Seed reproductive biology of the rare aquatic carnivorous plant *Aldrovanda vesiculosa* (Droseraceae)

ADAM T. CROSS¹,², LUBOMIR ADAMEC³, SHANE R. TURNER¹,², KINGSLEY W. DIXON¹,²,⁴ and DAVID J. MERRITT¹,²

¹School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley, Perth, WA 6009, Australia
²Kings Park and Botanic Garden, Fraser Avenue, Kings Park, Perth, WA 6005, Australia
³Section of Plant Ecology, Institute of Botany of the Czech Academy of Sciences, Dukelská 135, CZ-379 82 Trebon, Czech Republic
⁴Department of Environment and Agriculture, Curtin University, GPO Box U1987, Bentley, Perth, WA 6102, Australia

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Despite the unprecedented global decline in extant populations of *Aldrovanda vesiculosa* in the last century, little is known about the reproductive biology of this iconic aquatic carnivorous plant. This study aimed to investigate the role of seed-based reproduction in the ecology of *A. vesiculosa*, with particular focus on the interplay between the regulation of seed dormancy by temperature cues and the efficacy of exogenous ethylene gas to act as a germination stimulant, the desiccation capacity and long-term storage potential of seeds for conservation purposes. Sexual reproduction appears to be extremely limited in both natural and naturalized populations across three continents, with high variability in the success of flowering and seed set between sites and between seasons. Overall, flowering yielded few fertile fruit (6–19% of flowers producing fertile fruit) and seed viability was variable but generally low (29–88%). Fecundity appears to be influenced by seasonal climatic conditions and microhabitat characteristics. *Aldrovanda vesiculosa* possesses physiologically dormant seeds, with germination stimulated by exposure to ethylene gas (>90% germination) at 25 °C. Seeds appear sensitive to desiccation and sub-zero temperature storage, with no germination and markedly reduced embryo growth after storage of seeds for >1 month at 15 °C and 15% relative humidity or after short-term (24 h) storage at −18 °C. In the absence of significant conservation and restoration initiatives, the continuing decline of dystrophic freshwater wetland habitats globally leaves *A. vesiculosa* facing extinction. As the successful long-term storage of seeds appears unfeasible based on the approaches described in this study, other alternatives for germplasm conservation such as cryostorage of vegetative tissues or zygotic embryos must be considered for establishing long-term *ex situ* collections of critical germplasm. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016, 180, 515–529


INTRODUCTION

Few experimental studies provide empirical data on the germination ecology and storage capacity of the seeds and embryos of carnivorous plants (Baskin & Baskin, 2014), although many of these plants are considered to be of high conservation concern (Jennings & Rohr, 2011; Cross et al., 2013). Little is known generally about the reproductive biology of many species in this ecologically novel group, but like other charismatic plant groups such as orchids and cycads, carnivorous plants are under increasing pressure from habitat destruction and illegal collection (Jennings & Rohr, 2011). This paucity of information is an increasing global concern in the context of biodiversity conservation, with many carnivorous taxa currently assessed by the International Union for Conservation of Nature (IUCN) in
One example of a rare carnivorous species with a rapidly diminishing natural range requiring urgent conservation and management is *Aldrovanda vesiculosa* L. (Droseraceae), a rootless submerged carnivorous macrophyte with a disjunct global distribution (Elansary, Adamec & Storchová, 2010; Cross, 2012a). The only species known to have evolved snap-trap carnivory in the aquatic environment, *A. vesiculosa* has intrigued botanists since its discovery in 1696 (Darwin, 1876; Cross, 2012a). Most notably, its ecological novelty fascinated Charles Darwin, whose early experiments of carnivory and nutrient uptake in *A. vesiculosa* led to the conclusion that the foliage of the species was manifestly adapted for the capture of aquatic living prey such as water fleas and mosquito larvae (Darwin, 1876). The species once had an astonishingly expansive historical distribution, occurring naturally in nutrient-impoverished, oligo-mesotrophic and dystrophic (humic) wetland systems in Australia, Africa, Asia and continental Europe (Cross, 2012a).

Although broadly distributed, *A. vesiculosa* possesses a narrow ecological niche, appears highly sensitive to eutrophication (Kamiński, 1987a, b; Cross, 2012a) and is generally limited in suitable wetlands to specific, nutrient-poor, island-like microhabitats (Adamec, 1999a, 2005; Cross et al., 2015a). Subtle changes in the physico-chemical or biotic conditions in an inhabited wetland can lead to rapid local decline (Kamiński, 1987a, b; Adamec, 1999a, 2005; Adamec & Lev, 1999). Wetland degradation, primarily through eutrophication or drought, has resulted in a global collapse of *A. vesiculosa* populations within the last century (Cross, 2012a). The exact reasons why decline occurs in response to eutrophication remain partly unclear, but the species has become extinct in at least 11 of the 43 countries in which it is known from historical records to have occurred naturally (a total decline in abundance of c. 87%) and remains recently unverified in a further 21 countries (Cross, 2012a). Subsequently, the species has been recognized as Endangered B2ab (iii, v) by the IUCN (Cross, 2012b).

Developing effective conservation actions for rare taxa for which ecological requirements or seasonal biology may not be known requires, in part, an understanding of the phenology of seed development and maturation and the cues required for seed dormancy alleviation and germination. Likewise, establishing viable long-term *ex situ* germplasm collections relies on the development of adequate storage procedures (Merritt & Dixon, 2011). Factors related to germination biology are likely to be particularly pertinent for rare taxa for which ecological requirements or seasonal biology may not be known and are likely to have significant management implications. In such cases, it is important that seed biology data be presented in a biologically meaningful context, linking observed seed dormancy and germination characteristics to relevant environmental mechanisms such as seasonal temperature and moisture cues and natural germination promoters including light, temperature, smoke-related products, nitrates and ethylene (e.g. Merritt et al., 2007; Cross et al., 2013, 2015a, b; Baskin & Baskin, 2014; Long et al., 2014).

