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

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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

ADDITIONAL KEYWORDS: carnivorous plant – cold stratification – ethylene – flowering – freshwater wetlands – germination biology – rootless aquatic plant – seed dormancy.

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

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one of the categories of threat (Jennings & Rohr, 2011).

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 & Štorchová, 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 & Kovářová, 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 lurida* Wahlenb. (Cyperaceae), *Nuphar advena* (Ait.) Ait.f. (Nymphaceae), *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) among loose stands of *Eleocharis palustris* (L.) Roem. & Schult. (Cyperaceae), *Juncus bulbosus* (L.) (Juncaceae) and *Utricularia bremii* Köll, and a deeper (*c.* 50–90 cm) pool (SNL2) among 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 vouchered at the Institute of Botany of the Czech Academy of Sciences (Třeboň, 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 m^2) 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 CO_2 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 shrunken 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 $[(W_1 - W_d)/W_d] \times 100$, where W_1 and W_d 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,

$$B = \sum[(F_N/F_{\text{total}})/10]$$

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 (GA_3) or 0.7% (w/v) water agar after seeds had been exposed to 50 nmol ethylene gas (C_2H_4) 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 GA_3 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 (½MS) only or ½MS supplemented with plant growth regulators (PGRs) (1 μM zeatin and 3 μM GA_3; 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 × 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, GA_3, 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 $\log_{10}$ 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 $<4\ \mu g\ L^{-1}$) 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 × 0.95 ± 0.04 mm), with a substantial endosperm (1.12 ± 0.02 × 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 × 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%.

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

Country	Site	Year	Month	*N* (indivuals)	Shoot length (mm)	Whorls	Flowers	Branches	*N* (fruit)	Seeds per fruit		Seed fill (%)
										Mean	Range	
Australia	CLG	2010	January	141	95.6 ± 1.8a	13.0 ± 0.3a	1.3 ± 0.1a	0.8 ± 0.1a	16	4.3 ± 0.6a	1–8	88 ± 2a
		2011	January	36	77.9 ± 3.4b	14.1 ± 0.5a	1.7 ± 0.2b	0.8 ± 0.2a	99	6.9 ± 0.3b	1–11	65 ± 3b
		2012	January	74	121.8 ± 6.6c	13.2 ± 0.6a	1.7 ± 0.1b	1.0 ± 0.2a	177	7.8 ± 0.2c	1–12	29 ± 4c
		2013	January	80	146.2 ± 8.0d	15.0 ± 0.6a	1.9 ± 0.1b	0.9 ± 0.1a	27	4.0 ± 0.4a	1–8	32 ± 3c
North America	MVP	2013	June	149	103.2 ± 4.5a	20.2 ± 0.5a	2.3 ± 0.1a	1.2 ± 0.1a	12	5.3 ± 0.9a	1–9	54 ± 2a
	MCR	2013	June	39	147.2 ± 4.0b	28.5 ± 0.3b	2.7 ± 0.4b	1.3 ± 0.2a	20	5.5 ± 0.7a	1–9	76 ± 3b
Czech Republic	SNL1	2012	August	–	–	–	–	–	56	2.9 ± 0.2a	1–8	.
		2013	August	–	–	–	–	–	345	5.0 ± 0.1b	1–10	.
		2014	Mid July	40	105.9 ± 16.7a	18.5 ± 2.9a	1.1 ± 0.2a	1.2 ± 0.2a	–	–	–	–
			Late July	40	84.3 ± 13.3a	19.9 ± 3.1a	2.5 ± 0.4b	1.2 ± 0.2a	–	–	–	–
			August	40	81.9 ± 1.8b	19.3 ± 0.5a	2.8 ± 0.1b	1.3 ± 0.1a	112	3.3 ± 0.2c	1–9	.
	SNL2	2015	August	–	–	–	–	–	302	3.5 ± 0.1d	1–10	.
	KAR	2014	July	28	133.8 ± 25.3a	19.6 ± 3.7a	1.7 ± 0.3a	1.0 ± 0.2a	–	–	–	–
			August	40	79.0 ± 12.5b	15.6 ± 2.5a	2.4 ± 0.4b	0.9 ± 0.1a	–	–	–	–
		2015	September	–	–	–	–	–	300	3.5 ± 0.2	1–10	–

Apical and axillary branches were included. Means ± 1 SE are shown, with annotated lettering indicating significance in measured characteristics between measurement times ($P < 0.05$).

