ANNALS OF
BOTANY
Founded 1887

Morphophysiological dormancy in the basal angiosperm order Nymphaeales

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• **Background and Aims** Substantial evidence supports the hypothesis that morphophysiological dormancy (MPD) is the basal kind of seed dormancy in the angiosperms. However, only physiological dormancy (PD) is reported in seeds of the ANA-grade genus *Nymphaea*. The primary aim of this study was to determine the kind of dormancy in seeds of six species of *Nymphaea* from the wet–dry tropics of Australia.
• **Methods** The effects of temperature, light and germination stimulants on germination were tested on multiple collections of seeds of *N. immutabilis*, *N. lukei*, *N. macrosperma*, *N. ondinea*, *N. pubescens* and *N. violacea*. Embryo growth prior to hypocotyl emergence was monitored.
• **Key Results** Germination was generally <10 % after 28 d in control treatments. Germination percentage was highest at 30 or 35 °C for seeds exposed to light and treated with ethylene or in anoxic conditions in sealed vials of water, and it differed significantly between collections of *N. lukei*, *N. macrosperma* and *N. violacea*. Seeds of *N. pubescens* did not germinate under any of the conditions. Embryo growth (8–37 % in length) occurred before hypocotyl emergence (germination) in seeds of the five species that germinated.
• **Conclusions** Fresh seeds were dormant, and the amount of pregermination embryo growth in seeds of *N. lukei* and *N. immutabilis* was relatively small, while in seeds of *N. macrosperma*, *N. ondinea* and *N. violacea* it was relatively large. Thus, seeds of *N. lukei* and *N. immutabilis* had PD and those of *N. macrosperma, N. ondinea* and *N. violacea* had MPD. Overall, we found that seeds in the most phylogenetically derived clades within *Nymphaea* have MPD, suggesting that PD is the most likely basal trait within the Nymphaeales. This study also highlights the broad range of dormancy types and germination strategies in the ANA-grade angiosperms.

Keywords: Aquatic plants, basal angiosperms, embryo growth, morphophysiological dormancy, Nymphaeaceae, physiological dormancy, seed germination, wet–dry tropics

INTRODUCTION

Seed dormancy, defined as the restriction of germination under otherwise favourable conditions (Baskin and Baskin, 2004), allows seeds to survive periods of environmental stochasticity, maximizes the chances of seedling survival and reduces competition between siblings (Fenner and Thompson, 2005; Baskin and Baskin, 2014). Of the five classes of seed dormancy described by Baskin and Baskin (2004), morphophysiological dormancy (MPD) is considered to be the most ancestral type within the angiosperms (Baskin and Baskin, 2004; Willis *et al.*, 2014; Fogliani *et al.*, 2017). Seeds with morphological dormancy (MD) contain embryos that are differentiated, i.e. cotyledons and radicle can be distinguished, but they are underdeveloped and must grow prior to radicle emergence (Baskin and Baskin, 2004). Furthermore, in addition to MD seeds with MPD also have physiological dormancy (PD) (Baskin and Baskin, 2004).

Embryo morphology and development are ultimately linked to the different kinds of seed dormancy, and it is generally accepted that the rudimentary embryo is a basal seed trait in

angiosperms and that embryo size has increased over geological time (Martin, 1946; Nikolaeva, 1977; Forbis *et al.*, 2002; Finch-Savage and Leubner Metzger, 2006; Baskin and Baskin, 2014; Willis *et al.*, 2014; Friis *et al.*, 2015). Studies on seeds of both extant and extinct members of the ANA-grade angiosperms (*Amborella*, Nymphaeales and Austrobaileyales) revealed that seeds of *Amborella* and Austrobaileyales have underdeveloped embryos, and thus MPD or MD. However, except for seeds of *Trithuria* (Hydatellaceae) that possess an undifferentiated embryo and a specialized type of MPD (Rudall *et al.*, 2009; Tuckett *et al.*, 2010b; Fogliani *et al.*, 2017; Losada *et al.*, 2017), seeds of species of the Nymphaeales have been exclusively reported as having a fully developed (broad) embryo and PD (Martin, 1946; Baskin and Baskin, 2007).

Nonetheless, with the exception of the study by Baskin and Baskin (2007), empirical studies investigating whether embryos of seeds from the Nymphaeaceae grow prior to radicle emergence are absent. Baskin and Baskin (2007) examined seeds of nursery-grown plants of five members of *Nymphaea* (*N. capensis* var. *zanzibariensis*, *N. immutabilis*, *N. mexicana*, *N. micarantha*

and the hybrid *N.* 'Albert Greenburg') for evidence of embryo growth. Seeds of the three species that germinated contained differentiated embryos that increased only a little in length (3–14 %) prior to germination, which was not considered sufficient to warrant classifying the embryos as underdeveloped. Thus, the authors concluded that seeds of *Nymphaea* have only PD. These data were also considered in a recent study investigating the evolutionary context of MPD in *Amborella* (Fogliani *et al.*, 2017), which proposed that the transition from MPD to PD occurred in the common ancestor of the Cambombaceae and Nymphaeaceae, while also confirming support for MPD as basal.

The five taxa of *Nymphaea* studied by Baskin and Baskin (2007) represent <5 % of the total species richness within the genus *Nymphaea*, from across a small part of the otherwise wide geographical range of this cosmopolitan genus (Conard, 1905; Löhne *et al.*, 2008b). Thus, defining the dormancy and germination traits of species of *Nymphaea* more broadly and including species from environmentally unpredictable habitats where dormancy is central to regenerative success will strengthen our understanding of the evolution of seed dormancy in ANA-grade angiosperms.

In Australia, 18 species of *Nymphaea* occur throughout the wet–dry tropics in the northern part of the continent (Löhne *et al.*, 2009; Jacobs and Hellquist, 2011). This region is characterized by hot, wet monsoonal summers and warm, dry winters (Finlayson, 1999). Rainfall in the region is influenced by sporadic, tropical low-pressure systems and cyclones, which yield intermittent and often unpredictable amounts of precipitation (Taylor and Tulloch, 1985; Bureau of Meteorology, 2011). Therefore, many wetlands in the wet–dry tropics are transient or ephemeral and experience significant fluctuations in water level and temperature (Cowie *et al.*, 2000). *Nymphaea* species occupy a diverse range of these habitats in northern Australia, including perennial and ephemeral wetlands (Dalziell, 2016).

Our aim in this study was to identify the kind of dormancy and the germination requirements of representative species of *Nymphaea* from Australia to determine whether MD and/or MPD are present in extant members of the genus. Specifically, we addressed the following questions: (1) Are seeds water-permeable? (2) What are the optimal conditions for germination? (3) Do embryos grow under conditions considered optimal for germination? (4) Do seeds of the same species collected in different locations (collections) exhibit the same dormancy and germination responses?

MATERIALS AND METHODS

Species selection, seed collection and assessment of seed characteristics

Seeds of *N. immutabilis*, *N. lukei*, *N. macrosperma*, *N. ondinea*, *N. pubescens* and *N. violacea* were collected from wild populations over a 2-year period between March 2011 and August 2013 from multiple locations across northern Australia (Fig. 1). These species represent three of the four subgenera of *Nymphaea* (*Anecphya*, *Confluentes* and *Lotos*) in Australia and the diversity of habitat types across the entire distribution of *Nymphaea* in Australia (Table 1). For example, *N. ondinea* is restricted to a small area of north-west Australia in ephemeral, sandstone-based creeks of ultra-oligotrophic water, while *N. violacea* is found across northern Australia and was collected from both

ephemeral and permanent waterways comprising a range of water qualities. Multiple collections were made of *N. lukei*, *N. macrosperma* and *N. violacea* to examine among-population variation (if any) in dormancy traits and germination responses.

