



Seed dormancy alleviation by warm stratification progressively widens the germination window in Mediterranean climate Rutaceae

Michael Just^{A,*} , Adam T. Cross^{A,B} , Wolfgang Lewandrowski^{C,D}, Shane R. Turner^A, David J. Merritt^{C,D} and Kingsley Dixon^A

For full list of author affiliations and declarations see end of paper

***Correspondence to:**

Michael Just
School of Molecular and Life Science, Curtin University, Kent Street Bentley, WA 6102, Australia
Email: michael.just@curtin.edu.au

Handling Editor:

Noushka Reiter

Received: 15 July 2022
Accepted: 14 February 2023
Published: 8 March 2023

Cite this:

Just M *et al.* (2023)
Australian Journal of Botany, 71(2), 55–66.
doi:[10.1071/BT22076](https://doi.org/10.1071/BT22076)

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ABSTRACT

Context. In ecosystems where rainfall is episodic or highly seasonal, plant recruitment from a soil-stored seed bank occurs during periods of elevated soil moisture conducive to germination and seedling establishment. The release of seed dormancy in response to environmental conditions has significant consequences for the temperature window over which germination occurs, and as such the timing of germination can vary between years. **Aims.** We aimed to understand in seeds of two species of Rutaceae, *Diplolaena dampieri* and *Rhadinothamnus anceps*, how dormancy loss and germination timing is influenced by warm stratification. **Methods.** We tested the germination response to temperatures between 5 and 30°C following increasing durations (1–12 and 4–12 weeks) of warm stratification at 20, 25, or 30°C. **Key results.** Warm stratification for 1–8 weeks at 30°C progressively alleviated seed dormancy in *D. dampieri* and *R. anceps*, increasing germination proportion from ~0.1 to ~0.5 in both species. Stratification duration was optimal at 30°C for between 4 and 8 weeks depending on species. Warm stratification was not affected by water stress down to –0.8 Mpa. Application of aerosol smoke did not significantly improve germination, and heat treatments had a negative effect on final germination proportion. **Conclusions.** As dormancy was progressively alleviated, the range of temperatures that support germination increased for *D. dampieri* and decreased for *R. anceps*, allowing for confirmation of type 1 and type 2 non-deep physiological dormancy (PD), respectively. **Implications.** Arising from this conclusion, we suggest that in Mediterranean climates, type 1 and 2 PD dictate risk-taking and risk-avoiding ecological strategies by shifting the thermal requirements for germination towards that characteristic of the early- or mid-germination season. Classification of non-deep PD may offer a structured approach to predict how temperature requirements shift during dormancy loss, which will provide insight into seed germination response to year-to-year variation in seasonal environmental conditions.

Keywords: ecology, Mediterranean, physiological dormancy, restoration, Rutaceae, seed germination, stratification, thermal performance.

Introduction

In over 80% of angiosperms, seedling recruitment from soil seed banks is mediated by the alleviation of seed dormancy as well as the climatic and edaphic conditions that support germination (Gulden *et al.* 2004; Ooi 2010; Salazar *et al.* 2011; Baskin and Baskin 2014). Germinating seeds and newly established seedlings are vulnerable to rapid changes in environmental conditions, and thus the conditions required for seed germination, seedling emergence, and seedling establishment are usually far more restrictive than the long-term requirements for persistence of mature plants (Hardegreve *et al.* 2018). Seed dormancy is prevalent in ecosystems with seasonally dry periods, such as within Mediterranean climate regions (Merritt *et al.* 2007; Baskin and Baskin 2014; Turner *et al.* 2022), as it ensures germination occurs following the onset of seasonal

conditions favourable for seedling emergence and development (Baskin and Baskin 2014). Additionally, seed populations may exhibit bet-hedging strategies through the modulation of germination via variable seed dormancy status, spreading germination both spatially and temporally (Simons 2011). Bet-hedging reduces year-to-year risk of seedling mortality resulting from stochastic events and thus increasing the probability of seedling establishment in the medium to long term (Cross *et al.* 2015). How seed dormancy status fluctuates in response to climate, and how these changes influence recruitment timing, is of critical importance to better understand vegetation ecology and variability in recruitment strategy.

Physiological dormancy (PD) is the most common class of seed dormancy (Finch-Savage and Leubner-Metzger 2006). Seeds in the soil seed bank experience climate-driven seasonal variations in temperature and moisture and regulate their dormancy status in response to the varying environmental conditions to ensure germination occurs in periods when water is not limiting for seedling establishment (Merritt *et al.* 2007; Baskin and Baskin 2014). For seeds with PD, periods of warm to hot, dry conditions, which in many regions with Mediterranean climate typically occur over summer between the cooler and more mesic winter germination seasons (Merritt *et al.* 2007), can facilitate dormancy alleviation through the process of afterripening (Turner *et al.* 2009; Baskin and Baskin 2020). Seed dormancy alleviation can also occur under moist soil conditions (termed stratification) that occur between, in the lead up to, and during, the germination season as temperatures fluctuate in hydrated soils (Merritt *et al.* 2007). Stratification between temperatures of 0–10°C (cold stratification) typically alleviates dormancy in summer annuals (i.e. species germinating in early summer), and stratification at temperatures above 15°C (warm stratification) is generally associated with seed dormancy alleviation in winter annuals (i.e. species germinating in winter) (Baskin and Baskin 2014). While the conditions that alleviate physiological dormancy are generally understood, the specific regimes of temperature and moisture required for dormancy alleviation, and the effect that these conditions have on subsequent germination, remain unknown for most species.