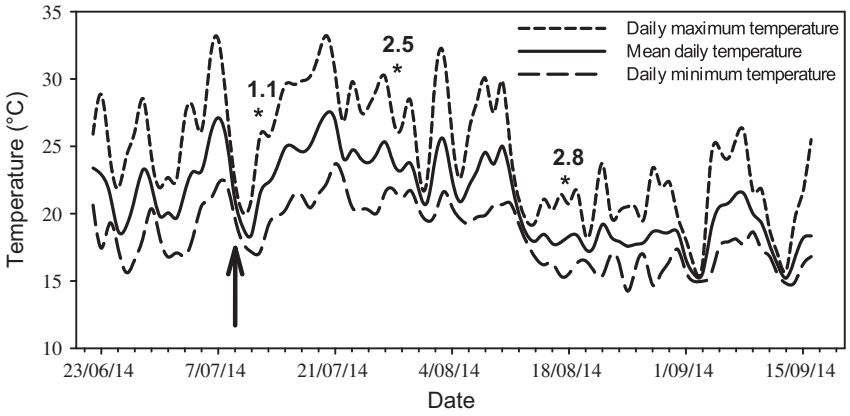

Figure 1. Average daily surface water temperature at Suchdol nad Lužnicí sand-pit (SNL1), South Bohemia, Czech Republic, during the *Aldrovanda vesiculosa* flowering season in 2014. The arrow indicates the onset of flowering on 8 July. Asterisks indicate sampling times for morphometric measurements, with annotated numbers indicating the average number of flowers per individual at each sampling point.

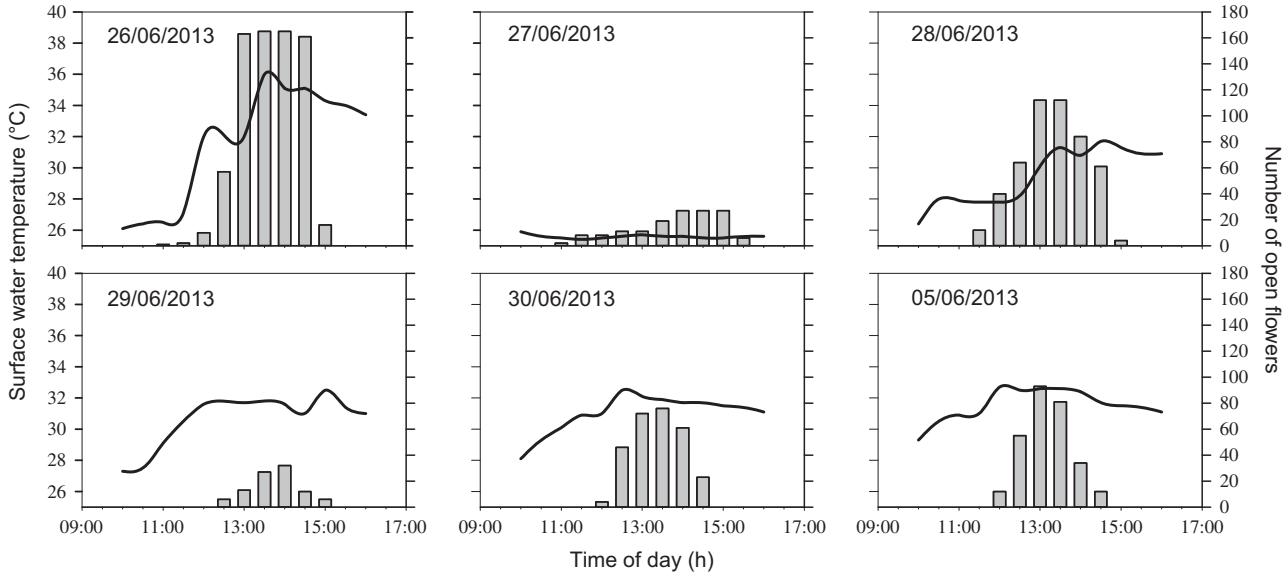

Figure 2. Daily surface water temperature between 10:00 and 16:00 h (lines) and the number of open flowers (bars) in a naturalized *Aldrovanda vesiculosa* population at Meadowview Pond (MVP), Virginia, USA, between 26 June and 5 July 2013. Clear conditions were observed on 26 and 30^{t} June and 5 July; all other days were partly cloudy.