Mean monthly minimum and maximum temperatures and rainfall for each collection site were obtained from the nearest Australian Bureau of Meteorology weather station for the three months prior to seed collection (Bureau of Meteorology, 2005, 2011, 2013). Mature seeds were collected at the time of natural dispersal, i.e. the pedicle had coiled and receded below the water line, fruits were dehiscing, and individual seeds were dark in colour with a fully intact aril. Unless otherwise stated (Table 1), whole seed heads were collected, kept moist and transported to Perth, Western Australia, in Glad® Snap Lock® bags. Upon arrival, seed heads were placed in shallow pans of water at room temperature (*c.* 24 °C) to allow fruits to dehisce naturally, except for collections of seeds that were cleaned in the field and dried for transport, which remained dry prior to experimentation. Sieves were used to separate the seeds from remaining plant material. Seeds were then blotted dry with paper towel, and initial seed fill was determined by X-ray analysis (Faxitron Specimen Radiography System MX-20 Cabinet, Tucson, AZ, USA). Seed viability was confirmed via a cut test (ISTA, 2017), whereby three replicates of 20 seeds were cut lengthways with a scalpel blade and visually inspected. Seeds with a fully formed, turgid, white embryo (lacking any conspicuous browning or necrotic areas) and endosperm/perisperm were considered viable. The remaining seeds were then passed through an aspirator (SELECTA BV Gravity Seed Separator 'Zig Zag', Netherlands) to separate filled and empty seeds. Once this operation was complete, seeds were placed back into pans of water at room temperature until the start of experiments (up to 2 weeks). Seed length and width were measured on 50 randomly chosen seeds of each collection using a dissecting microscope (Olympus SZX16, Japan) equipped with a camera and digital micrometer. Five replicates of 50 filled, intact seeds were weighed to estimate individual seed mass, which was then multiplied by 1000 to obtain 1000-seed mass.

Water uptake (imbibition)

To determine if seeds were water-permeable, three replicates of ≥0.05 g of pre-dried small seeds (or 15–25 individual large seeds) of each species were weighed (initial seed mass, at t_0) and then placed on a glass filter paper (84 mm; Advantec, Japan) inside a Petri dish under standard laboratory temperatures (approx. 22 °C). The filter paper was irrigated with reverse-osmosis (RO) water, and at each pre-determined time interval (1, 2, 5 and 30 min and 1, 2, 4, 6, 8, 24, 48, 72 and 96 h) seeds were removed from the Petri dish, blotted dry, weighed and placed back onto the moistened filter paper. Percentage moisture uptake was determined using the following equation:

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

where W_i is the mass of imbibed seeds at any given time (t_x) and W_d is the initial mass at t_0. For an additional three replicates of
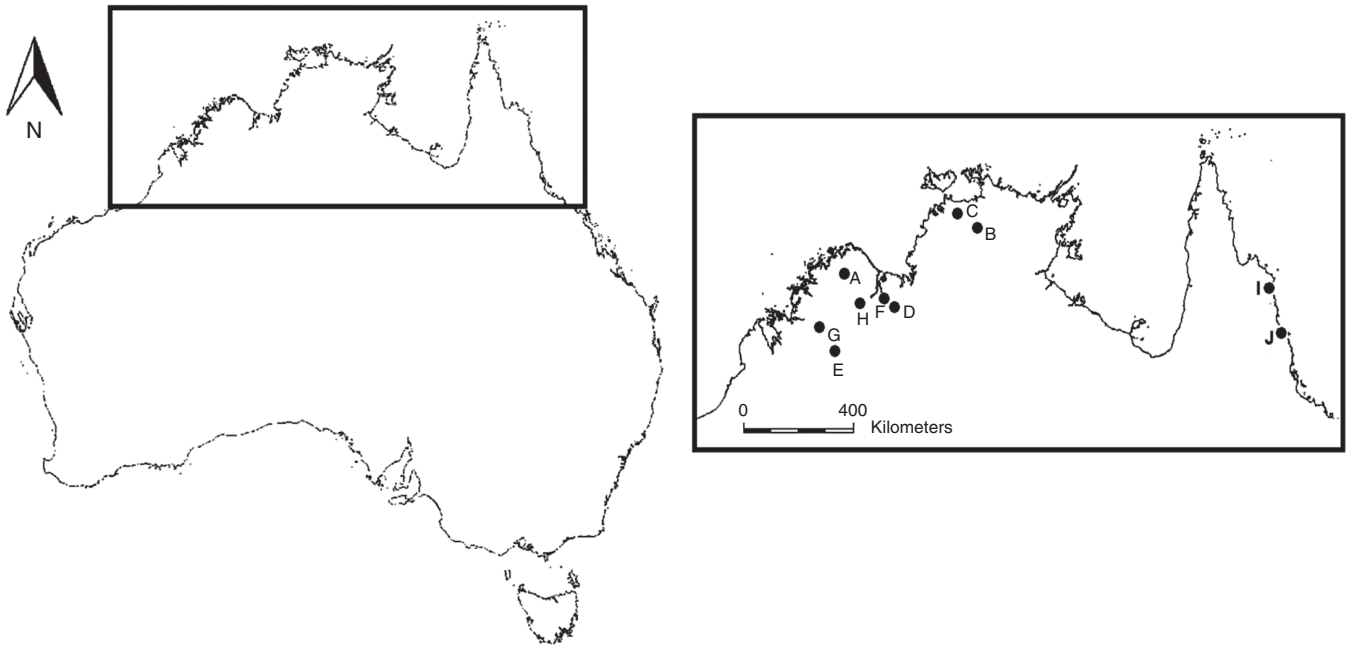
Fɪɢ. 1. Collection locations for *Nymphaea* seeds used in this study: (A) *N. ondinea* (NO1), *N. violacea* (NV1, NV2, NV3, NV6), (B) *N. pubescens* (NP1), *N. violacea* (NV4), (C) *N. macrosperma* (NM1), (D) *N. violacea* (NV5), (E) *N. lukei* (NL1), (F) *N. macrosperma* (NM2, NM3), *N. violacea* (NV7), (G) *N. lukei* (NL2), (H) *N. violacea* (NV8, NV9), (I) *N. immutabilis* (NI1), and (J) *N. violacea* (NV10).

≥0.05 g of seeds, the seed coat of each seed from each collection was scarified with a scalpel along the longitudinal axis and subjected to the same imbibition procedure.

Seed germination

The exogenous application of gibberellins to seeds with PD may be used to distinguish between non-deep and deep PD, whereby germination is either stimulated or not, respectively (Baskin and Baskin, 2004; Fogliani *et al.*, 2017). Assuming seeds of *Nymphaea* have a PD component to their dormancy, the application of gibberellic acid (GA_3) may promote germination. Additionally, the application of ethylene has been shown to promote germination in seeds of *Nymphaea* and is postulated to be produced when many individual seeds are crowded together under anoxic conditions (Else and Riemer, 1984). To determine the type of dormancy and assess the germination responses in the 18 collections of the six species of *Nymphaea*, we constructed a full-factorial experimental design to test the effects of GA_3, ethephon and crowding vs. no treatment on germination in light/dark (12/12 h) at 10, 15, 20, 25, 30, 35, 40 °C and 20/35 °C (12/12 h). Additionally, seeds of *N. immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM1), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV3) were subjected to the same germination stimulant × temperature design in total darkness for the duration of the trial. Each treatment consisted of four replicates of 25 seeds, except for the crowding treatment which consisted of one tube of 50 seeds. All seeds were used in germination experiments within 28 d of collection.