During the alleviation of non-deep PD, there is a shift in the base or ceiling temperatures that will support germination (i.e. the lowest and highest temperatures at which seeds will germinate), often referred to as a widening of the germination window (Finch-Savage and Leubner-Metzger 2006). The direction in which the base or ceiling temperature shifts determines the type of non-deep PD present, according to six types of PD split across two sublevels that have been currently classified (Baskin and Baskin 2014; Soltani *et al.* 2017; Baskin and Baskin 2021). How the germination window shifts in response to dormancy alleviation is unknown in most species with PD (Walck *et al.* 2011), although it is likely that conditions leading up to the germination season facilitate

dormancy alleviation and drive thermal requirements for germination to temperatures characteristic of the germination window. For example, in type 1 non-deep PD there is an increase in the ceiling temperature that will support germination (Baskin and Baskin 1989; Soltani *et al.* 2017). In environments with water availability restricted to the winter months, type 1 non-deep PD may represent a risk-taking strategy, pushing germination requirements towards warmer temperatures characteristic of the early germination window and providing an early establishment advantage, but an increased risk of mortality due to desiccation. Comparatively, type 2 non-deep PD, which is characterised by a lowering of the base temperature required for germination (Steadman and Pritchard 2004), may represent a risk-avoidance strategy, limiting seed germination to the cooler temperatures more common in the middle of the germination window when there is greater certainty of soil water stores sufficient for seedling establishment. Classification of non-deep PD type may have significant potential to predict how species recruitment will respond to year-to-year variation in the environmental conditions coinciding with the onset of the growing season.

In south-western Australia, a biodiverse region with a Mediterranean climate harbouring many species with PD, most species tend to produce seeds that germinate in late autumn through to early winter during a period where soil moisture has begun increasing but soil temperatures are still relatively warm (Turner *et al.* 2006; Merritt *et al.* 2007). Seeds of many species within this region such as *Acanthocarpus preissii* (Turner *et al.* 2006), *Lomandra preissii* (Merritt *et al.* 2007), *Persoonia longifolia* (Norman and Koch 2006; Chia *et al.* 2016), *Byblis gigantea* (Cross *et al.* 2013), and species of *Hibbertia* (Hidayati *et al.* 2012), produce seeds with PD or morphophysiological dormancy (MPD) (*Hibbertia* spp.) with dormancy fully or partially alleviated by warm stratification. Nevertheless, no studies to date have examined the effect of stratification on the shift in temperatures able to support germination during dormancy loss in south-west Australian species, having instead reported the effect on germination at single constant (Cross *et al.* 2013) or alternating incubation temperatures (Turner *et al.* 2006; Merritt *et al.* 2007; Chia *et al.* 2016). Also, there are few data on how species differ in their stratification duration and temperature requirements, or what the minimum threshold for hydration is before stratification ceases to occur; all of which have significant implications for the timing of dormancy loss and, therefore, the onset of the germination window and germination.

The period over which environmental conditions are conducive to seed germination and seedling establishment can vary between years (Saatkamp *et al.* 2019). While climatically mediated shifts in germination timing from year to year are studied infrequently in species from south-western Australia, germination timing of winter emerging species might be expected to follow similar patterns to those

from regions with similarly seasonal climate. For example, Sonoran Desert annuals are known to vary germination timing in response to water availability and temperature within and between seasons, demonstrating bet-hedging strategies that spread germination events temporally to increase overall survival (Gremer *et al.* 2016; Huang *et al.* 2016; Ten Brink *et al.* 2020). Investigation of the effect of warm stratification on dormancy loss and subsequent germination requirements may yield insights into how south-west Australian species vary germination timing in response to varying environmental conditions between years. Further, determination of stratification optima (optimum temperature and duration of stratification) may also identify methodology able to alleviate dormancy of species that have historically proven difficult-to-germinate for restoration purposes.

One group of difficult-to-germinate species also important in restoration species mixes in Australia is the Rutaceae. Efforts to understand the conditions required for seed dormancy alleviation and seed germination in species of south-west Australian Rutaceae have yet to be successful (Dixon *et al.* 1995; Roche *et al.* 1997; Rokich *et al.* 2002; Norman *et al.* 2006; Merritt *et al.* 2007; Maher *et al.* 2008). Many Rutaceae produce PD seeds (Commander *et al.* 2009; Martyn *et al.* 2009; Baskin and Baskin 2014; Mackenzie *et al.* 2016; Collette and Ooi 2017; Collette and Ooi 2020), although the depth and type of PD remains unknown or assumed for many Australian Rutaceae. In eastern Australia, some species of Rutaceae germinate readily only in the presence of smoke and/or following heat shock (Mackenzie *et al.* 2016; Collette and Ooi 2017). Field observations of Rutaceae emerging following wildfire and smoke application *in situ* in Western Australian ecosystems suggest heat and/or smoke also play a significant role in seed germination (Roche *et al.* 1997; Norman *et al.* 2006). However, the lack of success reported by previous studies employing these treatments on the Rutaceae (Dixon *et al.* 1995; Roche *et al.* 1997) suggests a requirement for warm stratification and/or afterripening to alleviate physiological dormancy and increase seed sensitivity to smoke. Identification of the hydrothermal thresholds required for dormancy alleviation will also provide insights into how seedling recruitment is mediated by environmental conditions of temperature and moisture, and how best to propagate and manage species.

To implement informed species and habitat management plans, and to time seeding in variable environments, a clear understanding of conditions that mediate dormancy loss and germination is required. Detailed knowledge of species-specific requirements of dormancy loss can provide methodology able to alleviate dormancy and facilitate seed germination for the purposes of land management and restoration. Here we sought to investigate the effect of temperature, duration, and water stress during warm stratification on the germination window of two previously unstudied south-west Australian Rutaceae, *Diplolaena dampieri* and *Rhadinothamnus anceps*. The overarching hypothesis was that dormancy would be

progressively alleviated over time in conditions suitable for warm stratification, thereby facilitating a widening of the germination window. It was also expected that stratification at 30°C would become less effective under increasing water stress, and that stratification below 30°C would be less effective than stratification at 30°C. Specifically, we sought to determine: (1) the class and type of seed dormancy present in study species; (2) investigate the interaction between water stress and warm stratification on seed dormancy alleviation and germination; (3) determine the effect of warm stratification temperature and duration on the temperatures over which seed germination occurs; and (4) interpret data to inform species management and restoration practice.

Materials and methods

Seed source

South-western Australia experiences a Mediterranean climate with cool wet winters and hot dry summers (Fig. 1), with most native species dispersing seed between November and February (Merritt *et al.* 2007). Mature seeds of *D. dampieri* and *R. anceps* were collected in late spring (November of 2020) from wild populations at various sites across south-west Western Australia. Seeds were pooled before being cleaned using a vacuum aspirator (SELECTA BV Gravity Seed Separator, The Netherlands) to separate seeds from loose plant debris, before storage under controlled conditions (15°C and 15% relative humidity) at the Western Australia Seed Centre, Kings Park and Botanic Garden, Perth, prior to experimental use.

