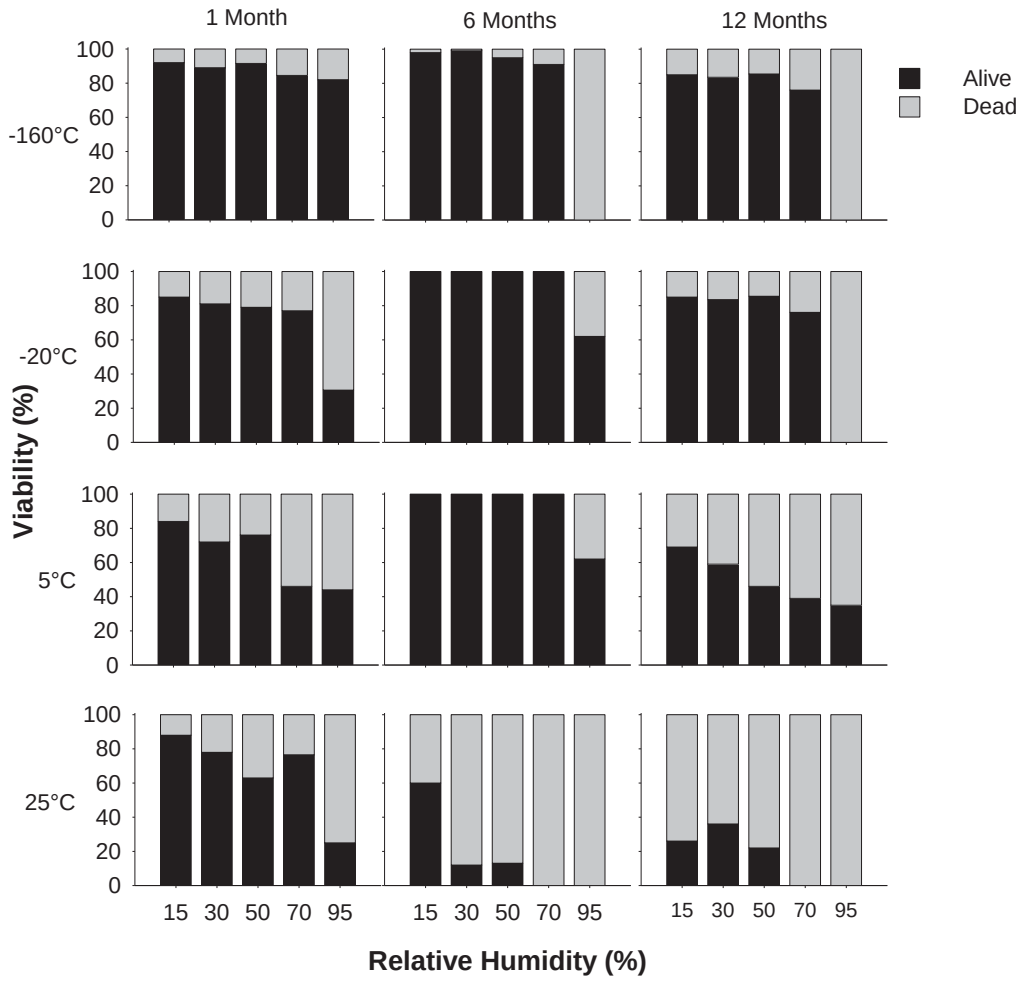

Figure 5.9: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea violacea* (NV10) seeds stored under experimental storage conditions. Seeds that had germinated, embryos were turgid, white and fully intact upon cut-testing or positively stained were considered to be alive.

Moisture sorption isotherms

Moisture sorption isotherms of seeds of *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea* were found to be relatively sigmoidal in shape (Fig. 5.10). Seeds of *N. immutabilis* equilibrated at 100 % RH were found to have the highest moisture content of all species test (41.0 %). Moisture content of *N. lukei* was 37.5 %; *N. macrosperma* was 33.0 %, while *N. violacea* ranged between 27.0 – 34.9 % (not all data not shown).

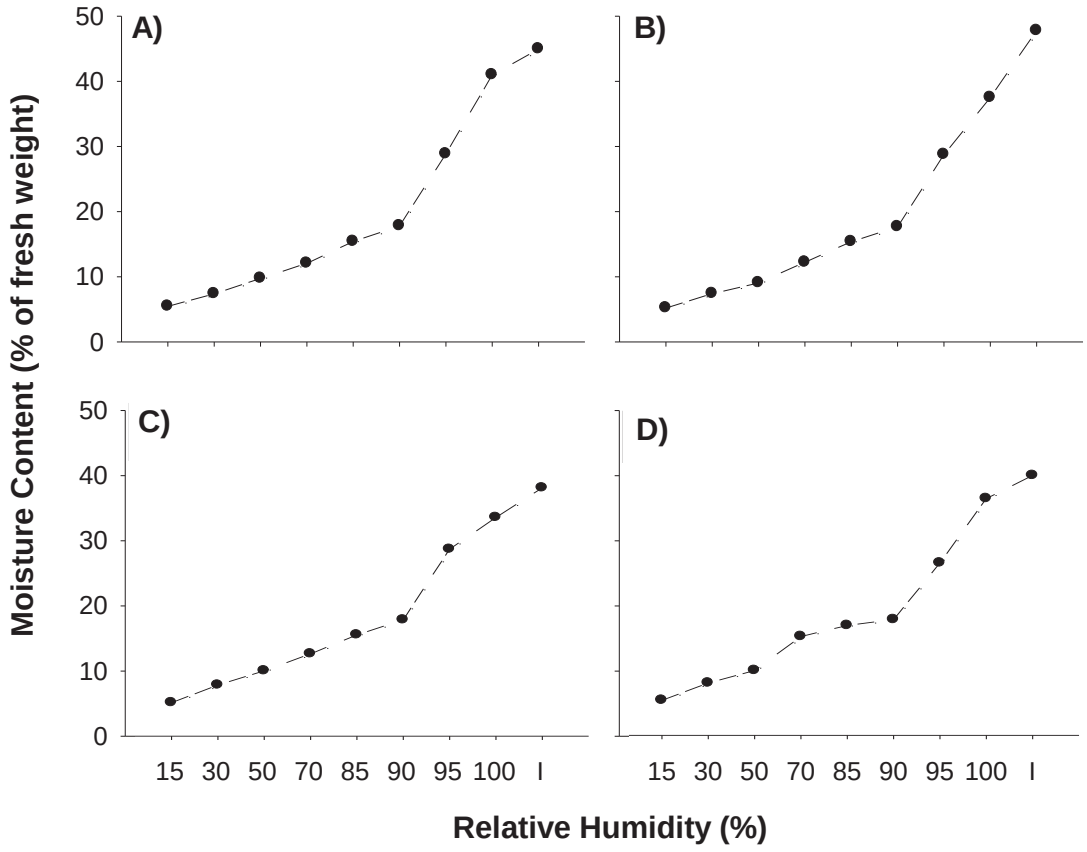

Figure 5.10: Moisture sorption isotherms determined at 20 °C for: A) *Nymphaea immutabilis* (NI1), B) *N. lukei* (NL2), C) *N. macrosperma* (NM3) and D) *N. violacea* (NV8).

Seed lipid content

Seed lipid content in all species and accessions was low, and ranged between 1.1 – 3.7 % on a dry weight basis. Lipid content by accession was: *N. immutabilis* (NI1) 2.8 %, *N. lukei* (NL3) 3.5 %, *N. macrosperma* (NM3) 3.1 %, and *N. violacea* NV8 1.1 %, NV9 1.8 % and NV10 1.9 %.

Differential scanning calorimetry (DSC)

Differential scanning calorimetry of seeds of *N. immutabilis*, *N. lukei, N. macrosperma* and *N. violacea* and after equilibration at ≥ 90 % RH (and imbibed seeds) showed thermal transitions associated with freezing and melting events (Fig. 5.11). The onset temperature of freezing peaks ranged between -18.5 °C and -24 °C while melting onset temperature ranged between -9.5 °C and -1.6 °C (data not shown). For seeds of all species at ≤ 85 % RH, no peaks were observed. No phase changes associated with lipids or other cellular components were evident in whole seeds between -50 °C and 30 °C.

The unfrozen water content (UWC) differed between species and accessions (Fig. 5.12). *Nymphaea macrosperma* NM3 had the highest UWC at 34.9 % MC. The UWC of *N. lukei* was 16.2 % and *N. immutabilis* 23.4 %. The three accessions of *N. violacea* showed UWCs of 26.2 % (NV8), 25.5 % (NV9) and 28.0 % MC (NV10) (not all data shown).

Lipids extracted from all species and accessions showed significantly different thermal profiles, both on cooling and rewarming curves (Figs. 5.13 and 5.14). The thermal profile from *N. immutabilis* showed one crystallisation event occurring at 11.7 °C; *N. lukei* NL2 also displayed one event at -4.5 °C; *N. macrosperma* NM3 showed two events at 29.2 °C and 11.8 °C; finally, *N. violacea* NV8 had three crystallisation events at 14.5 °C, -8.1 °C and -27.7 °C, *N. violacea* NV9 had two events at 25.8 °C and 2.3 °C and *N. violacea* NV10 had one event at 1.3 °C (Table 5.2). A phase change commonly occurred between 16.2 °C and 21.5 °C in lipid cooling profiles of *N. immutabilis, N. lukei* (NL2) and *N. violacea* (NV8) (Fig. 5.13). A melting peak was also detected in lipids of *N. macrosperma* (NM3) and *N. violacea* (NV9 and NV10) occurring between 23.2 °C and 29.2 °C (Fig. 5.14).

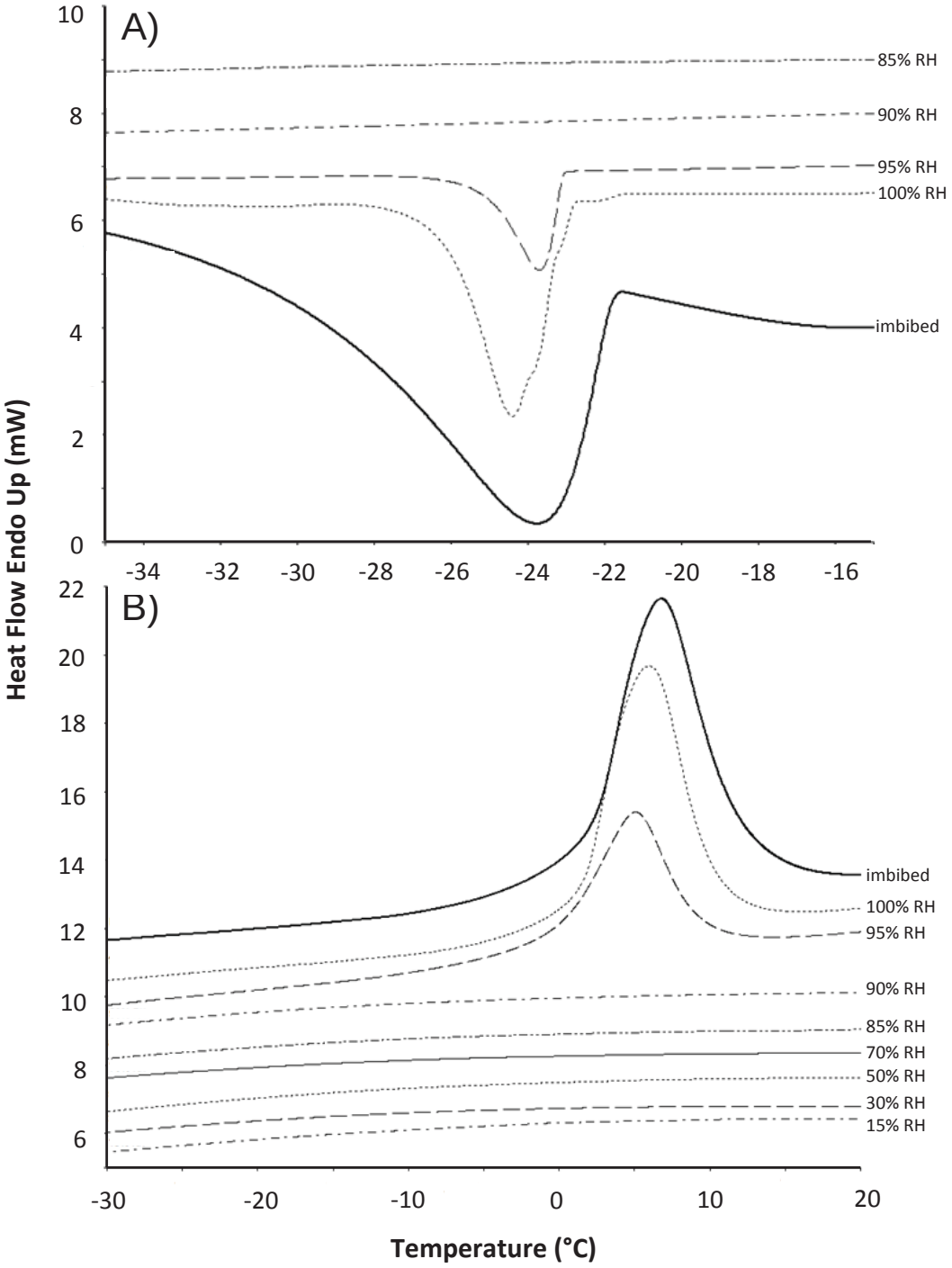

Figure 5.11: Representative A) cooling and B) warming thermograms of *Nymphaea violacea* (NV8 and NV9). Whole seeds were equilibrated over saturated and non-saturated solutions of lithium chloride to create relative humidities between 15- 95 % for a period of > 2 weeks. Imbibed seeds were imbibed in deionized water for 48 hours prior to experimentation. Each sample was cooled to -50 °C at 10 °C/min and rewarmed to 30 °C at 50 °C/min using differential scanning calorimetry.

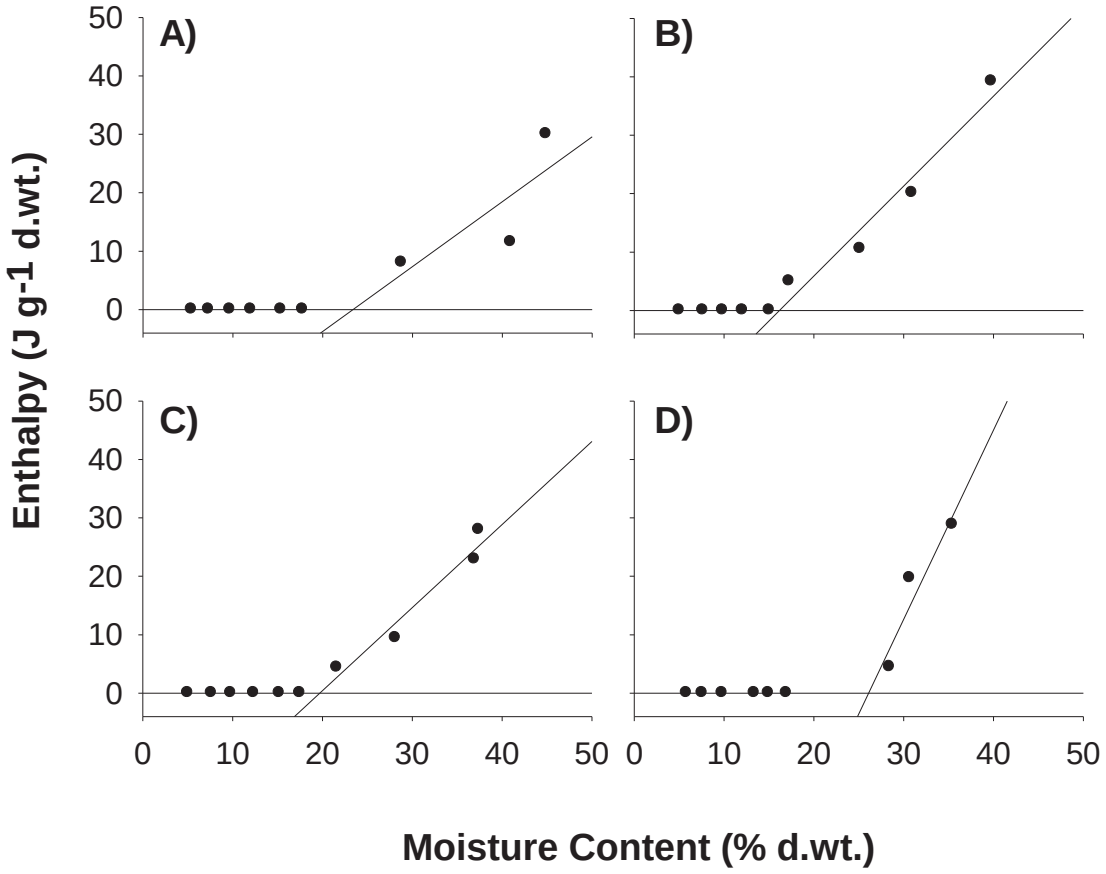

Figure 5.12: Determination of the unfrozen water content of A) *Nymphaea immutabilis* (NI1 = 23.4 %), B) *N. lukei* (NL2 = 16.2 %), C) *N. macrosperma* (NM3 = 34.9 %) and D) *N. immutabilis* (NI1 = 23.4 %). Transition enthalpy was determined by calculating the area under the peak from warming thermograms. The unfrozen water content was determined by the intersection of two regression lines fitted to the data.

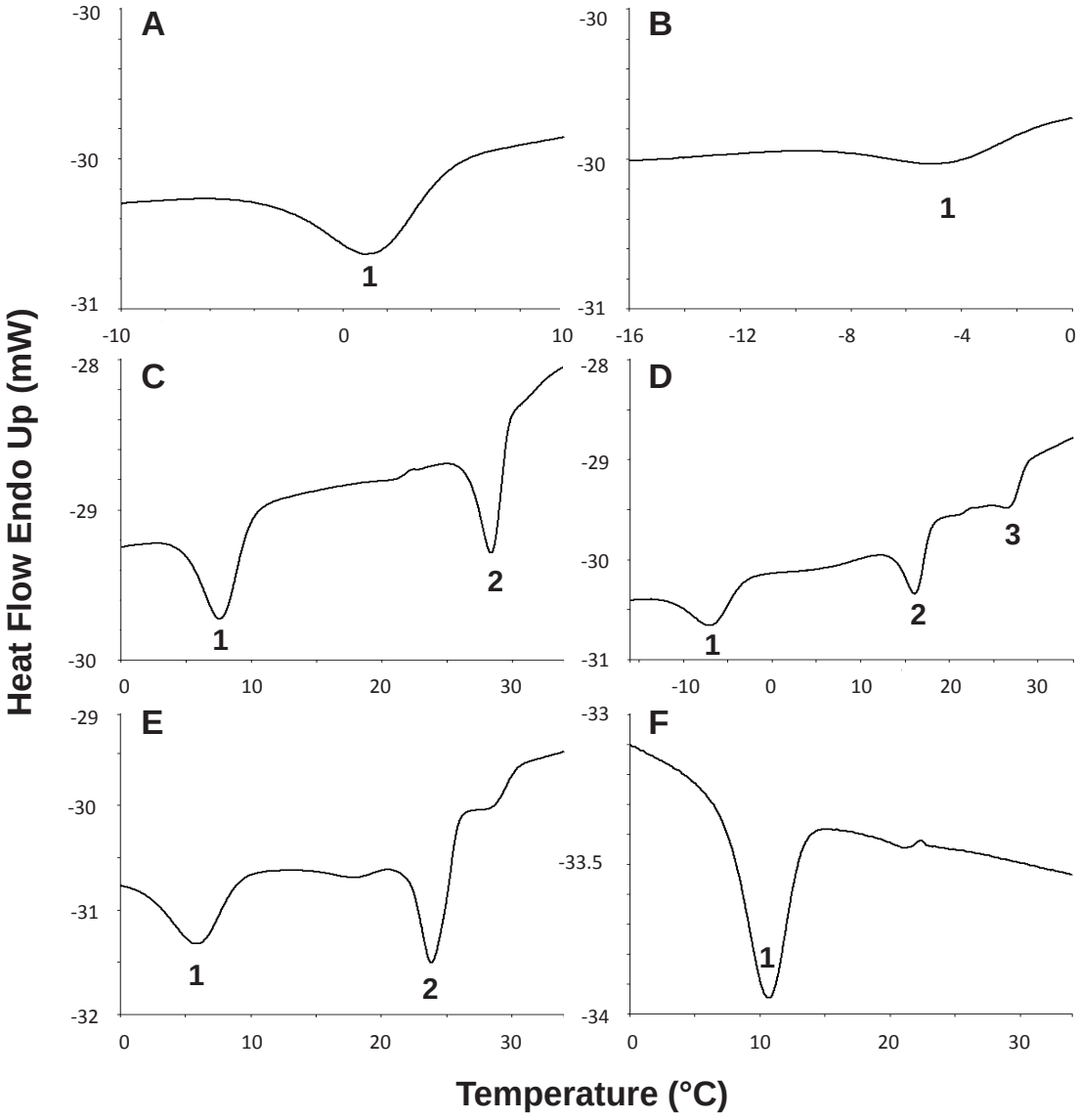

Figure 5.13: Cooling thermograms of lipids extracted from A) *Nymphaea immutabilis* (NI1), B) *N. lukei* (NL2), C) *N. macrosperma* (NM3), D) *N. violacea* (NV8), E) *N. violacea* (NV9) and F) *N. violacea* (NV10) Numbered peaks represent individual crystallisation events.

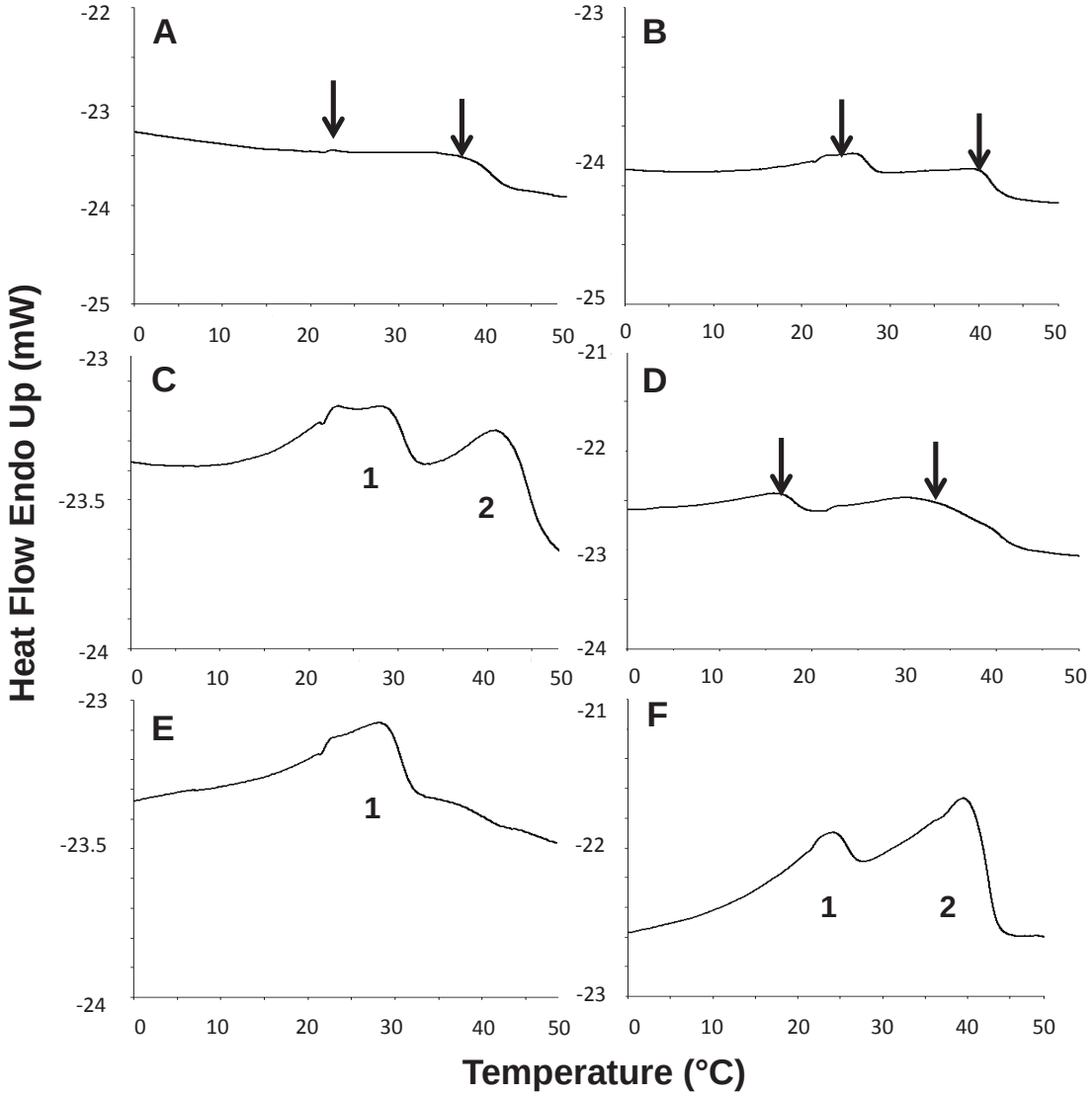

Figure 5.14: Warming thermograms of lipids extracted from A) *Nymphaea immutabilis* (NI1), B) *N. lukei* (NL2), C) *N. macrosperma* (NM3), D) *N. violacea* (NV8), E) *N. violacea* (NV9) and F) *N. violacea* (NV10) Numbered peaks represent individual crystallisation events..

