

1 **Germination ecology of the endangered species *Asterolasia buxifolia* (Rutaceae): smoke response**  
2 **depends on season and light**

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11 **Running title:** Germination ecology of *Asterolasia buxifolia* (Rutaceae)

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17 **Key words:** Seed dormancy, seed germination, seed ecology, riparian ecology, fire ecology,  
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21 **Summary text for Table of Contents**

22 Germination of fire-prone plant species can be complex. Understanding this key component of the  
23 regeneration niche can help elucidate mechanisms for persistence and inform conservation. Like  
24 many threatened species in fire-prone south-eastern Australia, *Asterolasia buxifolia* is restricted to  
25 riparian habitats. Germination ecology of this physiologically dormant family (Rutaceae) is not well  
26 understood. We found that seeds germinated only with smoke at winter temperatures. Darkness  
27 inhibited germination. These results help us understand why such species may be limited to riparian  
28 habitat.

29

30 **Abstract**

31 In fire-prone regions, many plant species rely on persistent seed banks for post-fire recovery.  
32 Understanding dormancy and germination cues is therefore important to predict population  
33 response. However, the germination ecology of species with physiologically dormant seeds in fire-  
34 prone regions is complex. We used the endangered species *Asterolasia buxifolia*, from riparian  
35 habitat in fire-prone south-eastern Australia, to investigate physiologically dormant seeds and their  
36 response to fire. We assessed whether fire cues alone promoted germination, or whether seasonal  
37 factors and light also played a role. Additionally, we tested the resilience of seeds to heat shock  
38 temperatures produced in soil during fire, in order to identify potential factors that restrict such  
39 species to fire refugia. Seeds only germinated at winter seasonal temperatures, and had an obligate  
40 smoke and light requirement. Heat shock treatments above 80°C slowed the germination rate.  
41 Smoke-related germination and the tolerance of *A. buxifolia* seeds to high fire-related temperatures  
42 demonstrated that recruitment dynamics can be driven by fire, however, germination is restricted to  
43 winter temperatures. This highlights the potential that changes to fire season may have on

44 population persistence. The slow germination rate caused by heat, and a light requirement, may  
45 contribute to restricting this species to riparian habitat.

46

## 47 **Introduction**

48 In fire-prone regions, many plant species rely on seeds stored within seed banks for post-fire  
49 recovery and population persistence (Whelan 1995). Dormancy and/or specific germination  
50 requirements allow the seeds to persist through the inter-fire period, with seeds germinating when  
51 conditions are favourable in the post-fire environment, where there is less competition and a  
52 necessity to recruit to replace individuals killed by the fire (Whelan 1995; Baskin and Baskin 2014).  
53 Fire cues such as heat and smoke can overcome dormancy and therefore allow germination from the  
54 seed bank (Keeley 1991; Ooi 2007; Merritt *et al.* 2007; **Invalid source specified**.. Hence,  
55 understanding dormancy mechanisms and germination cues is important for gaining a clear picture  
56 of seed bank and plant population dynamics in fire-prone regions.

57

58 In a study from the fire-prone heath and woodlands of south-eastern Australia, Ooi (2007) estimated  
59 that approximately 50% of all dormant shrub species had seeds with physical dormancy, while the  
60 rest had either physiological or morphophysiological dormant seeds, both of which have a  
61 physiological component (Merritt *et al.*, 2007; Ooi 2007; Baskin and Baskin 2014). However, when  
62 classifying only those shrub species listed as threatened (i.e. at some risk of extinction), the  
63 distribution of dormancy classes skewed heavily towards species with a physiological component to  
64 their dormancy (> 70%) (Ooi, unpubl. data). It is therefore possible that dormancy and germination  
65 cues play a role in determining why some species are rare or threatened in this, and other, fire-  
66 prone regions.