Seed characteristics and viability

Seed fill was determined for four replicate samples of 100 seeds using an X-ray (Autofocus X-ray cabinet, Faxitron, Tucson, USA) prior to further processing (initial seed fill)

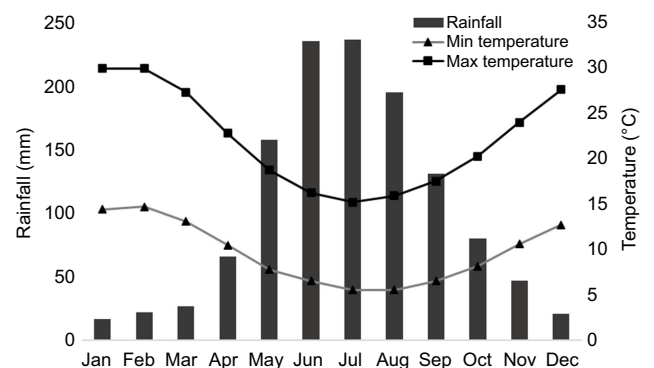


Fig. 1. Mean rainfall and temperature from 1935 to 2021 ($n = 84$) from the Australian Bureau of Meteorology weather station (site: 009538) at Dwellingup, Western Australia.

where damaged or unfilled seeds were removed (experimental seed fill). Seed weight (mg) was determined for four replicates of 25 filled seeds and the total weight divided by 25 to determine the mean individual seed weight.

To assist in classifying seed dormancy class, embryo morphology and water uptake were documented for both species using a Leica 205C camera with analysis in Leica Application Suite X (Leica Camera, Wetzlar, Germany). Sixty seeds were dissected longitudinally and embryos measured (μm) before defining embryo morphology according to Martin (1946). To assess whether seeds imbibe when exposed to water, a rudimentary assessment of testa permeability was conducted. Three replicates of 25 seeds were placed into Petri dishes lined with 84 mm germination paper (Advantec, Dublin, CA, USA) irrigated with deionised (DI) water. Each replicate was weighed prior to imbibition and again after 24 h of moist incubation, after being surface-dried using paper towels. Percentage water uptake was determined gravimetrically, based on the fresh weight of non-imbibed seeds, with the percentage increase in seed mass calculated as:

$$\text{Water uptake} = \frac{W_1 - W_d}{W_d} (100),$$

where W_1 and W_d are the mass of imbibed and dry seeds, respectively (*sensu* Turner *et al.* 2009).

Response to warm stratification following the application of fire cues

To determine the response of study species to fire cues known to promote germination of related species (Mackenzie *et al.* 2016), unimbibed seeds were treated with a 10 min application of aerosol smoke, heat or a combination of both smoke and heat. Four replicates of 25 seeds were used for all treatments. Heat treated seeds were exposed to dry heat in foil cups placed in a pre-heated electronic oven at 90°C for 10 min (Mackenzie *et al.* 2016). Seeds treated with smoke were placed into a 90 L plastic propagation tent before pumping in smoke generated by combusting 100 g of oat hay in a stainless-steel bee smoker for 10 min. In heat and smoke treatments, seeds were first exposed to heat before treatment with aerosol smoke. Heat, smoke, or heat and smoke treated seeds, as well as untreated seeds used as controls, were surface sterilised in a 4% (w/v) sodium hypochlorite (NaOCl) solution supplemented with several drops of Tween 80 (Sigma-Aldrich) for 30 min under alternating vacuum (−70 kPa) (Turner *et al.* 2018). Seeds were then rinsed three times in sterile deionised water (DI) before plating onto 0.7% (w/v) water agar and incubation at 10°C, 15°C, 20°C or 30°C under a 12-h photoperiod. To determine the effect of duration of warm stratification on response to fire cues and germination temperature four replicates of 25 seeds were first treated with either nothing (controls), heat, smoke, or smoke and heat as previously

described, and were then incubated at 30°C for 4, 8 or 12 weeks before being moved to either 10°C, 15°C or 20°C. Control seeds were maintained at 10°C, 15°C, 20°C or 30°C for the duration of the experiment. Germination was defined as emergence of the radicle to a length of >2 mm, and germination was scored once after 4 weeks to determine the proportion of primary dormancy as per Baskin and Baskin (2004), and again after 4 weeks incubation at the transferral temperature.

Identification of the optimal germination temperature following different stratification regimes

R. anceps was selected as a model species to further investigate the interactive effects of stratification and incubation temperatures on the germination response of seeds. Four replicates of 25 seeds were used for all treatments and were plated onto 0.7% (w/v) water agar under a 12-h photoperiod. Warm stratification has been defined as temperatures greater than 15°C, and an application duration of 4, 8, or 12 weeks can be used to determine the depth of physiological dormancy (Baskin and Baskin 2014). To determine if temperatures between 20°C and 30°C alleviate physiological dormancy untreated seeds were stratified at either 30°C, 25°C or 20°C for 1, 2, 4, 8 and 12 weeks before movement to either 5°C, 10°C, 15°C, 20°C or 25°C to complete the incubation process. As the germination window in south-west Australia typically proceeds with warmer drier conditions giving way to cooler, wetter conditions as the seasons move from summer through to winter (Merritt *et al.* 2007) seeds were only exposed to downwards shifts in temperature. Thus, seeds stratified at 30°C were moved to 5°C, 10°C, 15°C, 20°C and 25°C, those stratified at 25°C were moved to 5°C, 10°C, 15°C, 20°C and those stratified at 20°C were moved to 5°C, 10°C and 15°C. Seeds acting as controls were surface sterilised and plated as previously described before incubation at 5°C, 10°C, 15°C, 20°C, 25°C and 30°C for the entire duration of the experiment. Germination was defined as previously described and scored every 2 days for 60 days.

Interaction of warm stratification and water stress

To examine the interaction of warm stratification and water stress on seed germination, *R. anceps* seeds were surface sterilised as previously described. Following sterilisation seeds were dried overnight in a laminar flow cabinet before being placed onto germination papers in 90 mm Petri dishes irrigated with differing concentrations of polyethylene glycol 8000 (PEG) solution (10 mm per Petri dish) following Michel *et al.* (1983) to generate a range of water stresses (0 MPa, −0.1 MPa, −0.2 MPa, −0.4 MPa and −0.8 MPa). For each treatment, four replicates of 25 seeds were used.

