School of molecular and life sciences

Understanding the seed ecology of southwest Australian Rutaceae to improve restoration in a biodiversity hotspot

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Declaration

This thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Statement of Contribution by Others

The study presented in Chapter 2 is preparation for submission for publication within the peer reviewed literature.

I contributed 90% to this paper, including conceptualization, methodology, data collection, analysis, and visualisation, writing and revision. ST, AC, DM, and KD contributed to conceptualisation, methodology, writing, review and editing for each study.

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I contributed 85% to this paper, including conceptualization, methodology, data collection, analysis, and visualisation, writing and revision. ST, AC, DM, KD, and Dr Wolfgang Lewandrowski contributed to conceptualisation, methodology, writing, review and editing for each study.

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1.1 Introduction

Ecological restoration aims to assist the recovery of ecosystems that have been damaged, degraded, or destroyed by human activities (McDonald *et al.* 2016; McDonald and Williams 2009). As human-mediated ecological degradation continues to impact ecosystems around the world, the need for restoration continues to grow, with government, non-government organisations (NGOs), industry, and private individuals choosing or being required to repair degraded ecosystems for a range of reasons (Gann *et al.* 2019; McDonald *et al.* 2016). In more recent years there has been an increase in the number of organisations required to undertake restoration as part of their operations (Erickson *et al.* 2018; Koch 2007a; Rokich 2016). An increase in the requirement for ecological restoration, coupled with the willingness of peoples and companies to partake, has facilitated an increase in the number and scale of currently operating and planned restoration projects. Development of science and practice driven by the need for restoration has led to improved capabilities able to return ecosystem function however, significant research is still required to ensure biodiverse restoration throughout Australia.

In Australia, it is estimated that 38% of native forests have been lost since European colonisation, and much of the remaining forest is severely degraded (Bradshaw 2012). In southwestern Australia the ecological impact of vegetation loss has been magnified by the richness of regional biodiversity, with over 4000 plant species considered endemic to the region (Myers *et al.* 2000). Less than 10% of the region's pre-European colonisation vegetation remains, representing a loss of 310,000 km^2 of native ecosystems (Bradshaw 2012). In some areas this loss of vegetation is near-complete– over 93% of original vegetation in the Avon Wheatbelt Interim Biogeographic Region

(IBRA) has been cleared since 1940 (Saunders 1989), including up to 97% in some areas (Saunders *et al.* 1993). While much of the damage has already been done, land clearing is by no means a past occurrence and an estimated 2, 800ha of land was cleared in Western Australia (WA) between 1995 and 2005 (Bradshaw 2012; Cross *et al.* 2020a). The vast scale of land degradation, coupled with the increase in the desire and urgency for ecological restoration, has facilitated a growing need for innovation and research into effective and large-scale ecological restoration in Western Australia.

Seeds are the primary tool for reintroducing plant species into degraded ecosystems (Broadhurst *et al.* 2015a; Cross *et al.* 2020b; Merritt and Dixon 2011). The increasing scale of restoration required around the world has facilitated a massive and growing demand for native seeds (Merritt *et al.* 2007; Merritt and Dixon 2011; Nevill *et al.* 2018). Hundreds, if not thousands, of tonnes of seeds are required to meet the requirements of restoration currently planned and being undertaken in Western Australia (WA) alone (Merritt and Dixon 2011), and the restoration activities of regulated industries such as mining are often legally required to achieve biodiversity targets (Hancock *et al.* 2020). While ecological constraints to seed sourcing and dormancy alleviation define capability, financial and technological constraints set the limits of what is realistic in ecological restoration (Miller and Hobbs 2007). The reality of many restoration projects is that species selected for return need to be based on those species that are most likely to establish and thrive (Cross 2021; Erickson and Halford 2020). Seed mixes prepared for ecological restoration tend to exclude species where dormancy alleviation requirements are unknown, where seed germination is problematic or challenging, or species for which little ecological information is available (as seen in Maher *et al.* 2009). These factors can result in species bias in restoration programs (Broadhurst *et al.* 2015b), skewing species compositions towards those that are most

easily returned (McDonald *et al.* 2016) or physiologically favoured (Cross *et al.* 2021; Cross and Lambers 2021; Zhong *et al.* 2021) rather than those that best reflect the reference ecosystem. Species bias in this manner places higher demand on fewer species, which is of particular concern in Australia where more than 90% of seed is sourced from natural vegetation already under significant pressure (Broadhurst *et al.* 2015a; Broadhurst *et al.* 2015b). Such heavy reliance on a limited pool of species may compromise restoration outcomes, while creating uncertainty within restoration programs as questions of ongoing seed supply arise in response to increasing anthropogenic seed collection and climate change (Broadhurst *et al.* 2015b). An increasing demand for seed, coupled with limited capability to use many species with challenging seed dormancy alleviation requirements, means a significant increase in our understanding of seed dormancy is required.