Prior to testing, fresh seeds were sterilized in a 2 % (w/v) solution of calcium hypochlorite ($Ca(OCl)_2$) with two drops of surfactant (Tween 80) for 30 min under alternating vacuum (10 min on/off/on) and then rinsed three times in sterile RO water. For control treatments, seeds were sown in 90-mm-diameter, sterile Petri dishes containing sterile 0.7 % (w/v) water agar, while the GA_3 treatment consisted of water agar containing 0.29 mᴍ GA_3 (Sigma, Australia). The ethephon treatment was administered by placing seeds inside a nylon mesh bag and immersing it in a 20 mᴍ solution (Ethrel® Bayer Crop Science, Australia) for 24 h (at room temperature, approx. 24 °C) prior to sowing the seeds on 0.7 % (w/v) water agar. All Petri dishes were wrapped four times in polyethylene film to prevent excessive evaporation. To test the effect of crowding on germination, seeds were placed in a 10-mL plastic tube (TechnoPlas, Australia) filled with 10 mL of sterile RO water. For dark treatments, the Petri dishes or the 10-mL tubes containing seeds were immediately doubly wrapped with aluminium foil and placed in a light-excluding box for the duration of the experiment. For the light treatment, seeds were incubated in alternating light/dark conditions under a daily 12-h photoperiod of 30 µmol m^{-2} s^{-1}, 400–700 nm, cool white fluorescent light.

Seeds of *N. immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM1), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV3) incubated in the presence of light were scored daily for germination for the first 2 weeks and weekly thereafter for a total of 100 d. Dark treatments were only scored after 8 weeks. All other species and collections were scored daily for germination for the first 2 weeks and weekly thereafter for an additional 6 weeks. Seeds were considered to have germinated upon hypocotyl (*sensu* Haines and Lye, 1975) emergence ≥2 mm. At the conclusion of each experiment, all non-germinated seeds were subjected to a cut test to assess viability (ISTA, 2017). Seeds with a fully formed, turgid, white embryo,

TABLE 1. *Seed collection details for all seeds used in experiments in this study*

Species	Subgenus	Endemism	Collection	State	Collection location (GDA 94)	Habitat	Date	Dried	Mean rainfall (mm, ± s.e.)	Mean minimum temperature (°C, ±s.e.)	Mean maximum temperature (°C, ±s.e.)
Nymphaea immutabilis	*Anecphya*	Cosmopolitan	NI1	QLD	S15°25′35.7″, E145°9′14.5″	Large, permanent swamp (Barrett's Lagoon)	Jul-13	Y	47.8 ± 25.2	21.5 ± 0.9	26.8 ± 0.4
Nymphaea lukei	*Confluentes*	Endemic WA	NL1	WA	S17°34′47.6″ E126°04′59.6″	Small, ephemeral, sandstone based creek	May-12	N	90.5 ± 90.5	18.3 ± 3.3	33.9 ± 1.2
			NL2	WA	S16°43′0.2″, E125°27′35.8″	Small, semi-permanent, sandstone-based creek	Jun-13	N	31.4 ± 16.9	17.9 ± 1.6	31.0 ± 0.9
Nymphaea macrosperma	*Anecphya*	Cosmopolitan	NM1	NT	S12°42′47.9″, E131°38′17.7″	Permanent river oxbow (Mary River)	Jun-11	Y	55.9 ± 55.9	20.1 ± 1.9	31.9 ± 0.8
			NM2	WA	S15°35′35.7″, E128°16′47.9″	Ephemeral creek running into permanent swamp	May-12	N	98.2 ± 97.0	21.7 ± 2.3	33.9 ± 0.8
			NM3	WA	S15°35′35.7″, E128°16′47.9″	Ephemeral creek running into permanent swamp	May-13	N	40.1 ± 20.1	23.5 ± 1.3	36.1 ± 0.7
Nymphaea ondinea	*Confluentes*	Endemic WA	NO1	WA	S14°48′30.1″, E126°33′01.1″	Small, ephemeral, sandstone-based creek	Mar-11	N	485.0 ± 159.5	22.4 ± 0.1	30.8 ± 0.2
Nymphaea pubescens	*Lotos*	NT, QLD	NP1	NT	S12°53′27.8″, E132°30′59.7″	Large permanent swamp (Yellow Water, Kakadu National Park)	Jun-11	Y	55.9 ± 55.9	20.1 ± 1.9	31.9 ± 0.8
Nymphaea violacea	*Confluentes*	Cosmopolitan	NV1	WA	S14°46′33.1″ E126°30′52.6″	Small permanent swamp	May-11	N	151.6 ± 77.8	17.7 ± 2.9	29.7 ± 0.4
			NV2	WA	S14°47′18.8″, E126°31′47.5″	Ephemeral creek	May-11	N	151.6 ± 77.8	17.7 ± 2.9	29.7 ± 0.4
			NV3	WA	S14°48′23.9″, E126°31′47.5″	Ephemeral swamp	May-11	N	151.6 ± 77.8	17.7 ± 2.9	29.7 ± 0.4
			NV4	NT	S12°53′27.8″, E132°30′59.7″	Large permanent swamp (Yellow Water, Kakadu National Park)	Jun-11	N	38.4 ± 38.4	15.7 ± 2.7	29.7 ± 1.1
			NV5	WA	S15°56′46.5″, E128°56′56.6″	Ephemeral creek	Jun-11	N	55.9 ± 55.9	20.1 ± 1.9	31.9 ± 0.8
			NV6	WA	S14°46′33.1″, E126°30′52.6″	Small permanent swamp	Mar-12	N	336.1 ± 92.0	17.4 ± 2.8	31.1 ± 0.9
			NV7	WA	S15°35′35.7″, E128°16′47.9″	Ephemeral creek running into permanent swamp	May-12	N	98.2 ± 97.0	21.8 ± 2.1	33.3 ± 0.9
			NV8	WA	S15°51′39.8″, E127°14′20.8″	Small, ephemeral creek	Jun-13	N	34.1 ± 18.3	17.9 ± 1.6	31.0 ± 0.9
			NV9	WA	S15°51′39.8″, E127°14′21.0″	Small, ephemeral creek	Jun-13	N	34.1 ± 18.3	17.9 ± 1.6	31.0 ± 0.9
			NV10	QLD	S16°54′55.3″, E145°22′20.6	Small, ephemeral wetland adjacent to roadside	Jul-13	Y	8.9 ± 3.1	16.7 ± 0.8	25.7 ± 0.6

Cosmopolitan species are common throughout Australia, while endemic species are restricted to Western Australia (WA), the Northern Territory (NT) or Queensland (QLD). The majority of seed collections were kept damp during transport, except collections NI1 and NV10, which were spread in a thin layer and sun dried for 10 d, and NM1, NP1 and NV4, which were dried over silica gel for 14 d prior to transport. Rainfall and temperature data were obtained from the nearest Bureau of Meteorology weather station and averaged for the three months prior to seed collection.

perisperm and endosperm were considered to be viable. Empty, necrotic or soft seeds were considered to be non-viable and excluded from further analysis. As such, germination data presented here are based on the number of viable seeds.

Embryo morphology

To confirm that the classification of *Nymphaea* embryos was broad (Martin, 1946) and that they were differentiated, 20 seeds each of *N. immutabilis* (NI1) *N. lukei* (NL1 and NL2), *N. macrosperma* (NM3), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV7–NV10) were kept for 24 h on moist filter paper. The embryo was then excised under a microscope using a scalpel and a pair of fine forceps. Seeds and embryos were visually assessed for shape and differentiation.