Table 5.2: Melting and warning peaks (°C) and total enthalpy (J/g) calculated from thermograms of lipids extracted from multiple accessions of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM3) and *N. violacea* (NV8, NV9, NV10).

	NI1	**NL2**	**NM3**	**NV8**	**NV9**	**NV10**
Cooling						
Lipid crystallisation peak 1 (°C)	11.7 ± 2.1	-4.5 ± 0.1	11.8 ± 2.1	-27.7 ± 6.3	2.3 ± 1.8	1.3 ± 3.1
Enthalpy peak 1 (J/g)	10.0 ± 2.0	5.9 ± 0.6	7.4 ± 0.4	2.7 ± 1.3	3.1 ± 0.2	4.6 ± 0.7
Lipid crystallisation peak 2 (°C)	-	-	29.2 ± 0.4	-8.1 ± 1.2	25.8 ± 1.9	-
Enthalpy peak 2 (J/g)	-	-	6.7 ± 0.8	3.0 ± 0.4	3.5 ± 0.5	-
Lipid crystallisation peak 3 (°C)	-	-	-	14.5 ± 1.2	-	-
Enthalpy peak 3 (J/g)	-	-	-	1.4 ± 0.6	-	-
Warming						
Lipid melt peak 1 (°C)	-	-	23.2 ± 1.5	-	23.4 ± 1.7	29.2 ± 0.4
Enthalpy peak 1 (J/g)	-	-	8.9 ± 1.2	-	1.7 ± 0.9	9.6 ± 0.8
Lipid melt peak 2 (°C)	-	-	41.6 ± 3.1	-	39.8 ± 2.6	-
Enthalpy peak 2 (J/g)	-	-	6.9 ± 0.8	-	-	-
Phase transition temp 1 (°C)	21.5 ± 0.3	20.1 ± 4.1	-	16.2 ± 2.1	-	-
Phase transition temp 2 (°C)	36.6 ± 0.9	39.5 ± 1.8	-	30.8 ± 2.9	-	-

Comparative longevity

Longevity, as determine by P_{50}, ranged between 0.9 days (*N. immutabilis*) to 24.8 days (*N. violacea* NV9) (Table 5.3, Fig 5.15). Due to low initial germination *N. lukei* and *N. macrosperma* had P_{50} values of 0. Longevity differed significantly between accessions of *N. violacea* ($P < 0.01$), with P_{50} values ranging between 5.3 and 24.8 days (Table 5.3). No significant differences were detected in σ between species ($P = 0.7$; data not shown).

Table 5.3: Estimates of K_i and $1/\sigma$ and P_{50} for four accessions of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM3) and three accessions of *N. violacea* (NV8, NV9 and NV10) under standard experimental (comparative longevity) storage conditions of 45 °C at 60 % RH.

Species	Accession	P_{50}	K_i
Nymphaea immutabilis	NI1	0.91	-0.07
Nymphaea lukei	NL2	0	-1.12
Nymphaea macrosperma	NM3	0	-0.08
Nymphaea violacea	NV8	19.5	2.27
Nymphaea violacea	NV9	24.8	2.62
Nymphaea violacea	NV10	5.3	0.23

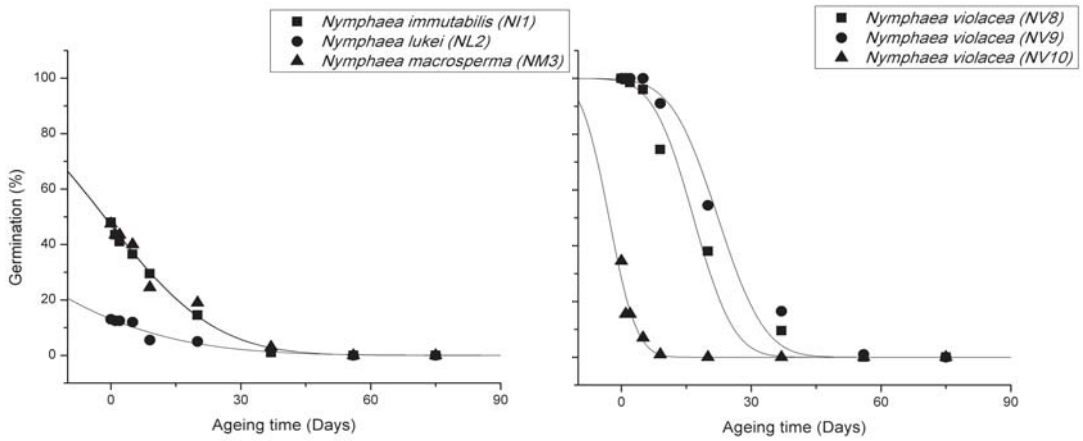

Figure 5.15: Seed survival curves of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM3) and three accessions of *N. violacea* (NV8, NV9 and NV10). Survival curves were plotted via probit analysis under standard experimental ageing conditions (comparative longevity 60 % RH and 45 °C).

DISCUSSION

Seeds of Australian Nymphaea are desiccation tolerant

All species of *Nymphaea* (*N. immutabilis, N. lukei, N. macrosperma* and *N. violacea*) showed tolerance to drying to 15 % RH (seed water content of approximately 5 %). While the total percentage germination decreased after drying in a number of species, subsequent viability testing confirmed that all ungerminated seeds were still viable. The decrease in germination following drying may simply be attributed to an induction of dormancy caused by drying, as previously described in other species (Hong *et al.* 1998; Pritchard 2004). The fact that these species of *Nymphaea* are tolerant to desiccation provides further evidence to support the hypothesis that desiccation tolerance is the ancestral trait, and evolved in the basal angiosperms (Dickie and Pritchard 2002; Tweddle *et al.* 2003). While the evolution of desiccation tolerance in seeds of an aquatic species may appear counterintuitive (Dickie and Pritchard 2002), this study, amongst others (e.g. *Trithuria* in Tuckett *et al.* (2010c)) reveals that the trait is present in a number of basal aquatic species inhabiting ephemeral habitats. The species of *Nymphaea* we have examined here occur through northern Australia's wet-dry tropics, which are characterised climatically by unpredictable cyclonic rainfall, followed by a period of drought (Finlayson 1999). The ability to withstand drying certainly presents a distinct advantage for aquatic species inhabiting ephemeral wetlands with transient water availability, allowing seeds to persist in the soil seedbank through the dry season (Dickie and Pritchard 2002). The reports that *N. alba* and *N. tuberosa* (Guppy 1897; Ewart 1908; Smits *et al.* 1989; Hay *et al.* 2000; Estrelles *et al.* 2004), are recalcitrant may therefore represent a subsequent loss in desiccation tolerance, as these species occupy temperate and perennially inundated wetlands of the northern hemisphere. Therefore, it seems likely that desiccation tolerance in seeds is a polymorphic trait within the Nymphaeaceae that has changed in response to the stability of water availability.

Australian Nymphaea display orthodox storage behaviour

All species tested in this study appear to display orthodox storage behaviour, as seeds were desiccation tolerant, and longevity was generally greater at colder storage temperatures (-20 °C or -160 °C), when compared with storage above 0 °C (25 °C or 5

°C). While the seeds of *N. immutabilis, N. lukei* and *N. macrosperma* were significantly reduced in their ability to germinate after any time in storage, subsequent viability testing revealed that ≥ 60 % of seeds survived at least one month in storage below 0 °C, when pre-equilibrated at low RHs. The ability of two accessions of *N. violacea* (NV8 and NV9) to germinate was not affected by storage when pre-equilibrated to low RHs, and high viability was maintained through the 12-month storage period when stored at RHs ≤ 30 %. The germination response of the third accession of *N. violacea* was more variable, however maintained viability of ≥ 80 % after one month of storage at 15 % RH. This decline in germination after storage, as with seeds that have been desiccated, may be attributable to the induction of dormancy (Hong *et al.* 1998; Pritchard 2004) which is further impeded by our relative inability to completely overcome dormancy in seeds of many Australian *Nymphaea* species (Chapters 3 and 4 of this thesis).

Viability declined significantly over the 12-month storage period for seeds of *N. immutabilis, N. lukei* and *N. macrosperma,* especially in seeds pre-equilibrated to high RHs (70 and 90 %) prior to storage. Additionally, higher temperatures (5 °C and 25 °C) appear to be more detrimental to viability than cold temperatures (- 20 °C and -160 °C), though the least detrimental conditions to viability are species specific. After 12 months in storage, the viability of seeds of *N. immutabilis* was greatest (55 %) when equilibrated at 15 % RH and stored at -160 °C (Fig. 5.4), while *N. lukei* and *N. macrosperma* showed the least viability loss at 15 % RH at -20 °C (55 % and 33 % respectively; Figs. 5.5 and 5.6). Therefore, investments should be made in refining the current cryopreservation protocols, as well as investigating the potential use of other methods such as vitrification, or encapsulation dehydration which have proven useful for other short-lived species (Merritt *et al.* 2014a).

The viability of *N. violacea* seed accessions remained higher than the other species tested over the test period, as viability was > 80 % after 12 months in all accessions when pre-equilibrated to any RH < 95 %, when stored below 0 °C (Figs 5.7- 5.9). However, viability did vary between the accessions, particularly at warmer temperatures. For example, NV10 showed a significant decline in viability at equilibrated RHs ≥ 75 % after one month in storage (Fig. 5.7), along with a significant decline at high temperatures, even at low RHs, while accessions NV8 and NV9 did not

(Figs 5.8 and 5.9). This variability between seed lots is not unusual, and has been reported to occur in a number of species and may be attributed to a number of factors (e.g. genetics or environmental fluctuations such as temperature and moisture) experienced during maturation and harvest (Pritchard 2004; Walters *et al.* 2010).

While representing a small fraction of total seed mass, lipids extracted from seeds of *Nymphaea* tested in this study showed a number of unusual phase transitions and melting events upon cooling and rewarming (Figs 5.13 and 5.14, Table 5.2). Detailed investigations in to triacylglycerol crystallisation in the genus *Cuphea* (intermediate storage behaviour) have shown freezing lipids contribute to fatal injuries sustained in seeds that are subsequently imbibed following low-temperature storage, whilst the lipids remain in the gel phase (Crane *et al.* 2003; Crane *et al.* 2006). However, given that low-temperature storage did not reduce the viability of *Nymphaea,* (when compared with seeds stored above 0 °C) lipid crystallisation must not contribute to the reduction in viability detected at temperatures below 0 °C in seeds of *N. immutabilis, N. lukei* and *N. macrosperma*. Furthermore, the unfrozen water content of seeds of all species was determined to be ≥ 16.2 % (*c.* ≥ 90 % eRH at 20 °C; Figs. 5.10 and 5.12), indicating that the decrease in germination of *N. immutabilis, N. lukei* and *N. macrosperma* and *N. violacea* (NV10) observed at low eRHs (Figs. 5.4- 5.6 and 5.9) cannot be attributed to damage from the formation of ice. Therefore, the reason for this decline in viability cannot be confirmed beyond stating that seeds are short-lived, despite these species presenting orthodox storage behaviour.

Seeds of Nymphaea are short-lived

The comparative longevity of *Nymphaea* species varied substantially, with different accessions of *N. violacea* varying in longevity by an order of magnitude (P_{50} ranging between 5.3 and 22.3 days) while seeds of *N. immutabilis* were extremely short-lived with a P_{50} of 0.9 days. Seeds of all *Nymphaea* examined here are relatively short-lived when compared with the global dataset of seed longevity, and in particular when compared with terrestrial Australian species- for example members of the Myrtaceae that have been tested in comparative longevity experiments, have a mean P_{50} of 366 days (Crawford *et al.* 2007; Probert *et al.* 2009; Hay *et al.* 2010).

While it has been postulated that that seeds of basal angiosperms may be short-lived in storage due to their evolution in moist environments, and seeds having a high internal moisture content (Dickie and Pritchard 2002; Tweddle *et al.* 2003; Probert *et al.* 2009), only one previous study has sought to investigate this claim. Tuckett *et al.* (2010b) found that the seeds of the genus *Trithuria* (Hydatellaceae) were not short-lived in comparison with other vernal pool aquatics, with P_{50} estimates ranging between 24 – 44 days. Our results suggest that *Nymphaea* are quite short-lived when compared with *Trithuria* and may provide some support for the initial hypothesis that basal species (at least within the Nymphaeaceae) are short-lived as suggested by Probert *et al.* (2009). However, it should also be noted that the longevity of seeds was also short when equilibrated at at low RH (15 % or 30 %) and stored at higher temperatures (i.e. 5 °C and 25 °C). Furthermore, it must be noted that intrinsically low germination in *N. immutabilis, N. lukei* and *N. macrosperma* as a consequence of seed dormancy resulted in low initial K_i values, and is likely to have contributed to their poor performance in the comparative longevity experiment. As such, future assessments of longevity in these species should base comparisons of probit model-fit on viability data.

Conclusions

Overall, given that three of four species of *Nymphaea* examined in this study display orthodox seed storage behaviour, but are relatively short-lived under conventional seed banking conditions. As such, the application of rigorous cryogenic storage protocols should be considered if *ex situ* conservation of any of these species is deemed necessary (Li and Pritchard 2009). Furthermore, the complications associated with the induction of dormancy during storage, and the difficulties in determining seed viability simply emphasises many of the problems faced when storing seeds of wild species. We have also highlighted significant complexities and variation in storage behaviour within and between species of *Nymphaea,* which may be attributed to a range of pre- or post-harvest conditions. Therefore, practitioners using seeds of *Nymphaea ex situ* should take this natural but often unpredictable variation into account when conducting any future *ex situ* conservation efforts.

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CHAPTER 6

IMPACT OF INCREASING SALINITY ON GERMINATION AND EARLY SEEDLING DEVELOPMENT OF *NYMPHAEA* L. (NYMPHAEACEAE) FROM NORTHERN AUSTRALIA'S FRESHWATER WETLANDS

ABSTRACT

Background and aims: Low-lying, coastal, freshwater floodplains and wetlands in northern Australia are at risk of increased saltwater intrusion associated with global sea-level rise. These wetlands support vast number of freshwater plant species, and current predictions suggest that many ecosystems will be severely affected by increasing water salinity. Waterlilies (*Nymphaea*) are iconic in northern Australia, and their loss from these wetlands will be detrimental, both ecologically and culturally. As such, we aimed to determine whether increasing salinities would significantly effect seed germination and early seedling growth in three widely distributed *Nymphaea* (*N. immutabilis, N. macrosperma* and *N. violacea*) species, and one geographically restricted species (*N. lukei*).

Methods: Seeds were subjected to a gradient of salinities (0, 50, 100, 200, 300, 400 and 500 mM NaCl), and the impact on seed germination noted. Total seedling biomass and length was determined after no further germination occurred for two weeks. In order to test the ability of ungerminated seeds to recover, seeds were placed in fresh water until no further germination occurred.

Key results: Seed germination, total seedling biomass and total seedling length decreased significantly at salinities ≥ 100 mM NaCl. Upon recovery in fresh water, only one accession of *N. violacea* showed a full recovery. No differences were detected between the responses of the geographically widespread species and the geographically restricted species; however, some variation between species was noted.

Conclusions: Seeds and early seedlings of *N. immutabilis, N. macrosperma* and *N. violacea* are sensitive to increasing water salinity. The majority of species and accessions showed a decrease in germination and early seedling biomass with any increase in water salinity. Significant increases in the salinity of coastal freshwater wetlands will likely result in a decrease in juvenile recruitment of all *Nymphaea* species.

Key words: conservation, climate change, saltwater intrusion, aquatic angiosperms, wet-dry tropics, seed germination.

INTRODUCTION

Wetlands and their biota are among the most highly threatened in the world (Hay *et al.* 2000). With rising pressure on freshwater resources from the increasing global human population, coupled with anthropogenic alteration (modified habitats, draining, eutrophication and introduced weeds) and the predicted impact of climate change (reduced rainfall, altered hydrological regimes and rising sea levels) the reduction in freshwater aquatic species diversity is set to further decrease (Amezaga *et al.* 2002). While in Australia, there has been an increasing shift toward the inclusion of important wetlands within reserves at the local, state, national and international level, protection in reserves alone will not mitigate all threatening processes, particularly in the face of a changing climate.

Northern Australia's wet-dry tropics encompass a vast area of coastal wetlands, rivers and floodplains that are currently at risk from climate change (Hennessy *et al.* 2007; Steffan *et al.* 2013; Steffan *et al.* 2014). Low-lying areas are particularly susceptible to saltwater inundation associated with rising sea levels and the increasing intensity of storm surges accompanying tropical cyclones and monsoonal lows (Eliot *et al.* 1999; Cowie *et al.* 2000a; Hughes 2003; Winn *et al.* 2006; Finlayson *et al.* 2009; BMT WBM 2010; Catford *et al.* 2013) (Figure 1, Appendix 7). Current data from the UNESCO World- Heritage listed Kakadu National Park, which covers approximately 195,000 hectares of low-lying floodplain (Eliot *et al.* 1999), has shown that salt water intrusion in the East Alligator River region has already contributed to a nine-fold expansion of saline mud flats and a reduction of freshwater *Melaleuca* fringed wetlands by more than 60 % (Winn *et al.* 2006; Steffan *et al.* 2014). Additionally, the Mary River floodplain (adjacent to the western border of Kakadu NP) has experienced saltwater intrusion resulting in 17,000 hectares of freshwater floodplain transitioning to mangrove swampland (Knighton *et al.* 1991; Mulrennan and Woodroffe 1998). Calculations show that global sea levels have risen by up to 3.2 mm/ year since 1993 (Church and White 2011) and current projections for Australia predict an average sea-level rise by up to 82 cm by 2100 (CSIRO and Bureau of Meteorology, 2015). Given that the majority of northern Australia's freshwater floodplains and wetlands are less than two metres above sea level, the consequences of rising sea levels and greater frequency of storm tides on these unique systems could be wide-spread and catastrophic (Steffan *et al.* 2014).

Therefore, our ability to predict the distribution of vulnerable freshwater wetland species under climate change scenarios is essential for effective, ongoing, and adaptive management practices. However, such predictions must be based on data investigating individual species' tolerances to environmental change and salinity in order to best manage at-risk populations (Hughes 2003).

Numerous studies from the USA have focussed on the impact of saltwater intrusion caused by land subsidence, shipping channels and floodwaters associated with hurricanes on aquatic plants in freshwater marsh ecosystems (McKee and Mendelssohn 1989; Flynn *et al.* 1995; Day Jr *et al.* 2007), and an increasing number of authors are recognising the threat posed to coastal freshwater wetlands through global sea-level rise (Allen *et al.* 1996; Baldwin and Mendelssohn 1998; Baldwin *et al.* 2001; Neubauer 2013). In general, while freshwater aquatic plant species may differ in their sensitivity and tolerances to increasing salinity (Haller *et al.* 1974), they are unable to persist indefinitely at salinities of ≥ 5 g L^{-1} (Nielsen *et al.* 2003a; Nielsen *et al.* 2003b; Brock *et al.* 2005; Nielsen *et al.* 2007).

In Australia, the predominant focus of the scientific literature has been on the salinity tolerance of freshwater plant species in semi-arid lakes and rivers affected by secondary salinization due to extensive land clearing (Hart *et al.* 1991; James and Hart 1993; James *et al.* 2003; Nielsen *et al.* 2003a; Nielsen and Brock 2009). Even at lower levels of salinity, growth of plants in these ecosystems may be inhibited and seedling recruitment is often significantly reduced. For example, James and Hart (1993) assessed the impact of increasing salinity on four common Australian freshwater macrophytes (*Myriophyllum crispatum, Eleocharis acuta, Potamogeton tricarinatus* and *Triglochin procera*) and found that while species differ in their thresholds to salinity tolerance, significantly reduced growth was observed at salinities in excess of 1,000 mg L^{-1} (total dissolved salts in seawater is approximately 35,000 mg L^{-1}). Sensitivity to salinity can also differ at different developmental life stages, for example seeds and seedlings may be more vulnerable to increasing salinity than mature adult plants (Nielsen *et al.* 2003a; Nielsen *et al.* 2003b). Under small changes in the level of salinity, seed germination and early seedling size of freshwater aquatics has been shown to either increase or decrease, but this response is variable between species (Nielsen *et al.* 2003b). However, the

results from all of these studies conclude that any salinity increase above a threshold value will result in a decrease in germination, reduced seedling biomass eventual necrosis and death; overall reducing plant recruitment in salinity-affected areas (Nielsen *et al.* 2003a).

When considering the potential impact of oceanic saltwater intrusion on freshwater aquatics in coastal regions, it is also important to take into account the spatial and temporal variability associated with salinization events. Events of saltwater intrusion may be restricted by low tides or high precipitation in wetland catchments, which may act to flush the system with fresh water, overall reducing water salinity (Flynn *et al.* 1995). As such, freshwater wetlands may only be exposed to a short period of salinization before returning to fresh water. Seeds of freshwater aquatic species represent a critical life stage as they act as a major source of gene flow and population persistence across intervening, hostile conditions (Fenner and Thompson 2005). Therefore, it is important to understand the ability of seeds to recover in fresh water in order to assess whether they are able to survive and persist after short-term exposure to increased salinity. The ability of seeds to recover after a period of exposure to increased salinity has been widely studied under laboratory conditions in agricultural species, native halophytes and other wild species with a coastal distribution (Ungar 1962; Woodell 1985; Debez *et al.* 2004; Necajeva and Ievinsh 2008; Guja *et al.* 2010). Overall, four germination responses to increasing salinity and subsequent freshwater recovery have been diagnosed; two resulting in reduced germination after incubation in salt, the others resulting in equal or increased germination after recovery (Guja *et al.* 2010). However, the majority of studies elucidating the effect of increasing salinity and subsequent recovery of seeds (or whole plants) of freshwater aquatics have done so in field or glasshouse trials in combination with experimental hydrological regimes, and the independent effect of salinity is often not considered [e.g. (Flynn *et al.* 1995; Baldwin and Mendelssohn 1998; Brock *et al.* 2005; Nielsen and Brock 2009; Neubauer 2013)].

Eighteen species of the basal genus *Nymphaea* (Nymphaeaceae), occupy the freshwater wetlands of the wet-dry tropics in northern Australia. While a number of species in the genus are restricted to inland wetlands, many species inhabit low-lying coastal

floodplains (Cowie *et al.* 2000b; Jacobs and Hellquist 2011). *Nymphaea* play an important role within these wetlands by providing in-stream sediment stabilisation, a reduction in turbidity and habitat for aquatic animals and food for migratory waterbirds (Russell-Smith 1995; Finlayson and Woodroffe 1996; Sainty and Jacobs 2003; Finlayson *et al.* 2006). Additionally, *Nymphaea* are culturally important to Indigenous Australian's who occupy the northern floodplains, as they traditionally serve as a food source through the dry season (Issacs 1987; Brockwell *et al.* 1995; Karadada *et al.* 2011). The loss of these plants through saltwater intrusion could therefore prove detrimental, both ecologically and culturally. No studies have previously investigated the potential impact of increasing salinity on seeds and seedlings of coastal freshwater plants in northern Australia, or on the impact of increasing salinity on seeds and seedlings of *Nymphaea.* As such, we tested the sensitivity of germinating seeds and early seedlings to increasing concentrations of sodium chloride, and hypothesised that:

1) Seeds and seedlings of Australian *Nymphaea* will be intolerant of increasing salinity beyond 100 mM of NaCl (brackish water approx. 5 g L^{-1}), and will show a significant reduction in germination and early-seedling size.
2) Once removed from the salinity treatment, un-germinated seeds would germinate in fresh water.

Furthermore, we compared the tolerances of common, widely distributed species occurring through the coastal tropics with a short-range endemic species with specific habitat requirements to test whether:

3) Geographically widespread *Nymphaea* seeds would be more tolerant to increasingly saline conditions and be able to recover more successfully than geographically restricted species.