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 ± 0.10. Following 72 h of imbibition *A. vesiculosa* seeds were found to have increased in mass by 14 ± 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

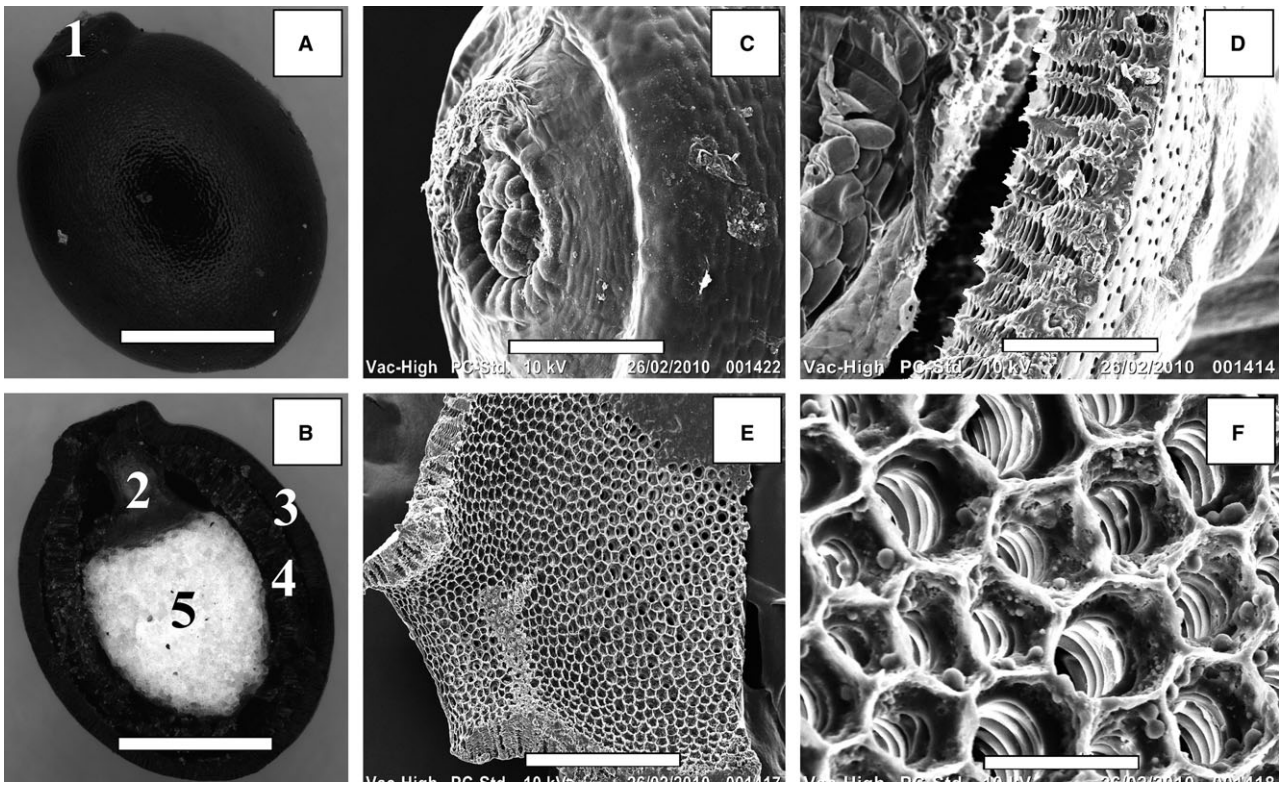

Figure 3. Photographs of intact (A) and longitudinally dissected (B) *A. vesiculosa* seeds; high-resolution scanning electron micrographs detailing seed structures including the operculum and exostome (C), cross-section of the exotesta and pitted external seed surface (D), honeycomb-like internal surface 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.