Although information pertaining to the success of sexual reproduction and dispersal in *A. vesiculosa* is largely anecdotal, fecundity appears to be linked strongly to microhabitat suitability (Cross et al., 2015a) and flowering is reported to be sporadic, uncommon and to rarely result in fertile fruit (Cross, 2012a). Although it has been suggested that a minimum period of high water temperature (>25 °C) may be required to initiate flowering in temperate populations of *A. vesiculosa* (Adamec & Tichý, 1997), the link between seasonal conditions and flowering remains unclear. Although herbarium specimens and published records suggest that *A. vesiculosa* is capable of producing flowers at all inhabited latitudes from northern Russia to south-western Western Australia (Cross, 2012a), most authors note that in temperate regions in particular flowers remain closed and fail to produce fertile fruit (Adamec & Tichý, 1997; Okada, 1998; Adamec, 1999b). This may be a result of the strong propensity for rapid asexual vegetative reproduction in *A. vesiculosa* (Adamec, 1999a, 2000; Adamec & Lev, 1999; Adamec & Kovárová, 2006), which is a common characteristic of many aquatic macrophytes and may imply a significantly reduced role for sexual reproduction in the species (Barrett, Eckert & Husband, 1993; Philbrick & Les, 1996).

A general lack of access to viable seeds means the mechanisms regulating germination remain unresolved for *A. vesiculosa*. However, Droseraceae include some species with physiologically dormant seeds such as *Dionaea muscipula* Ellis ex L. and *Drosera anglica* Huds. (Garnett, 1981; Baskin et al., 2001; Baskin & Baskin, 2014). Dormancy in physiologically dormant seeds is regulated primarily by changes in temperature and moisture and is often alleviated by warm (≥15 °C) or cold (0–10 °C) stratification depending on the climate of origin (Baskin & Baskin, 2014). Additional chemical cues (i.e. stimulants) may be required to promote germination once dormancy has been alleviated (e.g. Merritt et al., 2007; Chiwocha et al., 2009). Recent studies have implicated ethylene as a significant germination stimulant for numerous plant taxa with
physiologically dormant seeds, particularly several aquatic macrophytes (Kepczynski & Kepczynska, 1997; Lin, Zhong & Grierson, 2009; Cross et al., 2014, 2015b), and the presence of ethylene in the aquatic environment is considered intrinsic to various aspects of the growth and development of freshwater plants (Osborne, 1984). Consequently, the interplay between seed dormancy regulation by temperature and the efficacy of germination stimulants such as ethylene is an important consideration in resolving the seed germination ecology of aquatic taxa such as A. vesiculosa and improving the use of the seeds of these species for conservation purposes.

In this study we investigated the mechanisms responsible for regulating dormancy and stimulating germination in seeds of A. vesiculosa and examined sexual reproductive characteristics of the species in terms of flowering and seed production. The aims of this study were to: (1) confirm the presence and classify the kind of dormancy possessed by A. vesiculosa seeds; (2) determine the germination response of A. vesiculosa seeds to different light and temperature cues, particularly cold stratification; (3) elucidate the efficacy of exogenous ethylene as a germination stimulant; (4) determine the storage behaviour of A. vesiculosa seeds to inform long-term storage approaches for ex situ conservation; (5) examine the flowering characteristics of plants and analyse the environmental conditions preceding flowering; and (6) examine fruit production and seed set as an indicator of sexual reproductive success between natural, naturalized and cultivated populations from three continents (Australia, Europe and North America). The long-term outlook for A. vesiculosa throughout its natural range appears to be increasingly bleak in the face of continued wetland degradation (Adamec, 2005; Cross, 2012a; Cross et al., 2015a) and determining the role played by seed-based reproduction in the global ecology of the species is critical to ensuring the success of future conservation and management initiatives such as ex situ seed banking.

MATERIAL AND METHODS

STUDY SITES AND MORPHOMETRIC MEASUREMENTS

Observations of flowering, morphometrics and seed set were undertaken at one natural and four naturalized A. vesiculosa populations on three continents between 2010 and 2015: (1) Cape le Grande (CLG), a small native population from Esperance, south-western Western Australia (33°50'58.1"S, 122°12'44.9" E), growing in a shallow (c. 40 cm) seasonally inundated dystrophic swamp among Baumea articulata (R.Br.) S.T.Blake (Cyperaceae) and under trees including Melaleuca cuticularis Labill. and Eucalyptus cornuta Labill (Myrtaceae); (2) Meadowview Pond (MVP), an extensive naturalized population (plants originating from Kyoto, Japan) in Virginia, USA (38°08'45.5"N, 77°23'16.8"W), growing in a shallow (c. 20–40 cm) mesotrophic swampy pond among Carex alata Torrey and Carex lirida Wahlenb. (Cyperaceae), Nuphar advena (Ait.) Ait.f. (Nymphaeaceae), Sagittaria latifolia Wild. (Alismataceae) and Typha latifolia L. (Typhaceae); (3) Meadow Creek (MCR), a large naturalized population (plants originating from Kyoto, Japan) in Virginia, USA (38°08'44.1"N, 77°23'08.6"W), growing in a shallow (c. 15–30 cm) mesotrophic marshland with Utricularia purpurea Walt. and Utricularia inflata Walt. (Lentibulariaceae) among scattered Peltandra virginica (L.) Schott (Araceae), Nuphar advena and Nymphaea odorata Aiton subsp. odorata (Nymphaeaceae); (4) Suchdol nad Lužnicí sand-pit (SNL), a small naturalized population (plants originating from Hungary) in South Bohemia, Czech Republic (48°55'08.5"N, 14°53'03.1"W), growing in two adjacent oligotrophic sand-pit pools (a shallow (c. 25–50 cm) pool (SNL1) along loose stands of Eleocharis palustris (L.) Roem. & Schult. (Cyperaceae), Juncus bulbosus (L.) (Juncaceae) and Utricularia bremii Köl., and a deeper (c. 50–90 cm) pool (SNL2) along loose stands of Eleocharis palustris, Typha latifolia, Juncus bulbosus and Potamogeton natans L. (Potamogetonaceae); and (5) Karštejn (KAR), a small naturalized population (plants originating from eastern Poland) in South Bohemia, Czech Republic (49°08'20.8"N, 14°47'59.2"W), growing in a shallow (c. 20–40 cm) dystrophic fen lake (after fen extraction) among loose stands of Carex rostrata Stokes (Cyperaceae), Phragmites australis (Cav.) Steud. (Poaceae) and scattered Potamogeton natans.

Pressed specimens and ex situ living collections of each population are vouched at the Institute of Botany of the Czech Academy of Sciences (Trebon, Czech Republic: SNL, KAR), Kings Park and Botanic Garden (Perth, Western Australia: CLG) and Meadowview Biological Research Station (Virginia, USA: MVP, MCR).