1.1.1 Seed dormancy

In most global ecosystems except tropical forests, seed dormancy is more prevalent than non-dormancy (Baskin and Baskin 2020). Seed dormancy is a critical part of plant ecology, ensuring that seed germination is timed to the season best suited for sustained growth and development of seedlings. Poorly timed germination can result in the death of young seedlings and, therefore, seed dormancy is under immense selection pressure and has evolved numerous complex mechanisms (Baskin and Baskin 2014). The seed dormancy classification system by Baskin and Baskin, (2004) divides dormancy into 1) morphological, which is maintained by an immature embryo at the time of dispersal and a requirement for embryo growth prior to germination (Baskin *et al.* 2020); 2) physical, in which a water impermeable fruit or seed coat prevents seed imbibition, thus restricting water from the embryo (Erickson *et al.* 2016); 3) physiological, whereby concentrations of endogenous phytohormones within the

seed prevent germination (Hoyle *et al.* 2008); 4) morphophysiological, in which a combination of an immature embryo and concentrations of phytohormones restrict embryo growth (Just *et al.* 2019); and 5) combinational, in which a water impermeable layer restricts water to the embryo while concentrations of hormones restrict embryo growth (Turner *et al.* 2006b) (Table 1.1).

In Western Australia, the seed dormancy mechanisms of many species are unknown or inferred (Bell, 1999; Merritt, 2007; Baskin and Baskin, 2014). Some species germinate readily after pre-treatment whilst others continue to pose a problem for seed-based restoration. Typically, studies into seed germination have focused on single species (Turner and Dixon, 2009; Turner *et al.*, 2009a) or small groups (Turner *et al.*, 2009b; Hidayati *et al.* 2012; Commander *et al.* 2009), classifying seed dormancy (Table 1) on a case-by-case basis and progressively building our knowledge of the larger ecosystem. This process has identified families and species that produce seeds that are difficult or impossible to germinate *ex situ* (e.g., under laboratory conditions) and many of these intractable seeds possess a component of physiological dormancy (PD) (Merritt *et al.* 2007).

For this thesis the term “intractable” refers to the inability to alleviate seed dormancy and promote germination predictably and reliably. In this thesis, “intractable” is applied exclusively to species possessing a component of physiological seed dormancy. While seeds possessing physical or morphological seed dormancy can form considerable barriers to establishment when sown in restoration, the mechanisms that support dormancy alleviation in these species are generally well understood (Baskin and Baskin 2014), and, consequently, low rates of germination success are often due to confounding factors of viability, treatment application or chronological requirements for embryo growth and maturation prior to radicle emergence (Cross *et al.* 2020b).

Often, following further study physiologically dormant species previously cited as intractable (i.e., *Lysinema ciliatum, Acanthocarpus pressii, Anigozanthos manglesii, Persoonia longifolia*) have proven to be responsive to the application of smoke (Dixon *et al.* 1995), warm stratification (Turner *et al.* 2006a), heat (Tieu *et al.* 2001a) or seasonal fluctuations of temperature and moisture (Chia *et al.* 2016) and as a consequence have entered commercial horticultural production systems. This highlights both the anthropogenic nature of the term; a species said to be intractable may be described as such because insufficient study has been done to elucidate the mechanisms responsible for dormancy alleviation and germination stimulation for a substantive component of the pure live seed.

Table 1.1 The hierarchical system of dormancy classification by Baskin and Baskin (2014).

Dormancy class[1]	Subdivisions[1]	Characteristics[1]
Morphological		- Immature embryos that grow prior to germination - Does not include seeds with undifferentiated embryos
Physical	Requires subdivision[1]	- One or more water impermeable layers of palisade cells in the seed or fruit coat
Physiological	Non- deep •Types 1-6[1]	- Excised embryo produces normal seedling - Gibberellic acid (GA) overcomes dormancy - Cold (0-10˚C) or warm (>15˚C) stratification breaks dormancy - Seeds may after-ripen in dry storage - Scarification may promote germination
	Intermediate	- Excised embryo produces normal seedling - GA overcomes dormancy in some, but not all, species - 2-3 months of cold stratification breaks dormancy - Dry storage can shorten the stratification period
	Deep	- Excised embryo produces abnormal seedling - GA does not overcome dormancy - 3–4 months of cold stratification breaks dormancy
Morphophysiological	See [1]	- Shares characteristics with morphological and physiological dormancy
Combinational	Non-deep •Types 1- 2[2]	- Shares characteristics with physical and physiological dormancy