All plates were incubated at constant 30°C for 4 weeks, determined as the optimum stratification treatment for promoting dormancy alleviation from previous experiments. Following stratification, all germinated seeds were removed, with the remaining ungerminated seeds rinsed in sterile DI water and transferred to fresh germination papers irrigated with sterile DI water. Seeds were then incubated at 20°C, determined to be the optimum temperature for supporting germination in previous experiments. Germination was defined as previously described and scored every 2 days until no new germination had been observed for 25 days.

Statistical analyses

All analyses were conducted in the R statistical environment (R Core Team 2013). We used binomial generalised linear modelling (GLM) to assess the influence of three germination temperatures (10°C, 15°C and 20°C), four stratification durations (0, 4, 8, 12 weeks) and treatment applications of fire cues (heat and aerosol smoke) on germination success. The full model with interactions was fitted, followed by a stepwise reduction to simplify the final model. The final model was fitted with a logit-link function and a binomial error structure and analysed to determine the main effects of germination temperature, stratification duration and treatment on germination success following 4 weeks incubation at the final temperature. The *plot_models* function from the 'sjPlot' package (Lüdecke 2018) were used to visually represent model coefficients from the GLMs.

Germination modelling

Germination response over time for each temperature was assessed using curvilinear log-logistic germination models (Ritz *et al.* 2013). The 'drc' package (Ritz *et al.* 2016) was used to fit a three-parameter log-logistic function to germination data:

$$\text{Germination} = \frac{G_{\max}}{1 + \left(\frac{\text{Time}^b}{t_{50}}\right)}$$

Above, G_{\max} is the upper limit for germination, with the lower limit for germination rate assumed to be 0, t_{50} is the time required for germination to reach 50% from G_{\max} , and b is the slope of the germination function at t_{50} . A full model

was created for the number of germinated seeds divided by the number of seeds incubated for all temperature and stratification regimes. The *anova* function was used to assess the explanatory power of stratification duration, germination temperature and water stress as factors influencing t_{50} and G_{\max} , versus a model without stratification duration, germination temperature and water stress. Due to poor germination proportion following stratification at temperatures below 30°C (<0.25), 20 and 25°C stratification regimes were removed from the analysis (Supplementary Fig. S1).

Thermal performance

Model fits for final germination proportion for thermal response data were estimated using Beta, Yan and Hunt and Broken-Stick thermal performance functions described by Asbury and Angilletta (2010), Yan and Hunt (1999) and Yeager and Ultsch (1989) respectively. The AIC function was used to assess the explanatory power of each model and the final model selected accordingly based upon the lowest Akaike information criterion (AIC) value (Table S1). The final model was that described by Yan and Hunt (1999):

$$G_{\max} = R_{\max} \left(\frac{T_{\max} - T}{T_{\max} - T_{\text{opt}}} \right) \left(\frac{T}{T_{\text{opt}}} \right)^{\frac{T_{\text{opt}}}{T_{\max} - T_{\text{opt}}}},$$

where G_{\max} is the maximum germination proportion at any temperature (T), T_{opt} is the optimum temperature for germination at the peak of the performance function, T_{\max} is the limit of thermal tolerance and R_{\max} is the asymptotic maximum germination rate at T_{opt} .

Results

Seed characteristics and seed fill

Study species had an individual seed weight between 1.8 and 10.3 mg with seed fill >70% upon collection (Table 1). Seeds of both species possessed fully developed, linear embryos (Martin 1946) and significantly increased in mass by up to 26% after 24 h of imbibition indicating water movement into seeds (Table 1).

Table 1. Seed traits of the two Rutaceae species examined in this study.

Species	Collection location in Western Australia	Individual seed weight (mg)	Initial seed fill (%)	Experiment seed fill (%)	Water uptake (%)	Primary dormancy (%)
<i>Diplolaena dampieri</i>	Myalup	10.25 ± 0.22	72 ± 2	97 ± 1	19 ± 3	88
<i>Rhadinothamnus anceps</i>	Torbay	1.86 ± 0.01	94 ± 1	98 ± 1	21 ± 2	86

Seed viability was determined by X-ray analysis when the seeds were obtained (initial) and again following removal of unfilled seeds (experimental).

Response to warm stratification following the application of fire cues

Untreated control seeds incubated at 10, 15 or 20°C germinated to a maximum proportion of 0.22 for *D. dampieri* and 0.12 for *R. anceps*. Both species showed a significant increase in germination response with increasing duration of warm stratification up to four to eight weeks (*D. dampieri*; log-odds ratio = 12.89, probability ratio = 0.93, $Z = 11.07$, $P < 0.001$ and *R. anceps*; log-odds ratio = 9.25, probability ratio = 0.90, $Z = 11.42$, $P < 0.001$), with a three and four-fold increase in germination of *D. dampieri* and *R. anceps* respectively (Figs 2, 3). The main effect of germination temperature was also significant for both species, with decreasing germination temperature positively correlated with germination success for *D. dampieri* (log-odds ratio = 1.15, probability ratio = 0.54, $Z = 9.44$, $P < 0.001$) and negatively correlated with germination success for *R. anceps* (log-odds ratio = 0.89, probability ratio = 0.47,

$Z = -8.99$, $P < 0.001$). Fire cues had no significant positive effect on seed germination for either species (Fig. 3), although heat pre-treatments had a negative effect on the outcome of germination for both species (*D. dampieri*; log-odds ratio = 0.52, probability ratio = 0.34, $Z = -4.13$, $P < 0.001$; *R. anceps*; log-odds ratio = 0.42, probability ratio = 0.73, $Z = -2.13$, $P = 0.033$).