MATERIALS AND METHODS

Species selection and seed collection

Seeds of four species were selected for inclusion in this study based on their distribution and collection location (Table 6.1). Three species are geographically widespread in Australia; *Nymphaea immutabilis* Hook.*, N. macrosperma* Merr & L. M. Perry, and *N. violacea* Lehm., while the fourth, *N. lukei* S. W. L. Jacobs & Hellq. has a restricted range and is only found in a small area of the Kimberley region of Western Australia (Jacobs and Hellquist 2011). Additionally, two separate collections of *N. violacea* were included, one from low-lying coastal Western Australia, the other from inland (higher elevation) Queensland (Fig. 6.1). Seeds were collected over a period of three years between March 2011 and August 2013. Mature seeds were collected at the point of natural dispersal. Whole seed heads were collected, kept moist (approximately 30 mL of water was placed in the bottom of each bag) and transported to Kings Park, Perth in Glad® Snap Lock® bags. Upon arrival, seed heads were left in shallow pans of water to naturally dehisce at room temperature (c. 24 °C). All seeds were separated from chaff using an aspirator (SELECTA BV Gravity Seed Seperator "Zig Zag", Netherlands) or by hand. Initial seed fill was determined by x-ray analysis using a Faxitron Specimen Radiography System MX-20 Cabinet.

Exposure to salinity and subsequent recovery

Four replicates of 50 seeds per treatment were placed in 10 ml plastic screw-cap tubes (TechnoPlas, Australia) and irrigated with 10 ml of 0 (control), 50, 100, 200, 300, 400 or 500 mM (equivalent to seawater) of sodium chloride (NaCl, Sigma Aldrich Australia). The treatment whereby seeds are incubated under crowded conditions is explained in Chapter 3 of this thesis, and acts to stimulate germination. Tubes were then incubated at 35 °C and seed germination (as determined by protrusion of the radicle to ≥ 1mm) was scored weekly until no further germination occurred in controls for 2 weeks, as per Guja *et al.* (2010). In order to assess the ability of ungerminated seeds to recover from exposure to NaCl, all seeds that did not germinate within this initial incubation period were then removed from their tubes, rinsed three times in 100 mL DI water, and immediately transferred to 10ml tubes filled with 10 mL DI water. Seed germination

following transfer from NaCl to DI water was then scored every 3- 4 days until no further germination had occurred for at least two weeks.

Effect of increasing salinity on early seedling growth

In order to elucidate the impact of NaCl exposure on early seedling growth, all seedlings produced at the completion of the NaCl experiment (prior to recovery) were subjected to morphological analysis. Any remaining fragments of seed coat were removed prior to analysis. Groups of seedlings were placed inside custom-built Perspex tray filled with water and analysed on a flat bed scanner (WinRHIZO, Regent Instruments Inc., Sainte-Foy, Canada) for total length and then oven-dried at 75 °C for at least 3 days in order to obtain total biomass per replicate.

Statistical analysis

Germination data were analysed using a generalized linear model (GLM) with an inbuilt logit-link function in R. To determine the effect of increasing salinity, we first compared germination over the range of sodium chloride concentrations before initiation of the recovery experiment. To elucidate the effect of recovery in fresh water, we compared germination at the start and end of the recovery period via GLM. Species were initially included as a factor in the model, and when found to be significant, further GLMs were conducted within species, for increasing salinity.

In order to determine lethal thresholds of NaCl for seed germination and early seedling growth traits (biomass and length), data were analyzed by fitting the following three parameter Weibull curve available from the dose response curve package '*drc*' (Ritz and Streibig 2005):

$$F\ (x, b, d, e) = (\exp d)(-\exp(b(\log(x) - \log(e))))$$

Whereby (d) is the upper limit or maximum response of the curve, (b) is proportional to the slope, in response to NaCl (x), and (e) is the lethal threshold inducing a 50 % reduction (LC_{50}) in the response of the curve (Ritz 2009). Curve selection was based on the log-likelihood and AIC when comparing to other linear and non-linear functions (Ritz and Streibig 2005). In order to compare LC_{50} values between species and

accessions, the 'compParm' function was used to determine differences at a significance level of $P < 0.05$, through an approximate ratios t-test (Ritz 2009).

Table 6.1: Species of *Nymphaea* used in this study, abbreviated seed lot number, a description of the collection site, species distribution in Australia, GPS coordinates of the collection location and the elevation (in meters) at the collection site. Brackish definition: between 100-250 mM total dissolved salts.

Species	Seedlot No.	Description of collection habitat	Distribution	Collection location (GDA 94)	Elevation (m)	Water salinity at collection location
Nymphaea immutabilis	NI1	Drainage ditch. Coastal	NT, QLD	S15°25'35.7", E145°9'14.5"	22	Brackish
Nymphaea lukei	NL2	Small, ephemeral, sandstone based creek. Inland	Restricted- WA	S16°43'0.2", E125°27'35.8"	403	Fresh
Nymphaea macrosperma	NM3	Large coastal billabong. Coastal	Cosmopolitan	S15°35'35.7" E128°16'47.9"	9	Fresh
Nymphaea violacea	NV9	Large coastal billabong. Coastal	Cosmopolitan	S15°51'39.8" E127°14'20.8"	275	Fresh
	NV10	Large swamp. Inland		S16°54'55.3" E145°22'20.6"	391	Fresh

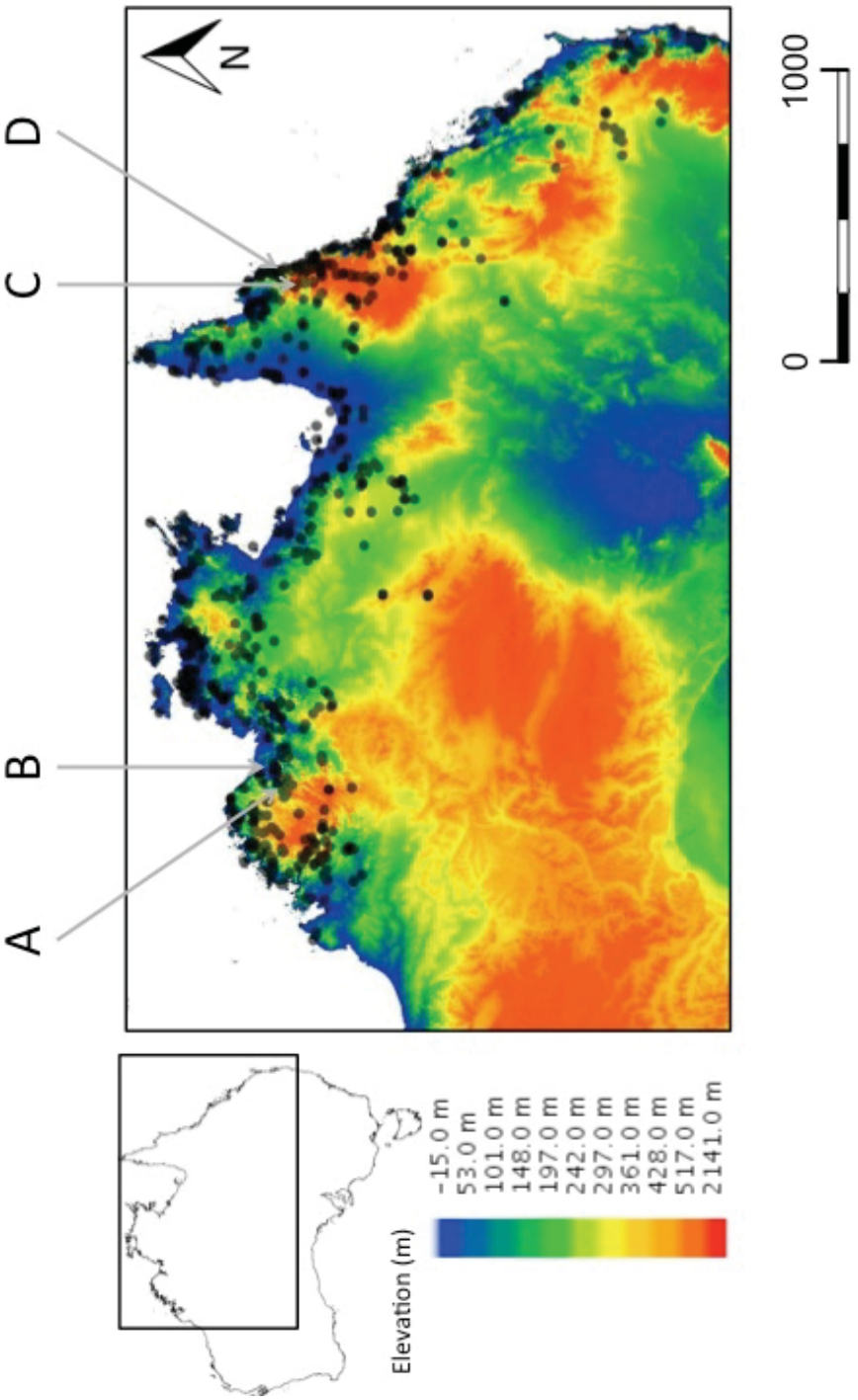

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Figure 6.1: Digital elevation model of Australia showing currently distribution of the genus *Nymphaea* (•), sourced from the Atlas of living Australia (Atlas of Living Australia 2015). Collection locations of A) *Nymphaea lukei* (NL1); B) *N. macrosperma* (NM3), *N. violacea* (NV9); C) *N. violacea* (NV10) and D) *N. immutabilis* (NI1).

RESULTS

Exposure to salinity and subsequent recovery

The exposure of seeds to a range of NaCl concentrations significantly reduced ($P < 0.001$) germination in all species and accessions tested, however the response was specific to each accession (Fig. 6.2). Seeds of *N. immutabilis* (NI1), *N. lukei* (NL2) and *N. macrosperma* (NM3) exposed to salt concentrations between 50- 200 mM NaCl still germinated, although to significantly lower ($P < 0.04$) percentages when compared with the 0 mM NaCl control. These three species were found to tolerate concentrations of 223- 257 mM of NaCl before germination was reduced by 50 % (Table 6.2). *Nymphaea violacea* (NV9) did not show a significant ($P = 1.0$) reduction in germination when exposed to a NaCl concentration of 50 mM, however once exposed to a NaCl solution ≥ 100 mM, germination ceased or was significantly reduced, resulting in a 50 % reduction in germination at a concentration of 124 mM NaCl (Table 6.2). Seeds of *N. violacea* (NV10) did not germinate under any NaCl treatment, despite showing high germination (> 75%) in the control treatment. Both accessions of *N. violacea* showed a 50 % reduction in germination at significantly ($P < 0.01$) lower NaCl concentrations compared with the other three species (Fig. 6.3; Table 6.2).

When comparing between species and accessions, the concentration of NaCl required to reduce seed germination by 50 % (LC_{50}) was significantly higher in seeds of *N. immutabilis* (257 mM), *N. lukei* (224 mM) and *N. macrosperma* (227 mM) compared to both accessions of *N. violacea* (NV9 = 121 mM, NV10 = 0.2 mM; Table 6.2).

Once seeds had been transferred from the NaCl treatment to pure water, seeds of all species (and accessions) in all NaCl treatments displayed some level of recovery (Fig. 6.2). Only one accession, *N. violacea* NV9, displayed 100 % recovery in all NaCl concentrations, with 100 % germination occurring after 20 days in all the recovery treatment (Fig. 6.2). No other species or accession showed complete recovery to the total germination obtained in the 0 mM NaCl controls (Fig. 6.2).

The initial concentration of NaCl that seeds of *N. immutabilis* and *N. macrosperma* were exposed to was significant ($P < 0.001$) in determining final germination at the end of the recovery period, with germination inversely proportional to NaCl concentration. Germination after recovery was significantly different at 100 - 500 mM (all $P < 0.04$) for *N. immutabilis* and 200 – 500 mM (all $P < 0.02$) for *N. macrosperma*, when compared with germination in the control. Both *N. lukei* and *N. violacea* NV10 showed the greatest recovery of germination in seeds exposed to high salinities between 400-500 mM, however this was not statistically significant. However seeds of *N. violacea* NV10 were particularly sensitive to salinity, with all NaCl concentrations showing at least a 39.5 % decrease in germination after the recovery period.

Effect of increasing salinity on early seedling growth

Overall, the effect of increasing salinity concentration on early seedling length and total biomass decreased predictably in a sigmoidal fashion, similar to the decline in germination (Fig 6.3). Total biomass per replicate was greatest in *N. immutabilis* (0 mM NaCl = 117.4 mg) and *N. macrosperma* (0 mM NaCl = 94.5 mg) and least in *N. violacea* NV10 (0 mM NaCl 9.5 mg), which represents a decreasing gradient of seed size (Chapter 3 of this dissertation). Total seedling length was also greatest in the larger seeded species (Fig. 6.3). However, there were inter-specific differences in tolerance thresholds to NaCl concentration. The total concentration of NaCl resulting in a 50 % reduction in total biomass was greatest in seedlings of *N. macrosperma* (219 mM NaCl), which was significantly higher ($P < 0.01$) than all other species and accessions (≤ 167.3 mM NaCl; Table 6.2). The LC_{50} NaCl concentration for total seedling length was lowest in seedlings of *N. violacea* (NV9 and NV10, 90 – 97 mM NaCl respectively).

When the mean LC_{50} for each measured parameter (germination, biomass and total length) was compared; no significant differences were detected ($P = 0.9$, Table 6.2). However, significant differences between species were detected. For example, *N. immutabilis* (NI1) displayed a significantly higher LC_{50} NaCl concentration for germination (257 mM), when compared with seedling biomass (134 mM) or length (186 mM), while *N. violacea* NV9 displayed a higher LC_{50} NaCl concentration for total biomass (167 mM), compared with germination (121 mM) or length (97 mM; Table 6.2).

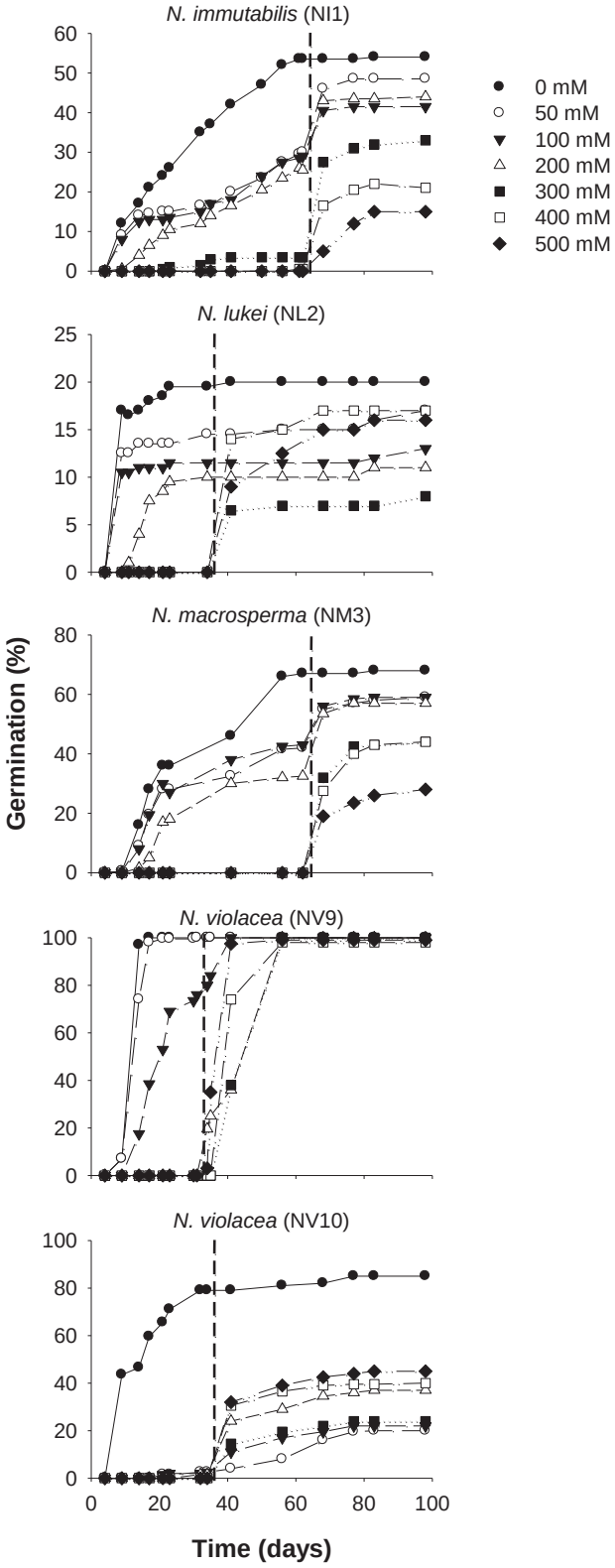

Figure 6.2: Germination (±SE) of seeds of *Nymphaea immutabilis, N. lukei, N.* macrosperma and two accessions of *N. violacea* subjected to a range of NaCl concentrations (0, 50, 100, 200, 300, 400 and 500mM NaCl). Once germination remained stable for two weeks, ungerminated seeds were transferred to sterile DI water (indicated by dashed line).

Table 6.2: Estimate of the inception point *e* from a dose response curve, which estimates the concentration of NaCl (mM) required to reduce germination, biomass, seedling length and seedling area by 50% in four species of *Nymphaea* (lethal concentration LC_{50}). Asterisks for LC_{50} refer to the model goodness of fit, while asterisks for mean LC_{50} refer to an ANOVA in means. P < 0.05 *, P < 0.01 **, P < 0.001 ***. Tolerance of salinity sensitivity based on the following categorisation; low $LC_{50} \geq$ 200 mM; moderate $LC_{50} \geq$ 100 mM < 200 mM, high < 100 mM.

Species	Accession	LC_{50} ± SE (Estimated by *e* in mM NaCl)	Salinity sensitivity
Germination			
Nymphaea immutabilis	NI1	257.0 (8.6) ***	Low
Nymphaea lukei	NL2	223.5 (9.8) ***	Low
Nymphaea macrosperma	NM3	227.3 (9.7) ***	Low
Nymphaea violacea	NV9	121.4 (9.5) ***	Moderate
Nymphaea violacea	NV10	0.22 (10.1)	High
	Mean	***165.8 (52.9)***	***Moderate***
Biomass			
Nymphaea immutabilis	NI1	134.1 (11.1) ***	Moderate
Nymphaea lukei	NL2	160.0 (56.9) **	Moderate
Nymphaea macrosperma	NM3	219.8 (11.9) ***	Low
Nymphaea violacea	NV9	167.3 (150.4)	Moderate
Nymphaea violacea	NV10	0.59 (81.3)	High
	Mean	***134.4 (39.8)***	***Moderate***
Length			
Nymphaea immutabilis	NI1	186.2 (8.4) *	Moderate
Nymphaea lukei	NL2	141.8 (5.9) **	Moderate
Nymphaea macrosperma	NM3	116.7 (9.5)***	Moderate
Nymphaea violacea	NV9	97.1 (19.5) ***	High
Nymphaea violacea	NV10	90.1 (44.4)	High
	Mean	***126.4 (19.5)***	***Moderate***

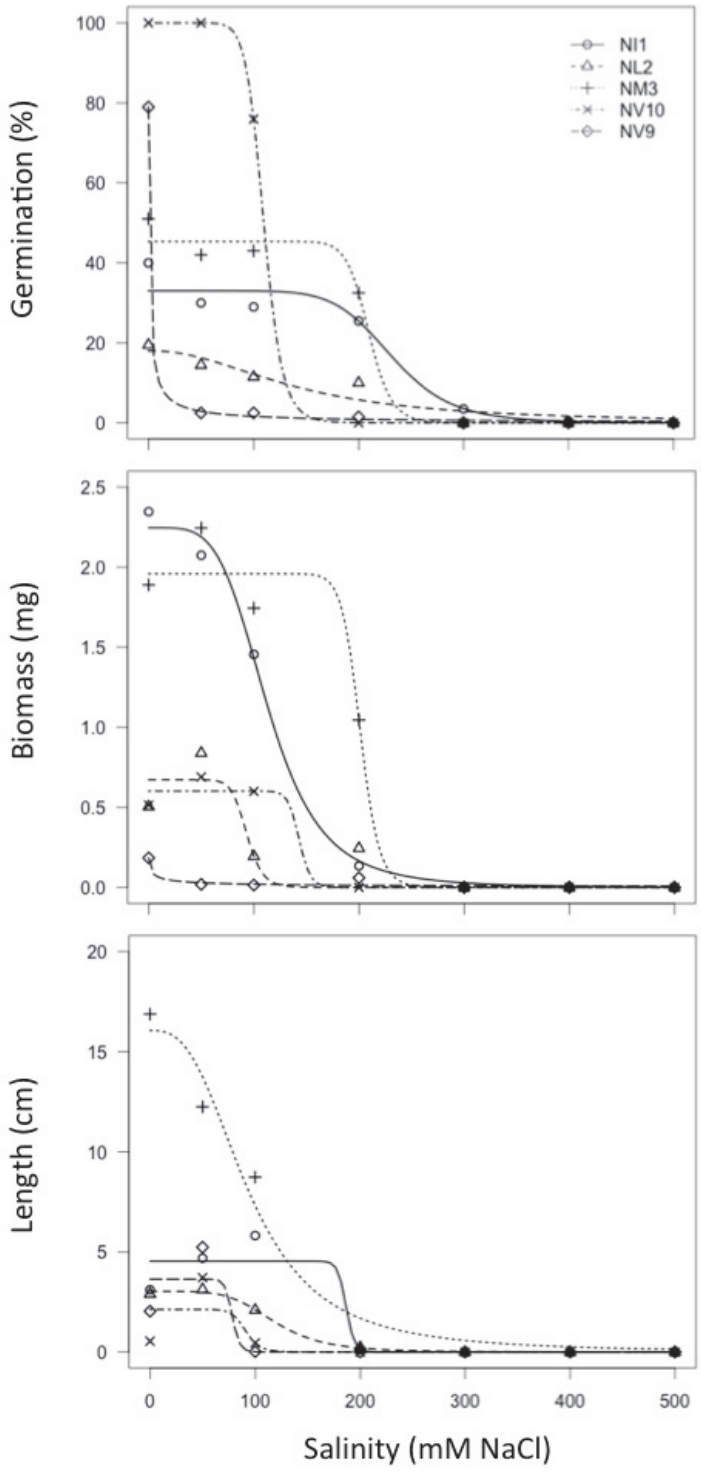

Figure 6.3: Impact of increasing NaCl concentrations on germination and early seedling morphology of four species (totalling five accessions) of *Nymphaea*, examined via a Weibull function. Germination (%), total biomass (mg) of pooled replicates, total seedling length (cm) of pooled replicates of *Nymphaea immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM3) and *N. violacea* (NV9 and NV10) is plotted against increasing NaCl. Seeds were retained under saline conditions until no further germination occurred in controls for 2 weeks (NV9 = 34 days, NL2, NV10 = 34 days and NI1, NM3 for 62 days).

DISCUSSION

Understanding the tolerance of coastal, freshwater aquatic plants to changing water salinity is essential for their successful conservation and management, especially when faced with the impacts of climate change. Many low-lying freshwater floodplains and wetlands in northern Australia are currently being or will be affected by saltwater intrusion from rising sea levels and an increase in the severity and frequency of storm tides (BMT WBM 2010; Steffan *et al.* 2014; CSIRO and Bureau of Meteorology 2015). Combined with the expected change in timing, duration and intensity of monsoonal rainfall and winds (Figure 1, Appendix 7), freshwater wetland species are faced with an ever-decreasing area of suitable habitat to persist in or disperse to. As such, we assessed the impact of increasing salinity on seed germination and early seedling size (biomass and length) on four species of Australian *Nymphaea,* and the ability of seeds to recover from a period of saline conditions.

Impact of increasing salinity on seeds and seedlings

All of the species of *Nymphaea* tested in this study showed a significant decrease in germination at all NaCl concentrations ≥ 100 mM, with the majority of species and accessions showing a significant decline in germination at concentrations ≥ 50 mM. Thus we can accept our first hypothesis and conclude that for the majority of species and accessions, NaCl concentrations ≥ 50 mM result in a significant reduction in germination. The majority of literature exploring the salinity tolerance of freshwater aquatic plants supports this finding, as seed germination is reduced in the majority of species at salinities above 100 mM (approximately 1 g L^{-1}) (Nielsen *et al.* 2003b; Brock *et al.* 2005).

However, the germination response at increasing salinities was variable between species. For example in seeds of *N. immutabilis,* only failed to germinated at salinity concentrations ≥ 300 mM, while seeds of *N. violacea* (NV10) failed to germinate at salinities ≥ 50 mM (Fig. 6.2). Seeds of *N. immutabilis* were collected from plants growing in a coastal area in almost brackish water, while seeds of *N. violacea* were collected from plants inland, and in fresh water. As such, this difference in salinity tolerance may be attributed to an acclimation to higher salinities in seeds of *N. immutabilis*. Previous authors have noted that prior exposure or a gradual increase in

salinity may result in an increased tolerance to salinity stress to adult plants (McKee and Mendelssohn 1989), and seeds may respond similarly. However, seeds of *N. lukei* (short-range, inland endemic collected in fresh water) still showed germination at 200 mM of NaCl (though significantly reduced from the 0 mM control), which does not support our third hypothesis that the short-range endemic species would be less tolerant to increasing salinity than the other species tested. Therefore, further research is required to confirm which species of Australian *Nymphaea* may be more susceptible to increasing water salinity.