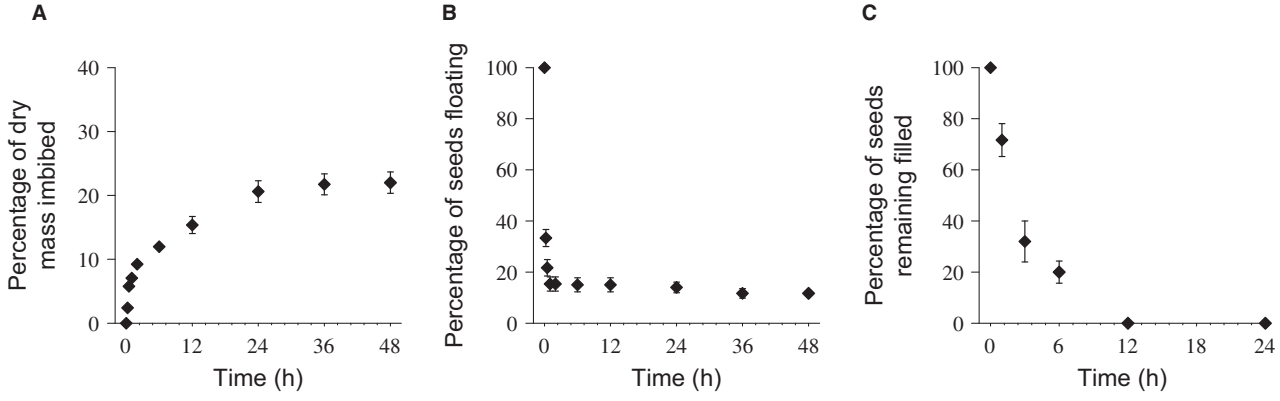

Figure 4. *Aldrovanda vesiculosa* seed characteristics. A, imbibition (percentage uptake of water through the testa) of freshly collected seeds over 48 h; B, buoyancy of freshly collected seeds over 48 h under laboratory conditions; and C, seed viability (expressed as the percentage of seeds remaining filled) over 24 months of burial in sediments at a natural site in south-western Western Australia.

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

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

Incubation temperature (°C)	Treatment		
	Water agar	2.89 mM GA_3	50 nM ethylene
5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
15	0.0 ± 0.0	1.0 ± 1.0a	3.0 ± 1.9a
20	2.0 ± 1.2a	3.0 ± 1.0a	12.8 ± 4.6b
25	32.9 ± 0.8a	13.0 ± 3.4a	94.0 ± 0.5b
30	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
35	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5–25	32.5 ± 1.0a	20.8 ± 10.8a	90.7 ± 5.5b

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 ($P < 0.05$).

(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 GA_3 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 (62.7 ± 0.4%; $P = 0.082$). 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 3. Growth potential (in %; mean ± SE) of *Aldrovanda vesiculosa* embryos excised from freshly collected seeds, and after storage

Storage conditions	Media type	Storage duration				
		Freshly collected	24 h	1 month	3 months	12 months
15 °C & 15% RH	½MS only	70.0 ± 1.1a	N/A	39.0 ± 0.9a	N/A	0.0 ± 0.0
	½MS + 1 μM Z & 3 μ GA_3	91.0 ± 0.7b	N/A	77.0 ± 6.5b	N/A	12.0 ± 0.9a
−18 °C	½MS only	70.0 ± 1.1a	39.0 ± 0.3a	N/A	0.0 ± 0.0	N/A
	½MS + 1 μM Z & 3 μM GA_3	91.0 ± 0.7b	82.0 ± 4.1b	N/A	0.0 ± 0.0	N/A

Embryos were incubated at 22–24 °C for 8 weeks on ½MS agar and ½MS water agar supplemented with PGRs (GA_3 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 & Kovářová, 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 & Kovářová, 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 CO_2 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 CO_2 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 CO_2 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 exotesta 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.