Optimal germination temperature following variable stratification duration

Compared with unstratified control seeds, germination proportion in *R. anceps* seeds warm-stratified for any duration was 2- to 5-fold higher and occurred over a wider range of temperatures (Fig. 4). Increasing durations of warm stratification widened the germination window in *R. anceps*, allowing seeds to germinate at either 5 or 10°C, which was not seen for seeds in control groups (Fig. 4). Maximum germination was achieved at 25°C in control groups and at 25, 25, 20,

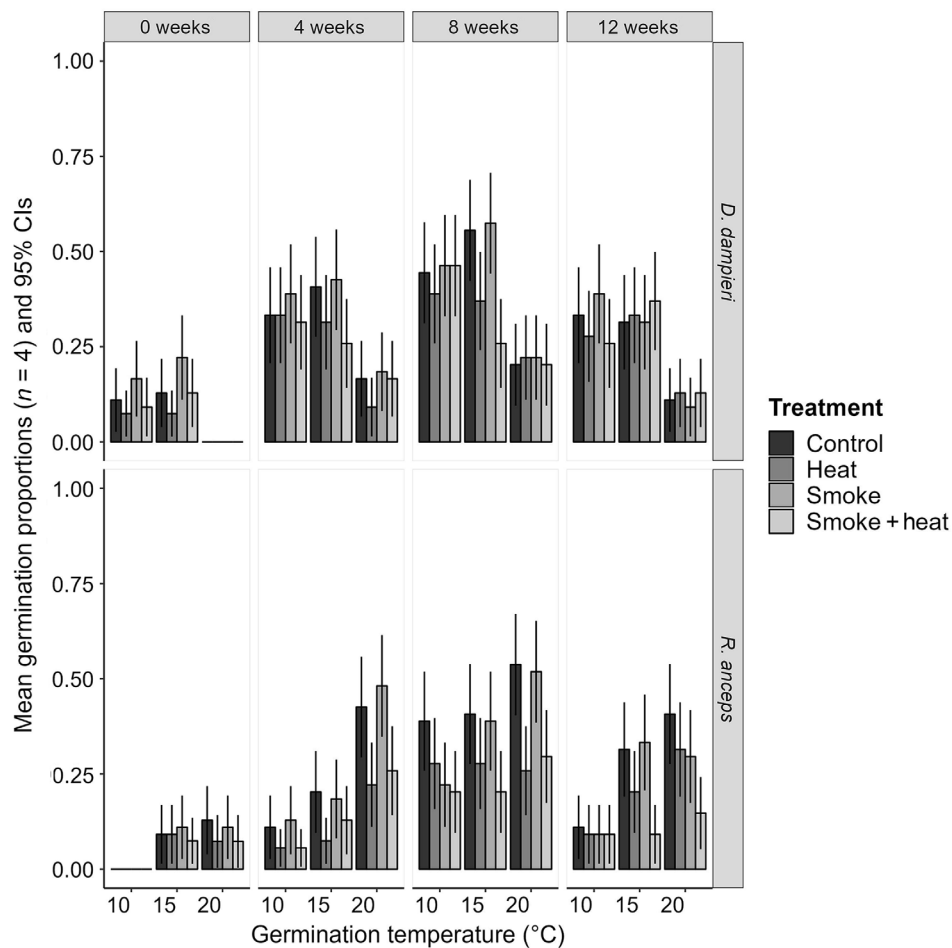


Fig. 2. Germination proportion ($\pm 95\%$ confidence interval) for two species of Rutaceae from southwest Western Australia subjected to combinations of fire cues followed by warm stratification (4, 8 and 12 weeks) at 30°C prior to incubation at 10, 15 or 20°C in alternating 12/12 h light. CIs, confidence intervals.

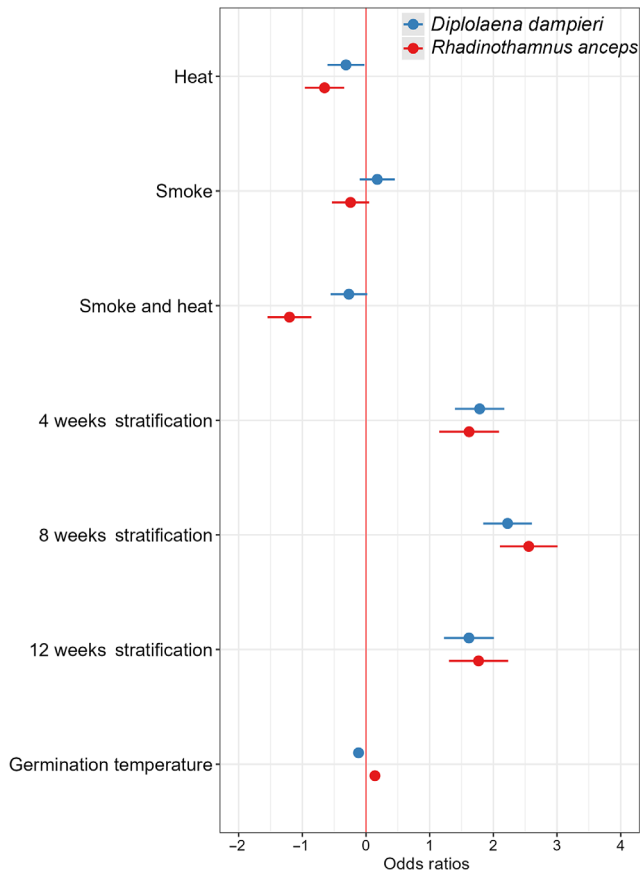


Fig. 3. Summary Forest plot from GLM showing odds ratio estimates (+95% confidence intervals) for germination response of *Diplolaena dampieri* (blue) and *Rhadinothermus anceps* (red) to smoke and heat cues, duration of warm stratification or increasing germination temperature. The intercept for the regression (red line) was determined as untreated seeds incubated under identical conditions. Positive odds ratios indicate a positive effect of treatment (to the right of intercept) while negative ratios indicate a negative effect of treatment (left of intercept).

20 and 20°C after 1, 2, 4, 8 and 12 weeks of warm stratification, respectively. Germination rate increased with increasing duration of stratification and was fastest at 20°C for all treatments excluding controls, where germination speed was similarly fastest at 25 and 30°C (Fig. 4). Models applied to seeds stratified at 20–25°C were unable to converge due to poor germination (<0.25) and have been excluded from analysis (Fig. S1).