Increasing salinity also had a predictable impact on early seedling size, as a reduction in biomass and length was observed in all species and accessions with increasing salinity (Fig. 6.3) and seedling size was reduced by 50 % in the majority of species at NaCl concentrations ≤ 180 mM. However, similar variation between species was observed when compared with seed germination. It should also be noted that due to persistent germination, seedlings of *N. immutabilis* and *N. macrosperma* were allowed to grow for a longer period of time compared with *N. lukei* and *N. violacea,* thus contributing to their greater overall biomass and length. Furthermore, as *N. violacea* NV10 did not germinate at salinities ≥ 50 mM (and subsequently no seedlings were produced), the dose response curve fit was poor, and contributed to the very low LC_{50} values obtained for this accession.

While many studies have addressed the impact on seed germination and seedling growth under increasing salinities [e.g. (Ungar 1962; Woodell 1985; Nielsen *et al.* 2003b; Greenwood and McFarlane 2006; Nielsen *et al.* 2007)], this study represents the first to consider the impacts on seeds and seedlings of *Nymphaea*, and the first to assess a species from the monsoonal tropics of northern Australia.

Ability of seeds to recover from salinity stress

Once seeds were removed from the salinity treatments and placed into pure water (recovery treatment), all species (with the exception of *N. violacea* NV9), failed to completely recover to the same levels of germination observed in the controls (0 mM NaCl). While *N. immutabilis* conformed to the expected inverse relationship between increasing salinity and decreasing ability to recover, *N. lukei* and *N. violacea* (NV10) showed slightly increased recovery in seeds initially treated at higher salinities (Fig.

6.2). The results observed in this study broadly fit within three (a-c) of the germination responses to salinity and recovery as described by Guja *et al.* (2010), in that the germination of *Nymphaea* seeds subjected to NaCl treatments and subsequent recovery never exceeded the germination response in the control. Many previous studies have reported similar findings [e.g. (Woodell 1985; Necajeva and Ievinsh 2008)] as the majority of species have seeds that can withstand at least a short period of increasingly saline water and the fact that there was some level of recovery in the majority of *Nymphaea* tested here highlights the possibility of persistence in response to short-term saltwater inundation.

A geographically restricted species does not show lower tolerance to increased salinity

Our results do not support our third hypothesis that geographically restricted species would be less tolerant of increasing salinity. *Nymphaea lukei* is restricted to small, ephemeral, freshwater creek lines to the north-west of the King Leopold Range in the Kimberley region in Western Australia and is notably absent from low-lying floodplains and billabongs (Jacobs and Hellquist 2011). Seeds of *N. lukei* germinated and developed into early seedlings at higher salinities when compared with both accessions of *N. violacea*. However, it must be noted that total germination of *N. lukei* seeds never exceeded 20 % under any treatment, including the 0 mM NaCl control. *Nymphaea violacea* NV10 showed a high overall sensitivity to salinity, with a significant decrease in germination, total seedling biomass and length at all salinities exceeding 50 mM NaCl. While *N. violacea* is geographically widespread and found in floodplain billabongs through Western Australia, the Northern Territory and Queensland, *N. violacea* NV10 was collected from a small population inhabiting a creek line, approximately 70 kms from the coast. As such, further investigations should focus on testing a much wider range of provenances in order to fully test this hypothesis.

Limitations of this study and considerations for future experimental design

While this study has provided the first empirical evidence of the salinity tolerances of Australian *Nymphaea* seeds and seedlings, we recognise that our experimental design is somewhat limited. Saltwater intrusions will also likely result in increasing water turbidity and an increase in flooding duration, depth and intensity (McKee and

Mendelssohn 1989; Flynn *et al.* 1995), which when combined, pose a severe threat to many freshwater aquatic species. For example, increasing the duration of exposure to saltwater not only decreased the mortality of shoots and leaf blades of the submerged freshwater aquatic *Vallisneria americana* from the Caloosahatchee Estuary in South Florida, but also acted to decrease the ability of the plant to recover, once transferred back to fresh water (Doering *et al.* 2001). The present study only assessed the ability of seeds to recover once germination did not further increase for a period of two weeks, as such, all salinity treatments lasted for up to 65 days, which may have had a significant impact on total germination, seedling size and ability to recover when compared with shorter exposure times. As such, future studies investigating the salinity tolerance of *Nymphaea* in Australia should use a range of inundation times to determine the capacity of adult plants, juveniles and seeds to cope with short salinisation events and to assess their full potential of recovery in fresh water.

Additionally, saltwater intrusion events caused by rising sea levels are likely to occur more gradually than sudden storm-tide related events. Therefore, the gradual exposure to increasingly saline water may allow for some level of acclimatisation (Atwell *et al.* 1999). For example, the grass species *Panicum hemitomon* and *Leersia oryzoides* occurring in the freshwater marsh communities in coastal Louisiana were found to be relatively salt tolerant when allowed to osmotically adjust at low salinities for a week prior to immersion in higher salinities (McKee and Mendelssohn 1989). As such, future experimental designs should consider replicating the gradual shift from fresh water to increasingly saline water in order to assess the ability of *Nymphaea* to osmotically acclimatise.

Flooding by salt water can act to change soil redox potentials in anaerobic soils, mobilising the production of hydrogen sulphide and ammonium which can cause plant mortality at high concentrations (McKee and Mendelssohn 1989; Flynn *et al.* 1995). As such, the impact of flooding with saltwater should be investigated in a range of coastal wetland soils from northern Australia in order to understand the full ecosystem ramifications of intrusion events.

Implications for conservation and conclusion

While this study is not comprehensive for all Australia *Nymphaea*, (or within a range of genotypes of individual species) and is not inclusive of factors other than increasing sodium chloride concentrations, it does provide some insight into the susceptibility of *Nymphaea* to the risks associated with saltwater intrusion. Four of the species tested here demonstrate a 50 % reduction in germination and early seedling size at salinities in excess of 200 mM, and for the vast majority of species and accessions tested, a significant decrease was also detected at salinities ≥ 50 mM. As such, even small increases in the salinity of coastal freshwater ecosystems in northern Australia are likely to result in a decrease in seed germination and seedling recruitment. Therefore, other conservation measures, both *in situ* (e.g. physical restriction of saltwater inundation) and *ex situ* (e.g. seed storage) need to be considered for the ongoing maintenance of susceptible *Nymphaea* populations. This study also serves to demonstrate the significant lack of understanding we have on increasing salinity in tropical, freshwater aquatic species in Australia and highlights the need for further study on these, and other species and ecosystems at risk from saltwater intrusion events.

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CHAPTER 7

GENERAL DISCUSSION

*"The highly nutritious seed heads of the three water lilies (*Nymphaea *spp.),* ***andem****,* ***yalgey*** *and* ***marjarkarlang****…which were major staple foods, were probably even more important to the traditional economy than yams"*

- Authors Sally Brockwell, Robert Levitus, Jeremy Russell-Smith and Peter Forrest discussing the importance of *Nymphaea* as 'bush tucker' to Aboriginal Australians inhabiting Kakadu National Park (Brockwell *et al.* 1995).

INTRODUCTION

The importance of freshwater wetlands and the services they provide to the global community cannot be overestimated (Chapter 1). However, threats from a myriad of sources (Chapter 1) are acting to decrease the number of these precious ecosystems, which subsequently reduces the diversity, abundance and distribution of the freshwater plant species that occupy them. While conserving species *in situ* is the priority for many conservation biologists, the ever-diminishing quantity of suitable habitat furthers the need for *ex situ* conservation for many at-risk species. However, a thorough understanding of individual species biology, including reproductive biology, is essential in order to ensure the success of such measures.

Northern Australia's wetlands are some of the most pristine and distinctive freshwater systems on the planet. These monsoonally driven environments are characterised and shaped by extremes in climate – torrential cyclonic rains and high temperatures dominate the summer, while drought and fire typify the long, warm winters (Chapter 1). As such, the freshwater biota of the region is uniquely adapted to the ever changing 'boom' and 'bust' of water availability. Yet, despite these adaptations to the harsh natural environment, rising pressures from increased urbanisation, pastoralism, introduced species and climate change, threaten to reduce the capacity of these wetlands to support aquatic biodiversity (Kennard 2011).

Water lilies (*Nymphaea*) are an important component of the northern Australian wetlands, and are valued not only ecologically, but also culturally and socially (Chapter 1). As such, their maintenance and management is vital, given the increasing risk of large-scale habitat degradation. In this thesis, I aimed to better understand the reproductive biology of Australian *Nymphaea* and develop appropriate *ex situ*

conservation strategies, in case they be required. Specifically, I aimed to address the following:

1. Characterise and investigate seed dormancy in representative species of Australian *Nymphaea.*
2. Examine the optimal conditions (temperature, light, germination stimulant) for successful seed germination.
3. Investigate methods of overcoming dormancy *ex situ* and under natural conditions and understand natural seed bank persistence.
4. Evaluate seed desiccation tolerance and seed storage behaviour to suggest the most effective method of storing seeds *ex situ.*
5. Assess the potential implications of climate change on seed germination of a number of species by evaluating the relative impact of increasing sea level rise on seed germination and early seedling vigour.

Additionally, I aimed to address whether different seed accessions of the same species would respond similarly to *ex situ* germination testing and storage conditions and to assess any inherent variability between seed collections. As such, where possible, multiple accessions of a single species were included in all experiments. These aims were addressed experimentally in sequence through Chapters 3- 6 of this dissertation.

In Chapter 1, I provide an extensive analysis of the current state of knowledge surrounding four key areas, which are central to the themes covered in this thesis, and the wider implications of this research to conservation and management of aquatic plants:

1) Wetlands – I address their classification, hydrology, flora and threats at a global scale, and then specifically focus on the wetlands of northern Australia. The first section of the general introduction aims to provide a global and regional context for the thesis, and treats wetland flora holistically in conjunction with wetland functioning.

2) The genus *Nymphaea* – Part 2 of the general introduction provides an overview of the genus *Nymphaea*. The first and only assessment of the genus as a whole is the monograph published in 1905 by Henry Conard (1905). As such, this section of my thesis provides one of the most comprehensive, modern appraisals of the genus in relation to; (i) their position as basal angiosperms, (ii) the current taxonomy of the genus, (iii) the world-wide distribution of the genus, including the most up-to-date species distribution list (Appendix 1) and the first review to reveal Australia's prominence as a centre of endemism and diversity, (iv) an assessment of flowering and pollination strategies as a basis for the study of seed biology, (v) common dispersal mechanisms, (vi) their ethnobotanical significance, particularly with respect to indigenous Australians, (vii) horticulture, and (viii) the conservation status of the genus as a whole.

3) Seed dormancy and germination - synthesises the current understanding of seed biology and its evolution (in specific relation to aquatic and basal angiosperms), offers the most likely types of dormancy in the genus *Nymphaea* and hypothesises about the potential climatic and ecological controls of seed germination in tropical aquatics.

4) *Ex situ* seed storage and seed longevity – I assess the literature for information regarding the *ex situ* seed conservation of aquatic species, and their likely longevity in storage.

Chapter 2 offers a description of the study species used in this dissertation, and general methodologies for seed collecting and handling. *Nymphaea* occurring in Australia show a large degree of phenotypic plasticity, and the intention of this chapter is to provide images of the plant population each main seed collection was taken from, and the botanical identification I have assigned. In the case of misidentification or change in nomenclature, this chapter will provide a reference for future readers.

SUMMARY OF FINDINGS

This main body of this thesis represents the first comprehensive evaluation of dormancy and germination biology of tropical *Nymphaea*. Additionally, it is the first assessment of the amenability of *Nymphaea* seeds to *ex situ* seed banking and storage, and the first to provide evidence of the need for *ex situ* conservation, due to the impacts of rising sea levels associated with global climate change.

Classification of seed dormancy

Chapter 3 addressed the first two aims, and sought to classify the type of dormancy possessed by Australian members of the Nymphaeaceae, and to understand which environmental conditions (light availability, temperature and the action of germination stimulants) were most critical to dormancy break and germination. The findings from this chapter are summarised in Table 7.1, and represents the largest study of its type in the Nymphaeaceae. Seeds of six species of *Nymphaea* (*N. immutabilis, N. lukei, N. macrosperma, N. ondinea N. pubescens,* and *N. violacea*) were found to not possess physical dormancy, as all species readily imbibed water, and nicking of the seed coat did not significantly increase the total percent water uptake. Examination of the embryos of each species revealed them to be broad in shape and differentiated, with rudimentary radicle and cotyledons present; findings supported by the current body of literature (Martin 1946; Schneider and Ford 1978; Baskin and Baskin 2007; Baskin and Baskin 2014). However, upon measuring changes in embryo size prior to germination, embryos of *N. immutabilis, N. lukei, N. macrosperma, N. ondinea,* and *N.violacea* increased in size on average by 22 % prior to radicle emergence. Additionally, total germination after 28 days incubation at an optimal temperature (30- 35 °C) resulted in ≤ 60 % germination across all species. As such, these species are considered to have morphophysiological dormancy (MPD), as described by Baskin and Baskin (2004; 2014). Therefore, this is the first conclusive evidence to suggest the presence of MPD within the *Nymphaea*, the Nymphaeaceae, and in seeds with broad embryos and is new information for this basal angiosperm family. Current hypotheses predict that MPD is the basal trait within the angiosperms, and that PD and PY dormancy types are more derived (Forbis *et al.* 2002b; Willis *et al.* 2014). Given the placement of the *Nymphaea* within the ANA-grade angiosperms, the evidence presented here that several Australian *Nymphaea* possess MPD supports this hypothesis.

Another finding of this chapter was that the average embryo to seed ratio (E:S ratio) in Australian *Nymphaea* (0.23), is much larger than the average E:S ratio of the Nymphaeaceae as a whole (0.036) as reported by Forbis *et al.* (2002b). However, my findings are in congruence with the E:S ratios reported by Baskin and Baskin (2007) who also found E:S ratios between 0.16 and 0.3 for a number of *Nymphaea* species. Furthermore, inspection of fossilised embryos of the extinct *Susiea newsalemae* gen. et sp. nov. [Nymphaeaceae, most closely related to the extant genus *Euryale* (Taylor *et al.* 2006)], shows an embryo cavity, likely containing an embryo similar in proportion to the endosperm/perisperm to the findings of the present study, and Baskin and Baskin (2007). The study by Forbis *et al.* (2002b) predominantly used the drawings by Martin (1946) of the internal seed morphology of numerous species in order to calculate mean E:S ratios in the Nymphaeaceae. Martin's (1946) classification system however included the genera *Cabomba* and *Brasenia,* which are members of the Nymphaeales but belong to their own family, Cabombaceae. These data were then incorporated by Forbis *et al* (2002b) into the angiosperm phylogenetic tree and averaged across major families and clades, revealing that a small E:S ratio in the basal trait, while a large E:S ratio is more derived. However, Forbis *et al.* (2002b) do highlight the need to further investigate E:S ratios in a range of basal taxa, due to large variation within the group studied so far. Also, it should be noted that the number of *Nymphaea* included in the Martin (1946) publication is small, compared with the number of extant taxa. Future studies seeking to investigate the evolution of embryo size in the angiosperms should further investigate both extinct (from fossil evidence) and extant members of the *Nymphaea,* given this discrepancy.

In Chapter 3 of this thesis, I suggested that that members of the *Nymphaea* occurring in Australia may differ significantly in terms of optimal conditions for germination when compared with species from the northern hemisphere, due to the significantly different climatic conditions in northern Australia. Through germination studies under a range of temperatures, I found that seeds of *N. immutabilis, N. lukei, N. macrosperma, N. ondinea N. pubescens,* and *N. violacea* germinated optimally in a narrow temperature window between 30- 35 °C. Outside of this optimal range, germination decreased significantly, and was completely absent at temperatures ≤ 25 °C (Table 7.1). These temperatures are significantly higher than the global average of 24 °C due to the vast majority of these data being collected in the temperate regions of the USA and Europe (Baskin and Baskin 2014). A recent paper investigating the seed biology and

germination ecology of other wetland aquatics (*Eriocaulon Portulaca,* and *Myriophyllum*) occurring in the Kimberley region of Western Australia has also found that these species preferentially germinate at temperatures ≥ 30 °C (Cross *et al.* 2015). Therefore, given the distribution of *Nymphaea* across northern Australia, it is likely that the majority of these species will display a similar preference for high temperatures.

Additionally, seeds of the six species of *Nymphaea* did not show significant germination in the dark, and germinated preferentially when receiving 12 hours of light per day. Many seeds of aquatic species germinate to higher percentages when exposed to a period of light, when compared with complete darkness (Thompson and Grime 1983; Schütz and Rave 1999; Kettenring *et al.* 2006; Tuckett *et al.* 2010a; Carta *et al.* 2013; Cross *et al.* 2015). For example, the majority of species inhabiting ephemeral pools in Mediterranean (Tuckett *et al.* 2010a; Carta *et al.* 2013) and tropical systems (Cross *et al.* 2015) show significantly reduced germination when incubated in complete darkness. This preference or requirement for light can be attributed to the phytochrome system (Pons 1991), which acts to ensure seed germination occurs on or close to the soil surface, allowing a soil seed bank to accumulate until a disturbance event exposes seeds to the light (Grime 1981; Carta *et al.* 2013). Furthermore, this mechanism may act to inhibit germination at depths not conducive to seedling establishment, as red light, which acts to stimulate germination, does not generally penetrate to a depth greater than five meters (Atwell *et al.* 1999). Therefore, it would seem likely that the majority of Australian *Nymphaea* would have a similar light requirement.

One interesting finding was that these species of *Nymphaea* also responded to the seed 'crowding' phenomenon as reported by Else and Riemer (1984) (Fig. 1). The crowding treatment, whereby ≥ 50 seeds are placed in a small vial filled with water, was by far the most successful germination treatment tested in this study. Else and Reimer (1984) attributed the success of this treatment to the endogenous production of ethylene, which acts as a germination stimulant in some species, including *N. odorata*. Using four (*N. immutabilis, N. lukei, N. macrosperma* and *N. violacea*) species from this study, I sought to investigate whether seeds of Australian *Nymphaea* endogenously produced ethylene.[1]

[1] Vials were placed in a 35 °C incubator and the headspace sampled daily for a period of two weeks. Subsequent GC- FID analysis of the headspace gasses produced under these crowded conditions from seeds of four species of Australian *Nymphaea* confirmed that ethylene was being produced by the seeds, however due to time constraints, this was not included in this thesis at the time of submission.

Table 7.1: summary of dormancy classification and germination characteristics of the six species of *Nymphaea* tested in this thesis. Under optimal germination stimulating treatment 'Crowd' refers to the crowding treatment investigated in Chapter 3 of this thesis.

Species	Seeds readily imbibe water?	Does precision nicking increase germination ?	Embryo shape	Mean fresh seed E:S raio	Differen -tiated embryo ?	Embryo growth prior to radicle emergence?	Dorma -ncy type	Optimal temperature for germination (°C)	Optimal germinatio n stimulating treatment	Greater germinatio n response in light vs. dark	Variation between accessions ?
Nymphaea immutabilis	Y	N	Broad	0.19	Y	Y	MPD	30-35	Crowd	Light	N/A
Nymphaea lukei	Y	N	Broad	0.23	Y	Y	MPD	30-35	Crowd	Light	Y
Nymphaea macrosperma	Y	N	Broad	0.22	Y	Y	MPD	30-35 [b]	Crowd [b]	Light	Y
Nymphaea ondinea	Y	N	Broad	0.29	Y	Y	MPD	30-35	Crowd	Light	N/A
Nymphaea pubescens	Y	N	Broad	0.20	Y	N/A [a]	PD [a]	N/A [a]	N/A [a]	N/A [a]	N/A
Nymphaea violacea	Y	N	Broad	0.23	Y	Y	MPD	30-35	Crowd	Light	Y

[a] *Nymphaea pubescens* did not germinate under any conditions tested in this thesis. As such, embryo growth was not observed and the optimal conditions for germination were not resolved.

[b] One accession of *Nymphaea macrosperma* (NM1) was less dormant than the other accessions tested and germinated over a wider range of temperatures, with and without the addition of a germination stimulant. As such, this optimal temperature and treatment is the average treatment across the three accessions tested.

Figure 7.1: Seeds of *Nymphaea violacea* displaying the 'crowding' phenomenon. On the far right, 10 individual seeds have been placed in 5 mL of water (no germination), while on the far left, 300 seeds have been placed in 5 mL of water (98 % germination).

Ethylene is produced endogenously by the embryonic axis in non-dormant embryos of a number of agriculturally important plant species, including *Chenopodium album* (Saini *et al.* 1985; Saini *et al.* 1987; Matilla 2000). Furthermore, a number of species including *Tageles erecta, Raphanus salivus, Tnticum aestivum* and *Catharanthus roseus* have been shown to require endogenously produced ethylene for germination to proceed (Lalonde and Saini 1992). Also, this endogenous production of ethylene can also act to stimulate germination in dormant neighbouring seeds of *Oryza sativa* a process termed promotive alleleopathy Takeuchi *et al.* (2001).

Ethylene can also be produced in permanent and ephemeral wetlands by microbial activity in anoxic sediments, particularly those high in organic matter (Esashi and Leopold 1969; Smith and Russell 1969a; Smith and Restall 1971; Abeles 1973; Devai and Delaune 1996; Cross *et al.* 2014). Ethylene production in short-lived tropical rock pool soils has been shown to occur in conjunction with periodic inundation or wetting events, followed by an increase in germination from the sediment seed bank of a number of aquatic plant species (Cross *et al.* 2014). However, when compared with permanently inundated wetland soils (Smith and Restall 1971), ethylene production in these extremely shallow and transient systems is low. It has been hypothesised that the stimulatory effect of ethylene on aquatic seed germination may act to detect suitable water levels for subsequent seedling establishment (Baskin and Baskin 2014; Cross *et al.* 2014). However, given that *Nymphaea* occur reasonably permanent wetlands (for at least five months of the year), the timing of germination to inundation events is still important but less critical when compared with aquatic species in rock pool communities. Therefore, I propose that ethylene may also serve to act as a 'sediment

detection system' for seeds of *Nymphaea,* ensuring seed germination does not occur in the water column and instead occurs in the presence of ethylene producing soil, in humus-rich microsites, allowing seedlings to take root and establish. Given the potential for endogenous (by seeds) and exogenous (by wetland sediments) ethylene production, both may be hypothesised to both play a role (in combination with temperature and moisture cues), in the dormancy break and germination stimulation of Australian *Nymphaea* (Fig. 7.2).

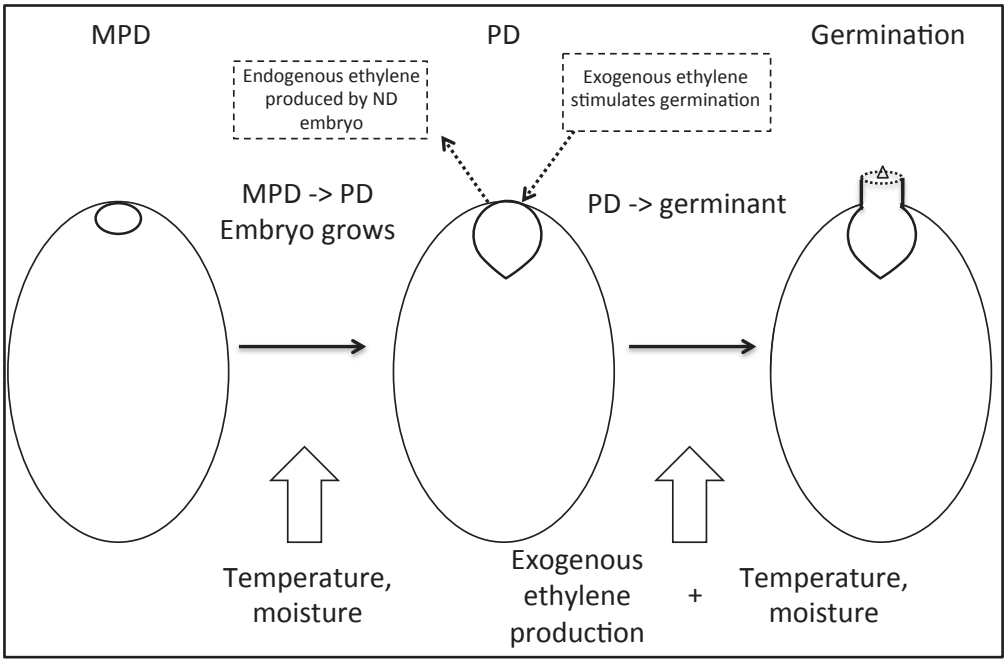

Figure 7.2: Hypothesised physiological mechanisms involved in overcoming MPD in seeds of Australian *Nymphaea.* The correct combination of temperature and moisture cues allow embryo growth to occur, while the combination of exposure to ethylene (either endogenously produced by non-dormancy seeds, or exogenously produced by microbial activity in anaerobic, waterlogged soils) and the optimal temperature and moisture conditions overcome PD and promote germination. Smaller dashed arrows and boxes (endogenous ethylene production) represent a far smaller input compared with exogenous ethylene inputs (larger arrows).