Embryo growth

Embryo growth was measured for all species but due to limited seed numbers only for the following collections: *N. immutabilis* (NI1), *N. lukei* (NL1 and NL2), *N. macrosperma* (NM3), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV3, NV8–NV10). To determine whether embryo growth occurred prior to hypocotyl emergence, 100 seeds of each species/collection were placed in 10-mL plastic tubes filled with 10 mL of sterile RO water and incubated at either 30 or 35 °C (previously determined optimal temperature for germination of each seed collection) under light/dark conditions (described above). After approximately 6 h, 20 seeds from each collection were removed, their embryos were excised and total embryo length and the internal length of the seed (the seed coat excluded) were measured; these served as 'fresh seed' measurements (Baskin and Baskin, 2007). The remaining seeds were inspected daily for 10 weeks, and any seeds with a split in the seed coat (indicating imminent germination) were removed and measured. Seeds where the hypocotyl had protruded (germinated) were removed from the vials and excluded from further analysis.

Statistical analyses

The germination data followed a binomial distribution and were analysed with binomial logistic regressions (GLMs), using the 'glm' function fitted with a logit-link function, followed by a likelihood ratios test in R (R Core Team, 2017). To determine the effects of temperature (continuous factor), light vs. constant dark (categorical factor) and germination stimulant (categorical factors), germination data were analysed across *N. immutabilis* (NI1), *N. lukei* (NL2), *N. macrosperma* (NM1), *N. ondinea* (NO1), *N. pubescens* (NP1) and *N. violacea* (NV3). To determine the effects of temperature and germination stimulant within species, germination data were analysed between each accession of *N. lukei* (NL1 and NL2), *N. macrosperma* (NM1–NM3) and *N. violacea* (NV1–NV10). All statistical effects are shown for main factors only. Embryo length and embryo to seed length ratio (E: S) were compared between fresh seeds and seeds that showed a split in the seed coat and were analysed with linear models using the '*lm*' function in R (R Core Team, 2017). Prior to analysis all embryo growth data

were checked for homogeneity to satisfy model assumptions via a graphical analysis of the residuals. When the data did not meet the model assumptions, the data were log-transformed. Pairwise comparisons (Holm's) were then used to determine significance ($P < 0.05$) between fresh and split seeds (imminent germination).

RESULTS

Seed characteristics and quality assessment

Nymphaea immutabilis and *N. macrosperma* (subgen. *Anecphya*) had the largest (length and width) seeds and *N. violacea* and *N. ondinea* (subgen. *Confluentes*) the smallest. However, some variation within species (between collections) was noted (Table 2). Seed mass was also variable within species, particularly for collections of *N. macrosperma*, which varied between 7.96 and 18.07 g (1000 seeds)$^{-1}$. Initial seed fill of each species ranged from 64 to 100 % (Table 2).

Water uptake (imbibition)

Seeds of all six species took up water readily over the 96-h period of imbibition, and average moisture uptake after 96-h was 45.6 % (± 1.5 %) for *N. immutabilis*, 38.8 % (± 1.6 %) for *N. lukei*, 47.1 % (± 1.2 %) for *N. macrosperma*, 49.0 % (± 1.6 %) for *N. ondinea*, 45.9 % (± 2.1 %) for *N. pubescens* and 48.7 % (± 7.1 %) for *N. violacea*. For seeds scarified prior to imbibition, the final percentage moisture uptake was not significantly different from that of non-scarified seeds ($P = 0.9$, data not shown).

Seed germination

Under dark conditions, overall percentage germination was low (0–19 %) in all species and collections, and most of the germination occurred in treatments with light (Fig. 2; Supplementary Data Table S1, $P < 0.001$ for all species). Seeds of *N. pubescens* did not germinate in any of the treatment combinations tested. Temperature had a significant effect on germination ($P < 0.001$) as no seeds of any species germinated in light at <25 °C and germinated mostly at 30 and 35 °C. Seeds of *N. ondinea* and *N. macrosperma* germinated at 40 °C (<60 %). No seeds of any species germinated at 20/35 °C. Germination of *N. immutabilis*, *N. lukei* and *N. ondinea* seeds in the control treatment (H_2O) never exceeded 20 %, whereas germination of *N. macrosperma* seeds reached a maximum of 70 % at 35 °C.

The crowding treatment produced the highest germination ($P < 0.001$ for all species) at 30 °C (*N. macrosperma* 74 %) or 35 °C (*N. immutabilis* 46 %, *N. lukei* 71 %, *N. ondinea* 99 % and *N. violacea* 71 %). Seeds of *N. lukei* (at 30 and 35 °C) treated with GA_3 germinated to higher percentages (23 and 25 %, respectively) than those in the control. However, the application of GA_3 did not significantly increase germination of *N. immutabilis*, *N. macrosperma*, *N. ondinea* or *N. violacea* seeds ($P > 0.05$). Overall, at optimal temperatures, ethephon and GA_3 significantly increased germination percentages

TABLE 2. *Mean seed length, width, 1000-seed mass and initial seed fill of all collections of the six species of* Nymphaea.

Species	Collection	Seed length (mm) (mean ± s.e.)	Seed width (mm) (mean ± s.e.)	1000-seed mass (g)	Seed fill (%)
Nymphaea immutabilis	NI1	4.64 ± 1.30	2.74 ± 0.07	18.89	99
Nymphaea lukei	NL1	2.54 ± 0.05	1.96 ± 0.03	3.84	97
	NL2	2.35 ± 0.06	1.67 ± 0.05	3.47	98
Nymphaea macrosperma	NM1	4.05 ± 0.07	3.35 ± 0.06	18.07	92
	NM2	3.00 ± 0.10	2.61 ± 0.07	7.96	95
	NM3	3.38 ± 0.07	2.85 ± 0.07	11.16	98
Nymphaea ondinea	NO1	1.81 ± 0.04	1.46 ± 0.03	0.81	64
Nymphaea pubescens	NP1	1.93 ± 0.06	1.61 ± 0.06	2.49	99
Nymphaea violacea	NV1	1.90 ± 0.06	1.58 ± 0.05	1.89	74
	NV2	1.73 ± 0.06	1.21 ± 0.05	1.45	98
	NV3	1.68 ± 0.03	1.23 ± 0.03	1.6	100
	NV4	1.52 ± 0.13	0.91 ± 0.08	3.19	80
	NV5	1.58 ± 0.07	1.15 ± 0.06	1.11	95
	NV6	1.19 ± 0.01	0.80 ± 0.01	3.8	88
	NV7	1.60 ± 0.05	1.13 ± 0.04	0.75	68
	NV8	1.62 ± 0.06	1.19 ± 0.05	1.14	99
	NV9	1.57 ± 0.04	1.10 ± 0.04	1.1	99
	NV10	1.56 ± 0.05	1.08 ± 0.03	0.94	98

($P < 0.001$) in seeds of *N. immutabilis*, *N. lukei*, *N. ondinea* and *N. violacea.* Maximum germination after 28 d of incubation at the apparent optimal temperatures (30–35 °C) was about 45–75 % for all species, except for seeds of *N. ondinea*, which germinated to 80–100 % in the crowding treatment (Fig. 3).