To compare the morphological characteristics of flowering individuals between sites, the shoot length and the number of whorls bearing mature traps, branches, unopened flower buds of any stage and flowers of flowering individuals were measured periodically throughout the flowering season at MVP, MCR, SNL1 and KAR (see Adamec, 1999b; Adamec & Lev, 1999). Brown and senescing shoot segments were not measured. Apical and axillary branches were included. Measurements were taken on 25 and 30 June 2013 at MVP and MCR (N = 100 and 40, respectively), 13 and 27 July and 17 August 2014 at
SNL1 (N = 40 in all cases) and 20 July and 23 August 2014 at KAR (N = 28 and 40, respectively). Due to the size of populations at MVP and MCR, measurements were undertaken on all flowering individuals in 50 randomly selected 0.5 × 0.5-m quadrats along shore–shore (north–south) transects at these sites (see Cross et al., 2015a), and at SNL1 and KAR measurements were made on plants randomly selected from small (c. 3 m²) microsites exhibiting the most prolific flowering.

Fertile fruits (capsules) were collected from four populations at the end of each flowering season and opened to determine the presence and number of seeds produced per capsule. Only mature seeds (black with a hard testa) were counted (see Cross, 2012a). Fruits were collected from CLG in 2010, 2011, 2012, 2013 and 2014, from MVP and MCR in 2013, from SNL1 in 2012, 2013 and 2014, and from SNL2 and KAR in 2015.

Surface water temperature during peak flowering periods was measured at 10-min intervals using a datalogger at SNL1 between 22 June and 16 September 2014 (Temperature Minikin; EMS Brno, Czech Republic) and manually at 30-min intervals between 10:00 and 16:00 h at MVP between 26 June and 5 July 2013 (TPD39 Digital Stem Thermometer; Omega). To examine the link between water temperature and flowering, the total number of flowers open at MVP at each measurement time was counted. Analytical determination of water chemistry parameters was conducted at each sample location at each time of measurement, measuring electrical conductivity, pH, total alkalinity and the concentration of ammonium- and nitrate-nitrogen and phosphorus (Appendix). The concentration of dissolved CO₂ was calculated from pH and total alkalinity values where possible.

CULTIVATION AND SEED COLLECTION

Individuals were collected from CLG in 2009 and maintained in ex situ cultivation in Perth, Western Australia (sensu Adamec, 2000; Cross, 2012a). Plants were grown in 150-litre containers filled with rainwater, among 40–60% cover of the native Australian sedge Baumea articulata, approximating natural habitat at the CLG site. Water depth was maintained between 5 and 20 cm by topping up with reverse-osmosis water as required. Mature fruits were collected from cultivated individuals of A. vesiculosa each season, with seeds used for experimental studies collected between January and March 2012. Due to the conservation status and low numbers of viable seeds produced by remaining natural populations, all seeds utilized in experiments originated from cultivated plants. The ex situ population (c. 20 000 plants) is larger than most sampled field sites and inbreeding is not considered to be a significant issue given the highly clonal nature of natural A. vesiculosa populations (Adamec, 1999a; Adamec & Kovářová, 2006; Cross, 2012a; Cross et al., 2015a).

Additionally, previous studies have suggested that A. vesiculosa possesses extremely low levels of genetic variation within and between populations (Maldonado San Martin et al., 2003; Elansary et al., 2010, A. T. Cross et al., unpubl. data). Fruits were transported intact back to the laboratory to prevent desiccation prior to experiments.

SEED AND EMBRYO CHARACTERISTICS

Seed quality in seeds collected from CLG, MVP and MCR and from ex situ-cultivated plants was determined for three replicates of 100 seeds from each collection via X-ray analysis (MX-20 digital X-ray cabinet; Faxitron). Seeds were scored as filled if the endosperm was fully developed, not shrunk or retracted from the testa and showed no sign of internal damage. Seed moisture content was determined gravimetrically for fresh seeds and seeds stored dry at 15 °C and 15% relative humidity (RH) for 1 month following the methods of International Seed Testing Association [ISTA] (1999). The length and width of all 300 seeds and the length and width of each embryo were measured digitally during X-ray analysis (Faxitron DX 1.0; Faxitron). Seed coat and internal structure were analysed under high-magnification using a scanning electron microscope (JCM-5000 Neoscope; Jeol), with seeds placed on carbon tape and mounted on a 1-cm metal stub before being coated with a thin layer of gold under vacuum (2 min duration, K550X Sputter Coater; Emitech) to enhance electron imagery. Samples were analysed at 10 kV under high vacuum.

To assess the water permeability of the seed coat, three 0.05-g replicates of freshly collected, filled seeds were immediately placed into small (2.5 × 2.5 cm) nylon mesh bags. Each bag was weighed, filled with seeds and placed in a Petri dish lined with filter paper irrigated with deionized (DI) water. The bags of seeds were weighed at time 0 and after 1, 2, 5, 15 and 30 min and 1, 1.5, 2, 4, 6, 12, 24, 48 and 72 h of imbibition, after they had been gently patted dry on paper towels before each measurement. Percentage water uptake of seeds was determined gravimetrically based on the fresh weight of non-imbibed seeds after subtracting the weight of the bags, with the percentage increase in seed mass calculated by [(Wᵣ − Wᵦ)/Wᵦ] × 100, where Wᵣ and Wᵦ are the mass of imbibed and dry seeds, respectively (sensu Turner et al., 2006, 2009).

Seed buoyancy was assessed by placing three replicates of 100 filled seeds into 850-mL plastic
containers (110 mm diameter × 110 mm height) containing 700 mL of DI water (sensu Guja, Merritt & Dixon, 2010; Cross et al., 2015b). Containers were incubated under ambient conditions (22–26 °C). The water level was kept constant and the water surface agitated gently at each recording period by gently shaking the containers. The number of seeds that remained floating was recorded at time 0 and after 2, 15 and 30 min and 1, 2, 6, 12, 24, 36 and 48 h. Seed buoyancy (B) is the sum of the floating fraction at each successive interval of time divided by the time elapsed during the interval,

\[ B = \sum \left( \frac{F_N}{F_{total}} \right) / 10 \]

where \( F_N \) is number of seeds floating after \( N \) min and \( F_{total} \) is the total number of seeds. Thus, values of \( B \) range from 0 (all seeds sank immediately) to 1 (all seeds remained floating after 48 h).