Additionally in Chapter 3, I also investigated whether different seed collections (accessions collected from different locations or from the same location at a different time) responded differently *ex situ*, with regard to germination. While conforming to broad patterns in germination response (i.e. seeds generally germinated at temperatures between 30- 35 °C), subtleties and complexities between accessions of *N. lukei, N. macrosperma* and *N. violacea* were found. For example, viable *N. violacea* NV1 (collected in Western Australia in May 2011) did not germinate under any conditions, while similarly viable *N. violacea* NV5 (collected in the Northern Territory in June 2011) germinated at temperatures between 25- 40 °C in combination with a range of germination stimulants.

There is increasing acceptance within the field of seed biology that different populations of the same species, distributed through space or time, may produce seeds with differing levels of dormancy and specific germination requirements (Andersson and Milberg 1998; Donohue 2009; Baskin and Baskin 2014). For example, different populations of the same species may be more or less dormant (Roach and Wulff 1987), show different patterns of germination (Andersson and Milberg 1998) or differ in their response to the application of germination stimulants (Tieu *et al.* 2001; Gorecki *et al.* 2012). These differences may be attributed to the maternal/offspring genotype or phenotype, in combination with environmental factors such as precipitation, elevation, latitude, competition and soil nutrients, amongst others (Roach and Wulff 1987; Baskin and Baskin 2014). As such, this natural variation is important to take into consideration when working with seeds of *Nymphaea*, especially for the purposes of *ex situ* conservation. Future studies in this field should consider testing multiple accessions from a number of collection locations in order to fully understand the breadth of variability within a given species.

Defining environmental factors for overcoming seed dormancy

In Chapter 4 of this thesis, I expanded the investigation of temperature and water availability as the critical driving factors behind dormancy loss and germination, in order to understand how these processes occur *in situ*, thus addressing my third overall aim.

Relatively few studies have focused on the dormancy break and germination requirements of seeds in the wet- dry tropics, and fewer still on floating-leaved aquatic macrophytes (Baskin and Baskin 2014). The wet-dry tropics of northern Australia are characterised by hot wet summers and warm dry winters (McQuade *et al.* 1996; Bowman *et al.* 2010). In this region, many ephemeral wetlands remain completely dry for the 8- month 'dry' period, and perennial wetlands experience a significant reduction in their total water budget (Finlayson and van der Valk 1995; Finlayson and Woodroffe 1996). As such, the periodicity of precipitation and availability of water becomes an important limiting factor to dormancy break, successful germination (Cross *et al.* 2015) and subsequent seedling establishment for aquatic plant species in northern Australia.

For *Nymphaea* species occupying ephemeral wetland systems, flowering and fruit set occur in conjunction with water availability [e.g. *N. lukei* and *N. ondinea* (Jacobs and Hellquist 2011)], with non-dormant seeds germinating and establishing rapidly (Chapter 3). For species occupying permanent wetlands, flowering and fruit set generally occurs during the dry season. Dormant seeds remain in the soil seed bank and experience decreasing overnight temperatures, and an increase in the diurnal temperature range though the dry wet season (Chapter 4). Once the wetland is dry (or the perimeter is dry in perennial systems), dormant seeds may further after-ripen in the soil seed bank over the winter period (e.g. *N. lukei,* DAR, Chapter 4). Overnight temperatures start to rise during spring (September), which may potentially cause a period of warm stratification in the soil seed bank. Sporadic rainfall may start to occur as early as October, however reliable rainfall does not occur until December (Garnett and Williamson 2010), hence creating a series of wetting and drying events within the wetland (results of WDC- *N. lukei* and *N. violacea* NV1, Chapter 4). By this time, seeds are predicted to be relatively non-dormant (seed burial trial, Chapter 4), and as wetland soils become waterlogged, ethylene is produced and germination stimulated (Fig. 7.3 and Chapter 3).

In order to understand the optimal temperatures of overcoming dormancy in Australian *Nymphaea,* I chose to firstly to assess temperature records for all collection sites in Australia during the month of collection (Bureau of Meteorology 2005, 2011), in conjunction with an experiment testing the germination response of the widely distributed *N. violacea* to alternating day and night temperatures (on a gradient between 0- 40 °C).

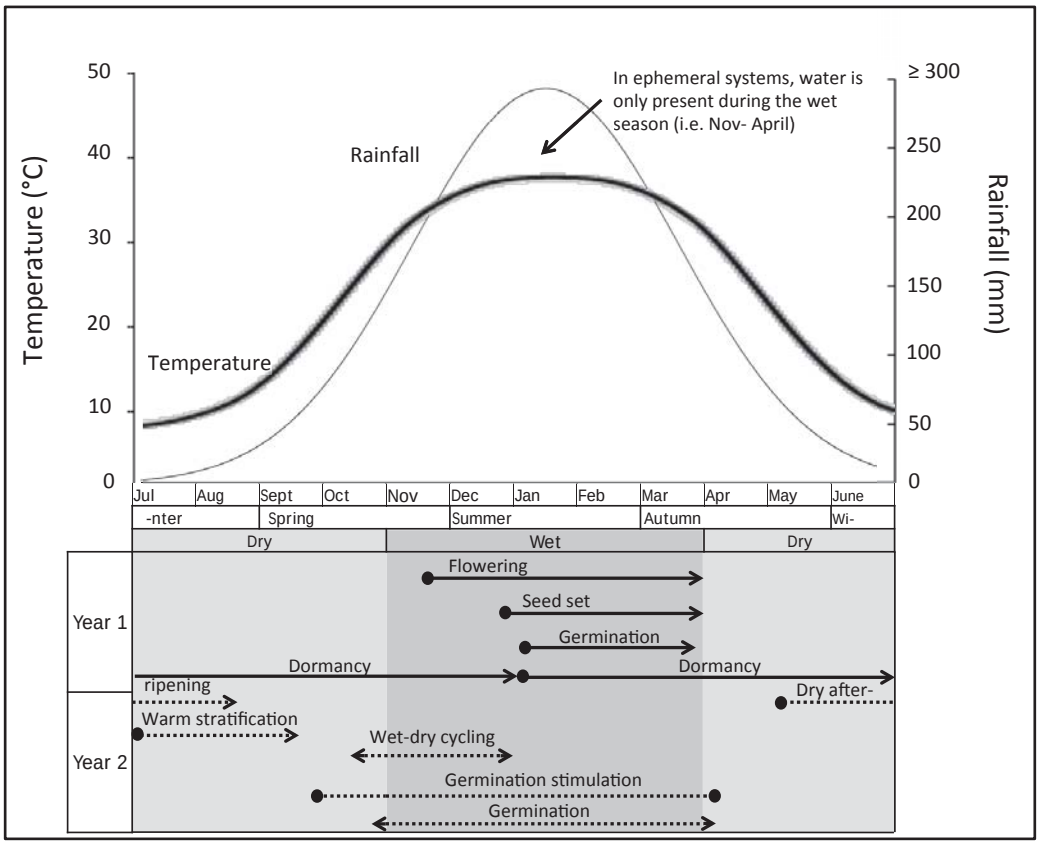

Figure 7.3: Potential progression of dormancy loss in Australian *Nymphaea*, from the time of flowering and seed set in the first year, to germination in the second year. In ephemeral wetlands, *Nymphaea* species occupying ephemeral wetlands flower during December to March, which is followed by fruit set and germination of non-dormant seeds. Dormant seeds settle in the sediment seed bank. During this time, it can be hypothesised that seeds experience a period of dry after-ripening as wetlands dry back (or in the case of perennial wetlands water levels recede, exposing the seed bank on the wetland margins). This may be followed by a period of warm stratification as temperatures begin to rise at the onset of spring. Intermittent and unpredictable rainfall often commences in October- November, causing a series of wetting and drying events in the sediment seed bank. As rainfall becomes more consistent and reliable, wetland water levels rise, sediments become waterlogged and anoxic, and ethylene produced from microbial activity is released into the soil, potentially stimulating germination in dormant *Nymphaea* seeds. *Nymphaea violacea* has been shown to have a relatively transient seed bank (Chapter 4 of this thesis) species has a transient seed bank, as a large proportion of seeds die during the first year, however under the right conditions, seeds can persist for > 12 months (P_{50} of 367 days). This finding further supports the hypothesised series of environmental cues controlling dormancy loss and germination, as germination and establishment are likely to occur in the first and second years following seed dispersal. Modified from Merritt *et al.* (2007).

The results from the latter showed that seeds germinated to high percentages when incubated at high temperatures (> 25 °C), which corresponds to temperatures experienced during the wet summer (December to March). Accordingly, I designed warm and cold stratification and move-along experiments (Baskin and Baskin 2003) to simulate the natural series of temperatures seeds of Australian *Nymphaea* would experience in the progression from summer to winter and *vice versa*. I also used the conventional technique of dry after-ripening (DAR) to mimic a potential period of winter drought, while cyclical periods of wetting and drying (wet/dry cycling = WDC (Hoyle *et al.* 2008a)) were used to mimic the sporadic and intermittent rains usually associated with the onset of the wet season (Bureau of Meteorology 2011).

Neither the stratification nor 'move along' experiments produced any significant germination, as germination across all treatments was < 5 %. As such, it can be conclusively stated that seasonal changes in temperature alone is not the critical factor influencing dormancy loss in seeds of Australian *Nymphaea*. The DAR and WDC treatments significantly increased germination in seeds of *N. lukei* (a short-range endemic of ephemeral creeks), with WDC completely overcoming dormancy after 4- 6 fortnightly wetting and drying cycles. However, both treatments failed to overcome dormancy or significantly increase germination in the other two species tested (*N. macrosperma* and *N. violacea*, widely distributed through the wet-dry tropics of Australia). Given these results, in combination with the results from Chapter 3, I conclude that the factors controlling dormancy and germination in these species are complex, and I propose that the interplay of multiple environmental cues, including exogenously produced ethylene, are likely to contribute to regulation dormancy loss and germination (Fig. 7.3).

The timing of inundation and flooding events also influences seed germination in temporary and ephemeral wetland systems (Casanova and Brock 2000; Brock *et al.* 2003; Deil 2005; Brock 2011; Carta *et al.* 2013). For example, the Palo Verde Marsh, an ephemeral coastal freshwater marsh system in Costa Rica found that seeds of *Nymphaea amazonum* emerged from the soil seed bank more readily under simulated drawdown conditions, when compared with completely flooded conditions (Osland *et al.* 2011). As such, future studies investigating the germination response of Australian *Nymphaea* should consider trialing experimental water levels including; i) intermittent

wetting and drying, ii) drawdown conditions and iii) flooding on sediment samples collected from a range of northern Australian wetlands. These inundation events should also be tested in conjunction with temperature regimes, depth (light) and ethylene, matching the natural fluctuations experienced at the onset of the wet season to adequately determine the combinational impact of temperature and flooding on seed germination and dormancy in Australian *Nymphaea*.

Seed storage behaviour

The evolution of the ability of seeds to desiccate to low internal moisture contents (and thus be amenable to *ex situ* storage) has been discussed widely in the literature. It has been proposed that the evolution of this trait is basal within the angiosperms (Dickie and Pritchard 2002; Tweddle *et al.* 2003), and recent evidence showing that seed of the basal genus *Trithuria* (Hydatellaceae) are tolerant of drying, support this hypothesis (Tuckett *et al.* 2010a). Given the placement of the Nymphaeaceae within the ANA-grade angiosperms (Qiu *et al.* 1999), it would likely follow that *Nymphaea* would also be tolerant of desiccation, although previous investigations have shown that *N. alba* and *N. odorata* are highly sensitive to desiccation (Else and Riemer 1984; Smits *et al.* 1989). However, taking into account the genus' distribution through many transient wetlands in Australia, and the results of dry burial from Chapter 3 (in which seeds were most persistent under dry conditions), I suggested that Australian *Nymphaea* would be tolerant of desiccation to 3- 7 % internal moisture content (Chapter 4). Upon testing their tolerance to desiccation, this study found that seeds of *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea* were able to survive drying to an internal moisture content of approximately 5 % with no loss in viability. Therefore, these species are desiccation tolerant, and this further supports the hypothesis that desiccation tolerance is the basal trait.

Prior to this thesis, no other studies have examined the seed storage behaviour in *Nymphaea*, beyond assessing desiccation tolerance. Testing of seed storage behaviour revealed *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea* to be orthodox, in that they were amenable to desiccation and survived subsequent low temperature storage (-20 °C and -160 °C). However, some complexities in their storage behaviour were revealed, as viability decreased significantly during time in storage under all experimental conditions (25, 5, -20 and -160 °C and RHs 15, 30, 50, 70 and 95 %),

suggesting seeds may be short-lived. The longevity of *N. immutabilis, N. lukei,* and *N. macrosperma* seeds in rapid ageing experiments (comparative longevity) was extremely short, with P_{50} values of < 1 day. While viability was more consistently maintained in seed of *N. violacea*, their longevity in storage was also short, and variable between accessions with P_{50} values ranging between 5 and 25 days. In comparison with a range of data sets investigating the comparative longevity of seeds globally (Probert *et al.* 2009; Hay *et al.* 2010; Tuckett *et al.* 2010b; Mondoni *et al.* 2011; Merritt *et al.* 2014b), *Nymphaea* are relatively short-lived in storage. As such, if *ex situ* storage for Australian members of the *Nymphaea* is deemed necessary, further investigations should focus on the cause of this rapid viability decline and the development of improved storage procedures to increase longevity.

Preliminary results I have obtained from the assessment of fatty acid constituents of *N. immutabilis* and *N. lukei* have shown that the ratio of C14/C10 saturated fatty acids exceeds 7.6 (Table 7.2, data not presented in the body of this thesis). Ratios > 2 have previously been shown to cause damage during sub-zero temperature storage of *Cuphea* species (Crane *et al.* 2003; Crane *et al.* 2006). In order to overcome triacylglycerol crystallisation issues in *Cuphea*, seeds can be warmed to 45 °C immediately after being removed from cold storage. Therefore, future studies investigating the storage behaviour of *Nymphaea* should consider an assessment of the seeds' fatty acids in order to determine whether this is the cause of viability decline and determine whether similar rewarming procedures may overcome this issue.

Given the short-lived nature of seeds of *Nymphaea,* investment should be made in the manufacture of appropriate seed cryopreservation protocols, with the addition of cryoprotectants or stepwise cooling procedures. For example, many orchid (Orchidaceae) species display a similar response to seed storage as *Nymphaea,* in that they are orthodox but short-lived under conventional seed banking conditions. A number of methods including vitrification, encapsulation-dehydration and encapsulation-vitrification of seeds, originally produced for desiccation-sensitive material, have been successfully employed for the *ex situ* conservation of some short-lived orchid species (Sommerville *et al.* 2008; Hirano *et al.* 2009; Galdiano *et al.* 2012; Gogoi *et al.* 2013; Merritt *et al.* 2014a).

In this study, accessional variation in seed longevity was observed between the three collections of *N. violacea* (NV8, NV9 and NV10). Similar variation in seed longevity has been reported for a number of terrestrial Australian species [*Minuria* (Asteraceae), *Plantago* (Plantaginaceae) and *Wahlenbergia* (Campanulaceae) (Kochanek *et al.* 2009)], in which σ for *Plantago* and P_{50} for *Wahlenbergia* increased with increasing annual rainfall at the site of collection (Kochanek *et al.* 2009; Kochanek *et al.* 2010). As such, further investigation is required to determine whether environmental conditions experienced by the maternal population at time of collection are responsible for the differences between accessions in terms of longevity in *Nymphaea.* Furthermore, the results from the comparative longevity experiment presented in this thesis are likely to have been affected by seed dormancy, i.e. low germination producing low K_i values. Therefore it is likely that seed viability has been underestimated in this experiment and future assessments of comparative longevity should be made on a combination of germination and viability data.

In Chapter 5, I have also reported that seed moisture sorption isotherms are relatively sigmoidal in shape. However, testing below 15 % eRH is required in order to confirm this statement, particularly given that the shape of this relationship has been hypothesised to have some bearing on the amenability of seeds to desiccation and storage (Vertucci and Leopold 1987). For example, sorption isotherms of orthodox seeds are sigmoidal in shape, whereas recalcitrant seeds produce a curve more hyperbolic in shape (Leopold and Vertucci 1986; Vertucci and Leopold 1987).

Recent publications (Merritt and Dixon 2011; Walck *et al.* 2011; Hay and Probert 2013) have highlighted the need for increasing the capacity of restoration and conservation seed banks for wild plant species, particularly given the impacts of global climate change. However, there are obvious difficulties when working with wild seeds in that viability is often difficult to predict and their seed biology and storage behaviour is often completely unknown (Fig. 7.4). Furthermore, the issues of dormancy and successful germination significantly impact on our ability to assess the effectiveness such conservation measures.

Table 7.2: Total percent fatty acid analysis using GC-MS. Lipids were extracted from seeds of *N. immutabilis* and *N. lukei* using the modified Blight and Dyer extraction protocol outlined in Merritt *et al.* (2005). C10 = Decanoic acid, C14 = Tetradecanoic acid, C15 = Pentadecanoic acid, C16:0 = Hexadecanoic acid, C16: 1, C17 = Heptadecanoic acid, unsaturated fatty acids are: C18 = Octadecanoic acid, C20 = Eicosanoic acid, C22 = Docosanoic acid, C23 = Tricosanoic acid and C24 = Tetracosanoic acid.

	C10:0	C14:0	C15:0	C16:0	C16:1	C16:1 2	C17:0	C18:0	C18:1	*C18:1	C18:2	C18:3	C20:0	C20:1	*C20:1	C20:2	C20:3	C22:0	C22:1	C23:0	C24:0
N. immutabilis	0.0	0.3	0.2	14.9	0.2	0.1	0.1	0.1	13.7	6.1	0.8	24.4	3.4	17.4	0.5	5.7	0.7	0.1	9.3	0.1	0.2	1.9
N. lukei	0.1	0.6	0.4	26.6	0.5	0.3	0.0	0.3	5.5	9.3	1.1	39.8	5.5	3.0	0.6	0.3	0.5	0.0	2.0	0.2	0.3	3.3

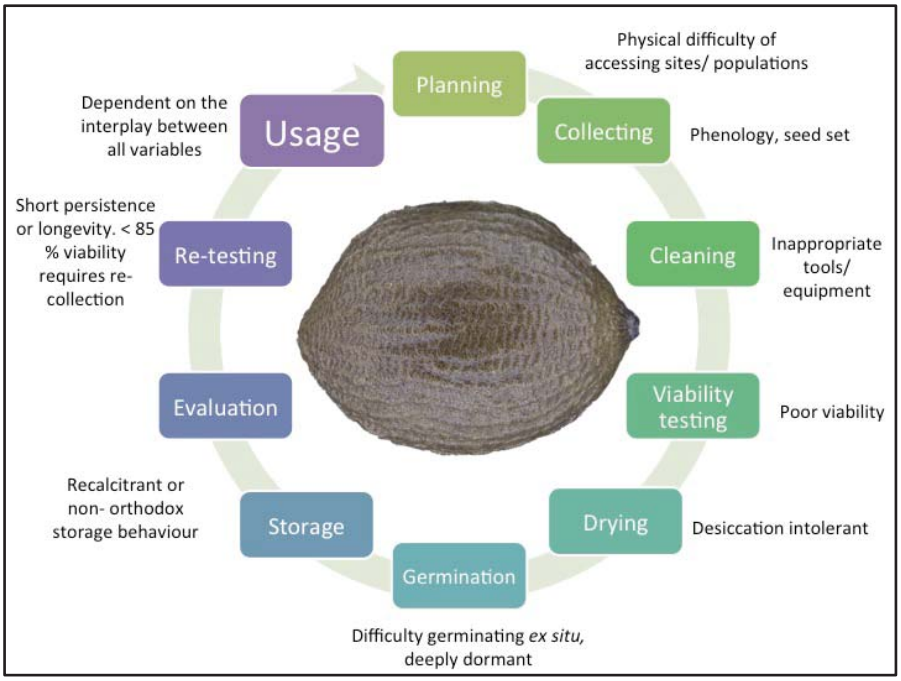

Figure 7.4: Steps involved in successfully banking seeds and some commonly encountered issues, particularly when working with wild species. Modified from (Terry *et al.* 2003; Merritt 2006).

Impact of increasing salinity on germination and growth

The final aim of this thesis was to "assess the potential implications of climate change on seed germination" of a number of Australian species of *Nymphaea*. In Chapter 6, I assessed the impact of increasing salinities on seed germination and early seedling vigour in four species of Australian *Nymphaea.* I found that salinities ≥ 192 mM NaCl reduced the overall germination, biomass and length of seedlings in *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea* by ≥ 50 %. Additionally, some variation was observed in the salinity tolerances of different accessions of *N. violacea*, with mean LC_{50} scores differing significantly (between 30.3 mM and 128.6 mM NaCl).

Given current predictions on the impact of climate change to the coastal wetlands of northern Australia, a rise in global sea levels is by far the greatest risk to these ecosystems (Eliot *et al.* 1999; BMT WBM 2010; Bowman 2010; Catford *et al.* 2013; Steffan *et al.* 2014). Intrusion of saltwater into the low-lying coastal floodplains and

wetlands has already been reported in the Northern Territory and the UNESCO world heritage listed Kakadu National Park (Knighton *et al.* 1991; Mulrennan and Woodroffe 1998; Winn *et al.* 2006). While in Kakadu National Park there have been efforts made to reduce the risk of saltwater intrusion through the construction of small dams and levees (Cowie *et al.* 2000a), substantial engineering works will be required if global sea levels continue to rise by the predicted > 80 cm by 2100 (Church and White 2011). Though perhaps even more at risk are the remote and less accessible wetlands in the Kimberley Region of Western Australia and in the Gulf of Carpentaria (NT and QLD), which remain unstudied. As such *Nymphaea* occupying many of these coastal floodplains and wetlands will be exposed to increasingly saline conditions. Given the results of this study, I can conclude that any significant increase beyond 50 mM NaCl in the wetlands of northern Australia will result in a reduction in the overall recruitment of *N. immutabilis, N. lukei, N. macrosperma* and *N. violacea*.

PRIORITIES FOR FUTURE RESEARCH

While this thesis has made a significant contribution towards our understanding of the seed biology of Australian *Nymphaea*, further research in several key areas is required to ensure the successful conservation of the genus:

Seed biology

Due to the logistical difficulties associated with collecting seeds, I was only able to assess the general seed biology in six of 18 native Australian *Nymphaea* species, representing three of six subgenera (*Anecphya, Confluentes* and *Lotos*) from the genus. As such, in order to conclude that all Australian *Nymphaea* possess MPD, future seed collecting efforts should focus on unstudied species, particularly those occurring in Queensland, which are underrepresented in this thesis. Furthermore, as discussed in Chapter 1, very little is known about the seed biology of the genus as a whole, particularly those occurring in the tropical regions of Central and South America. As such, future research endeavours should prioritise the collection of these species,

including targeted collections for the subgenera *Nymphaea, Brachyceras* and *Hydrocallis*.

Additionally, given the variation between accessions with regard to dormancy level and germination behaviour, multiple accessions need to be investigated in order to capture this naturally occurring variation. Also, studies should focus on the application of multiple dormancy breaking treatments, such as WDC in combination with temperature stratification in order to adequately mimic the shift from warm, dry winters through to hot, wet summers.

Seed storage behaviour

In order to optimise *ex situ* seed storage protocols (as mentioned previously in this chapter), further investigations into the seed storage behaviour of *Nymphaea* should focus on the following key research areas:

- Assessment of desiccation tolerance in other species within the genus to determine the proportion and distribution of *Nymphaea* displaying desiccation tolerance.
- Assessment of a broader range of accessions to determine whether maternal environment is responsible for variation in storage behaviour and longevity of different seed collections.
- Investment in the production of other cryogenic *ex situ* storage protocols such as vitrification and encapsulation- dehydration.