67

68 Physiological dormancy is the most common dormancy type throughout the world and is primarily  
69 broken by stratification of the seed at seasonal temperatures or via dry after-ripening (Baskin and  
70 Baskin 2014). While our knowledge of physiological dormancy is well developed in a number of  
71 climate regions, such as in temperate and arid vegetation types (and particularly for many annual  
72 species), the understanding of how physiological dormancy is controlled in fire-prone vegetation is

73 still developing. From the studies that have been conducted, however, and from the behaviour of  
74 species in other vegetation types, it is likely that season and smoke may be important for  
75 physiologically dormant fire-prone species (Roche *et al.* 1998; Baker *et al.* 2005; Ooi *et al.* 2006;  
76 Thompson and Ooi 2010; Cross *et al.* 2013; Mackenzie *et al.* 2016). This relationship between smoke  
77 and season means that the timing of the fire event can have significant effects on recruitment  
78 success (Ooi 2010). Additionally, light may play a role in germination, although whether it is likely to  
79 have a positive, neutral or negative effect on Australian fire-prone species is largely unknown  
80 (Merritt *et al.* 2006; Downes *et al.* 2015).

81

82 Due to their topography, soil type and moisture, riparian zones can buffer against fire and therefore  
83 act as a refuge for fire sensitive species (Pettit and Naiman 2007). This is highlighted by the number  
84 of rare and threatened species that are restricted to riparian habitats (Naiman *et al.* 1993; Pickup *et al.*  
85 *al.* 2003; Ooi *et al.* 2006a; Pettit and Naiman 2007). One hypothesis for this is that species restricted  
86 to such areas do not maintain traits that are as tightly bound to fire, or the fire regime of the  
87 surrounding vegetation, and retract to riparian areas where they can persist via other drivers in  
88 addition to fire. **Invalid source specified..** For example, Pickup *et al.* (2003) found that dormancy  
89 mechanisms and seed bank dynamics of the endangered species *Grevillea rivularis* were related to  
90 disturbance more generally, and could therefore respond to both flooding and fire. Additionally,  
91 other studies have found that high soil moisture can be critical for successful recruitment of species  
92 in these habitats (Dwire and Kauffman 2003; Jackson and Sullivan 2009). Identifying the drivers of  
93 seed dormancy and germination can therefore help to develop an understanding of the reasons for  
94 rarity and the mechanisms for persistence within riparian fire-prone systems. In particular, the  
95 magnitude of germination response with or without the presence of fire cues can inform how tightly  
96 bound species are to fire-related dynamics (Ooi *et al.* 2014), while the tolerance of seeds to high  
97 temperatures can ascertain whether seeds respond to fire or simply tolerate it (Ramos *et al.* 2016).  
98 Riparian habitat fires are often less intense and soil moisture is high (DeBano 2000; Dwire and

99 Kauffman 2003), which translates to lower soil temperatures than the surrounding vegetation  
100 matrix. Species may therefore show a lower resilience to heat shock treatments than similar species  
101 from a dry habitat.

102

103 In our study, we investigated the germination ecology of *Asterolasia buxifolia* (Rutaceae) a  
104 threatened species from south-eastern Australia, a highly fire-prone environment with an aseasonal  
105 rainfall climate. This species is representative of numerous other threatened species from fire-prone  
106 habitats, many of which are restricted to riparian areas. It is likely to have physiological dormancy  
107 (Martyn *et al.* 2009), and therefore may have some type of seasonal germination requirement. Little  
108 is currently known of the ecology of *A. buxifolia*, or indeed of a number of threatened physiologically  
109 dormant species from fire-prone regions, limiting the potential for implementing effective  
110 management and conservation initiatives.

111

112 To gain a better understanding of the recruitment dynamics of physiologically dormant species in  
113 riparian systems, and how they are related to fire, the germination response and resilience of seeds  
114 to heat shock temperatures generated by fire were investigated for *A. buxifolia*. The main aims of  
115 this study were to address the following questions:

116

- 117 (i) What type of dormancy does *Asterolasia buxifolia* exhibit?
- 118 (ii) Are there seasonal temperature requirements of germination for this species and, if  
119 so, which seasonal temperatures are important and how do they interact with fire  
120 cues such as heat and smoke? Is there any evidence suggesting fire is necessary for  
121 recruitment or that seeds are resilient to heat?
- 122 (iii) What conditions produce the highest levels of germination and can this be utilised  
123 for *ex situ* conservation collections?