**Seed germination biology**

To assess the germination response of *A. vesiculosa* seeds to a range of temperature, light and germination stimuli, freshly collected seeds were plated onto 0.7% (w/v) water agar only (control), 0.7% (w/v) water agar containing 2.89 mM gibberellic acid (GA3) or 0.7% (w/v) water agar after seeds had been exposed to 50 nmol ethylene gas (C2H4) for 24 h. Methods of ethylene exposure followed Cross et al. (2014, 2015b). Four replicates of 25 seeds for each treatment were placed in incubators at constant 5, 10, 15, 20, 25, 30 or 35 °C under a 12-h photoperiod and in constant darkness (plated in darkness and wrapped in aluminium foil). Germination (proto-root > 1 mm) was scored weekly for 8 weeks in light/dark treatments, but only after 8 weeks in dark treatments. Upon completion of the experiment, all non-germinated seeds were cut-tested to determine viability. Seeds with a firm, white endosperm and embryo were judged to be viable. Germination percentages are thus based on the number of viable seeds.

To investigate the impact of cold stratification on seed dormancy alleviation, replicates of 25 freshly collected seeds were prepared as described previously on water agar, water agar containing GA3 and on water agar after exposure to ethylene gas. Four replicates for each treatment were incubated at constant 5 or 25 °C with a 12-h photoperiod for 16 weeks, with a further four replicates similarly incubated at 5 °C for 8 weeks before transfer to 25 °C for a further 8 weeks. Germination was scored at the conclusion of the full 16-week incubation period and all non-germinated seeds were cut-tested to assess viability as previously described.

To determine whether embryo growth occurs inside seeds prior to radicle emergence, and thus if the seeds have morphological/morphophysiological dormancy (Baskin & Baskin, 2014), 100 seeds were placed in 90-mm Petri dishes on 0.7% (w/v) water agar after exposure to 50 nmol ethylene gas for 24 h (sensu Cross et al., 2014, 2015b), before being sealed with plastic cling wrap and incubated at 25 °C on a 12-h photoperiod. Prior to incubation and after each week for 8 weeks, ten seeds were randomly selected and dissected, with the seed and embryo length for each measured under a dissecting microscope equipped with an ocular micrometer to determine the embryo length to seed length (E:S) ratio.

**Seed storage behaviour**

To provide an assessment of seed storage behaviour, the germination response of freshly collected seeds was tested after 1, 3, 6 and 12 months of storage in a controlled environment room maintained at 15 °C and 15% RH. Additional replicates of seeds were hermetically sealed in laminated aluminium foil bags after 2 weeks of drying at 15 °C and 15% RH and stored at −18 °C, with germination response tested after 24 h, or after 3 months. Four replicates of 25 seeds from each treatment were removed at each time period, before being incubated as described previously on water agar after exposure to ethylene gas for 24 h. Seeds were incubated at constant 25 °C with a 12-h photoperiod, with germination assessed after 8 weeks.

Stored seeds were further examined to determine the growth potential of embryos following desiccation and subsequent sub-zero storage. Embryos were extracted and cultured from freshly collected seeds, seeds that had been stored dry at 15 °C and 15% RH for 1, 3 or 12 months and seeds that had been stored at −18 °C for 24 h or 3 months after drying to stable moisture content (5%, determined gravimetrically) for 2 weeks at 15 °C and 15% RH. Four replicates of ten seeds for each treatment were surface sterilized in 2% (w/v) calcium hypochlorite [Ca(OCl)2] solution for 30 min and then rinsed three times in sterile water inside a sterile laminar flow unit. Each seed was carefully dissected under a binocular microscope, with embryos excised and placed onto a filter paper inside sterile Petri dishes irrigated with either 10 mL of half-strength Murashige & Skoog (1962) liquid basal medium (%MS) only or %MS supplemented with plant growth regulators (PGRs) (1 μM zeatin and 3 μM GA3; Sigma Industries). The pH of all media was adjusted to pH 6.0. Embryo cultures were maintained at 22–24 °C for 8 weeks, in darkness for the first 2 weeks followed by a 16-h/8-h light–dark cycle.
 SEED LONGEVITY IN SEDIMENT

To assess the longevity of seeds in the sediment seed bank, five replicates of 100 filled seeds were sealed in small (5 x 5 cm) stainless steel mesh bags and buried at 1 cm depth in sediment at CLG in January 2011. Bags were exhumed after 1, 3, 6, 12 and 24 months, with remaining seeds counted and seed fill determined by X-ray analysis as previously described. All filled seeds were incubated as described previously on water agar only after exposure to ethylene gas for 24 h. Seeds were incubated at constant 25 °C with a 12-h photoperiod, with germination assessed after 8 weeks, and all non-germinated seeds were cut-tested to assess viability as previously described.

DATA ANALYSIS

Binary logistic regression (SPSS Statistics 23; IBM) was used to assess the main and interaction effects of light, temperature, GA3, ethylene, stratification and storage treatment on seed germination and the main and interaction effects of PGRs and storage conditions on the growth of excised embryos. One-way ANOVA with Tukey post-hoc tests were used to assess the variation in morphological and flowering characteristics (shoot length and the number of whorls, branches, flowers, fruits and seeds) between each of the sampled sites and between seasons. Linear regression models were fitted to examine the relationship between shoot length and the number of whorls and branches on the number of flowers per individual at KAR, MCR, MVP and SNL1 during the peak flowering period (late June to mid August). Where necessary, data were log10 transformed to help meet the assumption of normality and equal variance. All statistical tests were conducted using the 95% confidence interval (CI), with significance determined at $P < 0.05$. Data are presented as mean ± 1 SE of the raw data unless stated otherwise.

RESULTS

MORPHOMETRIC MEASUREMENTS

Morphological data were gathered for 707 flowering individuals of A. vesiculosa (Table 1). The number of flowers per individual ranged from one to five, varied between sites (d.f. = 712, $F = 18.6$, $P < 0.001$) and was negatively correlated with shoot length ($B = -0.005$, $P = 0.022$) and positively correlated with number of whorls ($B = 0.043$, $P = 0.002$). The number of flowers per individual increased markedly from mid July to mid August at SNL1 and KAR (Table 1), corresponding to a slight reduction in the average length of individuals (d.f. = 40, $B = -0.091$, $P = 0.271$). The number of flowers and the number of branches per individual was inversely correlated across all sites (d.f. = 712, $B = -0.177$, $P = 0.032$) and a strong positive correlation was present between the number of flowers per individual and the number of mature whorls ($B = 0.557$, $P < 0.001$). In total, 1166 fertile fruits were collected from five sites throughout the 5 years of study (Table 1), containing 4671 seeds (one to 12 per fruit). The number of seeds per fruit varied significantly between sites and between seasons in each site (d.f. = 866, $P < 0.001$ in both cases) (Table 1). The production of fertile fruit appeared to be generally uncommon, with infertile fruit and aborted inflorescences accounting for c. 87% of all flowering at CLG, 94% at MVP and 81% at MCR.