Impacts of increasing salinity levels on freshwater plants and wetlands

Given the range of impacts saltwater intrusion may have on low-lying coastal freshwater wetlands future experiments should also consider the timing, duration, frequency and intensity of salinization events along with the impacts of flooding and increased sedimentation (Flynn *et al.* 1995; Doering *et al.* 2001; Neubauer 2013) on all life stages of *Nymphaea,* including adult plants. These experiments may take place under laboratory conditions, however the use of experimental mesocosms and field sites (transplant experiments) is strongly encouraged. Furthermore, the installation of long-term monitoring sites in regions such as Kakadu National Park would allow for

vegetation changes associated with intrusion events to be monitored before, during and after salinization events.

Given the results of such investigations, the limits to distribution of *Nymphaea* under saltwater intrusion events should be well established. As such, the use of mechanistic modelling programs such as CLIMEX, may allow for the prediction of the spatial distribution of freshwater aquatics such as *Nymphaea* under simulated intrusion events (Sutherst 1999). Such information would be invaluable to land management agencies that manage wetlands in the wet-dry tropics of northern Australia, so that areas and species most vulnerable to salinization events may be identified early and appropriate control measures deployed.

Non-destructive viability testing

Given the issues associated with viability testing of wild seed collections, such as limited seed numbers and the lack of standard viability-testing protocols, it is important to stress the need to find a relatively simple and cost-effective way of accurately testing seed viability non-destructively. While tetrazolium staining can be employed successfully for some species, it is destructive, highly subjective and time consuming to develop an adequate protocol (Gosling 2003; Ooi *et al.* 2004). Furthermore, other methods of determining viability tend can also be destructive, inaccurate and time consuming (Table 7.3). The assessment of headspace volatiles produced by dead or damaged seeds appears promising (Colville *et al.* 2012), however its cost would be quite prohibitive to small scale research facilities. Therefore, investment should be made in order to develop, cheap, accurate, non-destructive methodologies or procedures. For example, whole seed respiration, whereby CO_2 production or O_2 consumption can be measured in real-time may, with technological refinement prove to be one such technology (Garwood and Lighton 1990; Agelet *et al.* 2012; Xin *et al.* 2013). Such technologies will be broadly applicable across all industries working with wild seed.

Table 7.3: Current methods commonly used to assess seed viability. Modified from Gosling (2003).

Test	Advantage(s)	Disadvantage(s)
Germination	• Direct measure of germination	• Time consuming • May exceed the longevity of some short-lived or recalcitrant species
Cut-test	• Quick result • Cheap	• Indirect measure of germination • Destructive • Subjective to interpretation
X-ray	• Quick result • Non-destructive • Useful for wild species which may produce unfilled, damaged or predated seeds	• Does not truly assess viability, just assesses seed fill or the presence of living/damaged tissues without the addition of contrast agents • Indirect measure of germination • 2D representation of 3D material
Tetrazolium staining	• Relatively quick result (1-3 days) • Suited to deeply dormant species	• Indirect measure of germination • Destructive • Subjective • A skilled interpretation of the results is required • Labour intensive • Requires dexterous surgical skills
Excised embryo	• Relatively quick result (1-2 wks) • Suited to deeply dormant species	• Indirect measure of germination • Labour intensive • Requires dexterous surgical skills

Taxonomy

Despite the increasing number of publications on the subject in the last decade (Borsch *et al.* 2007; Jacobs 2007; Löhne *et al.* 2008a; Löhne *et al.* 2009; Borsch *et al.* 2011; Jacobs and Hellquist 2011), the taxonomy and phylogeny of Australian *Nymphaea* is not particularly well resolved. During the course of this research, I have observed significant morphological variation within single populations of what appear to be the same species, along with a number of populations that do not fit the descriptions of the 18 currently known species (not included in this PhD). I therefore predict that there will be a number of new species of *Nymphaea* yet to be discovered or described in Australia. As such, I believe that significant population-level sampling and continent-wide sampling is required in order to fully comprehend the genetic diversity of *Nymphaea* in Australia.

Through the course of compiling this thesis, I have also taken a series of SEM images of the seeds of some *Nymphaea* species (Appendix 3), which may provide a diagnostic tool for species identification, as floral morphology is so variable. However, it use is yet to be confirmed

Biogeography and drivers of rarity and endemism

Several species within the genus, such as *N. lukei* and *N. ondinea* from the Kimberley region of Western Australia, are short-range endemics with restricted distributions. Given the increasing pressures on many freshwater systems, it is important to understand the reasons behind these species' limited distributions, so they can be best managed in the future. As such, I propose the following questions be investigated:

- Are short-range endemics intrinsically restricted, or have external pressures reduced their current distribution?
- What is the predicted distribution of these species after the Last Glacial Maximum (LGM)?
- Do endemic species have specific habitat requirements or are they only restricted by their capacity to disperse (i.e. restricted by the matrix of land between waterways)?

The Kimberley region is already regarded as a centre of endemism for several reptiles and mammals (Pepper and Keogh 2014), and increasingly so for its floral diversity (Barrett, R. and Barrett M. pers. comms.). These high rates of endemism can be attributed to the high heterogeneity of the landscape that is physically bounded to the north, south and west (Pepper and Keogh 2014). While aquatic plants are generally widespread (Santamaria 2002), the fact that these barriers are in place lends itself to the hypothesis that the Kimberley may also be a centre for endemism in the *Nymphaea*.

Seed dispersal

Reproductive propagules of aquatic species are predominantly dispersed hydrochorously by the downstream movement of water (Santamaria 2002). Seeds of *Nymphaea* have been show to disperse in this fashion via an air-filled aril surrounding the seed that provides buoyancy (Conard 1905; Smits *et al.* 1989). Preliminary data I obtained during the course of this study showed significant variation in the floating-time between species (Fig. 7.5). Seeds may also be distributed internally or externally via animals (Figuerola and Green 2002; Calviño-Cancela *et al.* 2007). Given the desiccation tolerance of Australian *Nymphaea,* epizoochorous dispersal is entirely feasible. Both findings lead to a suite of questions regarding the long-distance dispersal of these species, and the reason behind the limited range of some species.

Surveying and conservation

In Australia, the wetlands of the wet- dry tropics relatively unstudied, particularly in the remote Kimberley region of Western Australia and the Gulf of Carpentaria in north Queensland. This is mainly attributed to the isolation of the region, and its inaccessibility, particularly during the wet season. Equally, the majority of the world's *Nymphaea* species occur in emerging socioeconomic areas where the level of surveying is inadequate. As such, future research should focus on understudied regions where taxonomic resolution of the genus is poor in order to gain an adequate understanding of species richness and distribution.

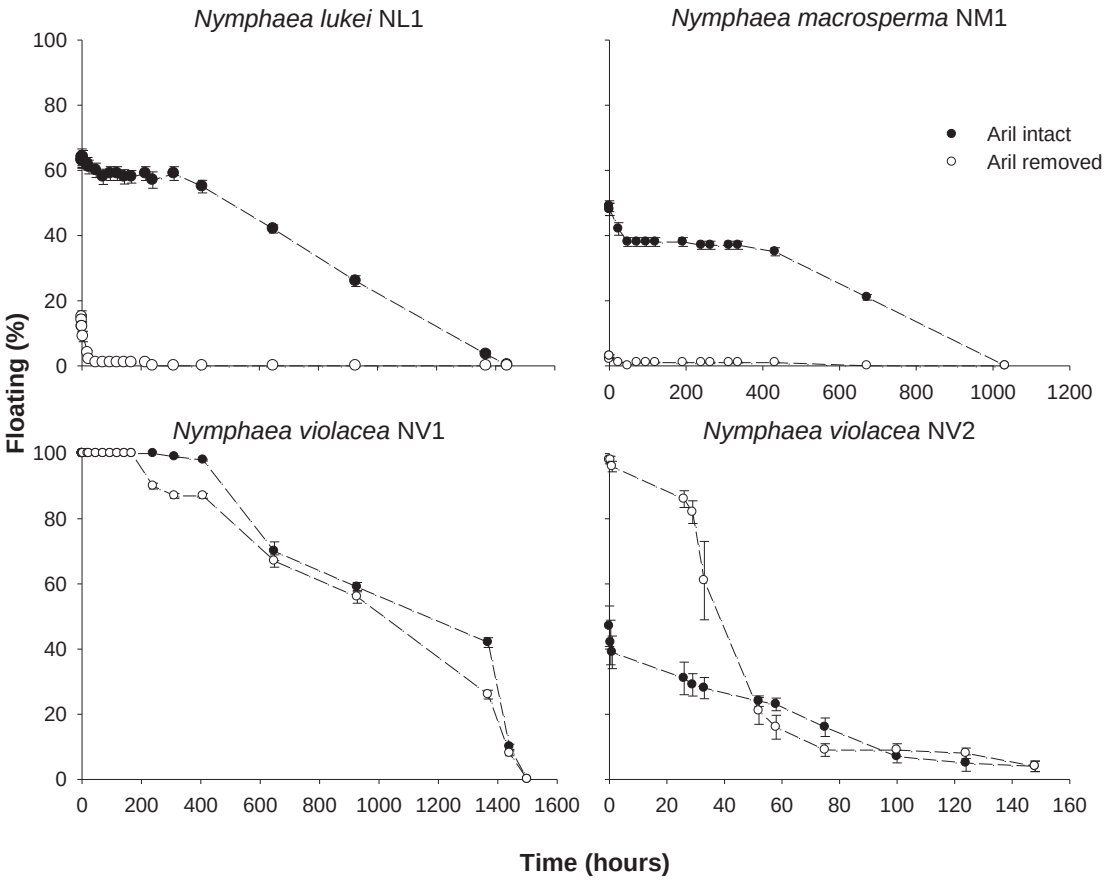

Figure 7.5: Percentage of *Nymphaea lukei, N. macrosperma* and *N. violacea* seeds floating in a 100 mL container of water, with and without the removal of the aril over time. The water in the containers was carefully stirred every day to break the surface tension and prevent seeds from resting against the sides of the container.

The use of unmanned aerial vehicles (UAVs) is becoming increasingly accessible to smaller-scale research facilities as costs are becoming less prohibitive. Such vehicles could allow for the remote collection of wetland imagery where access by land or foot is difficult or dangerous, which could be used to assess wetland size, and the abundance of floating-leaved macrophytes such as *Nymphaea* (Anderson and Gaston 2013).

Resources for managers, conservationists and the public

Current resources available to land-managers and the public about Australian *Nymphaea* are poor, and no comprehensive list of Australian species with images is available. Given the high levels of endemic *Nymphaea* in Australia, and the potential threats to northern Australia's wetlands, I believe the production of a publication (either in the form of a website, pamphlet or handbook) highlighting this information would be of use and interest to land managers, tourists and the general public.

CONCLUSION

Given the importance of freshwater wetlands and their vulnerability to increasing treats, it is timely that there be an increased focus on the conservation and management on these systems, including the aquatic plants that inhabit them. While waterlilies are cosmopolitan in distribution, and play important ecological, social and cultural roles in many countries worldwide, there has been little focus on these charismatic plants in the scientific literature. This thesis demonstrates the first comprehensive investigation into the seed biology of Australian native *Nymphaea,* their amenability to *ex situ* conservation and the potential risks they face from a changing climate. As such, this thesis provides crucial information for the effective conservation and management of this genus in Australia. However, as I have highlighted, more basic research is needed in order to ensure the success of such programs. While this thesis has specifically focussed on species occurring in Australia, I have also highlighted the lack of information and understanding of monsoonally driven freshwater ecosystems worldwide, and freshwater floating-leaved macrophytes that occupy them.

Australia is exceptionally fortunate to still have some of the most pristine, unique and intact natural wetlands remaining on the planet. Therefore, it is our responsibility to ensure their protection and management so that future generations may also experience the wonder and biodiversity of Australia's monsoon tropics.

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APPENDICES

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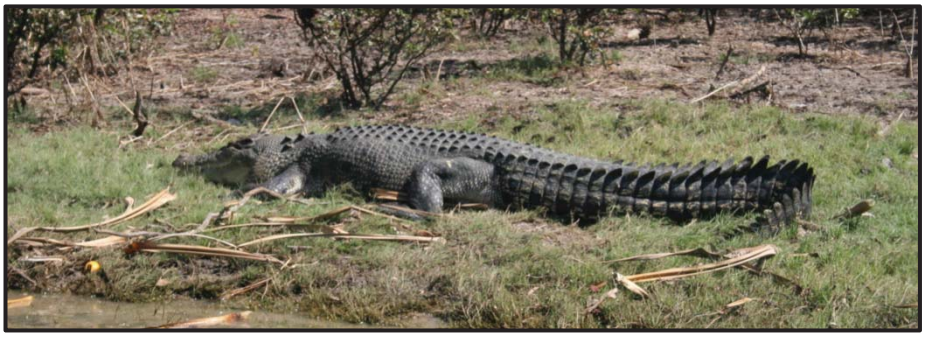

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APPENDIX 1

Current list of *Nymphaea* species and their global distribution

Preface

Currently, there is a no complete species list for the genus *Nymphaea,* and even less information is available about their worldwide distribution. In order to determine how Australia ranks in terms of species numbers and endemism, I have compiled the following lists to the best of my knowledge, given the information available to me at the time.

Table 1: Current species list for the genus *Nymphaea*. Information has been gathered from Löhne *et al.* (2008b) Jacobs and Hellquist (2006), Jacobs (2007), Jacobs and Hellquist (2011), the online database Germplasm Resource Information Network (USDA, ARS) and various other floras listed in Table 2, below. Subgenera are abbreviated as follows; Anecphya (A), Brachyceras (B), Confluentes (C) Hydrocallis (C), Lotos (L) and Nymphaea (N).

Species	Subgenus	Species	Subgenus
N. alba	N	*N. lingulata*	H
N. alexii	C	*N. lotus*	L
N. amazonum	H	*N. lukei*	C
N. ampla	B	*N. macrosperma*	A
N. atrans	A	*N. maculata*	u/k
N. belophylla	H	*N. malabarica*	H
N. caerulea	B	*N. mexicana*	N
N. calliantha	B	*N. micarantha*	B
N. candida	N	*N. minuta*	u/k
N. capensis	B	*N. muschleriana*	u/k
N. carpentariae	A	*N. noelea*	C
N. colorata	B	*N. nouchali*	B
N. conardii	H	*N. novogranatensis*	H
*N. daubenyana**	u/k	*N. odorata*	N
N. divaricata	u/k	*N. ondinea*	A
N. elegans	B	*N. ovalifolia*	B
N. elleniae	C	*N. oxypetala*	H
N. gardneriana	H	*N. pertersiana*	u/k
N. georginae	A	*N. potamophila*	H
N. gigantea	A	*N. prolifera*	H
N. glandulifera	H	*N. pubescens*	L
N. gracilis	B	*N. pulchella*	B#
N. guineensis	u/k	*N. pygmea*	u/k
N. hastifolia	C	*N. rubra*	L
N. heudelotii	B	*N. rudgeana*	H
N. immutabilis	A	*N. stuhlmannii*	B
N. jacobsii	A	*N. sulphurea*	B
N. jamesoniana	H	*N. tenerinervia*	H
N. kimberleyensis	A	*N. tetragona*	N
N. lasiophylla	H	*N. thermarum*	u/k
N. leibergii	N#	*N. vaporalis*	C
N. lekophylla	u/k	*N. violacea*	C

*Validity of this name is questionable
Validity of placement in this subgenus is questionable

Table 2: Current species distribution for the genus *Nymphaea*. Information has been gathered from Löhne *et al.* (2008b) Jacobs and Hellquist (2006), Jacobs (2007), Jacobs and Hellquist (2011), the online database Germplasm Resource Information Network (USDA, ARS), JSTOR plants, The Plant List and Tropicos. *Nymphaea alba* (alb), *alexia* (alx), *amazonum* (amz), *ampla* (amp), *atrans* (atr), *belophylla* (bel), *caerulea* (cae), *calliantha* (cal), *candida* (can), *capensis* (cap), *carpentariae* (car), *colorata* (col), *conardii* (con), *daubenyana* (dau), divaricata (div), *elegans* (ele), *elleniae* (ell), *gardeneriana* (gar), *georginae* (geo), *gigantea* (gig), *glandulifera* (gla), *gracilis* (gra), *guineensis* (gui), *hastifolia* (has), *heudelotii* (heu), *immutabilis* (imm), *jacobsii* (jac), *jamesoniana* (jam), kimberleyensis (kim), lasiophylla (las), *leibergii* (lei), *lekophylla* (lek), *lingulata* (lin), *lotus* (lot), *lukei* (luk), *macrosperma* (mac), *maculata* (mcl), *malabarica* (mal), *mexicana* (mex), *micarantha* (mic), *minuta* (min), *muschleriana* (mus), *noelea* (noe), *nouchali* (nou), *novogratinensis* (nov), *odorata* (odo), *ondinea* (ond), *ovalifolia* (ova), oxypetala (oxy), *pertersiana* (per), *potomophylla* (pot), *prolifera* (pro), *pubescens* (pub), *pulchella* (pul), *pygmea* (pyg), *rubra* (rub), *rudgeana* (rud), *stuhlmannii* (stu), *sulphurea* (sul), *tenerinervia* (ten), *tetragona* (tet), *thermarum* (the), *vaporalis* (vap) and *violacea* (viol). E= endemic, Ex= extinct, ? = questionable species location, *multiple subspecies, ** potential synonym of *caerulea* # hybrid % may be *micarantha* ^?, ^^? @ likely to be in other countries, ## maybe *nouchali*, $ potential synonym of *caerulea*, *** probably invasive elsewhere, red cells = alien species, green cells = native species, orange cells = naturalised species

Country	alb	alx	amz*	amp	atr	bel	cae	cal**	can	cap	car	col	con
Afghanistan													
Albania													
Algeria													
Angola													
Antigua and Barbuda													
Argentina													
Armenia													
Australia		E			E						E		
Austria									Ex				
Bahamas, The													
Bangladesh													
Belarus													
Belgium													
Belize													
Benin													
Bolivia													
Botswana													
Brazil													
Bulgaria													
Burkina Faso													
Burma													
Burundi													
Cambodia													
Cameroon													
Canada													
Cayman Islands													
Chad													
China													
Colombia													
Congo, DR													
Congo, Rep.													
Cook Islands													
Costa Rica													
Cote d'Ivoire													
Croatia													
Cuba													

Czech Republic													
Denmark													
Dominica													
Dominican Republic													
Ecuador													
Egypt													
El Salvador													
Eritrea													
Estonia													
Ethiopia													
Finland													
France													
French Guiana													
French Polynesia													
Gabon													
Gambia, The													
Georgia													
Germany													
Ghana													
Greece													
Grenada													
Guadeloupe													
Guatemala													
Guinea													
Guinea-Bissau													
Guyana													
Haiti													
Honduras													
Hungary													
India													
Indonesia													
Iran													
Ireland													
Israel													
Italy													
Jamaica													
Japan													
Jordan													
Kazakhstan													
Kenya													
Korea, North													
Korea, South													
Laos													
Latvia													
Lesotho													
Liberia													
Lithuania													
Madagascar										?			
Malawi													
Malaysia													
Mali													
Martinique													
Mauritania													
Mauritius										?			
Mexico													
Mongolia													
Montserrat													
Morocco													
Mozambique													

Namibia													
Nepal													
Netherlands													
Netherlands Antilles													
Nicaragua													
Niger													
Nigeria													
Norway													
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APPENDIX 2

Images of Australian wetland habitats

Preface

Though the course of this thesis, I frequently make reference to the diversity of wetland habitats in northern Australia. As such, I have compiled a series of images to demonstrate this diversity through the states of Western Australia, the Northern Territory and Queensland.

Kimberley Region of Western Australia

Plate 1: *Melaleuca viridifolia* fringing a small inland billabong containing *Nymphaea violacea*. Pool is fed from runoff from adjacent sandstone pavement. Water flowing out of this area forms a small, ephemeral creek.

Plate 2: Small, inland, sandstone based creek containing *Nymphaea ondinea*. Maximum depth 1.5m. Photo taken at the end of the wet season, March 2011.

Plate 3: Perennial inland billabong containing *Nymphaea violacea* fringed by *Melaleuca* and *Pandanus*

Plate 4: Perched inland billabong adjacent to the Morgan River, containing *Nymphaea violacea*

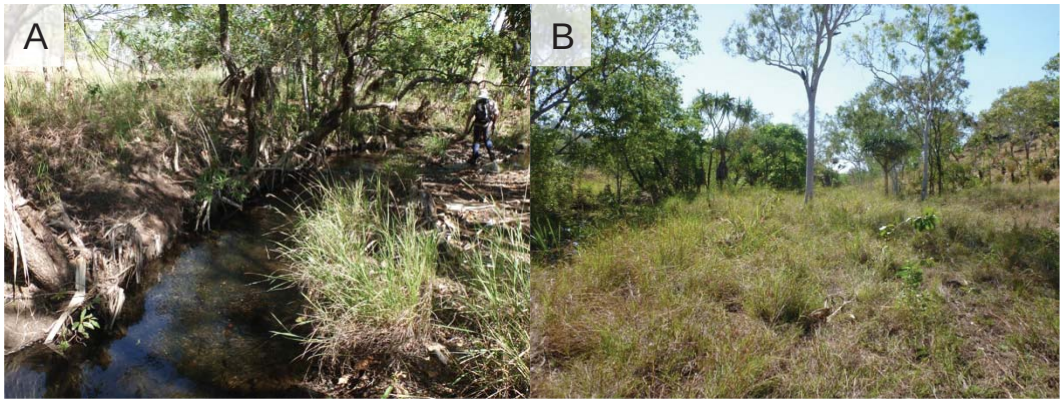

Plate 5: A) small, sandstone based creek and B) surrounding grassland and riparian vegetation including *Eucalyptus* and *Pandanus*. Contains *Nymphaea lukei*

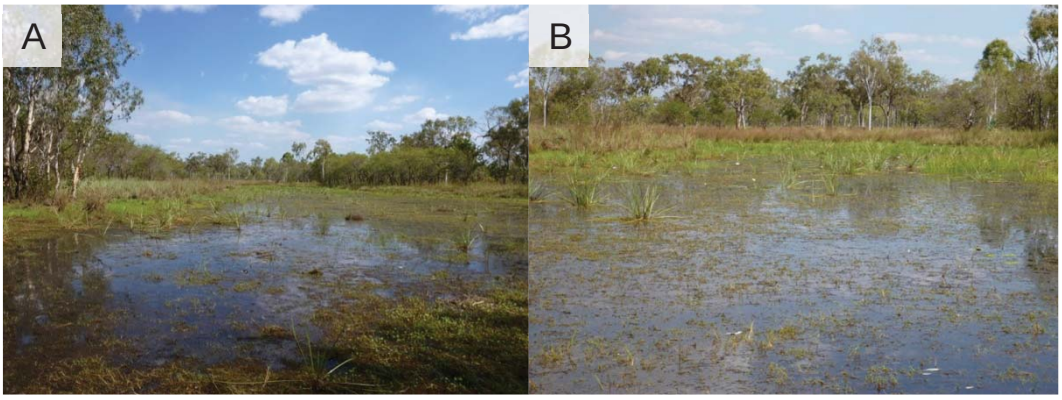

Plate 6: Perched black-soil depression (inland billabong) to the south of the Morgan River.

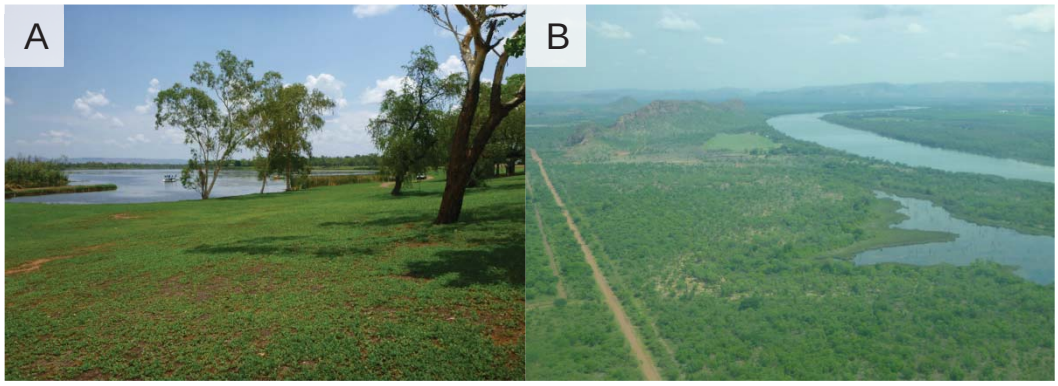

Plate 7: A) Lake Kununurra and B) Lake Kununurra, bottom right and the Ord River. Contains *Nymphaea violacea*.