124

## 125 **Methods**

### 126 *Study species and site*

127 *Asterolasia buxifolia* (Rutaceae) is a tall woody shrub, known from only one population in the Blue  
128 Mountains region of New South Wales (NSW), in south-eastern Australia. The species is listed as  
129 endangered under the NSW Threatened Species Conservation Act 1995. First discovered in the  
130 1830's it was later thought to be extinct, but was rediscovered in 2000 in a riparian zone (a  
131 transitional area between a water body and upland terrestrial area), with plants occurring no more  
132 than 20m from the river's edge (Mackinson 2002). Current estimates suggest there are around 2200  
133 individuals occurring along a 2-3km stretch of river (Collette 2016). The seeds of *Asterolasia buxifolia*  
134 are small and held in a soil-stored seed bank. As with other species from the family, seeds are  
135 initially ballistically dispersed and, based on observations of new seedlings occurring near ant nests,  
136 then undergo secondary dispersal via myrmecochory. Little is known of the phenology of the plant,  
137 although it has a short-term flowering season in late summer-early spring where small yellow  
138 flowers are produced and seeds formed shortly after pollination (Mackinson pers comm.).

139 The region where *A. buxifolia* occurs is highly fire-prone and has an aseasonal rainfall climate.  
140 However, the site of the population is long unburnt (> 40 years), and several seedlings have been  
141 observed emerging during the inter-fire period suggesting that multiple recruitment mechanism or  
142 strategies may be employed (Collette 2016). Additionally, anecdotal evidence suggests that the  
143 species may be an obligate seeder (i.e. standing plants are killed by fire), highlighting the importance  
144 of seed germination for population persistence post fire.

145 The population is located at approximately 750m above sea level, in the western region of the Blue  
146 Mountains. This altitude, along with distance from the coast, provides a climate that is cold in the  
147 winter months and potentially very hot in the summer months. The closest Australian Government  
148 Bureau of Meteorology weather station is located at Mt. Boyce, approximately seven kilometres  
149 away. The mean winter maximum/minimum temperatures are 10.2 /3.1°C, while the corresponding

150 mean summer maximum/minimum is 23.1/12.7°C. Rainfall occurs relatively evenly throughout the  
151 year (Fig. 1).

#### 152 *Seed collection*

153 Seed collection was carried out in September 2015, by bagging flowers on at least 30 mature  
154 individuals with high flower densities, spread throughout the population, after they had been  
155 pollinated and just before fruit set (a period of around 2 weeks). This allowed the seeds to reach  
156 maturity and then be captured in the bags prior to natural ballistic dispersal. A high seed fill was  
157 observed from initial assessment of the collected seed lot (~ 85%) (Frith *et al.* 2009). Seeds were  
158 then sorted via their weight using a Selecta zigzag gravity seed separator and unfilled seeds  
159 discarded, leaving mainly viable seeds in the seed lot used for experiments. Seeds were stored in a  
160 temperature and humidity controlled room set at 15°C and 15% humidity for six months before  
161 commencement of the experiments

#### 162 *Imbibition test and seed morphology*

163 To assess whether seeds were able to imbibe water, and therefore test if the seed coat was  
164 impermeable and played a role in controlling germination, an imbibition test was performed (Baskin  
165 and Baskin 2014). Twenty *A. buxifolia* seeds were chosen randomly from the collected seed lot, and  
166 were individually weighed with a set of A&D GR-202 scales to the nearest 0.01 mg. Seeds were then  
167 placed onto allocated areas of moistened filter paper. Every hour for 6 hours, seeds were removed  
168 and excess moisture dried off. Seeds were weighed and then returned to the filter paper. After the  
169 first 6 hours, seeds were left overnight to imbibe further and weighed again the next day, 24 hours  
170 after the experiment had commenced. At the end of the experiment, the change in seed weight over  
171 time was calculated. Imbibed seeds were also dissected under a dissection microscope to investigate  
172 their embryo type which helps to inform the type of dormancy that seed are likely to exhibit (Baskin  
173 and Baskin 2014).