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REFERENCES

Adamec L. 1995. Ecophysiological study of the aquatic carnivorous plant *Aldrovanda vesiculosa* L. *Acta Botanica Gallica* **142:** 681–684.

Adamec L. 1999a. Seasonal growth dynamics and overwintering of the aquatic carnivorous plant *Aldrovanda vesiculosa* at experimental field sites. *Folia Geobotanica* **34:** 287–297.

Adamec L. 1999b. Further notes on flowering and seed set of *Aldrovanda vesiculosa*. *Flytrap News* **12:** 9–12.

Adamec L. 2000. Rootless aquatic plant *Aldrovanda vesiculosa*: physiological polarity, mineral nutrition, and importance of carnivory. *Biologia Plantarum* **43:** 113–119.

Adamec L. 2003. Ecophysiological characterization of dormancy states in turions of the aquatic carnivorous plant *Aldrovanda vesiculosa*. *Biologia Plantarum* **47:** 395–402.

Adamec L. 2005. Ten years after the introduction of *Aldrovanda vesiculosa* to the Czech Republic. *Acta Botanica Gallica* **152:** 239–245.

Adamec L, Kovářová M. 2006. Field growth characteristics of two aquatic carnivorous plants, *Aldrovanda vesiculosa* and *Utricularia australis*. *Folia Geobotanica* **41:** 395–406.

Adamec L, Lev J. 1999. The introduction of the aquatic carnivorous plant *Aldrovanda vesiculosa* to new potential sites in the Czech Republic: a five-year investigation. *Folia Geobotanica* **34:** 299–305.

Adamec L, Tichý M. 1997. Flowering of *Aldrovanda vesiculosa* in outdoor culture in the Czech Republic and isozyme variability of its European populations. *Carnivorous Plant Newsletter* **26:** 99–103.

Amezaga J, Santamaria L, Green A. 2002. Biotic wetland connectivity – supporting a new approach for wetland policy. *Acta Oecologia* **23:** 213–222.

Barrett S, Eckert C, Husband B. 1993. Evolutionary processes in aquatic plant populations. *Aquatic Botany* **44:** 105–145.

Baskin CC, Baskin JM. 2014. *Seeds: ecology, biogeography and evolution of dormancy and germination*. San Diego: Academic Press.

Baskin CC, Milberg P, Andersson L, Baskin JM. 2001. Seed dormancy-breaking and germination requirements of *Drosera anglica*, an insectivorous species of the Northern Hemisphere. *Acta Oecologica* **22:** 1–8.

Chiwocha SDS, Dixon KW, Flematti GR, Ghisalberti EL, Merritt DJ, Nelson DC, Riseborough JM, Smith SM, Stevens JC. 2009. Karrikins: a new family of plant growth regulators in smoke. *Plant Science* **177:** 252–256.

Cross AT. 2012a. *Aldrovanda*, the waterwheel plant. Dorset: Redfern Natural History Publications.

Cross A. 2012b. *Aldrovanda vesiculosa*. The IUCN Red List of Threatened Species 2012: e.T162346A901031. UK, IUCN. Available at: http://dx.doi.org/10.2305/IUCN.UK.2012.RLTS.T162346A901031.en (accessed on 16 February 2016).

Cross AT, Cawthray GR, Merritt DJ, Turner SR, Renton M, Dixon KW. 2014. Biogenic ethylene promotes seedling emergence from the sediment seed bank in an ephemeral tropical rock pool habitat. *Plant and Soil* **380:** 73–87.

Cross AT, Merritt DJ, Turner SR, Dixon KW. 2013. Seed germination of the carnivorous plant *Byblis gigantea* (Byblidaceae) is cued by warm stratification and karrikinolide. *Botanical Journal of the Linnean Society* **173:** 143–152.

Cross AT, Skates LM, Adamec L, Hammond CM, Sheridan PM, Dixon KW. 2015a. Population dynamics of the endangered aquatic carnivorous macrophyte *Aldrovanda vesiculosa* at a naturalised site in North America. *Freshwater Biology* **60:** 1772–1783.