Thermal performance

The Yan and Hunt (1999) model was fitted across all temperatures tested following stratification at 30°C (Fig. 5). When fitted to maximum germination proportion the optimum temperature for germination (T_{opt}) was $25.5 \pm 1.7^\circ\text{C}$ in control groups and decreased significantly ($P < 0.05$ in all cases) with increasing duration of stratification (Table 2) between 2 and 8 weeks, where the minimum optimum temperature for

germination was estimated as $18.6 \pm 1.4^\circ\text{C}$ (Table 2). When compared to control seeds (0 weeks of stratification), the maximum germination proportion (R_{max}) increased significantly following 1 ($P = 0.048$, t value = -2.02), 2 ($P < 0.001$, t value = -6.5158) and 4 ($P < 0.001$, t value = -6.5878) weeks of stratification, with maximum germination (0.48 ± 0.03) achieved following 2 and 4 weeks (Table 2). For all stratification durations the T_{max} for germination was within the range 30.8–31.4°C (Table 2).

Interaction of warm stratification and water stress

Increasing water stress during warm stratification had no effect on maximum germination proportion or time until 50% of final germination (t_{50}) at 20°C ($P = 0.81$, $F = 0.63$). The maximum germination proportion achieved (\pm s.e.) was between 0.39 ± 0.03 and 0.44 ± 0.03 and the t_{50} between 4.3 ± 0.5 and 4.9 ± 0.4 days (Fig. 6).

Discussion

Warm stratification was found to be an effective treatment for promoting germination in two Rutaceae species growing in a Mediterranean climate, which germinated after several weeks of exposure to warm moist conditions followed by incubation at cooler temperatures. These results add to reports of warm stratification alleviating dormancy in previously difficult-to-germinate Mediterranean-type climate taxa and enhance our capacity to propagate these species under controlled conditions for use in restoration, horticulture, and amenity planting (Turner *et al.* 2006; Hidayati *et al.* 2012; Chia *et al.* 2016). The thermal thresholds for germination of both *D. dampieri* and *R. anceps* were seen to widen following warm stratification, while the optimum temperature for germination of *R. anceps* decreased significantly as dormancy was alleviated. The application of smoke, heat, or increasing water stress during stratification (to -0.8 MPa) had no effect on either dormancy alleviation by warm stratification or final germination proportion. A lack of smoke response for fresh seed of the two study species does not neatly align with previous records for Australian Rutaceae. Significant germination responses have been noted following the application of heat, smoke or karriginolide (KAR1) (Commander *et al.* 2009; Mackenzie *et al.* 2016), or the passage of fire (Roche *et al.* 1997; Norman *et al.* 2006), and soil stored seeds may respond differently than those studied here following the effects of seasonal afterripening and stratification (Mackenzie *et al.* 2021). It does however demonstrate the inconsistency of smoke response between species of Rutaceae and suggests that dormancy alleviation may reduce the requirement for smoke stimulated germination in the inter-fire period. Prior to this study it was unknown how seeds of *D. dampieri* or *R. anceps* alter their requirements for germination during

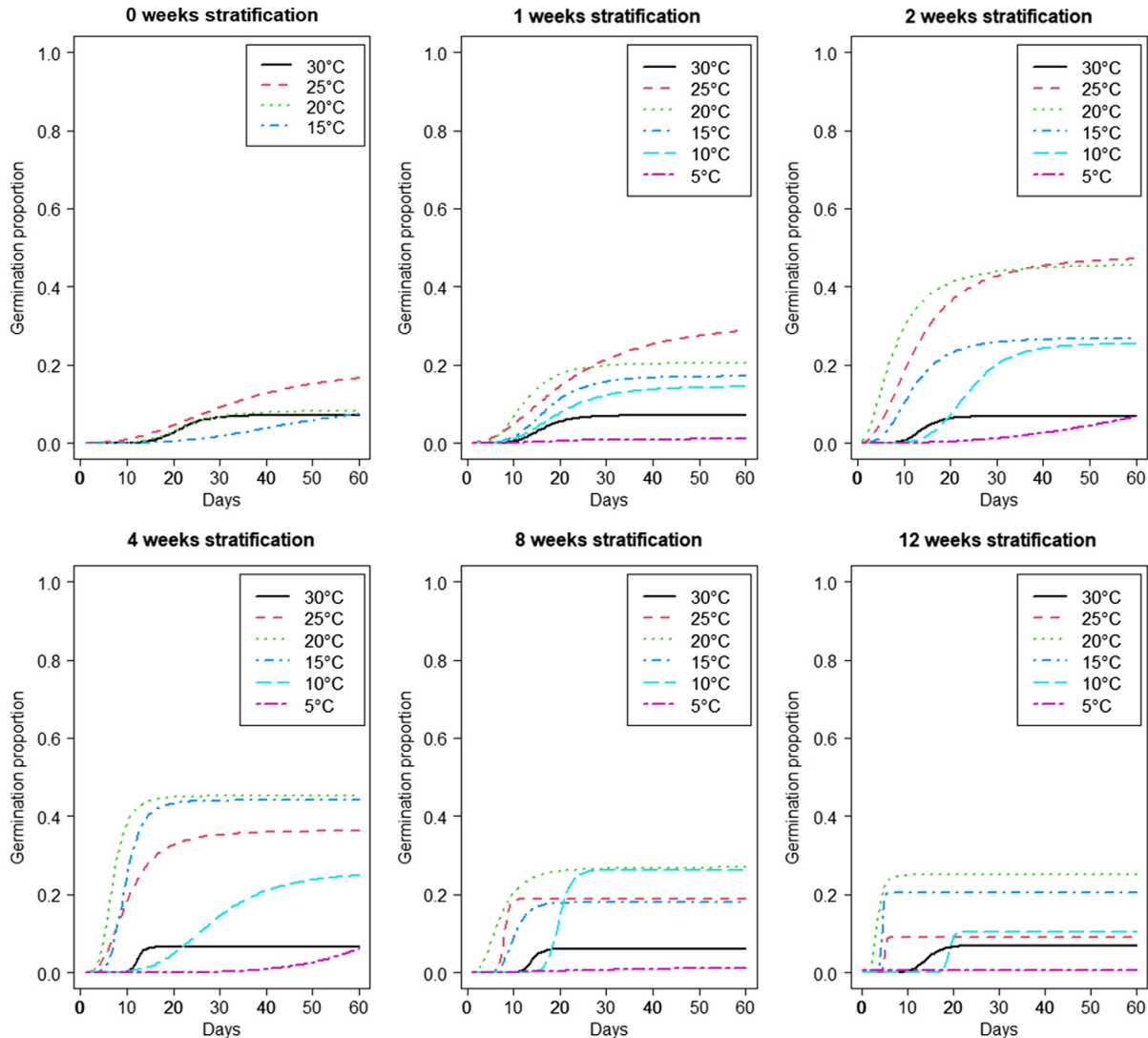


Fig. 4. Mean cumulative germination proportion for seeds of *Rhadinothamnus anceps* incubated at 5–30°C in alternating 12/12 h light/dark for 60 days following warm stratification at 30°C for increasing durations (from 1 to 12 weeks). Control seeds stratified for 0 weeks failed to germinate at 5 and 10°C.

dormancy alleviation and the results presented here provide potential evidence for bet-hedging traits expressed in the modulation of germination of these south-west Australian species.