Intraspecific differences in germination responses were evident between different within-seed collections of *N. lukei*, *N. macrosperma* and *N. violacea* (Fig. 4; Table S2). Germination percentage of *N. lukei* seeds was highest at 35 °C for both collections in the crowding treatment. However, seeds of NL1 germinated to 70 %, whereas those of NL2 germinated to <40 %. Seeds of *N. macrosperma* from the NM1 collection in the control germinated to 70 % at 35 °C, whereas no germination occurred in controls for the NM2 and NM3 collections. Germination in the crowded treatments was highest at 30 °C for NM2 (12 %) and at 35 °C for NM3 (58 %). For seeds of *N. violacea*, the NV1 collection did not germinate at any temperature regardless of the addition of a germination stimulant, whereas seeds of NV4 readily germinated at all temperatures and with most stimulants. With the exception of seeds from NV1, germination percentages of NV collections were highest in the crowding treatments at 30 or 35 °C. In collections NV5, NV8 and NV9, germination in crowded treatments was 100 % after 8 weeks at 35 °C, while germination in the control never exceeded 40 %.

Embryo morphology

Embryos of all six species were wider than long, and they were surrounded by a thin layer of endosperm, which in turn was surrounded by abundant perisperm. The embryos were thus considered to be broad, in accordance with Martin's (1946) classification system. Embryos generally took up less than 25 % of seed (minus seed coat thickness) length (Fig. 5). Embryos were fully differentiated; the hypocotyl and cotyledons could be distinguished.

Embryo growth

Prior to germination, embryos in seeds of *N. immutabilis*, *N. lukei* and *N. macrosperma* exhibited a small but significant ($P < 0.001$) increase in length of 10, 8 and 21 %, respectively, while embryos of *N. ondinea* and *N. violacea* increased by 37 and 32 %, respectively ($P < 0.001$) (Table 3; Fig. 5). E: S ratios increased significantly ($P < 0.01$) in all species and collections during incubation, except for NL2. The change in E: S ratio was greatest for embryos of *N. ondinea* seeds, which increased from 0.29 to 0.38.

DISCUSSION

The primary aim of this study was to determine whether MD and/or MPD is/are present in species of the Nymphaeaceae from the wet–dry tropics of Australia. We also aimed to define the range of conditions suitable for germination and to determine whether seed dormancy and germination responses varied between different populations of the same species. Because seeds of all *Nymphaea* species we tested readily imbibed water, the presence of physical dormancy was excluded. Seeds of all species and collections, except *N. macrosperma* NM1, not treated with a germination stimulant (i.e. controls), germinated to ≤13 % in 28 d; the seeds of *N. macrosperma* germinated to 70 %. Furthermore, germination in the controls was <17 % over the 8-week test period for all collections, with the exception of NM1 (70 %) and three of ten collections of *N. violacea* (30, 55 and 44 %), indicating that seeds within most populations were dormant.

Embryos in seeds were broad and fully differentiated, and embryo length in five of the six species that germinated increased 8–37 % prior to hypocotyl emergence (germination). Baskin and Baskin (2007) proposed that the small increase in embryo length (≤14 %) in three species of nursery-grown *Nymphaea* was not enough to consider the embryos as being underdeveloped. For seeds of *N. immutabilis* and *N. lukei*, in which embryo growth was only 10 and 8 %, respectively, we likewise conclude that the embryos are not underdeveloped and
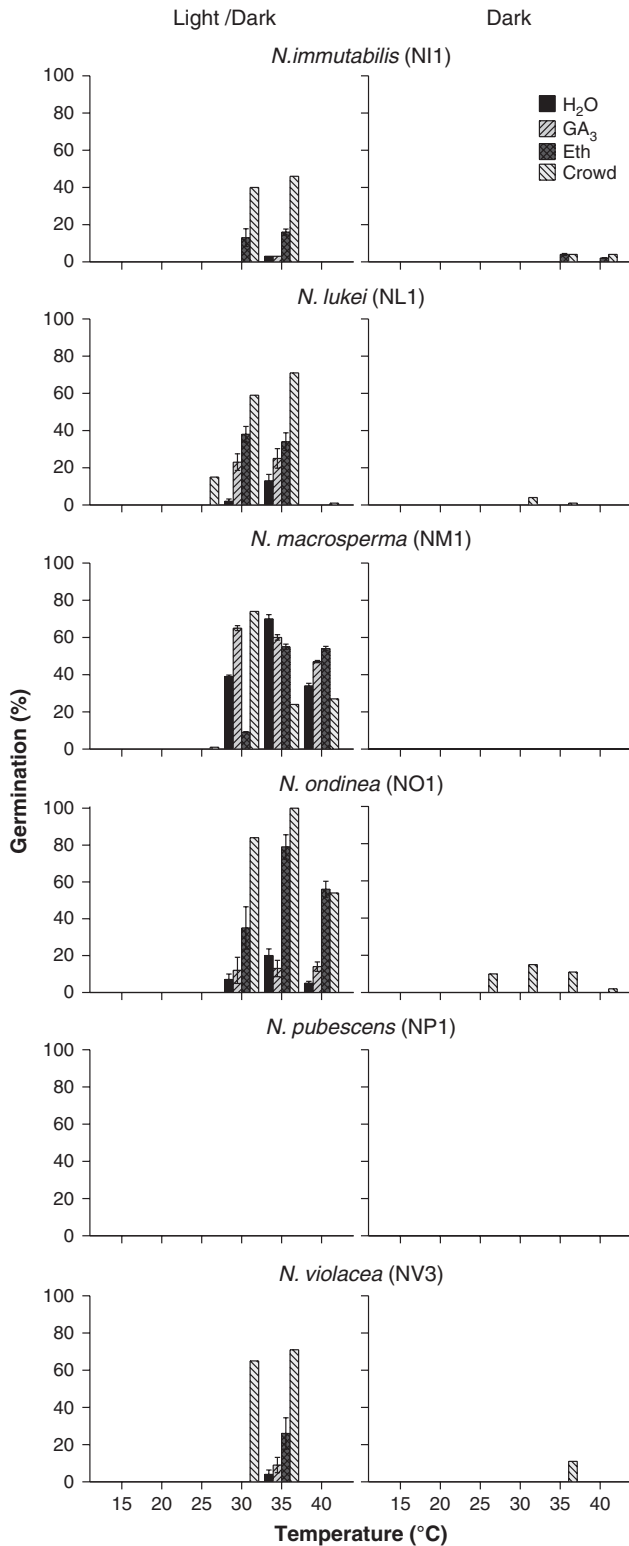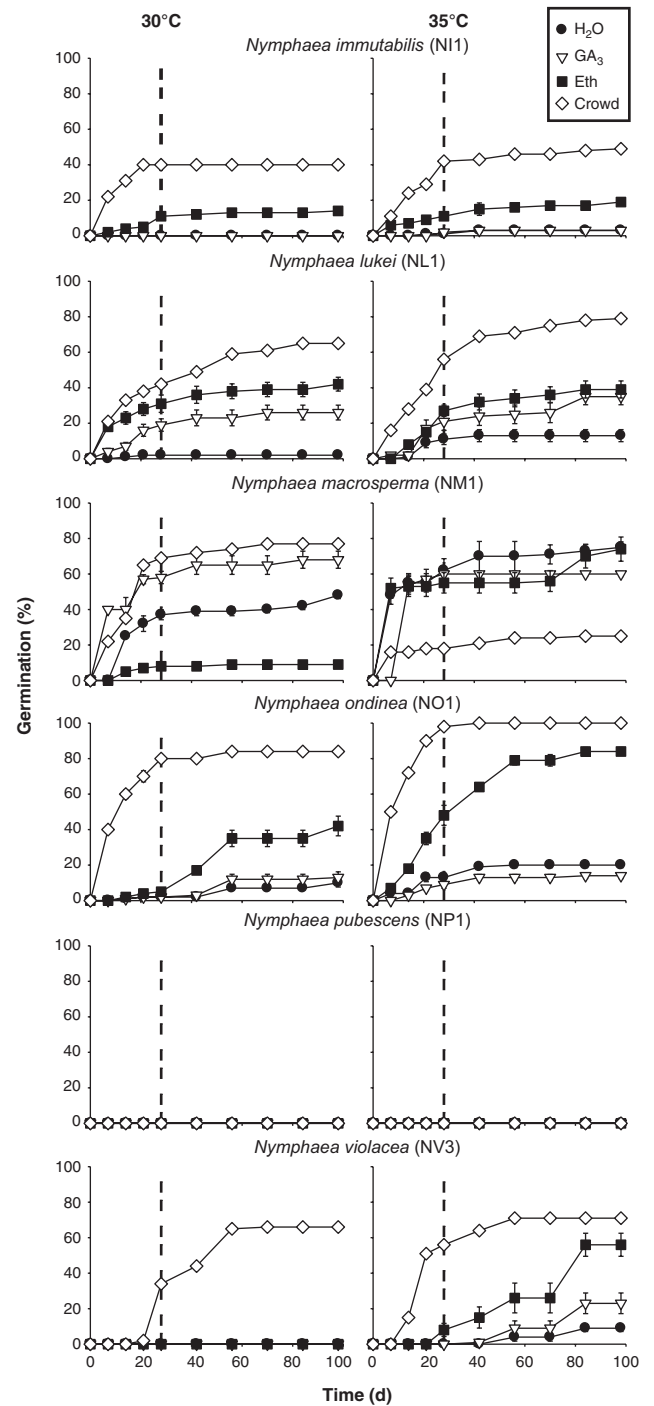
FIG. 2. Effect of H_2O (control), gibberellic acid (GA$_3$; 0.29 μM), ethephon (Eth; 20 mM) and crowding (Crowd) on total germination percentage (mean ± s.e.) of six *Nymphaea* species exposed to light/dark or full dark conditions at six temperatures after 8 weeks.