[1] Soltani *et al.* (2017) [2] Baskin and Baskin (2014)

1.1.2 Physiological seed dormancy

Physiological seed dormancy is the most evolutionarily conserved dormancy class (Finch-Savage & Leubner-Metzger, 2006; Graeber *et al.* 2012) and can be the most challenging for seed-based restoration, as its mechanisms are not sufficiently understood and techniques for its alleviation can be both specific and complex. Physiological dormancy is established during seed maturation as storage compounds accumulate, restricting embryo growth through the interplay of the phytohormones gibberellic acid (GA) and abscisic acid (ABA), which are mediated by conditions of temperature and moisture (Finch-Savage and Leubner-Metzger, 2006). Gibberellic acid and abscisic acid have key antagonistic rolls in the onset, maintenance, and loss of seed dormancy (Baskin and Baskin, 2014) with abscisic acid known as a positive regulator of dormancy in species from diverse evolutionary origins (Asterids; Petruzzelli *et al.* 2003, Monocots; McCarty, 1995, Rosids; Nambara and Marionpoll, 2005 and Gymnosperms; Feurtado *et al.* 2004; Corbineau *et al.* 2002). Abscisic acid inhibitors (Debeaujon and Koornneef, 2000; Grappin *et al.* 2000; Ali-Rachedi *et al.* 2004; Nambara *et al.* 2010) have demonstrated a requirement for *de novo* synthesis of abscisic acid in the maintenance of seed dormancy, while the exogenous application of gibberellic acid has been shown to stimulate and enhance germination in a wide range of species (Baskin and Baskin, 2014).

Physiological seed dormancy can also be imposed by seed covering material (mechanical dormancy) and chemical inhibition (chemical dormancy) (Baskin and Baskin, 2014). As climatic conditions alter the internal hormone balance within the seed, the range of conditions at which germination can occur increase, overcoming restrictions imposed by mechanical or chemical aspects. If environmental conditions overlap with the decrease in dormancy to stimulate germination, then germination can

proceed. However, if conditions are not favourable seeds may re-enter physiological dormancy in a phenomenon termed ‘secondary dormancy’ (Finch-Savage and Footitt, 2017). Thus, a defined physiologically dormant state does not exist. Rather, physiological dormancy exists along a spectrum, with the sensitivity to the germination environment changing as a function of variable ambient conditions (Finch-Savage and Leubner-Metzger, 2006; Just *et al.* 2018). The environment can have a range of effects on dormancy; however, the two primary factors controlling physiological dormancy loss are temperature and moisture. The mechanism by which environmental conditions drive dormancy loss is largely unknown (Graeber *et al.* 2012) although its effect is well documented across many different species (Turner *et al.* 2009; Commander *et al.* 2009; Commander *et al.* 2015; Commander *et al.* 2008; Cross *et al.* 2018) and can be seen in the currently recognised subdivisions (Table 1) of physiological dormancy (Washitani and Masuda, 1990).

Non-deep physiological seed dormancy can be overcome by exogenous application of gibberellic acid, and dormancy may be alleviated by either warm (>15°C) or cold (0–10°C) stratification (time under moist conditions). Intermediate physiological dormancy may be overcome by the application of gibberellic acid, while deep physiological dormancy cannot, and both have been shown to respond to 2–3 or 3–4 months of cold stratification respectively. Baskin and Baskin (2014) recognised five types of non-deep physiological dormancy based upon the work of Vegis (1963, 1964, 1973), and have expanded the classification scheme to include a sixth type (Soltani *et al.* 2017). The six types of non-deep physiological dormancy are based upon the patterns of temperature requirements for germination following the alleviation of non-deep physiological dormancy.