Mean daily surface water temperature during the late summer peak flowering period (late June to mid August) was 23.1 ± 2.4 °C at SNL1, with average daily minima and maxima 19.8 ± 2.0 and 27.0 ± 3.3 °C, respectively (Fig. 1). Flowering at SNL1 began on 8 July and was preceded by 5 days of increasing surface water temperature to a maximum of 28–33 °C (Fig. 1). Average daylight during the study period at MVP was 14 h and 44 min, with average surface water temperature 29.8 ± 0.4 °C, ranging from 25.4 to 36.0 °C. Open A. vesiculosa flowers at MVP were recorded only between 11:00 and 15:30 h, with peak flower opening occurring between 13:00 and 14:00 h daily (Fig. 2). The number of flowers open daily at MVP was positively correlated with increasing surface water temperature (d.f. = 77, $P = 0.01$). Water chemistry at sampled microsites harbouring prolific A. vesiculosa flowering was variable, but all were generally oligo-mesotrophic with low concentrations of N and P (usually <4 μg L⁻¹) during the peak flowering period (Appendix).

SEED AND EMBRYO CHARACTERISTICS

The dispersal unit of A. vesiculosa is a small, ovoid seed ($1.50 ± 0.04 \times 0.95 ± 0.04$ mm), with a substantial endosperm ($1.12 ± 0.02 \times 0.63 ± 0.01$ mm) surrounded by an inner integument, a palisade-like endotesta and a structurally complex honeycomb-like exotesta (Fig. 3). Seeds possess a basal, rudimentary embryo ($0.29 ± 0.05 \times 0.41 ± 0.04$ mm; Fig. 3). The fresh mass of individual seeds from CLG was $0.82 ± 0.01$ mg and moisture content at seed release was $34 ± 1\%$. The mass of individual seeds stored dry at 15°C and 15% RH for 1 month was $0.57 ± 0.01$ mg, with moisture content of $5 ± 2\%$. 

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Table 1. Morphological characteristics, fertile fruit production, seed production and seed fill of flowering *Aldrovanda vesiculosa* individuals at native and naturalized sites in Australia, North America and the Czech Republic

<table>
<thead>
<tr>
<th>Country</th>
<th>Site</th>
<th>Year</th>
<th>Month</th>
<th>N (individuals)</th>
<th>Shoot length (mm)</th>
<th>Whorls</th>
<th>Flowers</th>
<th>Branches</th>
<th>N (fruit)</th>
<th>Seeds per fruit</th>
<th>Mean</th>
<th>Range</th>
<th>Seed fill (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia</td>
<td>CLG</td>
<td>2010</td>
<td>January</td>
<td>141</td>
<td>95.6 ± 1.8a</td>
<td>13.0 ± 0.3a</td>
<td>1.3 ± 0.1a</td>
<td>0.8 ± 0.1a</td>
<td>16</td>
<td>4.3 ± 0.6a</td>
<td>1-8</td>
<td>88 ± 2a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2011</td>
<td>January</td>
<td>36</td>
<td>77.9 ± 3.4b</td>
<td>14.1 ± 0.5a</td>
<td>1.7 ± 0.2b</td>
<td>0.8 ± 0.2a</td>
<td>99</td>
<td>6.9 ± 0.3b</td>
<td>1-11</td>
<td>65 ± 3b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>January</td>
<td>74</td>
<td>121.8 ± 6.6c</td>
<td>13.2 ± 0.6a</td>
<td>1.7 ± 0.1b</td>
<td>1.0 ± 0.2a</td>
<td>177</td>
<td>7.8 ± 0.2c</td>
<td>1-12</td>
<td>29 ± 4c</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>January</td>
<td>80</td>
<td>146.2 ± 8.0d</td>
<td>15.0 ± 0.6a</td>
<td>1.9 ± 0.1b</td>
<td>0.9 ± 0.1a</td>
<td>27</td>
<td>4.0 ± 0.4a</td>
<td>1-8</td>
<td>32 ± 3c</td>
<td></td>
</tr>
<tr>
<td>North America</td>
<td>MVP</td>
<td>2013</td>
<td>June</td>
<td>149</td>
<td>103.2 ± 4.5a</td>
<td>20.2 ± 0.5a</td>
<td>2.3 ± 0.1a</td>
<td>1.2 ± 0.1a</td>
<td>12</td>
<td>5.3 ± 0.9a</td>
<td>1-9</td>
<td>54 ± 2a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCR</td>
<td>2013</td>
<td>June</td>
<td>39</td>
<td>147.2 ± 4.0b</td>
<td>28.5 ± 0.3b</td>
<td>2.7 ± 0.4b</td>
<td>1.3 ± 0.2a</td>
<td>20</td>
<td>5.5 ± 0.7a</td>
<td>1-9</td>
<td>76 ± 3b</td>
<td></td>
</tr>
<tr>
<td>Czech Republic</td>
<td>SNL1</td>
<td>2012</td>
<td>August</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>2.9 ± 0.2a</td>
<td>1-8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>August</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>345</td>
<td>5.0 ± 0.1b</td>
<td>1-10</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2014</td>
<td>Mid July</td>
<td>40</td>
<td>105.9 ± 16.7a</td>
<td>18.5 ± 2.9a</td>
<td>1.1 ± 0.2a</td>
<td>1.2 ± 0.2a</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Late July</td>
<td>40</td>
<td>84.3 ± 13.3a</td>
<td>19.9 ± 3.1a</td>
<td>2.5 ± 0.4b</td>
<td>1.2 ± 0.2a</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>SNL2</td>
<td>2015</td>
<td>August</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>302</td>
<td>3.5 ± 0.1d</td>
<td>1-10</td>
<td>–</td>
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<tr>
<td></td>
<td>KAR</td>
<td>2014</td>
<td>July</td>
<td>28</td>
<td>133.8 ± 25.3a</td>
<td>19.6 ± 3.7a</td>
<td>1.7 ± 0.3a</td>
<td>1.0 ± 0.2a</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
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<td>40</td>
<td>79.0 ± 12.5b</td>
<td>15.6 ± 2.5a</td>
<td>2.4 ± 0.4b</td>
<td>0.9 ± 0.1a</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