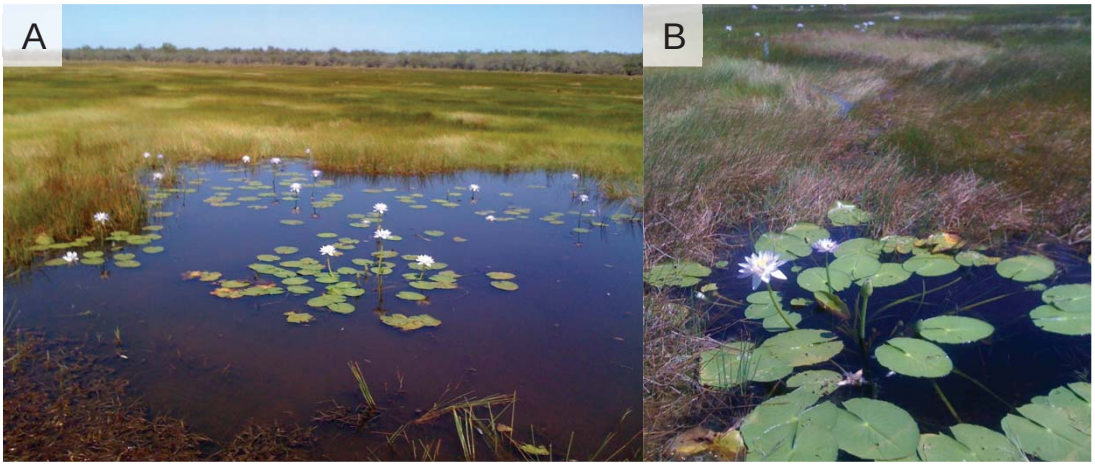

Plate 8: A) Lake Louisa, perched dune swamp containing B) *Nymphaea violacea*. Images courtesy of Kingsley Dixon.

Plate 9: Perched inland swamps, near Derby.

Plate 10: Sandstone based pool filled by a waterfall and creekline. Adcock Gorge. Contains *Nymphaea violacea* and *Nymphoides indica*.

Plate 11: Lake Gladstone, contained within a small reserve on Mount House station. Perched inland lake/ billabong. Contains *Nymphaea violacea* and an unidentified white *Nymphaea.*

Plate 12: Donkey Hole, Charnley River Station. Contains *Nymphaea violacea, N. lukei*.

Plate 13: Parry Creek Lagoon, Parry's Creek Nature Reserve. Contains *Nymphaea violacea* and *N. macrosperma.* Large coastal billabong.

Plate 14: Parry Creek Farm, large, open water coastal billabong, fringed with *Nymphaea violacea* and *N. macrosperma.*

Plate 15: small inland creek, recently burned, containing *Nymphaea violacea.*

Northern Territory

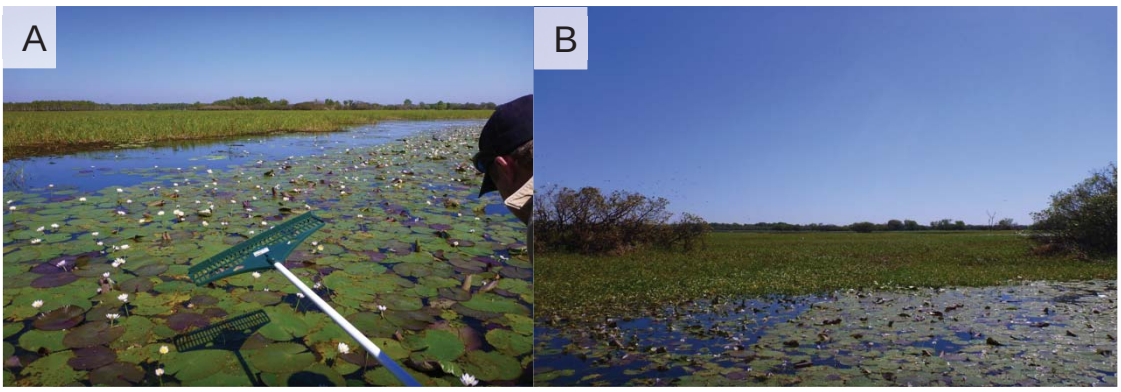

Plate 16: Yellow Water Billabong, Kakadu National Park. Large, coastal, open water billabong and floodplains. Contains *Nymphaea macrosperma, N. pubescens* and *N. violacea*.

Plate 17: Jim Jim Billabong, Kakadu National Park. Coastal billabong, containing *Nymphaea violacea* and *N. macrosperma*.

Plate 18: Large blacksoil billabong. Anbangbang Billabong, Kakadu National Park. Contains *Nymphaea violacea* and *N. macrosperma*.

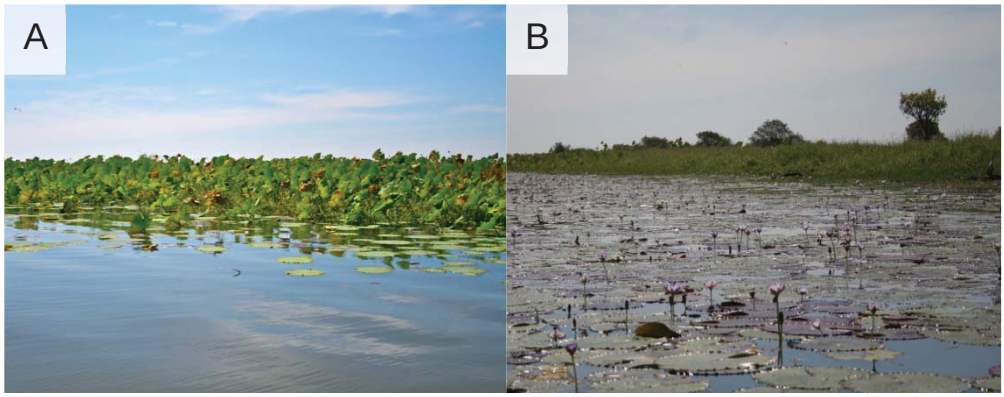

Plate 19: Corroboree Billabong, fringed by A) *Lotus* and B) *Nymphaea macrosperma* and *N. violacea*

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Plate 20: Roadside drainage ditch, near Mareeba Wetlands that had been recently burned. Contains *Nymphaea violacea*

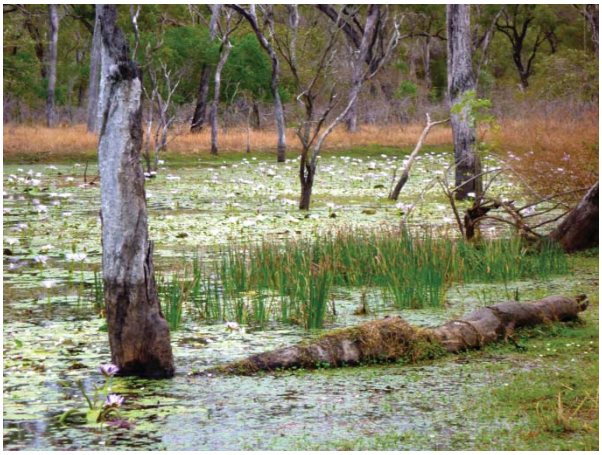

Plate 21: Perched, roadside, inland billabong. Contains *Nymphaea immutabilis*.

Plate 22: Large, coastal lagoon/billabong. Keating's Lagoon, Cooktown. Contains *Nymphaea immutabilis* and *N. violacea.*

Plate 23: Large stock dam, fringed with *Nymphaea immutabilis*.

Plate 24: Small, roadside ditch near Daintree. Contains a currently undescribed miniature *Nymphaea.*

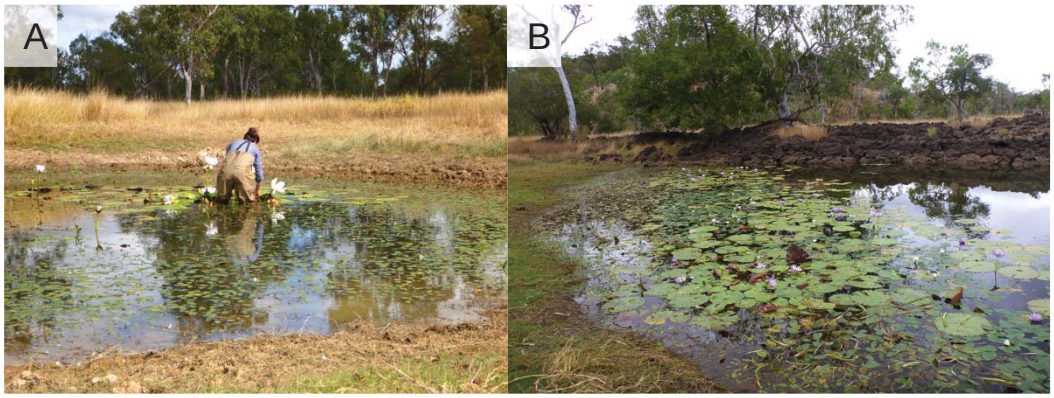

Plate 25: A) Cattle wallow, with *Nymphaea violacea* and small, roadside pond with *N. violacea*. Valley of Lagoons. Both images by Christine Best.

Plate 26: Large, inland lake near Mount Garnet. Contains *Nymphaea immutabilis*. Photo by Christine Best.

Plate 27: Roadside drainage ditch, Cairns, containing *Nymphaea immutabilis*.

APPENDIX 3

Imagery of selected Australian *Nymphaea* seeds

Species and accessions of *Nymphaea* used in this study

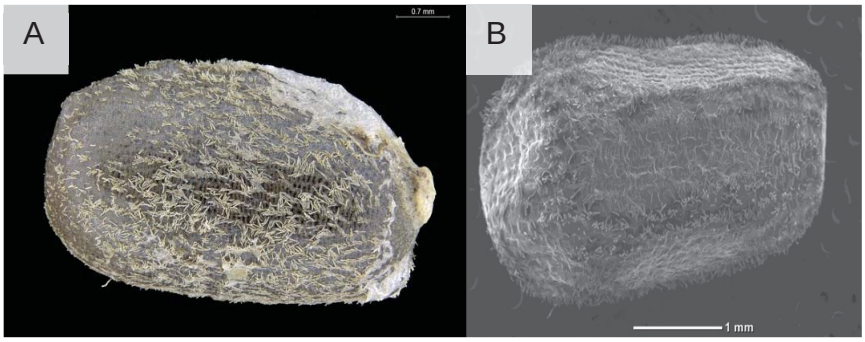

Plate 1: *Nymphaea immutabilis* NI1, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

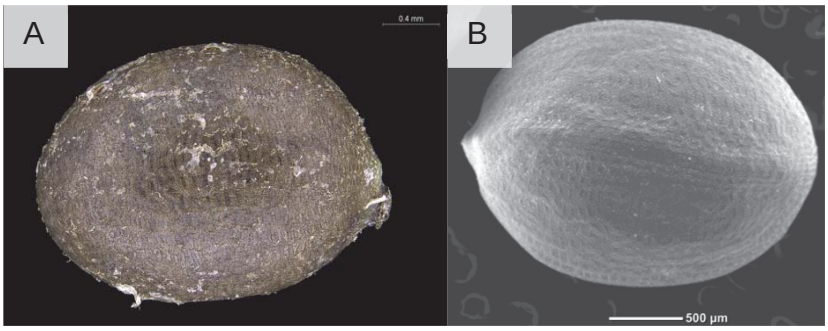

Plate 2: *Nymphaea lukei* NL2, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

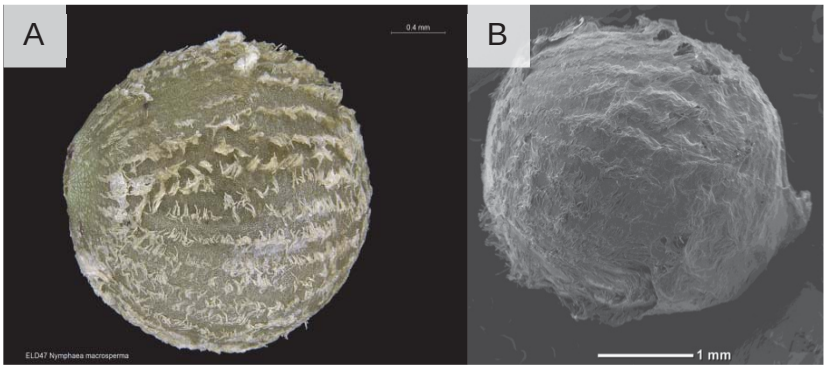

Plate 3: *Nymphaea macrosperma* NM2, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

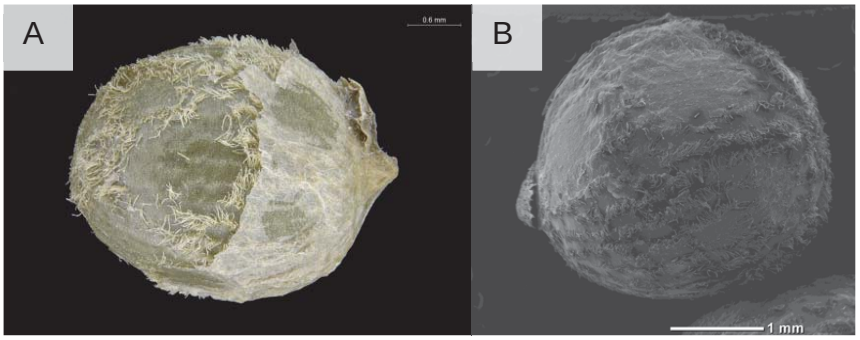

Plate 4: *Nymphaea macrosperma* NM3, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

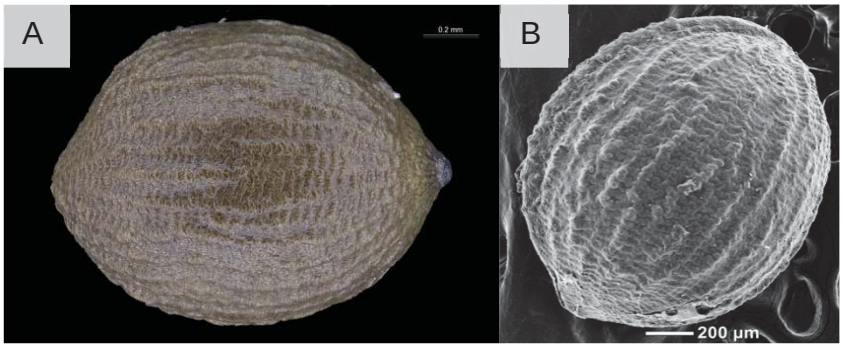

Plate 5: *Nymphaea ondinea* NO1, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

Plate 6: *Nymphaea pubescens* NP1, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

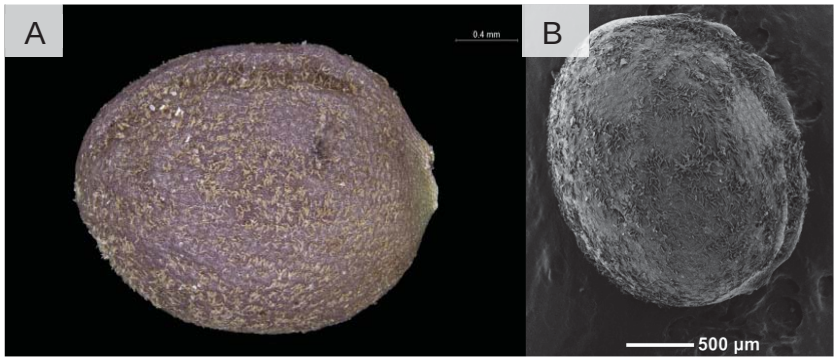

Plate 7: *Nymphaea violacea* NV1, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

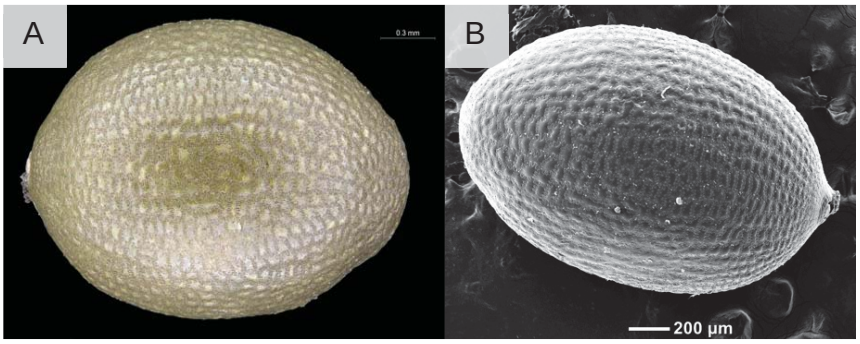

Plate 8: *Nymphaea violacea* NV2, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

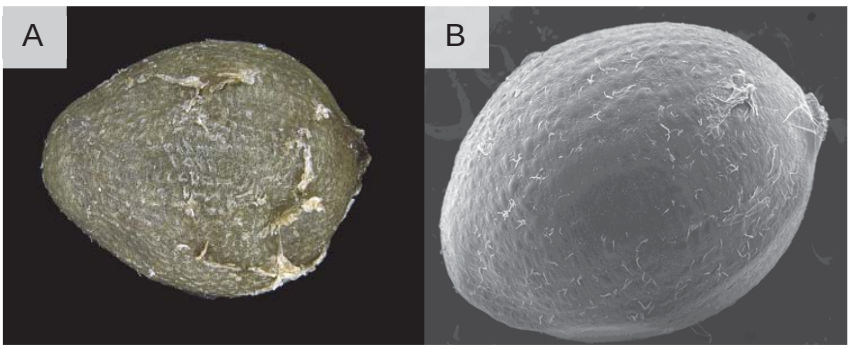

Plate 9: *Nymphaea violacea* NV7, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

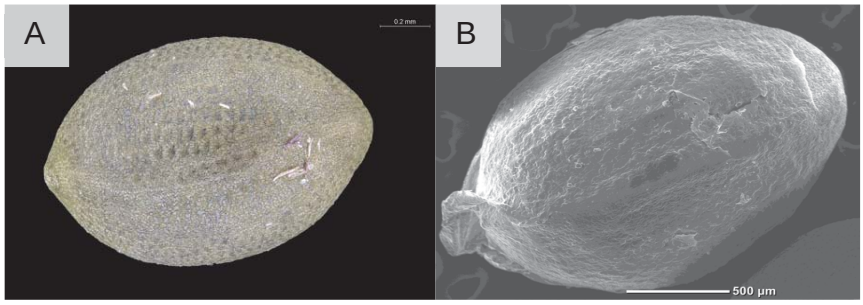

Plate 10: *Nymphaea violacea* NV8, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

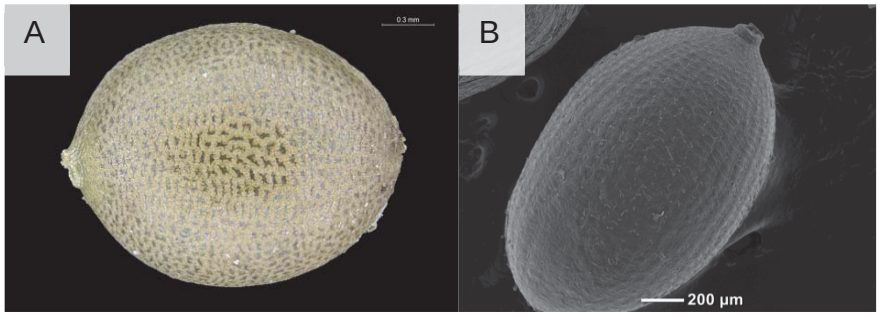

Plate 11: *Nymphaea violacea* NV9, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater.

Plate 12: *Nymphaea violacea* NV10. image taken using Leica M205C

Other Australian species of *Nymphaea*

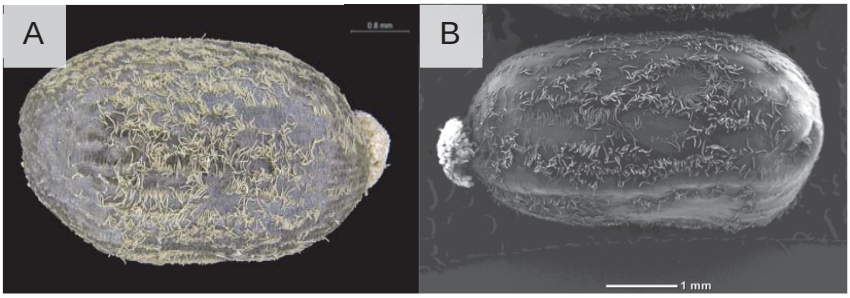

Plate 13: *Nymphaea atrans*, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater. Seeds courtesy of Andre Leu.

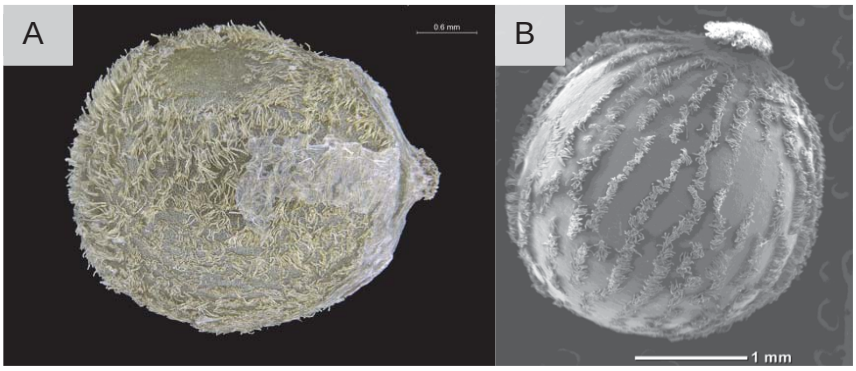

Plate 14: *Nymphaea carpentariae*, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater. Seeds courtesy of Andre Leu.

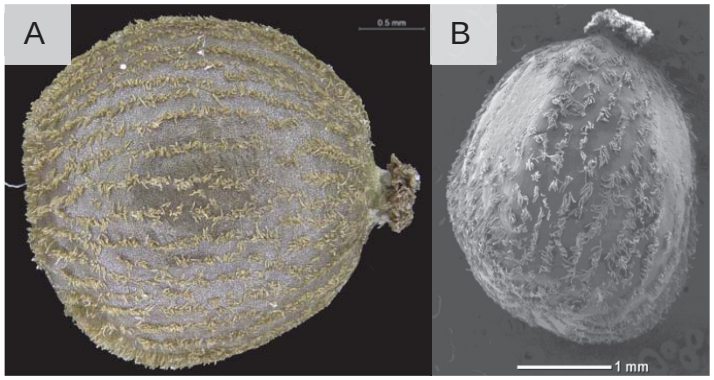

Plate 15: *Nymphaea georginae*, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater. Seeds courtesy of Andre Leu.

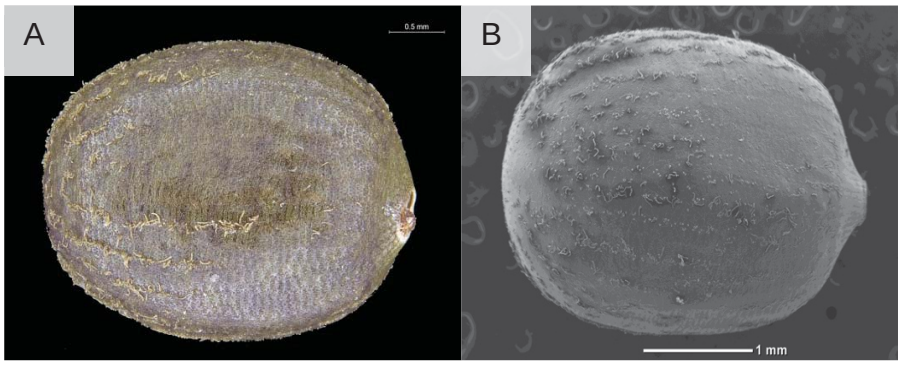

Plate 16: *Nymphaea gigantea*, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater. Seeds courtesy of Andre Leu.

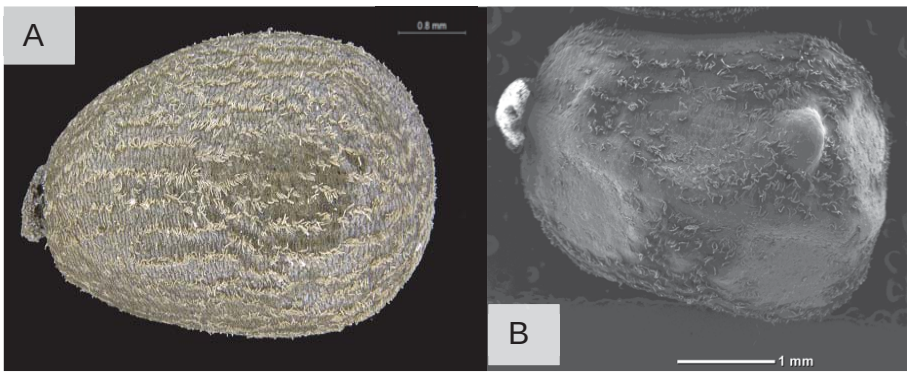

Plate 17: *Nymphaea jacobsii*, A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater. Seeds courtesy of Andre Leu.