174



175 *Germination experiments*

176 *Experiment 1: the effects of seasonal temperatures and fire cues on germination*

177 The seasonal requirements for germination, and how these interacted with fire cues, were  
178 investigated using a fully factorial experiment with the factors season, heat and smoke. Incubators  
179 were used to mimic seasonal differences, set at temperature cycles of 11/3°C, 18/6°C or 25/11°C,  
180 approximating winter, spring/autumn and summer conditions respectively for the study region. One  
181 incubator only was used per seasonal temperature treatment, and temperature cycles set within  
182 each incubator were monitored with Thermoline ibuttons. Temperatures were based on the average  
183 minimum and maximum temperatures for in winter, spring/autumn and summer based on Bureau  
184 of Meteorology data from the Mt. Boyce weather station (Fig. 1), which is within 10 km of the study  
185 site (although at slightly higher elevation than the study site). The experiment was established with  
186 four treatments at each seasonal temperature (control, smoke, heat, smoke + heat). Treatments  
187 were applied to four replicates (dishes) with 20 seeds in each dish. Smoke treatments consisted of  
188 irrigating dishes with smoke water, which had been made by burning leaf litter and passing the  
189 smoke produced through water for three hours via a vacuum pump. This solution was diluted to 2%  
190 before being applied to seeds, as this concentration had been shown to produce the highest  
191 response in a pilot study on species with a known smoke response (Catelotti unpubl. data). For the  
192 heat shock treatment, dry seeds were transferred to a 90 mm glass petri dish and placed in an oven  
193 set at 60°C, for a period of 10 minutes. Each replicate was heated separately (Thomas *et al.* 2003;  
194 Ainsley *et al.* 2008). Seeds were then transferred to plastic 90mm petri dishes lined with qualitative  
195 filter paper, where they were irrigated with distilled water (or smoke water) until the filter paper  
196 was consistently moist. Heat shock was applied prior to smoke treatments.

197 For seeds that were initially irrigated with smoke water, all subsequent irrigation was done with  
198 distilled water so they were only exposed to the smoke treatment once. Germination trials were  
199 conducted in three light, humidity and temperature controlled Thermoline incubators running on

200 12/12-hour day/night cycles at each of the seasonal temperatures. Temperatures were additionally  
201 monitored by ThermoChron iButtons placed in each incubator to ensure temperatures were  
202 maintained. Replicate dishes were checked every week for new germination and moistened with  
203 distilled water as required. Germination was scored on emergence of the radicle. The germination  
204 experiments continued for a total of 11 weeks, until the rate of germination had slowed, with less  
205 than one seed germinating per week. Germination percent was calculated based on the assumption  
206 of 100% viability, as an initial cut test revealed that seeds had a very high initial viability close to this  
207 level. This and all subsequent cut tests were performed using hydrated seeds under a dissecting  
208 microscope. Seeds were scored as inviable if there was no embryo present, or the embryo had lost  
209 structural integrity and/or had become brown in colour. Inversely, they were scored as viable if the  
210 embryo and endosperm was intact, and a white colour was maintained. Cut tests provide a quick  
211 and accurate assessment of seed viability (Ooi *et al.* 2004a).

#### 212 *Experiment 2: resilience to increasing heat shock and germination under dark conditions*

213 The optimal seasonal temperature for germination was established from Experiment 1 (winter  
214 temperature cycles of 11/3°C) and there was an obligate requirement for smoke. Therefore, to  
215 assess the effect of heat and dark treatments, two experiments were conducted at these winter  
216 temperatures. Firstly, to investigate the effect of heat, seeds were subjected to treatments ranging  
217 from 40°C, increasing by 20°C increments to 120°C. The high temperatures are towards the upper  
218 limit of those experienced beyond 1 cm depth in the soil during fire (Auld and Bradstock 1996; Ooi *et*  
219 *al.* 2014). As before, four replicates of 20 seeds were used for each treatment level and heat shock  
220 was applied for 10 minutes. Smoke treatments were then applied to all seeds. After treatments,  
221 replicates were placed in the incubator at 11/3°C. The control treatment for this experiment  
222 consisted of replicates which had the smoke treatment applied but no heat. The impact of heat was  
223 assessed by quantifying increased or decreased germination and mortality in comparison to the  
224 control seeds.

225 Viability of all remaining seeds following germination trials was tested by cut tests as described  
226 above. To confirm that our assessment of viability was robust, a tetrazolium test was performed on  
227 100°C treated seeds. The ungerminated seeds from each of the four replicate dishes were cut, and if  
228 they appeared viable, then placed into one of four sample jars filled with a 1% tetrazolium solution.  
229 After four days in a dark cupboard at laboratory temperatures (approximately 22°C), seeds were  
230 extracted from the solution and cut open under a dissection microscope where their colour was  
231 assessed. Viable seed tissue stains pink or red in tetrazolium solution (Ooi *et al.* 2004a). The results  
232 from the tetrazolium test were consistent with the cut tests.