Apical and axillary branches were included. Means ± 1 SE are shown, with annotated lettering indicating significance in measured characteristics between measurement times (*P* < 0.05).
The embryo is fully developed at seed maturity in *A. vesiculosa*, with no significant embryo growth occurring prior to emergence of the proto-root ($d.f. = 19$, $F = 158$, $P = 0.696$). The E:S ratio in seeds from CLG was 0.35/C6 0.10. Following 72 h of imbibition *A. vesiculosa* seeds were found to have increased in mass by 14/C6 3 mg (16%) over their initial dry mass prior to hydration. Cut tests undertaken on these seeds following the completion of the imbibition experiment confirmed that water had indeed moved into the interior of the seeds, as the endosperm in imbibed seeds appeared soft and moist compared with the endosperm of non-hydrated seeds that was much denser and granulated in appearance following dissection. *Aldrovanda vesiculosa* seeds are poorly buoyant ($B = 0.18$), and most sank immediately (66 ± 8%). Only 12 ± 3% remained floating after 48 h (Fig. 4). Seed fill varied markedly between both seasons and sites, ranging from 30 to 90% (Table 1).

**SEED GERMINATION BIOLOGY**

A significant positive interaction effect was present between temperature and ethylene gas with respect to germination success ($P = 0.006$). For seeds
incubated at 25 °C under a 12-h photoperiod, germination was significantly higher following exposure to ethylene (94%), as compared with seeds on water agar (33%). Germination was not enhanced by cold stratification (32.5%), but seeds exposed to ethylene after cold stratification exhibited high germination.

Figure 3. Photographs of intact (A) and longitudinally dissected (B) *A. vesiculosa* seeds; high-resolution scanning electron micrographs detailing seed structures including the operculum and exostome (C), cross-section of the exotesta and pitted external seed surface (D), honeycomb-like internal surface of the exotesta (E) and spiral thickening on the radial walls of exotesta cells (F). Numbering indicates location of seed structures: 1, operculum; 2, rudimentary embryo; 3, exotesta; 4, palisade-like endotesta; 5, starchy endosperm. Scale bars indicate 1 mm in A and B, 100 μm in C and D, 200 μm in E and 20 μm in F.

Figure 4. *Aldrovanda vesiculosa* seed characteristics. A, imbibition (percentage uptake of water through the testa) of freshly collected seeds over 48 h; B, buoyancy of freshly collected seeds over 48 h under laboratory conditions; and C, seed viability (expressed as the percentage of seeds remaining filled) over 24 months of burial in sediments at a natural site in south-western Western Australia.
3 months at collected controls following ethylene exposure RH for 1 month was slightly lower than in freshly
° 30° on water agar, and at temperatures between 15 and

Growth potential (in %; mean Table 3. Germination of seeds stored dry at 15 °C and 15% RH for 1 month was slightly lower than in freshly collected controls following ethylene exposure

(91%) (Table 2). Germination in other treatments and at other temperatures was minimal or absent, with limited germination observed at 20 and 25 °C on water agar, and at temperatures between 15 and 30 °C following treatment with GA₃ or ethylene (Table 2). No germination was observed for seeds incubated in darkness for any treatment (data not shown).

Seed storage behaviour
Germination of seeds stored dry at 15 °C and 15% RH for 1 month was slightly lower than in freshly collected controls following ethylene exposure and no germination was observed in seeds stored dry for 3, 6 or 12 months and no germination was observed in seeds stored at −18 °C for 24 h or 3 months.

Normal growth and high growth potential (>90%) was observed in embryos excised from freshly collected seeds cultured on both growth media (Table 2). The growth potential of excised embryos cultured on ½MS medium declined by 31% after 1 month of dry storage or after 24 h of dry storage at −18 °C (Table 3), but growth was similar to that of embryos from freshly collected seeds (c. 80%) when embryos were cultured with growth hormones. Embryo growth after 12 months of dry storage was negligible and was observed only in embryo cultures with growth hormones (c. 12%) and no embryo growth was evident after 3 months of storage at −18 °C in either medium. The addition of PGRs significantly enhanced embryo growth potential in all storage treatments (P < 0.001).

SEED LONGEVITY IN SEDIMENTS
Seed fill in seeds stored in the sediment seed bank at CLG declined from 100% (prior to burial) rapidly to c. 20% after 6 months (Fig. 4), with high levels of fungal attack (abundant hyphae were observed) resulting in many seeds rotting during burial. No filled seeds were recovered from bags exhumed after 12 or 24 months. Although all filled exhumed seeds failed to germinate, all possessed an intact and undamaged embryo when cut-tested.

DISCUSSION
This study presents the first examination of sexual reproductive biology in the rare carnivorous macrophyte A. vesiculosa and the first observation of

Table 2. Germination (mean ± SE) of freshly collected Aldrovanda vesiculosa seeds in response to temperature cues and chemical stimuli

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Treatment</th>
<th>Water agar</th>
<th>2.89 mM GA₃</th>
<th>50 nM ethylene</th>
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<tbody>
<tr>
<td>5</td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 1.0a</td>
<td>3.0 ± 1.9a</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>2.0 ± 1.2a</td>
<td>3.0 ± 1.0a</td>
<td>12.8 ± 4.6b</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>32.9 ± 0.8a</td>
<td>13.0 ± 3.4a</td>
<td>94.0 ± 0.5b</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>5–25</td>
<td></td>
<td>32.5 ± 1.0a</td>
<td>20.8 ± 10.8a</td>
<td>90.7 ± 5.5b</td>
</tr>
</tbody>
</table>

Seeds were incubated under alternating light/dark conditions (dark results not shown) for 16 weeks at constant temperatures, and for 8 weeks at 5 °C before incubation for a further 8 weeks at 25 °C in cold stratification treatments. Annotated lettering indicates between-treatment significance for each temperature or storage method (P < 0.05).