FIG. 3. Effect of H_2O (control), gibberellic acid (GA$_3$; 0.29 μM), ethephon (Eth; 20 mM) and crowding (Crowd) on cumulative germination percentage (mean ± s.e.) of six *Nymphaea* species exposed to 12/12-h light/dark regime at optimum temperature (30 °C and 35 °C) range. Dashed vertical line indicates 28 d of incubation, used to classify dormancy type.

that the seeds have PD. Compared to seeds of most other species that have MPD/MD, this amount of embryo growth is small. For example, underdeveloped linear embryos in members of

the Campanulaceae (Baskin *et al.*, 2005) increased 87–179 % prior to radicle emergence. On the other hand, embryos in seeds of *Viburnum odoratissimum* (Adoxaceae) (Baskin *et al.*, 2008) increased by approx. 300 %, and those in seeds of the basal angiosperm *Schisandra arisanensis* increased by 360 % (Chien *et al.*, 2011) prior to germination. However, in some seeds with MD/MPD, there is a relatively small amount of embryo
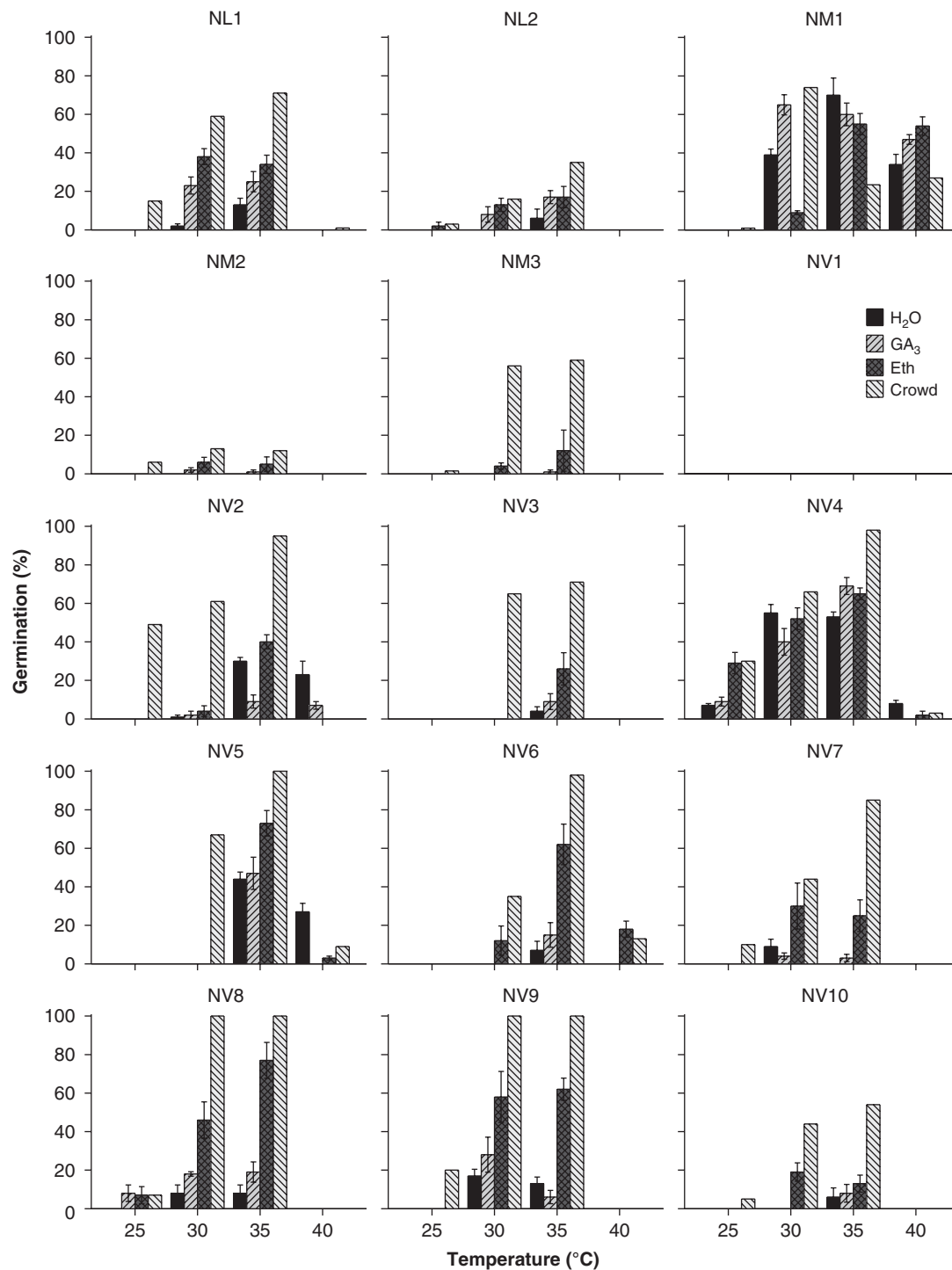
FIG. 4. Effect of H_2O (control), gibberellic acid (GA$_3$; 0.29 μM), ethephon (Eth; 20 mM) and crowding (Crowd) on total germination percentage (mean ± s.e.) of multiple collections of *Nymphaea lukei* (NL1, NL2), *N. macrosperma* (NM1–NM3) and *N. violacea* (NV1–NV10) after 8 weeks of incubation in light at 25, 30, 35 or 40 °C.

growth. For example, in a recent study of the basal angiosperm *Austrobaileya scandens*, embryo development and growth continues following seed dispersal and prior to germination, and longitudinal sections of the seed shows that embryo growth is minor prior to the emergence of the radicle (Losada *et al.,* 2017). Furthermore, Erickson *et al.* (2017) found that embryos in seeds of *Wahlenbergia tumidifructa* (Campanulaceae)

increased in size by 27.5 % prior to radicle emergence, and thus were considered to have MD (Erickson *et al.,* 2016).