Cross AT, Turner SR, Renton M, Baskin J, Dixon KW, Merritt DJ. 2015b. Seed dormancy and persistent sediment seed banks of ephemeral freshwater rock pools in the Australian Monsoon Tropics. *Annals of Botany* **115:** 847–859.

Darwin C. 1876. *Insectivorous plants*. New York: D. Appleton and Company.

Dorofeev PI. 1968. *Ob oligotsenovoi flore Zaural'ia.Paleontologicheskii Zhurnal* **2:** 111–119.

Elansary H, Adamec L, Štorchová H. 2010. Uniformity of organellar DNA in *Aldrovanda vesiculosa*, an endangered aquatic carnivorous species, distributed across four continents. *Aquatic Botany* **92:** 214–220.

Galka M, Bergonzini L, Williamson D, Majule A, Masao C, Huguet A. 2015. Macrofossil evidence of Late Holocene presence of *Aldrovanda vesiculosa* L. in central-eastern Europe (Poland) and East Africa (Tanzania). *Quaternary International* **386:** 186–190.

Garnett K. 1981. Cultivation and propagation of insectivorous plants. *Combined Proceedings of the International Plant Propagators Society* **31:** 316–324.

Goodlass G, Smith KA. 1978. Effect of pH, organic matter content and nitrate on the evolution of ethylene from soils. *Soil Biology and Biochemistry* **10:** 193–199.

Guja LK, Merritt DJ, Dixon KW. 2010. Buoyancy, salt tolerance and germination of coastal seeds: implications for oceanic hydrochorous dispersal. *Functional Plant Biology* **37:** 1175–1186.

Hong TD, Ellis RH. 1996. *A protocol to determine seed storage behaviour*. Rome: International Plant Genetic Resources Institute.

International Seed Testing Association [ISTA]. 1999. International rules for seed testing. *Seed Science and Technology* **27**(Suppl)**:** 1–335.

Jackel U, Schnell S, Conrad R. 2004. Microbial ethylene production and inhibition of methanotrophic activity in a deciduous forest soil. *Soil Biology and Biochemistry* **36:** 835–840.

Jennings DE, Rohr JR. 2011. A review of the conservation threats to carnivorous plants. *Biological Conservation* **144:** 1356–1363.

Kamiński R. 1987a. Studies on the ecology of *Aldrovanda vesiculosa* L. I. Ecological differentiation of *A. vesiculosa* population under the influence of chemical factors in the habitat. *Ekologia Polska* **35:** 559–590.

Kamiński R. 1987b. Studies on the ecology of *Aldrovanda vesiculosa* L. II. Organic substances, physical and biotic factors and the growth and development of *A. vesiculosa*. *Ekologia Polska* **35:** 591–609.

Kepczynski J, Kepczynska E. 1997. Ethylene in seed dormancy and germination. *Physiologia Plantarum* **101:** 720–726.

Ladygina N, Dedyukhina EG, Vainshtein MB. 2006. A review on microbial synthesis of hydrocarbons. *Process Biochemistry* **41:** 1001–1014.

Lin Z, Zhong S, Grierson D. 2009. Recent advances in ethylene research. *Journal of Experimental Botany* **60:** 3311–3336.

Long RL, Gorecki MJ, Renton M, Scott JK, Colville L, Goggin DE, Commander LE, Westcott DA, Cherry H, Finch-Savage WE. 2014. The ecophysiology of seed persistence: a mechanistic view of the journey to germination or demise. *Biological Reviews of the Cambridge Philosophical Society* **90:** 31–59.

Maldonado San Martin A, Adamec L, Suda J, Mes T, Štorchová H. 2003. Genetic variation within the endangered species *Aldrovanda vesiculosa* (Droseraceae) as revealed by RAPD analysis. *Aquatic Botany* **75:** 159–172.

Merritt D, Turner S, Clarke S, Dixon K. 2007. Seed dormancy and germination stimulation syndromes for Australian temperate species. *Australian Journal of Botany* **55:** 336–344.

Merritt DJ, Dixon KW. 2011. Restoration seed banks – a matter of scale. *Science* **332:** 424–425.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with Tobacco tissue cultures. *Physiologia Plantarum* **15:** 473–497.