Table 3. Growth potential (in %; mean ± SE) of Aldrovanda vesiculosa embryos excised from freshly collected seeds, and after storage

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Media type</th>
<th>Freshly collected</th>
<th>24 h</th>
<th>1 month</th>
<th>3 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 °C &amp; 15% RH</td>
<td>½MS only</td>
<td>70.0 ± 1.1a</td>
<td>N/A</td>
<td>39.0 ± 0.9a</td>
<td>N/A</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>½MS + 1 μM Z &amp; 3 μ GA₃</td>
<td>91.0 ± 0.7b</td>
<td>N/A</td>
<td>77.0 ± 6.5b</td>
<td>N/A</td>
<td>12.0 ± 0.9a</td>
</tr>
<tr>
<td>−18 °C</td>
<td>½MS only</td>
<td>70.0 ± 1.1a</td>
<td>39.0 ± 0.3a</td>
<td>N/A</td>
<td>0.0 ± 0.0</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>½MS + 1 μM Z &amp; 3 μ GA₃</td>
<td>91.0 ± 0.7b</td>
<td>82.0 ± 4.1b</td>
<td>N/A</td>
<td>0.0 ± 0.0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Embryos were incubated at 22–24 °C for 8 weeks on ½MS agar and ½MS water agar supplemented with PGRs (GA₃ and zeatin) following excision, after storage for up to 12 months at 15 °C and 15% RH, or after storage for up to 3 months at −18 °C following drying for 2 weeks at 15 °C and 15% RH. Annotated lettering indicates between-treatment significance for each storage treatment (P < 0.05).
ethylene gas as a powerful germination stimulant for seeds in an aquatic carnivorous plant. In addition, this study provides strong support to conclusions of previous work that *A. vesiculosa* is principally reliant upon vegetative propagation for reproduction, with reproduction from seed of secondary importance for this species (e.g. Adamec, 1999a, b; Adamec & Kovárová, 2006; Cross et al., 2015a). All five sampled populations across three continents were characterized by limited fertile fruit production and modest seed set, and seed fill from CLG, MVP and MCR was generally poor (Table 1). The seeds of cultivated plants originating from a native Mediterranean south-western Western Australian population displayed specific germination requirements (exposure to exogenous ethylene), a restricted germination window in terms of temperature optima and limited seed persistence (Table 3).

A reduced capacity to reproduce sexually is a common characteristic of aquatic plants, particularly highly clonal macrophytes (Barrett et al., 1993; Santamaria, 2002). Numerous studies have emphasized the rapid vegetative growth potential of *A. vesiculosa* under optimal conditions (Adamec, 1999a, 2000; Adamec & Lev, 1999; Adamec & Kovárová, 2006) and it is evident that this strategy has been ecologically successful for the species both in temperate and in subtropical zones. The historical distribution of *A. vesiculosa* included populations in at least 43 countries across four continents (Cross, 2012a) and the colonization of suitable habitat by few individuals has resulted in populations numbering in the thousands to millions of individuals as highlighted from several sites in North America, a continent with no native records for this species (Adamec, 2005; Cross et al., 2015a). Previous studies have noted that flowering in naturalized *A. vesiculosa* populations is sporadic, rarely results in fertile fruit and is unpredictable between seasons (Adamec & Tichý, 1997; Adamec, 1999a), including the failure to locate a single fertile fruit in an examination of >1000 flowering individuals at an introduced location in the Czech Republic (Adamec, 1999b). The low rates of fertile fruit production observed in our study are consistent with previously reported values of 8–50% fruiting success for *A. vesiculosa* in the Czech Republic and Japan (Adamec & Tichý, 1997; Okada, 1998). Similarly, studies have emphasized the variability in seed set within and between fruit of various populations: one to ten seeds per fruit (mostly four or five) from introduced plants in the Czech Republic originating from Poland (Adamec, 1999b), one to 14 seeds per fruit (mean 8.7) from a native population in the Danube Delta, Romania (Adamec, 1999b), and one to nine seeds per fruit from Japanese individuals (Nakano, 1992; Okada, 1998). The pollination mechanism of *A. vesiculosa* remains unknown, but both straight-style and curved-style flowers have been observed in various populations around the world (Cross, 2012a) and all available evidence suggests that the species is autogamous, setting seeds via self-pollination only in flowers possessing curved styles (Okada, 1998). Further study is needed to understand the breeding biology of *A. vesiculosa*.

Anecdotal evidence suggests that the onset of flowering may be cued by periods of high irradiance or periods of increased surface temperature, possibly in combination with other factors such as high CO₂ concentration and enhanced prey capture percentage (Cross, 2012a). The strong link found in this study between increased surface water temperature and number of flowers opening daily for one population may support this hypothesis and also suggests that reproductive success may be influenced by seasonal climatic conditions. Additionally, the onset of flowering at SNL1 followed a week of increasing surface water temperature, and it appears likely that flowering in temperate *A. vesiculosa* populations such as those studied in the Czech Republic is cued by a period of warm summer weather resulting in daily surface water temperature >25 °C. Fecundity appears to be linked to microhabitat characteristics in *A. vesiculosa*, particularly water depth and competition from other aquatic plants (Cross et al., 2015a) and thus flowering success may be influenced by a combination of parameters. Chemical factors in the water column such as the concentration of dissolved CO₂ and humic acids are considered to be critical to the growth and development of *A. vesiculosa* (Kamiński, 1987a, b; Adamec, 1995, 1999a, 2000). However, it is not known to what extent these factors influence the development of flowers or seeds (e.g. KAR, SNL1 and SNL2 in Appendix) or to what extent this development is influenced by variation in the nutrition obtained from captured prey. Our results suggest a reproductive trade-off exists in *A. vesiculosa*, with the development of flowers corresponding to a slight reduction in shoot elongation, markedly reduced production of axillary branches and an increased number of whorls. Future study is required to determine the ecological cues for flowering in *A. vesiculosa*, examine whether variation in factors such as nutrient availability and ambient CO₂ account for the marked variability in reproductive success between seasons and populations, and assess the apparent trade-off between sexual and vegetative reproduction.