233 Secondly, to investigate the effect of light on germination, we established a factorial experiment also  
234 at the 11/3°C temperature cycle, using the factors smoke (+/-) and light (+/-). Due to limited  
235 remaining seeds, we used three replicates of 20 seeds for treated seeds, and three replicates of 14  
236 seeds for the control in this experiment. Smoke treatments were applied as above, while dishes  
237 assigned to dark treatments were wrapped in two layers of aluminium foil.

238

### 239 *Statistical analysis*

240 For the imbibition test, the percentage of weight change was plotted against time. The final average  
241 (n=20) weight after 24 hours was then compared to the initial average weight using a paired T test.  
242 The data were tested for normality using a Shapiro-Wilk W test and was found to be normally  
243 distributed. For Experiment 1, there was an obvious effect of winter temperature observed (< 2%  
244 germination at any other seasonal temperature). The germination data were analysed within season  
245 using a 2 factor (heat and smoke) GLM with a binomial distribution and logit link function. For  
246 Experiment 2, both germination and final viability data were analysed using a 1 factor (heat) GLM  
247 with a binomial distribution and a logit link function. For both germination experiments there was no  
248 evidence for overdispersion of data. Post-hoc Tukey's HSD tests were performed in order to assess  
249 differences between treatment levels.

250 To test for the rate of germination in Experiment 2, t50 (time to reach 50% of total seeds  
251 germinated) values were calculated by plotting the percentage of seeds that germinated in each  
252 replicate against time, then fitting a linear model and solving the equation of the line to calculate the  
253 x intercept when y=50. A 1 factor (heat) ANOVA was then performed on the t50 data. Prior to  
254 analysis, the data were tested for normality and no transformations were required. All analyses were  
255 performed using the JMP pro 11.0.0.0 (SAS Institution Inc.) statistical package.

256

## 257 **Results**

### 258 *Imbibition test and seed morphology*

259 Mean seed weight significantly increased after 24 hours by 0.25 mg (~ 11% increase) from 2.26 mg  
260 to 2.51 mg (t=2.44, df=19, p=0.001). Seeds were endospermic and contained a linear embryo, which  
261 extended along the full length of the seed.

### 262 *Germination experiments*

#### 263 *Experiment 1: the effects of seasonal temperatures and fire cues on germination*

264 Results from Experiment 1, investigating seasonal temperature response, showed that high levels of  
265 germination only occurred at the winter temperature treatments (11/3°C). Within the winter  
266 temperature treatment there was an interaction between smoke and heat ( $\chi^2 = 6.12$ , df = 1, p =  
267 0.013), with smoke increasing germination well above non-smoked seeds. The interaction appears to  
268 be from a small (5%) difference in germination for the smoke and smoke x heat treatments.  
269 Negligible germination occurred at all other seasonal temperature cycles (Fig. 2).

#### 270 *Experiment 2: resilience to increasing heat shock and germination under dark conditions*

271 The heat shock treatments significantly affected germination response ( $\chi^2 = 153.72$ , df = 4, p <  
272 0.001). Response to temperature treatments of 40°C and 60°C did not differ significantly from the  
273 control, with approximately 80% of seeds germinating. Germination was negatively affected by heat

274 shock treatments above 80°C, where it dropped to 66% and 6% after the 80°C and 100°C treatments  
275 respectively. No germination occurred after the 120°C treatment (Fig. 3). The rate at which  
276 germination occurred was also affected by the heat shock treatments. Time to 50% germination  
277 (t50) was able to be calculated for germination of seeds from the control, 40°C, 60°C and 80°C heat  
278 shock treatments only, due to negligible germination at the higher temperature treatments. The  
279 average t50 value (n=4) was 26 days for the control which progressively increased with temperature  
280 treatment to 34 days at 40 °C, 32 days at 60°C, and 55 days at 80°C. The t50 value for the 80°C  
281 treatment was significantly higher than all lower temperature rates, meaning germination took  
282 longer to occur ( $F = 19.50$ ,  $df = 3$ ,  $p < 0.001$ ) (Fig. 4).