Nakano T. 1992. Collection and germination of seeds of *Aldrovanda*. *Journal of the Japanese Insectivorous Plant Society* **43:** 21–25.

Okada H. 1998. Pollination system of *Aldrovanda vesiculosa* (Droseraceae), a critically endangered aquatic plant in Japan. *Makinoa N.S.* **7:** 93–100.

Osborne DJ. 1984. Ethylene and plants of aquatic and semi-aquatic environments: a review. *Plant Growth Regulation* **2:** 167–185.

Philbrick C, Les DH. 1996. Evolution of aquatic angiosperm reproductive systems. *BioScience* **46:** 813–826.

Prothero DR. 1994. The late Eocene–Oligocene extinctions. *Annual Review of the Earth and Planetary Sciences* **22:** 145–165.

Santamaria L. 2002. Why are most aquatic plants widely distributed? Dispersal, clonal growth and small-scale heterogeneity in a stressful environment. *Acta Oecologica* **23:** 137–154.

Smith AM. 1976. Ethylene in soil biology. *Annual Review of Phytopathology* **14:** 53–73.

Tuckett RE, Merritt DJ, Hay FR, Hopper SD, Dixon KW. 2010. Dormancy, germination and seed bank storage: a study in support of *ex situ* conservation of macrophytes of southwest Australian temporary pools. *Freshwater Biology* **55:** 1118–1129.

Turner SR, Commander LE, Baskin JM, Baskin CC, Dixon KW. 2009. Germination behaviour of *Astroloma xerophyllum* (Ericaceae), a species with woody indehiscent endocarps. *Botanical Journal of the Linnaean Society* **160:** 299–311.

Turner SR, Merritt D, Ridley EC, Commander LE, Baskin JM, Baskin CC, Dixon KW. 2006. Ecophysiology of seed dormancy in the Australian endemic species *Acanthocarpus preissii* (Dasypogonaceae). *Annals of Botany* **98:** 1137–1144.

VanCleemput O, El-Sebaay AS, Baert L. 1983. Evolution of gaseous hydrocarbons from soil: effect of moisture content and nitrate level. *Soil Biology and Biochemistry* **15:** 519–524.

Wing SL, Greenwood DR. 1993. Fossils and fossil climate: the case for equable continental interiors in the Eocene. *Philosophical Transactions: Biological Sciences* **341:** 243–252.

Yakubovskaya TV. 1990. On Tertiary *Aldrovanda* L. (Droseraceae) in Belorussia. *Doklady Akademii Nauk* **34:** 550–553.

Yakubovskaya TV. 1991. The genus *Aldrovanda* in the Pleistocene of the Belorussian SSR. *Botaničeskij Žurnal* **76:** 109–118.

Zeikus JG, Winfrey MR. 1976. Temperature limitation of methanogenesis in aquatic sediments. *Applied Environmental Microbiology* **31:** 99–107.

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 (Karštejn, KAR; Suchdol nad Lužnicí, SNL1 and SNL2); water samples in all cases were collected from microsites with prolific plant flowering

Country	Site	Date	Conductivity (mS m^{-1})	pH	Total alkalinity (meq L^{-1})	CO_2 (mM)	NH_4-N (μg L^{-1})	NO_3-N (μg L^{-1})	PO_4-P (μg L^{-1})
Australia	CLG	22 June 2010	89.5	6.29	–	–	0.9	0.1	0.1
North America	MVP	26 January 2014	–	5.72	–	–	0.1	0.3	0.5
	MCR	26 January 2014	–	5.70	–	–	0.1	0.2	0.2
Czech Republic	SNL1	13 July 2014	3.5	6.98	0.17	0.040	–	–	–
		27 July 2014	4.2	6.58	0.14	0.084	0.9	0.0	1.9
		17 August 2014	3.3	6.18	0.05	0.079	0.0	–	25.1
	SNL2	1 September 2015	35.8	6.60	0.07	0.042	40.8	0.0	3.5
	KAR	20 July 2014	17.0	6.54	0.97	0.520	–	–	–
		23 August 2014	14.0	6.56	0.76	0.380	116.0	–	23.9