Although sexual reproduction appears to play a limited role in the modern ecology of *A. vesiculosa*, it may have been a much more significant mechanism historically before the widespread loss of freshwater.
wetland habitats (Amezaga, Santamaria & Green, 2002). Habitat connectivity is likely to have been much greater in areas such as Europe before widespread wetland degradation of the last century (e.g. in Poland; Kamiński, 1987a) and dispersal may have occurred with a higher frequency than at present. The observed link between seasonal conditions and reproductive success may also imply a degree of susceptibility to climatic change in *A. vesiculosa*, although data suggest the species may respond more positively to warmer conditions than a cooler climate. The apparent tropical-climate affinity of *A. vesiculosa* may be reflective of the likely origin of *Aldrovanda* L. in the warm, equable early Eocene (Wing & Greenwood, 1993; Prothero, 1994; Cross, 2012a), a theory supported by the significant macrofossil evidence of *A. vesiculosa* and its ancestral taxa throughout Europe (e.g. Dorofeev, 1968; Yakubovskaya, 1990, 1991; Galka et al., 2015). The structural complexity of the exoesta in *A. vesiculosa* seeds is apparently a feature unique to this species and in addition to providing mechanical protection may facilitate the exchange of exogenous gases, such as ethylene, or endogenous gases regulating seasonal buoyancy in a similar manner to that observed in *A. vesiculosa* turions (Adamec, 2003).

The indehiscent fruits of *A. vesiculosa* are produced in late summer and may float on the water surface for several weeks following senescence from the main shoot (Adamec, 1999b; Cross, 2012a). Seeds are released into the water column as the fruit rots and most seeds appear to sink immediately to the sediment. Seed and embryo characteristics and germination biology results indicate that seeds of *A. vesiculosa* are physiologically dormant at maturity (sensu Baskin & Baskin, 2014). Freshly collected seeds possess a limited thermal window for germination, with germination occurring predominantly at 25 °C and cold stratification appears ineffective at promoting germination at other temperatures. Seeds are strongly responsive to the presence of exogenous ethylene and require exposure to light to germinate, light being a common requirement for germination of other macrophytes (Tuckett et al., 2010; Baskin & Baskin, 2014; Cross et al., 2015b). Ethylene in wetland sediments originates primarily from the decomposition of organic matter by soil microbes (Jackel, Schnell & Conrad, 2004; Ladygina, Dedyukhina & Vainshtein, 2006). Ethylene is likely to play a significant ecological role even at low levels (Smith, 1976) and particularly high rates of ethylene production have been recorded from habitats similar to those inhabited by *A. vesiculosa*: shallow freshwater wetlands with sediments rich in organic matter (Zeikus & Winfrey, 1976; Goodlass & Smith, 1978; VanCleemput, El-Sebaay & Baert, 1983; Jackel et al., 2004). Studies have previously implicated ethylene as a major driver of the timing and success of seed germination and seedling emergence in freshwater wetland habitats (Baskin & Baskin, 2014; Cross et al., 2014, 2015b), and it is likely to represent an important ecological stimulus for many aquatic species. However, it is notable that seeds that were apparently still viable following burial in sediment for 1 month or longer were not responsive to ethylene, unlike freshly harvested seeds. This suggests a more complex dormancy and germination behaviour for seeds that enter into, and persist in, the sediment. Furthermore, given the extensive distribution of *A. vesiculosa* from northern Russia to southern Australia and from equatorial Africa to Japan (Cross, 2012a) and the high levels of variability in reproductive success observed between seasons and between populations, it is possible that the seed germination response of the tested south-western Australian population may not be truly representative of the species throughout its entire range. Although flowering and seed set in European *A. vesiculosa* populations is relatively rare (Adamec & Tichý, 1997; Adamec, 1999b), future studies should attempt to examine whether other populations exhibit similar responses to chemical stimuli and whether northern temperate populations experiencing more pronounced, longer cold winters may exhibit a more marked response to cooler incubation conditions.

Although limited in extent, our embryo growth data suggest that seeds of *A. vesiculosa* are non-orthodox in storage behaviour (sensu Hong & Ellis, 1996), with seeds viability declining to near zero within 12 months (or earlier) of storage at 15 °C and 15% RH, or within 3 months of dry storage at −18 °C. These results suggest that seeds are probably not amenable to conventional seed banking practices (e.g. Merritt & Dixon, 2011). As the successful long-term storage of seeds appears unfeasible based on approaches described in this study, more viable alternatives of germplasm conservation such as the cryostorage of vegetative tissues or zygotic embryos must be explored.

Results from this study are of importance not only for determining the trajectory of future conservation and management initiatives focusing on *A. vesiculosa*, but they are also applicable to other ecologically sensitive clonal freshwater macrophytes displaying a reduced reliance on sexual reproduction. The conservation of rare or threatened clonal plants, particularly those possessing a highly stenotopic ecology such as *A. vesiculosa*, is likely to require a considerably different approach than is conventionally adopted for species producing abundant classically orthodox seeds. Many aquatic plants develop
extensive sediment seed banks, from which they are capable of recovering following the mitigation of disturbance such as unseasonable drought or eutrophication (Baskin & Baskin, 2014). However, it seems probable that species predominantly reliant upon vegetative propagation such as _A. vesiculosa_ that lack the resilience afforded by a persistent seed bank are likely to experience rapid decline and local extinctions following disturbance events. Management of _A. vesiculosa_ habitat must primarily focus on the preservation and rehabilitation of remaining suitable freshwater wetlands at a catchment scale to mitigate processes such as eutrophication and hydrological change (Cross et al., 2015a), with particular focus on the protection of suitable microhabitats and the maintenance of the numerous biotic processes governing water quality and biodiversity in these sensitive ecosystems (Amezaga et al., 2002). Developing an adequate understanding of the ecology of aquatic plants with particular habitat requirements such as _A. vesiculosa_ is intrinsic to the facilitation of successful conservation efforts for these species.

**ACKNOWLEDGEMENTS**

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Appendix  Water chemistry at sampled study sites of *Aldrovanda vesiculosa* in Australia (Cape le Grande, CLG), North America (Meadowview Pond, MVP; Meadow Creek, MCR) and the Czech Republic (Karstejn, KAR; Suchdol nad Lužnicí, SNL1 and SNL2); water samples in all cases were collected from microsites with prolific plant flowering

<table>
<thead>
<tr>
<th>Country</th>
<th>Site</th>
<th>Date</th>
<th>Conductivity (mS m⁻¹)</th>
<th>pH</th>
<th>Total alkalinity (meq L⁻¹)</th>
<th>CO₂ (mm)</th>
<th>NH₄-N (μg L⁻¹)</th>
<th>NO₃-N (μg L⁻¹)</th>
<th>PO₄-P (μg L⁻¹)</th>
</tr>
</thead>
<tbody>
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<td>North</td>
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<td>27 July 2014</td>
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<td></td>
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<td>17 August 2014</td>
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