283 Cut tests indicate that seed viability was significantly affected by heat shock treatments ( $\chi^2 = 47.04$ ,  
284  $df = 6$ ,  $p < 0.001$ ). Control seeds had a high viability of 98% and there was no significant negative  
285 affect until the 120°C treatment, where it dropped to an average of 40% (Fig. 3).

286 Seeds from the 100°C heat shock treatment that were placed in the tetrazolium solution stained  
287 well. Of the 22 seeds that were subjected to this test, 17 (77%) stained dark red indicating viability,  
288 and five (23%) had either no stain or pink uneven colouring, indicating inviability, which was  
289 consistent with the results from the cut tests (32/40 seeds (80%) viable).

290 Constant darkness completely inhibited germination. Smoke treatments did not overcome any  
291 inhibitory factors imposed by dark conditions. Smoke treated seeds in light conditions germinated to  
292 significantly higher levels than any of the other treatments ( $\chi^2 = 7.327$ ,  $df = 8$ ,  $p < 0.007$ ) (Fig. 5).

293

## 294 **Discussion**

295

296 The strong requirement for a smoke cue to promote germination, and the tolerance of *Asterolasia*  
297 *buxifolia* seeds to high fire-related temperatures, both clearly demonstrated that recruitment  
298 dynamics could be driven by fire. There was negligible germination without smoke, however seeds

299 only germinated when incubated at winter temperature cycles that are typical of the Blue  
300 Mountains region where the population is found. These results suggest that the species maintains  
301 fire-adaptive traits. Additionally, germination was relatively slow and seeds had a strong light  
302 requirement, meaning they would need to be imbibed at or near the soil surface for an extended  
303 period. Few areas within the landscape would maintain such conditions, and this, along with other  
304 factors such as fire frequency, may contribute to limiting the species ability to expand beyond the  
305 riparian zone.

306 The identification of a linear embryo, along with evidence suggesting that seeds could imbibe water  
307 without scarification, confirmed that *A. buxifolia* is a physiologically dormant species. The 11%  
308 increase in weight due to water up-take aligns with results from imbibition tests performed on other  
309 species from the Rutaceae family (Martyn *et al.* 2009). Very few past studies of physiologically  
310 dormant species have identified such a strong preference to a single seasonal temperature as a  
311 requirement for germination in Australian fire-prone systems. Almost no germination occurred at  
312 warmer temperatures, suggesting that emergence in the field would be highly restricted to cold  
313 temperature conditions. Mackenzie *et al.* (2016) found a similar, albeit less constrained preference  
314 for winter germination temperatures for a number of common *Boronia* species (also Rutaceae),  
315 whilst Ooi *et al.* (2006) found that three *Leucopogon* species (Ericaceae) required long periods of  
316 warm stratification to overcome physiological dormancy. The long period of warm stratification  
317 resulted in seedling emergence in late autumn or winter post-fire, regardless of earlier rainfall  
318 events (Ooi *et al.* 2004).

319 For species like *A. buxifolia*, with a strong seasonal preference and obligate smoke requirement  
320 occurring in an aseasonal rainfall climate, the season that a fire occurs in may be an extremely  
321 important determinant of first germination, then recruitment success (Ooi 2010). Cunningham  
322 (1984) reported that the natural fire season in the Blue Mountains study region occurs primarily in  
323 spring/summer, with very occasional late winter fires. The strong seasonal requirement, and the

324 relatively long period of incubation (8 weeks) to produce germination, could mean that a fire  
325 occurring in mid to late winter or spring could have a negative impact on *A. buxifolia* recruitment.  
326 Emergence would be delayed for up to a year before the winter temperatures required to promote  
327 germination occur (Mackenzie *et al.* 2016), and such species would face higher levels of competition  
328 from species with no seasonal requirement, such as resprouting plants and those with physical  
329 dormancy (Ooi 2010). Delayed emergence of physiologically dormant species due to fire season has  
330 been shown to cause a decrease in seedling emergence, survival and growth resulting from a  
331 presumable decrease in competitive ability, in fire-prone aseasonal rainfall regions (Ooi *et al.* 2004;  
332 Ooi 2010).