Fresh seeds of both study species were mainly dormant, with 0–0.14 germination proportion observed after 4 weeks of incubation in alternating light at 10°C, 15°C or 20°C. Developed linear embryos and the unrestricted uptake of water determines seeds of both species possess PD, and the positive rapid response to warm stratification indicates non-deep PD (Baskin and Baskin 2014). Notably, the shift in temperatures suitable for germination differed in these two species during dormancy alleviation. Following warm stratification, seeds of *D. dampieri* germinated under warmer conditions, which is typical of seeds with type 1 non-deep PD. In contrast, seeds of *R. anceps* exhibited a decrease in temperatures able to

support germination, which is often seen in seeds with type 2 non-deep PD (Soltani *et al.* 2017). Seeds released into the soil seed bank in south-western Australia experience a relatively brief period of warm stratification followed by a decrease in soil temperature, prior to significant increases in soil moisture characteristic of the germination window during the cooler winter months (Merritt *et al.* 2007). An increase in the ceiling temperature for germination during this period of warm stratification, as seen in *D. dampieri*, suggests seeds are seeking to germinate while temperatures remain relatively warm during mid to late autumn, rather than delay germination until the onset of cold winter conditions. Conversely, a decrease in the temperature threshold required for germination, like that of *R. anceps*, indicates that seeds are delaying germination until soil temperatures decrease to a level typical of the winter germination season.

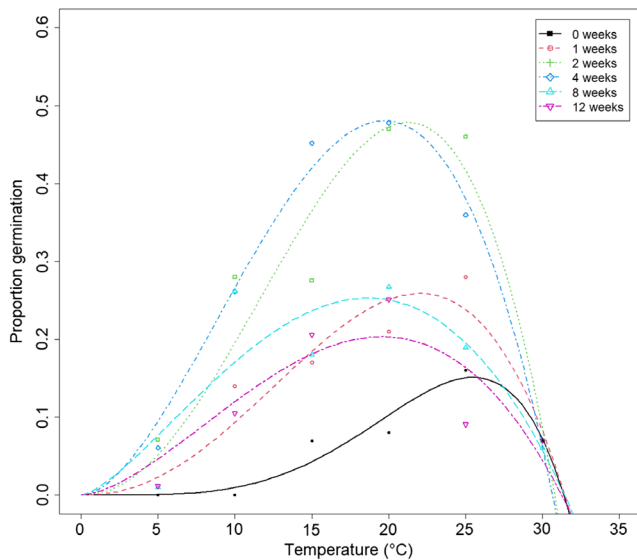


Fig. 5. Maximum germination proportion for *Rhadinothermus anceps* following warm stratification at 30°C for 1–12 weeks. Symbols represent the average maximum germination proportion estimate for seeds at 5–30°C incubation temperatures following increasing duration of stratification. Lines represent the permutations of the Yand and Hunt thermal performance curve fitted to the maximum germination proportion estimate (G_{\max}) for each stratification duration.

The contrasting types of non-deep PD that occur in *D. dampieri* and *R. anceps* might be alternative recruitment strategies responding to variation in the onset of the germination window. Seeds in the soil seed bank within south-west Australia are exposed to intermittent increases and decreases in moisture during autumn (specifically from April to May), and consistently high moisture conditions during winter and the beginning of spring (June to September) (Turner *et al.* 2006; Merritt *et al.* 2007). By germinating under the relatively warmer temperatures characteristic of the early germination window in autumn, *D. dampieri* may profit from a longer growing period, possibly yielding establishment and reproductive advantages (Donohue *et al.* 2010; Ten Brink *et al.* 2020; Miller *et al.* 2021) and a competitive benefit over later emerging individuals (Verdú and Traveset 2005; Ten Brink *et al.* 2020). However, the early stages of the germination window have a greater risk of mortality due to drought (Mercer *et al.* 2011; Donohue 2014; Thomson *et al.* 2017;

Ten Brink *et al.* 2020) as the soil moisture conditions driving dormancy release and early germination may not be consistently present post-germination to support effective seedling establishment (Harrison *et al.* 2018). In contrast, by germinating in cooler temperatures more characteristic of later in the season, seedlings of *R. anceps* are more likely to avoid early season drought and establish in more predictable and consistent rainfall and soil moisture conditions.

In the context of Mediterranean south-west Australia, type 1 non-deep PD (*D. dampieri*) appears to be a ‘high-risk high-reward’ strategy while type 2 non-deep PD (*R. anceps*) is a ‘risk-avoidance’ strategy, though the likely trade-off is elevated seedling competition (Duncan *et al.* 2019; Miller *et al.* 2021). How species possessing different types of non-deep PD respond to variable environmental conditions is an area of seed ecology that requires further research, particularly under current predictions for climate change. Identification of non-deep dormancy type offers a structured approach to species classification that will aid management and restoration practices and provide insight into the sensitivity of species to an altered germination window under a shifting climate.

Indeed, how species possessing these alternate germination strategies will respond to changes in temperature and moisture under current climate predictions is an area that requires further investigation. While it is generally accepted that altered seasonal cycles of air temperature will change, the relationship between seasons is expected to stay the same, i.e. summer will be warmer than winter (Hajek and Knapp 2022) and therefore warm stratification will still occur if sufficient rainfall is present. Comparatively, seasonal patterns in water availability are expected to change in regionally specific ways that require nuanced assessment of ecosystem vulnerability and ecological consequence (Hajek and Knapp 2022). Predictions for future (2030–2059) mean surface soil moisture across south-western Australia suggest a decrease in volumetric water content ($\text{m}^3 \text{ water m}^{-3} \text{ soil}$) when compared to historical records (1970–1999) (Andrys *et al.* 2017). Currently the seed moisture content needed to facilitate stratification in the lead up to the germination window is unknown. The mean base water potential required for germination of south-west Australian species has been estimated as 0.62 MPa (Dalziell *et al.* 2022), and while it was expected that dormancy loss by warm stratification would decrease as water stress approached

Table 2. Parameter estimates (\pm s.e.) from Yan and Hunt model fits for the final germination proportion of seeds of *Rhadinothermus anceps* warm-stratified at 30°C for 1–12 weeks.