In seeds of *N. ondinea* and *N. violacea* in the present study, the increase in embryo length prior to germination was 37 and 32 %, respectively, with growth occurring over a period of several days (Dalziell, 2016). Thus, we conclude that these embryos are underdeveloped and have MPD. We
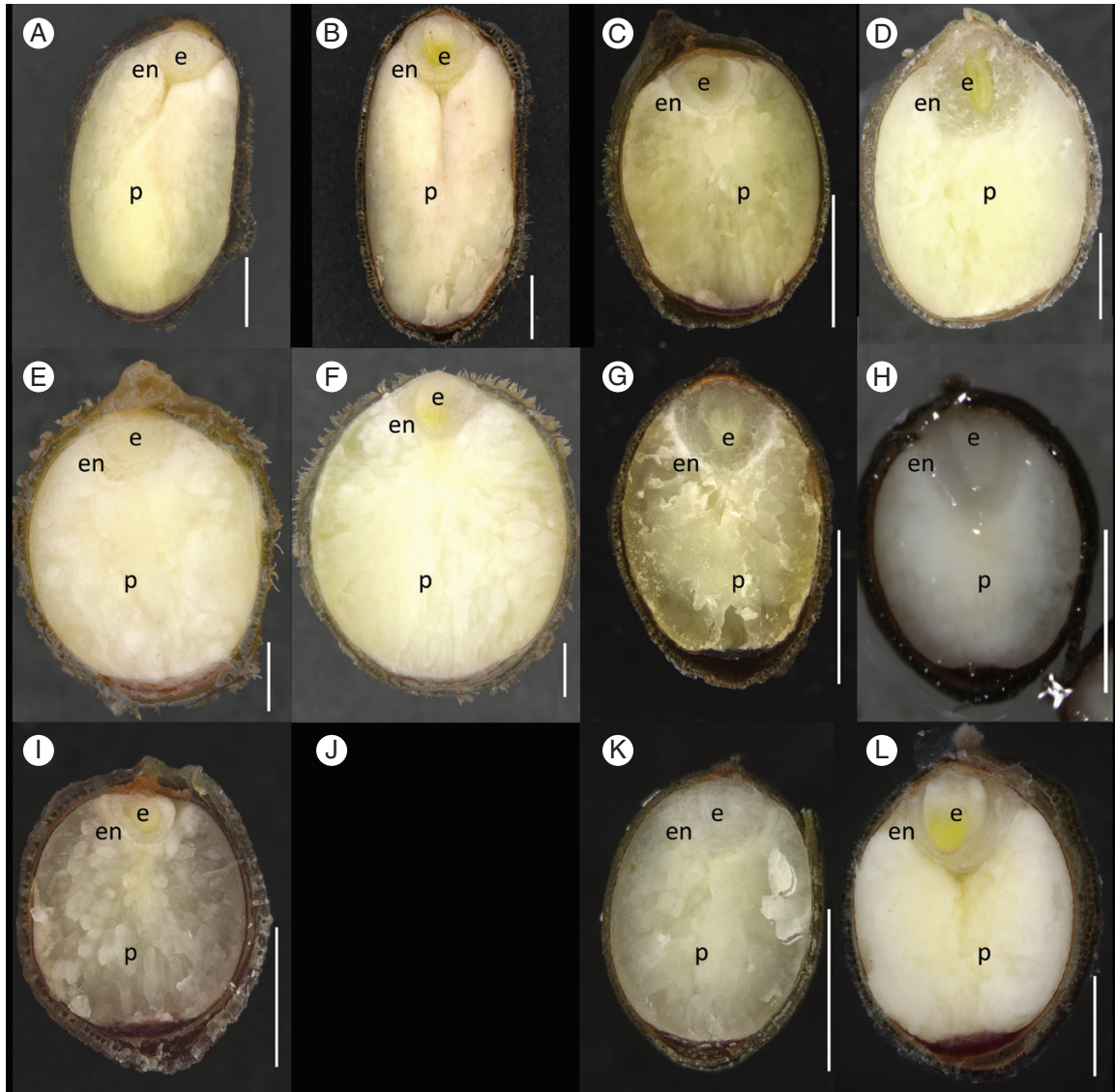
Fig. 5. Longitudinal sections of seeds of *Nymphaea* species prior to (A, C, E, G, I and K) and after a period of incubation (B, D, F, H and L), showing evidence of embryo growth: *N. immutabilis* (A and B), *N. lukei* (C and D), *N. macrosperma* (E and F), *N. ondinea* (G and H), *N. pubescens* (I and J: seeds did not germinate) and *N. violaceae* (K and L). e = embryo, en = endosperm, p = perisperm. Scale bar (white vertical line in each image) = 1 mm.

can further conclude that the level of MPD present in seeds is non-deep simple MPD, i.e. embryo growth occurs at temperatures suitable for warm stratification. Temperatures in northern Australian rarely fall below 10 °C (Bureau of Meteorology, 2005), and germination occurs only at high temperatures (30–35 °C), indicating that embryos only grow at temperatures suitable for dormancy break during warm (≥15 °C) stratification (Baskin and Baskin, 2004, 2014). The embryos of seeds of *N. macrosperma* (accession NM2) increased in length by 21 % prior to germination, indicating that seeds of this species also have some morphological component to their dormancy. As total germination in two of three collections of *N. macrosperma* (NM2 and NM3) was low without the addition of a germination-stimulating treatment, we conclude that the majority of seeds in these collections have non-deep simple MPD. Given the higher germination observed in the NM1 accession of

N. macrosperma seeds, it seems this population of seeds either has morphological (MD) dormancy only or the seeds are non-dormant.

The five species of *Nymphaea* that germinated in our study did so at significantly higher percentages in light than in darkness. This result is congruent with the findings of a range of studies showing that the majority of aquatic plants require light to germinate (Baskin and Baskin, 2014), including those that grow in temporary pools in the Mediterranean region (Carta *et al.*, 2013), south-west Western Australia (Tuckett *et al.*, 2010*a*) and the monsoon tropical Kimberley region in Australia (Cross *et al.*, 2015). The requirement for light may ensure that germination occurs at shallow enough depths of water to allow sufficient light penetration to support subsequent seedling establishment, or allows for germination of seeds in the soil seed bank in response to disturbance (Grime *et al.*, 1981; Pons, 1991). The five germinating species of *Nymphaea* in our study

TABLE 3. *Information on fresh seed embryos and embryos prior to hypocotyl emergence of six* Nymphaea *species; embryo type classified as per Martin (1946)*