333 Seed mortality of *A. buxifolia* in relation to heat shock treatments was similar to most species from  
334 fire-prone regions around the world, with high levels of seed mortality not occurring until  
335 treatments reached 120°C (Auld and O'Connell 1991; Keeley 1991; Tierney 2006; Moreira *et al.*  
336 2010; Ooi *et al.* 2014). This further suggests that *A. buxifolia* maintains characteristics indicating  
337 some level of adaptation to fire. Interestingly, germination reduction at 100°C for *A. buxifolia* was  
338 not related to seed mortality, with around 80% of seeds that didn't germinate maintaining viability.  
339 There is a building body of evidence showing that heat shock can increase germination of some  
340 physiologically dormant species in fire-prone regions. For example, a 70 - 80°C treatment can  
341 improve germination of the Australian species *Prostanthera eurybioides* (Lamiaceae), *Codonocarpus*  
342 *cotinifolius* (Gyrostemonaceae), *Boronia anemonifolia* (Rutaceae), *Epacris micropylla* and *E. paludosa*  
343 (Ericaceae) and several *Darwinia* (Myrtaceae), either alone or by interacting with smoke (Thomas *et*  
344 *al.* 2003; Baker *et al.* 2005; Ainsley *et al.* 2008; Auld and Ooi 2009; Mackenzie *et al.*, 2016). Dormant  
345 seeds from other continents continue this trend (e.g. Ribeiro *et al.* 2012), however, world-wide, few  
346 have reported a suppression of germination without loss of viability with heat shock, as we have  
347 found in this study.

348 It is difficult to assess the viability of deeply dormant seeds as cut tests have a level of subjectivity  
349 (Ooi *et al.* 2004a). It is therefore possible that rather than our heat shock treated seeds remaining  
350 viable and their germination suppressed, seeds were damaged, lowering the amount and rate of  
351 germination. However, if our viability results are accurate, as the results from our cut versus  
352 tetrazolium test data support, heat shock could have suppressed or delayed germination (i.e.  
353 germination could have occurred if seeds were given a longer period to do so). Heat shock can result  
354 in changes to seed proteins which play an important role in germination (Lu *et al.* 1995; Wood *et al.*  
355 2000; Finkelstein *et al.* 2008). The production of heat shock proteins (HSPs) (which act as molecular  
356 chaperones) increases dramatically under stressful temperatures, and these can prevent protein  
357 aggregation or potentially participate in refolding of denatured proteins after heat stress (Wang *et*  
358 *al.* 2004; Tripp *et al.* 2009), potentially delaying germination response. While HSPs are found in  
359 seeds, our understanding of their role in native species is limited (Feder and Hofmann 1999).

360

361 The relationship between fire refugia, such as that provided by riparian habitat, and threatened  
362 species, has often brought up the suggestion that life history traits of riparian species are not as  
363 closely bound to the fire regime of the broader environment. The obligate requirement for smoke  
364 and the resilience to fire-related temperatures displayed by *A. buxifolia* seeds suggests that  
365 recruitment of this species is fire-adapted, and that at this life-history stage fire is not limiting.  
366 However, the slow rate of germination, which declines even further after heat shock temperatures  
367 above 80°C, means that successful germination and seedling establishment would require very long  
368 periods of high soil moisture post-fire, of between four and ten weeks. In the study region, which  
369 consists of well-drained sandy soils, it is unlikely that soil moisture would regularly be retained for  
370 such periods away from watercourses, particularly at the shallow depths where most of the small-  
371 seeded *A. buxifolia* would be able to emerge from. The high levels of moisture associated with  
372 riparian habitat could therefore assist germination and the success seedling establishment and



373 survival (Bhattacharjee et al. 2008) and provide part of the explanation of the species restriction to  
374 the riparian zone.

375 The strong requirement for light may also provide a reason for restriction to moister riparian  
376 habitats. A light requirement is often associated with small-seeded species, to ensure that  
377 germination does not occur too deep within the soil profile (Bell 1999). However, soil moisture  
378 evaporates more quickly from near the surface (Huang *et al.* 2016), and the long periods with a  
379 moist top soil substrate required for germination would most likely be found close to stable  
380 watercourses in these habitats. This is one of few studies from eastern fire-prone Australia that have  
381 identified a light and smoke requirement for germination, which suggests that further study of other  
382 riparian species should include dark treatments.