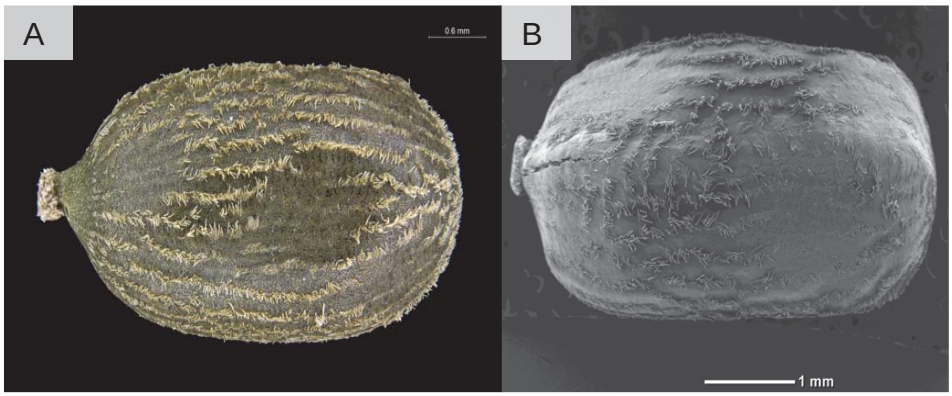

Plate 18: *Nymphaea jacobsii* 'Toomba', A) image taken using Leica M205C, B) Scanning electron microscope image (Jeol JCM-6000 SEM). Dried seeds were mounted on an aluminium scanning electron microscope (SEM) stub covered with carbon tape. The stub and samples were then coated with a thin layer of gold using an EMITECH K550X sputter coater. Seeds courtesy of Andre Leu.

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APPENDIX 4

Supplementary information to Chapter 3

Table 1: Time to 50 % germination (t50) as determined via Weibull function in R at 30 °C and 35 °C of *Nymphaea immutabilis, N. lukei, N. macrosperma, N. ondinea* and *N. violacea*. *Nymphaea pubescens* was not included in the analysis due to no germination occurring. Numbers in brackets represent binomial standard errors.

Species	Stimulant	Temperature (°C)	
		30	35
Nymphaea immutabilis	Control	-	29.0 (7.1)
	GA_3	-	27.2 (14.8)
	KAR_1	-	21.8 (3.6)
	Ethrel	21.1 (2.5)	51.5 (5.4)
	Crowd	6.7 (0.4)	14.9 (0.6)
Nymphaea lukei	Control	14.0 (2.6)	19.0 (2.1)
	GA_3	19.0 (1.0)	26.9 (5.6)
	KAR_1	20.8 (1.8)	16.4 (1.6)
	Ethrel	16.7 (4.3)	23.0 (1.6)
	Crowd	45.3 (15.5)	20.7 (1.4)
Nymphaea macrosperma	Control	13.6 (1.1)	35.3 (2.6)
	GA_3	7.9 (2.1)	11.3 (2.3)
	KAR_1	14.4 (3.2)	26.1 (2.6)
	Ethrel	13.7 (4.8)	< 7
	Crowd	12.6 (0.7)	< 7
Nymphaea ondinea	Control	50.1 (1.1)	20.1 (2.2)
	GA_3	45.2 (4.0)	21.6 (2.9)
	KAR_1	49.9 (2.1)	19.9 (3.5)
	Ethrel	42.7 (1.5)	26.4 (1.0)
	Crowd	7.8 (0.3)	7.5 (0.3)
Nymphaea violacea	Control	20.0 (2.2)	71.0 (31.4)
	GA_3	21.6 (2.9)	75.0 (15.3)
	KAR_1	19.9 (3.5)	40.0 (5.9)
	Ethrel	26.4 (1.0)	69.2 (3.2)
	Crowd	7.5 (0.27)	18.0 (0.6)

Table 2: Cardinal temperatures required to reach 50 % and 99 % germination within seed lots of *Nymphaea immutabilis, N. lukei, N. macrosperma, N. ondinea* and *N. violacea. Nymphaea pubescens* was not included in the analysis due to no germination occurring. g50 and g99 were calculated via a dose response curve in the '*drc'* package in R (Ritz and Streibig 2005).

Species	Seedlot	g50/ g99	H_20	GA	Ethrel	Crowd
N. immutabilis	NI1	g50	-	-	28.90 ± 1.8	28.8 ± 3.0
N. immutabilis	NI1	g99			32.5 ± 4.5	31.7 ± 4.8
N. lukei	NL1	g50	35.9 ± 175.3	28.3 ± 3.8	26.9 ± 4.0	28.6 ± 2.0
N. lukei	NL1	g99	48.1 ± 403	31.6 ± 4.4	28.8 ± 5.7	50.0 ± 11.3
N. lukei	NL2	g50	-	30.2 ± 1.3	28.2 ± 1.3	32.2 ± 3.5
N. lukei	NL2	g99	-	36.8 ± 15.2	36.8 ± 5.6	49.2 ± 15.5
N. macrosperma	NM1	g50	29.8 ± 0.5	26.9 ± 5.3	36.9 ± ??	26.1 ± 6.2
N. macrosperma	NM1	g99	34.2 ± 13.4	28.6 ± 7.3	51.2 ± ??	27.4 ± 14
N. macrosperma	NM2	g50	-	26.8 ± 5.4	27.0 ± 3.8	25.0 ± 0.3
N. macrosperma	NM2	g99	-	31.4 ± 13.1	29.8 ± 6.7	27.9 ± 9.8
N. macrosperma	NM3	g50	-	-	26.8 ± 2.9	27.7 ± 0.5
N. macrosperma	NM3	g99	-	-	31.9 ± 7.7	31.6 ± 0.9
N. ondinea	NO1	g50	31.1 ± 5.2	28.6 ± 31.4	30.2 ± 1.1	27.7 ± 9.7
N. ondinea	NO1	g99	39.0 ± 30.4	31.2 ± 30.0	34.2 ± 20.8	31.6 ± 7.1
N. violacea	NV2	g50	-	-	35.8 ± 0.5	26.7 ± 0.4
N. violacea	NV2	g99	-	-	46.2 ± 5.0	48.5 ± 2.9
N. violacea	NV3	g50	-	-	36.3 ± 2.4	29.8 ± 0.5
N. violacea	NV3	g99	-	-	42.7 ± 15.1	45.7 ± 3.5
N. violacea	NV4	g50	25.8 ± 2.4	30.1 ± 1.0	25.9 ± 0.7	28.9 ± 0.9
N. violacea	NV4	g99	28.0 ± 8.7	45.0 ± 5.6	39.8 ± 4.7	50.1 ± 5.3
*N. violacea**	NV5	g50	35.2 ± 0.3	35.1 ± 0.3	34.3 ± 0.8	27.8 ± 0.5
*N. violacea**	NV5	g99	39.9 ± 6.4	38.9 ± 10.8	37.7 ± 3.3	31.2 ± 2.8
N. violacea	NV6	g50	37.7 ± 34.6	35.8 ± 49.3	32.2 ± 8.1	31.7 ± 4.3
N. violacea	NV6	g99	47.2 ± 58.3	38.8 ± 63.0	39.4 ± 30.1	35.3 ± 14.2
N. violacea	NV7	g50	26.5 ± 5.6	26.9 ± 12.1	25.9 ± 3.6	31.1 ± 1.8
N. violacea	NV7	g99	29.4 ± 9.2	30.0 ± 22.8	28.0 ± 12.3	47.6 ± 9.1
N. violacea	NV8	g50	27.5 ± 22.3	25.4 ± 1.6	29.6 ± 0.6	26.4 ± 3.0
N. violacea	NV8	g99	29.8 ± 33.2	32.7 ± 15.3	40.9 ± 3.8	29.0 ± 8.8
N. violacea	NV9	g50	26.6 ± 24.8	26.5 ± 8.3	26.2 ± 7.0	25.8 ± 2.8
N. violacea	NV9	g99	28.0 ± 38.2	28.0 ± 12.7	30.6 ± 5.4	28.5 ± 13.1
N. violacea	NV10	g50	-	-	28.0 ± 0.8	26.8 ± 7.0
N. violacea	NV10	g99	-	-	35.2 ± 2.7	28.6 ± 9.8

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APPENDIX 5

Supplementary information to Chapter 4

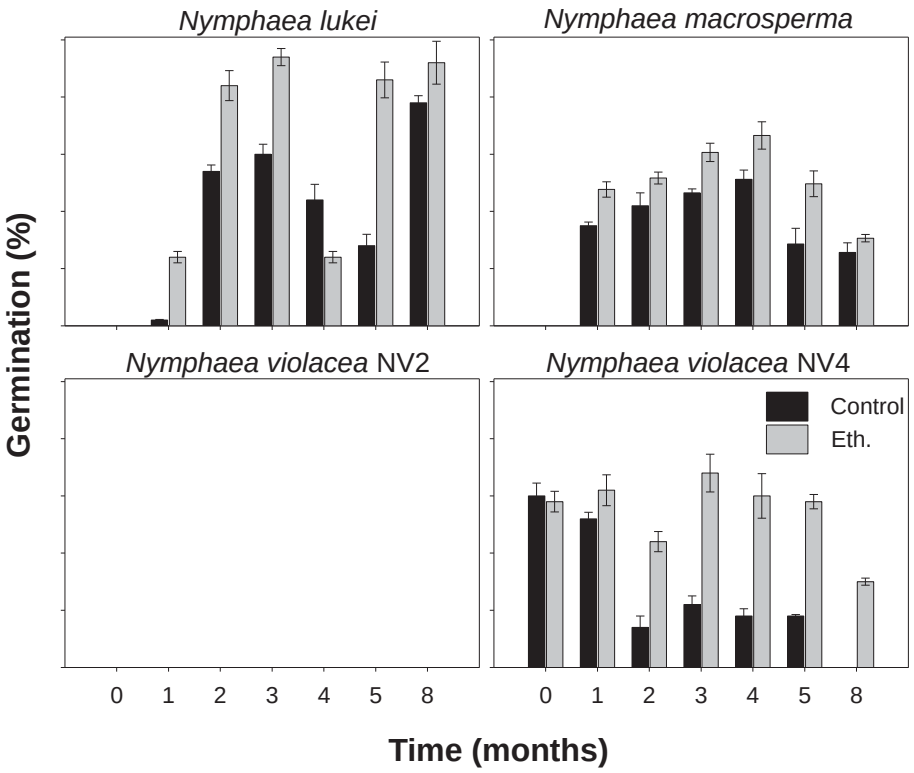

Figure 1: Germination of seeds of *Nymphaea lukei* (NL1), *N. macrosperma* (NM2) and two accessions of *N. violacea* (NV1 and NV4) after wet dry cycling in complete darkness. Seeds were placed in a FiBox electrical box containing a 50 % RH solution of LiCl at 35 °C for 12 days, then removed and placed on 0.7 % water agar for 2 days. This was repeated twice a month for 8 months. Seeds were extracted at 1, 2, 3, 4, 5 and 8-month intervals. Seeds were sown on plain water agar or pre-treated with Ethrel (20 mM) prior to being plated on plain water agar incubated with 12 hour light d^{-1} at 30 °C and scored for germination for 8 weeks.

APPENDIX 6

Supplementary information to Chapter 5

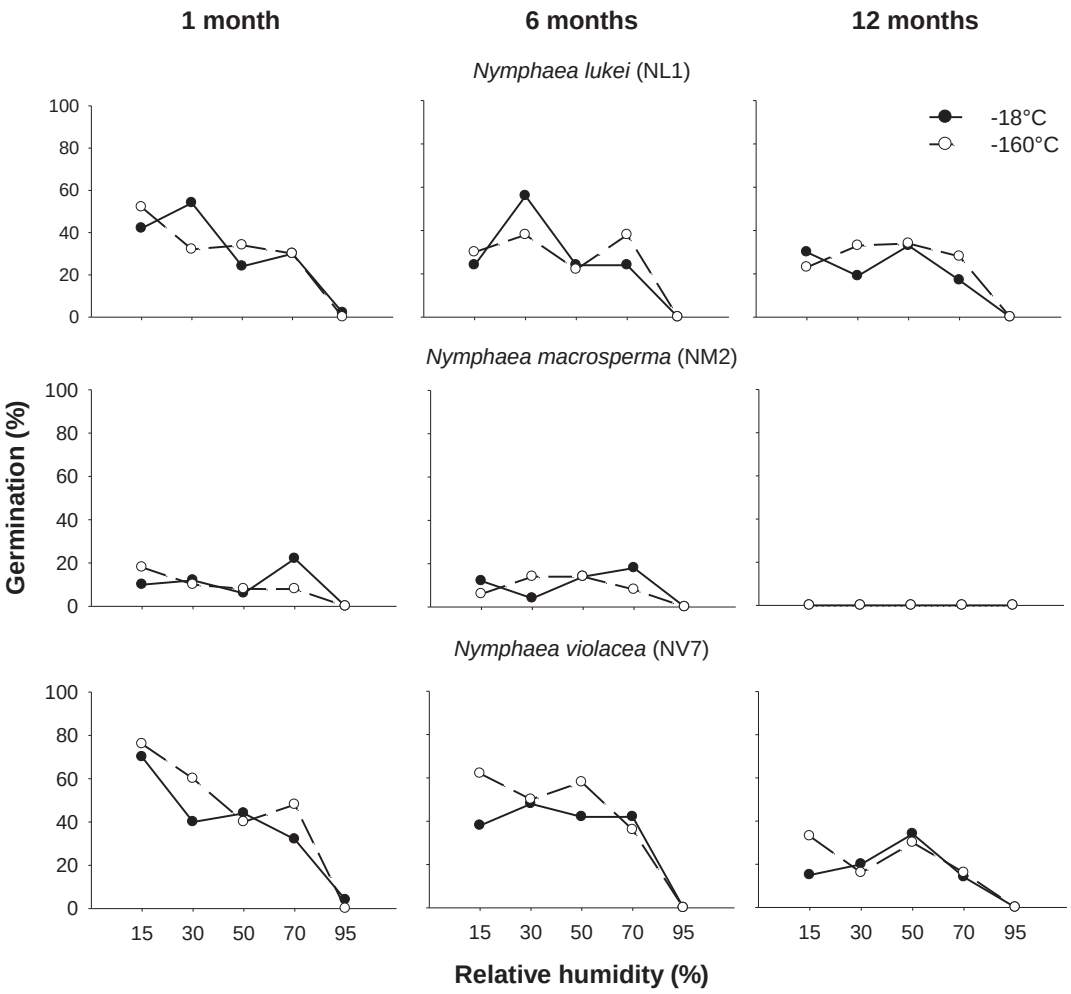

Figure 1: Germination of *Nymphaea lukei* (NL1) *N. macrosperma* (NM2) and *N. violacea* (NV7) in 2012 collections after 1, 6 and 12 months storage at -18 ^{o}C (black circles) or -150 ^{o}C (white circles) after being equilibrated at 15, 30, 50, 70 and 95 % RH.

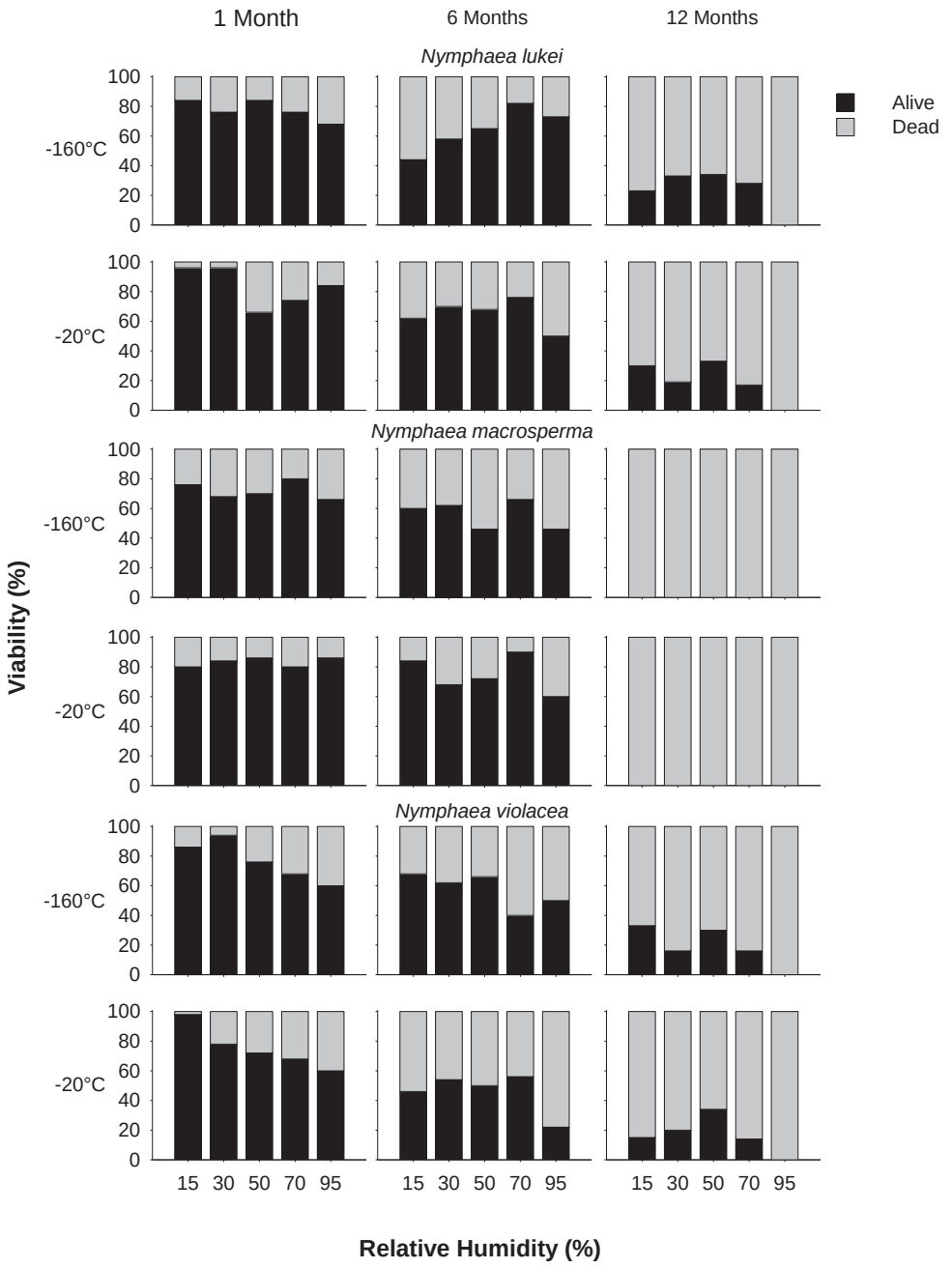

Figure 2: Percentage viability (as determined via cut-tests and tetrazolium staining) of *Nymphaea lukei* (NL1), *N. macrosperma* (NM2) and *N. violacea* (NV7) seeds stored under experimental storage conditions. Seeds that had germinated or were viable were considered to be alive.

Table 1: Melting and warning peaks (°C) and total enthalpy (J/g) calculated from thermograms of lipids extracted from *Nymphaea lukei* (NL1), *N. macrosperma* (NM2) and *N. violacea* (NV7).

	NL1	**NM2**	**NV7**
Cooling			
Lipid melt crystallisation 1 (°C)	-23.0 ± 1.5	25 ± 1.0	20.1 ± 0.6
Enthalpy peak 1 (J/g)	3.8 ± 0.1	3.6 ± 2.3	3.0 ± 1.3
Lipid crystallisation peak 2 (°C)	-17.3 ± 0.6	-	28.5 ± 0.3
Enthalpy peak 2 (J/g)	15.7 ± 0.1	-	1.5 ± 0.1
Lipid crystallisation peak 3 (°C)	22.1 ± 1.5	-	-
Enthalpy peak 3 (J/g)	0.3 ± 0.1	-	-
Warming			
Lipid melt peak 1 (°C)	-3.5 ± 0.6	-	-
Enthalpy peak 1 (J/g)	22.6 ± 5.5	-	-
Lipid melt peak 2 (°C)	-	-	-
Enthalpy peak 2 (J/g)	-	-	-
Phase transition temp 1 (°C)	-27.7 ± 2.3	21.5 ± 1.6	19.1 ± 1.1
Phase transition temp 2 (°C)	-	36.8 ± 3.4	34.7 ± 1.9

APPENDIX 7

Supplementary information to Chapter 6

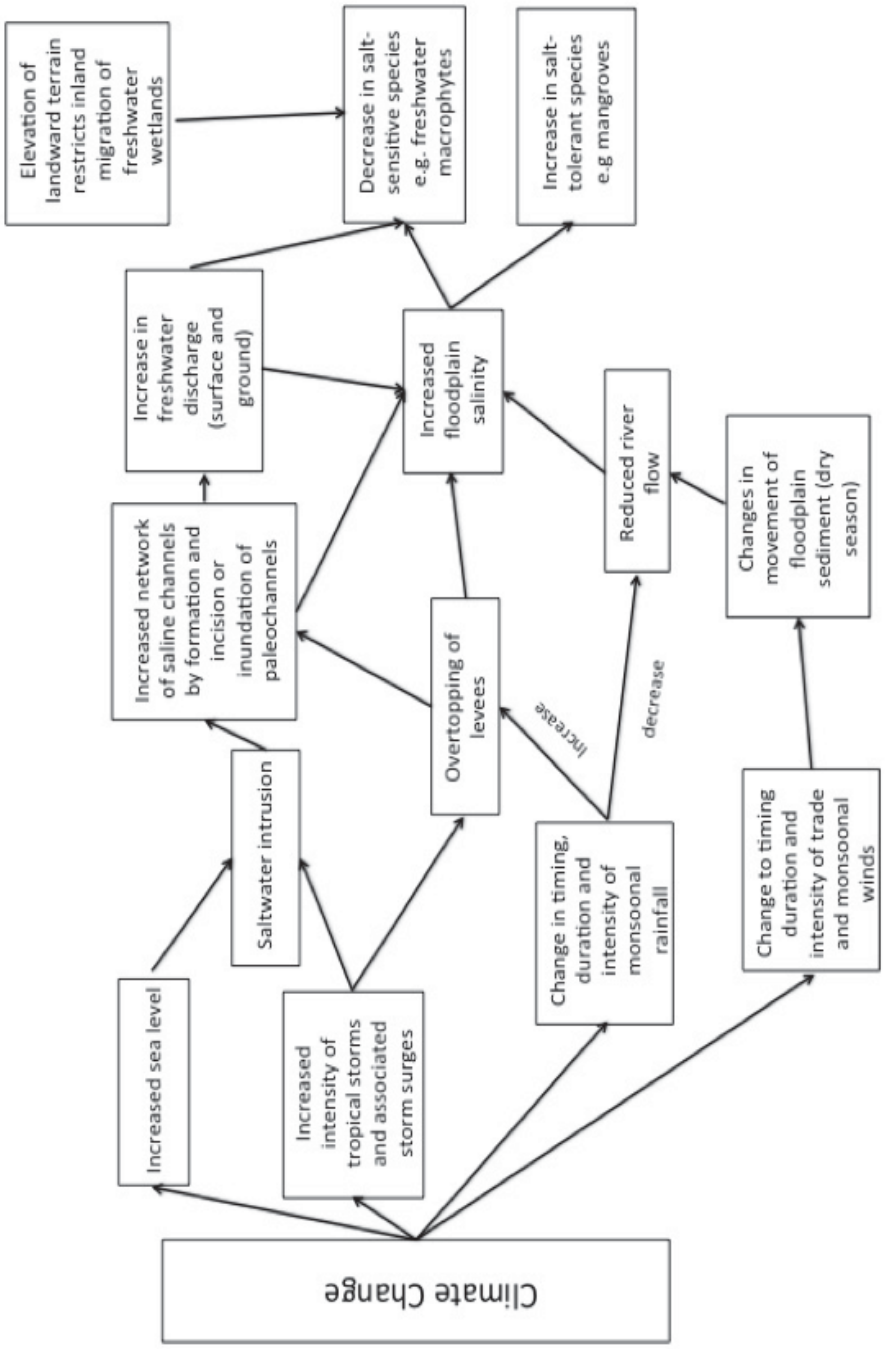

Figure 1: Conceptual model showing the potential impact of climate change and associated flow-on effects influencing the distribution and abundance of aquatic plants in northern Australia's coastal wet-dry tropics. Model modified from Catford *et al.* (2013) and Finlayson *et al.* (2009).

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