Trait	Parameter	Stratification duration (weeks)					
		0	1	2	4	8	12
Final germination	G_{\max}	0.15 ± 0.05	0.25 ± 0.04	0.48 ± 0.03	0.48 ± 0.03	0.25 ± 0.03	0.20 ± 0.03
	T_{opt}	25.46 ± 1.71	22.01 ± 1.35	21.24 ± 0.76	19.72 ± 0.70	18.65 ± 1.44	19.56 ± 1.59
	T_{\max}	31.38 ± 1.70	31.45 ± 1.21	30.8 ± 0.53	30.64 ± 0.55	31.44 ± 1.44	31.24 ± 1.56

G_{\max} , maximum germination proportion; T_{opt} , optimum temperature; T_{\max} , ceiling temperature.

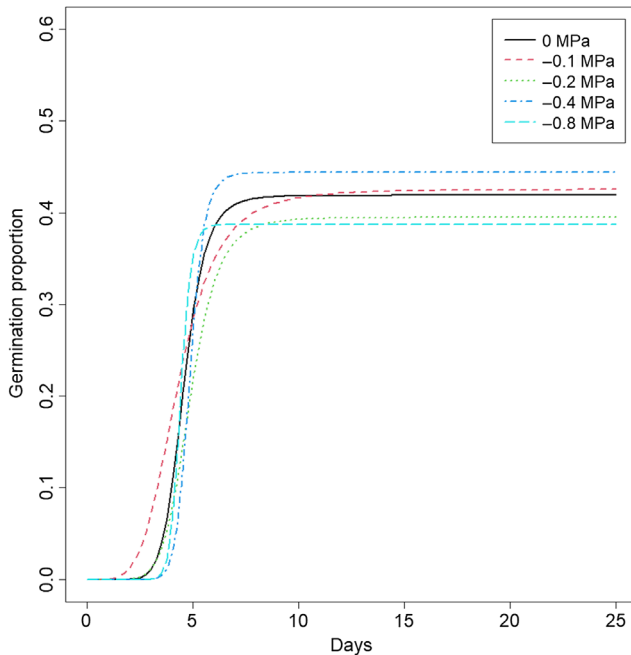


Fig. 6. Mean cumulative germination proportion for seeds of *Rhadinthamnus anceps* incubated in alternating 12/12 h light/dark at 20°C following the application of 4 weeks of stratification at 30°C in combination with a range (0 to -0.8 MPa) of water stresses.

this upper limit, our results show that warm stratification was unaffected by water stress down to -0.8 MPa. This result suggests that the base water potential required for stratification of south-west Australian species is lower than that required for germination, which makes intuitive sense considering autumn is drier than winter. Accurate estimates of dormancy alleviation in the lead up to the germination window will be required to determine how some PD species will respond to predicted reductions in soil moisture. A better understanding of the hydrothermal thresholds required for stratification in south-west Australian species is therefore a priority to ensure informed decisions are made regarding vegetation management and restoration.

Conclusion

These results have significant implications in seed ecology and practices seeking to propagate physiologically dormant species from seed. Fresh seed of both *D. dampieri* and *R. anceps* had optimum germination temperatures, which were significantly different before and after relatively short periods of warm stratification. Identification of differing optimum stratification requirements between sympatric species suggests the presence of dormancy alleviation strategies adapted to short to long periods of elevated soil moisture prior to the germination window. Wetting and drying cycles have also been shown to alleviate dormancy to proportions

greater than stratification alone (Hidayati et al. 2012; Chia et al. 2016; Lewandrowski et al. 2018). In combination with stratification mediated shifts of the thermal requirements for germination, these processes highlight the ability of seeds to sense their environment and regulate germination based on past climatic cues. It is likely that the difficulty of germination associated with our study species and other members of the Rutaceae in south-west Australia is due to the requirement for species-specific cues of temperature and moisture and suggests that no single treatment application is likely to be optimal across species. The moderate (~ 0.5) germination proportions achieved here suggest bet-hedging strategies are being employed to spread germination events between years, and that germination proportions may be increased by further investigation into regional climate signals that occur across years. To understand the dormancy and germination requirements of Rutaceae and other physiologically dormant species several stratification durations and temperatures need to be tested to ensure protocols are optimised and seed ecological requirements are more fully captured. Implications of these findings are three-fold; firstly, warm stratification should be applied to a greater number of taxa in south-western Australia as it is likely to increase the proportion of species able to be germinated readily. Secondly, identification of the type of non-deep physiological dormancy present in Australian taxa offers additional resolution in efforts to predict germination and seedling recruitment timing and response to environmental change. Finally, understanding the hydrothermal thresholds required for dormancy alleviation by warm stratification, including identifying the lowest effective water potential for stratification, should be pursued so as to contribute to understanding the effects of climate change on germination timing in south-west WA.

Supplementary material

Supplementary material is available [online](#).

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. All authors acknowledge that, regarding this manuscript, there are no conflicts of interest to declare.

Declaration of funding. This research was funded under the Australian Research Council Industrial Transformation Training Centre for Mine Site Restoration (IC150100041). This work was supported by Australian Research Council [grant number IC150100041].

Acknowledgments. The authors acknowledge the Wadjuk people of the Noongar nation, traditional owners of the land on which this work was undertaken and pay our respects to Elders past, present and emerging. The authors are grateful to Keith Smith (Formosa Flora) and Neroli Forster (Seed Shed) for their efforts in securing the seed required for this study.

Author affiliations

^ASchool of Molecular and Life Science, Curtin University, Kent Street Bentley, WA 6102, Australia.

^BEcoHealth Network, 1330 Beacon St, Suite 355a, Brookline, MA 02446, USA.

^CKings Park Science, Department of Biodiversity, Conservation and Attractions, 2 Kattidj Close, Kings Park, WA 6005, Australia.

^DSchool of Biological Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.