383 Despite the obligate requirement for a smoke cue, seedling emergence has been observed in the  
384 field even though the *A. buxifolia* population has not been burnt for over 40 years (Collette 2016).  
385 This shows that some, albeit small numbers, of seeds can germinate in the absence of fire. A possible  
386 reason for this is that physiologically dormant seeds often decrease their levels of dormancy over  
387 time via after-ripening, and are subsequently able to germinate under a broader range of conditions  
388 as they age (Finch-Savage and Leubner-Metzger 2006; Turner *et al.* 2009; Baskin and Baskin 2014).  
389 Although it is likely that a large proportion of the seed bank will maintain the requirement for a fire  
390 cue, the ageing process may allow some leakage from the seed bank via germination. This may  
391 contribute to maintaining a basal population presence in the absence of fire as older plants senesce,  
392 and other disturbance mechanisms, such as flooding, may contribute to recruitment opportunities  
393 (e.g. see Pickup *et al.* 2003). However, further long-term study is required to clearly demonstrate  
394 whether such recruitment alone is sufficient for viable population persistence.

395 Management of endangered species such as *A. buxifolia* is extremely important because of their  
396 restriction to a single population (Childs *et al.* 2010). Implementing prescribed fires is a key  
397 management tool in fire-prone areas, and understanding the outcomes after fire is critical. Obligate

398 seeders are particularly sensitive to changes to the fire regime (Keith 1996; Bowman *et al.* 2014).  
399 Results from our germination experiments indicate that it is not only fire frequency, often the usual  
400 focus of fire management plans, but also the season of fire which can have consequences for the  
401 persistence of physiologically dormant species like *A. buxifolia* because of the strong seasonal  
402 temperature requirement. Fire implemented in the late winter or spring period would potentially  
403 have a negative impact on *A. buxifolia*, due to the relative delay in emergence, however fires  
404 occurring naturally in summer or implemented in autumn would potentially reduce the relative  
405 delay of emergence. The majority of managed fires currently occur in cooler seasons to enable  
406 better control for safety purposes (McLoughlin 1998), but the timing within this prescribed fire  
407 season may have significantly different outcomes.

408 *Ex situ* conservation is an important platform for many endangered species across the world, with  
409 the goal of supporting the survival of species in the wild by having a back-up ‘insurance policy’ by  
410 storing their seeds (Cochrane *et al.* 2007). For physiologically dormant species in fire-prone regions  
411 such as *A. buxifolia*, germination can be complicated, and limit the utility of *ex situ* collections. A  
412 critical finding from this study is therefore identification that germination requires remarkably low  
413 winter season temperatures typical of the study region and a smoke cue, and that germination in the  
414 laboratory environment requires several weeks at such temperatures. This finding can be  
415 incorporated into germination protocols of *A. buxifolia ex situ* seed collections.

416

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554 **Figure captions**

555 Figure 1. Climate data from 1991-2017 from the Australian Government Bureau of Meteorology  
556 weather station at Mt. Boyce. Mean monthly minimum (—■—) and maximum (—◆—) temperatures  
557 represented by lines, and mean monthly rainfall is represented by bars.

558 Figure 2. The mean percentage germination of *Asterolasia buxifolia* seeds incubated at three  
559 different seasonal temperatures, using factorial applications of smoke and heat. Error bars represent  
560 1 standard error.

561 Figure 3. The mean percentage germination (□), viable but not germinated (■), and inviable (■)  
562 *Asterolasia buxifolia* seeds incubated at the winter temperature cycle. All temperature treatments  
563 have smoke applied. Control treatment has smoke but no heat-shock treatment.

564 Figure 4. Germination rates over the duration of the experiment, represented by the average  
565 percentage germination plotted against time (weeks) for each of the heat shock treatment  
566 temperatures applied. The 120°C heat shock treatment was excluded because no germination was  
567 recorded. Error bars represent 1 standard error.

568 Figure 5. The average percentage germination of *Asterolasia buxifolia* seeds incubated at winter  
569 temperature cycles (11°C day 3°C night), with factorial applications of light and smoke. Error bars  
570 represent 1 standard error.

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572