Species	Collection	Embryo type	Embryo differentiated?	Mean embryo length (mm) of fresh seed (mean ± s.e.)	Embryo length (mm) at hypocotyl emergence (mean ± s.e.)	E: S ratio of fresh seed (mean ± s.e.)	E: S ratio at hypocotyl emergence (mean ± s.e.)
Nymphaea immutabilis	NI1	Broad	Y	0.78 ± 0.02[a]	0.86 ± 0.01[b]	0.19 ± 0.01[a]	0.22 ± 0.002[b]
Nymphaea lukei	NL1	Broad	Y	0.53 ± 0.02[a]	0.61 ± 0.01[b]	0.22 ± 0.01[a]	0.27 ± 0.003[b]
	NL2	Broad	Y	0.58 ± 0.02[a]	0.59 ± 0.01[a]	0.24 ± 0.01[a]	0.26 ± 0.01[a]
Nymphaea macrosperma	NM2	Broad	Y	0.71 ± 0.02[a]	0.86 ± 0.01[b]	0.22 ± 0.005[a]	0.28 ± 0.002[b]
Nymphaea ondinea	NO1	Broad	Y	0.49 ± 0.01[a]	0.67 ± 0.01[b]	0.29 ± 0.01[a]	0.38 ± 0.004[b]
Nymphaea pubescens	NP1	Broad	Y	0.35 ± 0.01	NA	0.20 ± 0.01	NA
Nymphaea violacea	NV7	Broad	Y	0.43 ± 0.01[a]	0.55 ± 0.01[b]	0.25 ± 0.01[a]	0.30 ± 0.01[b]
	NV8	Broad	Y	0.38 ± 0.01[a]	0.48 ± 0.01[b]	0.23 ± 0.01[a]	0.31 ± 0.003[b]
	NV9	Broad	Y	0.38 ± 0.01[a]	0.47 ± 0.01[b]	0.23 ± 0.01[a]	0.31 ± 0.004[b]
	NV10	Broad	Y	0.33 ± 0.01[a]	0.50 ± 0.01[b]	0.22 ± 0.004[a]	0.32 ± 0.003[b]

Letters in superscript denote significant differences at $P < 0.05$.

germinated to the highest percentages at 30 and 35 °C; these are similar to the mean maximum temperatures (Table 1) seeds experience during maturation and dehiscence. Furthermore, *N. macrosperma* and *N. ondinea* germinated to >50 % at 40 °C in some treatments. Outside the optimal temperature range, particularly at lower temperatures, germination did not occur, or was reduced significantly in all species. Interestingly, no germination was recorded for any species when seeds were incubated at 20/35 °C. The suppression of germination under such a diurnal variation in temperature may allow seeds to avoid unseasonal germination during the onset of the dry season, when night temperatures and water availability decrease (Bureau of Meteorology, 2013). At optimal temperatures, crowding (*sensu* Else and Riemer, 1984) elicited the greatest germination response in the five species (and most collections) and the addition of ethephon also tended to increase germination. The increased germination response in the crowding treatment has previously been attributed to the endogenous production of ethylene by seeds, which stimulates germination (Else and Riemer, 1984; Yin *et al.*, 2009). In the natural environment, ethylene is produced under waterlogged or anaerobic conditions in both permanently (Smith and Restall, 1971; Zeikus and Winfrey, 1976) and ephemerally (Cross *et al.*, 2014) inundated wetland soils. The region of northern Australia from which our collections were made experiences summer monsoonal rainfall, and summer temperatures frequently exceed 35 °C with high overnight temperatures (McQuade *et al.*, 1996; Bureau of Meteorology, 2011). The strong germination response to high temperatures and ethylene suggests that seeds of these *Nymphaea* are cued to germinate and establish in the summer wet season, when water availability is at its peak. In ephemeral creeks, where water turbidity is low, and visibility is high (i.e. allowing for observations to be made), seedlings of *N. lukei*, *N. ondinea* and *N. violacea* often can be seen during the late wet season (February). While this region of northern Australia reliably receives rainfall during the summer wet season, the timing, duration and amount of precipitation is highly variable and often related to cyclonic events (Taylor and Tulloch, 1985; Peel *et al.*, 2007; Bureau of Meteorology, 2011), which has a significant impact on the hydrological regime of wetlands in

the region (Cowie *et al.*, 2000). Thus, seed dormancy delays germination for a period of time, which ensures that water is available for establishment either in the same or in a subsequent wet season (Tuckett *et al.*, 2010*a*, *b*; Cross *et al.*, 2015).

Depth of dormancy varied both between and within the collected populations of seeds of the *Nymphaea* species we tested. No seeds of *N. pubescens* germinated, suggesting that they were deeply dormant, whereas seeds of the other five species germinated. Furthermore, germination responses differed substantially between seed collections of *N. lukei*, *N. macrosperma* and *N. violacea*. Spatial and temporal variation in the degree of dormancy and germination requirements among seed populations is well established (Andersson and Milberg, 1998; Donohue, 2009; Baskin and Baskin, 2014). *Nymphaea violacea* seeds collected from the same location in different years (NV1 and NV6) had very different responses; seeds of NV1 did not germinate under any conditions (despite being deemed viable), while seeds of NV6 displayed 100 % germination under optimal conditions. Similarly, some seeds of *N. macrosperma* NM1 appear to be ND (i.e. those of NM1), but only a very low germination percentage was obtained for seeds of other accessions (NM2 and NM3). These responses may be attributed to a number of factors, including maternal/genetic or ecotypic effects between seed populations (see Baskin and Baskin, 2014, and references therein). Given the inter-seasonal variability and unpredictable nature of rainfall in the wet–dry tropics of Australia, along with the range of sizes of permanent and ephemeral wetlands *Nymphaea* species inhabit (Table 1), it seems likely that seeds from environments with greater unpredictability may produce more dormant seeds than those in predictable environments, thus ensuring population survival during adverse conditions. For example, seeds of NV4 collected from plants growing in a large, permanent water body were the least dormant of the *N. violacea* collections, while the majority of the other *N. violacea* collections came from ephemeral wetlands and these seeds were more dormant.

Our study highlights some diversity in the dormancy and germination traits in tropical, Australian *Nymphaea* species, particularly those occurring in unpredictable wetland ecosystems. Overall, our results lead to the conclusion that the ANA-grade

angiosperms encompass a range of seed dormancy classes, including MPD in *Amborella trichopoda* (Fogliani *et al.*, 2017) and *Austrobaileya scandens* (Losada *et al.*, 2017); the specialized form of MPD in *Trithuria austinensis* and *T. submersa* (Tuckett *et al.*, 2010*b*); PD in seeds of *Nymphaea immutabilis*, *N.* Albert Greenburg and *N. capensis* var. *zanzibariensis* (Baskin and Baskin, 2007); and the range of PD, MPD, MD and ND we have found here in *N. immutabilis*, *N. lukei*, *N. macrosperma*, *N. ondinea*, *N. pubescens* and *N. violacea*. However, there is still a significant lack of information about the kinds of dormancy in other extant genera within the Nymphaeales. To our knowledge, no studies have specifically investigated the kind of dormancy in seeds of *Cabomba*, *Brasenia* (Cabombaceae), *Barclaya*, *Euryale*, *Nuphar* or *Victoria* (Nymphaeaceae). Previous reports of PD in *Cabomba* and *Nuphar* have been based on inference from reports of germination observations or experiments (e.g. see Tarver and Sanders, 1977; Smits *et al.*, 1990, 1995). Furthermore, the current phylogenetic tree of the Nymphaeaceae is incomplete; this is a task made particularly challenging by the complex, reticulate patterns of evolution and high frequency of hybridity within certain groups of *Nymphaea*, and there being a general lack of information regarding the distribution and ecology of species (Löhne *et al.*, 2008*a*, 2009; Borsch *et al.*, 2011). As such, further research is required to resolve the evolution of PD/MPD within the Nymphaeaceae.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: Summary statistics (*z*-statistics and *P*-values) from binomial logistic regressions to determine the effects of GA$_3$, ethephon, crowding, light and temperature on germination of the six *Nymphaea* species studied. Table S2: Summary statistics (*z*-statistics and *P*-values) from binomial logistic regressions to determine the effects of GA$_3$, ethephon, crowding and temperature on germination of individual collections of *Nymphaea lukei*, *N. macrosperma* and *N. violacea*.

ACKNOWLEDGEMENTS

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