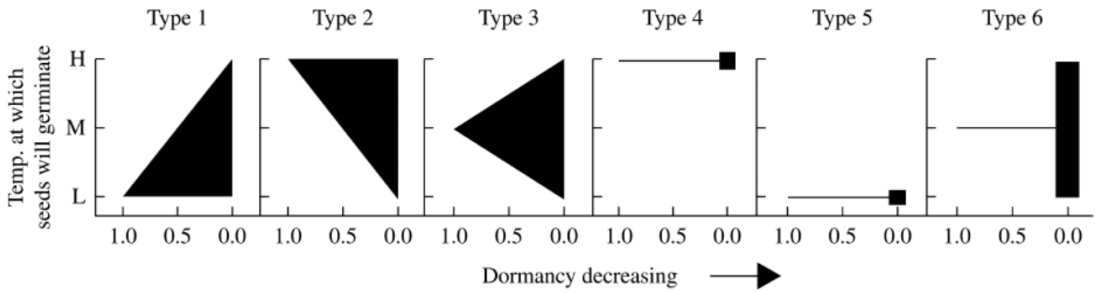

Figure 1.1. The six patterns of temperature requirements for germination as seeds come out of non-deep physiological dormancy as presented in Soltani *et al.* (2017).

As the depth of non-deep physiological dormancy decreases (moving left to right, X-axis, Fig 1.1) there is a shift in the temperatures able to support the germination process (Y-axis, Fig 1.1). In type 1 the temperature that can support germination is seen to increase as the depth of dormancy decreases. In seeds with type 2 physiological dormancy the opposite pattern occurs whereby seeds are only able to germinate at high temperatures, and the window widens as dormancy decreases, lowering the temperature at which germination can take place. Seeds with type 3 physiological dormancy is known from a limited number of species where dormancy restricts germination above or below an intermediate temperature, and both the maximum and minimum temperatures for germination increase/decrease as dormancy is alleviated. Finally, type 4, 5 and 6 physiologically dormant seeds will only germinate once dormancy has been adequately alleviated and will do so at specific high (type 4) and low (type 5) temperatures, or across a wide range of temperatures (type 6).

1.1.3 Germination

It is important to note the clear difference that exists between seed dormancy and seed germination (Thompson and Ooi 2010; Vleeshouwers *et al.* 1995). In the words of Vleeshouwers *et al.* (1995) "*dormancy is a seed characteristic, the degree of which*

defines what conditions should be met to make the seed germinate". That is to say, the deeper the dormancy within a seed, the narrower the range of conditions that will stimulate it to germinate (Hilhorst 1993). Therefore, a germination stimulant is a change in the environment, whereas dormancy alleviation is a change in the seed (Thompson and Ooi, 2010).

Generally, germination occurs during the wettest period of the year (Merritt *et al.* 2007) and the germination response to temperature depends on the historical range of the species, with imbibition at warm temperatures alleviating dormancy in winter annual species (type 2, Fig. 1.1) and imbibition at cold temperatures (type 1, Fig. 1.1) alleviating dormancy in summer annual species (Graeber *et al.* 2012). Species in fire-prone regions of South Africa (Brown, 1993), California (Keeley, 1984) and Australia (Dixon, Roche, and Pate, 1995) form soil stored seed banks which persists through the inter-fire period, progressively losing dormancy until germination is stimulated by a diverse range of conditions. Fire provides many cues that stimulate germination such as heat, Karrikins and cyanohydrins (Flematti *et al.* 2015); however, there exists a growing body of evidence suggesting that the direct passage of fire does not stimulate germination in many Australian species (Ooi, Auld and Whelan, 2006). The ecological mechanisms driving dormancy loss and germination of species known to have a strong response to heat and smoke are reasonably well understood (Tieu *et al.* 2001; Ooi, 2007; Ooi *et al.* 2012; Ribeiro *et al.* 2013). Direct heating of seeds may rupture seed coats of physically dormant species (Ooi *et al.* 2012), or alleviate physiological dormancy (Tieu *et al.* 2001a), and chemicals in smoke (Flematti *et al.* 2004) can stimulate germination of seeds that have lost dormancy. The post-fire environment is typified by elevated soil nutrients released from ash (Baker *et al.* 2005b), higher light and moisture conditions and a shift in diurnal temperature (LeMone *et al.* 2017), which

promote germination into a less competitive and resource rich environment. Species that do not have such an obvious response to heat and smoke (Dixon *et al.* 1995; Roche *et al.* 1997; Keeley and Fotheringham, 1998; Clarke *et al.* 2000; van Staden *et al.* 2000) make up a gap in our knowledge of germination ecology, evident in our inability to produce 'on-demand' seedlings of many wild species (Hancock, 2014). These species may require various stratification treatments, individual events specific to their ecology, or a combination of sequential conditions that occur in soil to alleviate dormancy before germination can be stimulated.

1.1.4 Intractable species

To reach biodiverse restoration targets dormancy alleviation and germination stimulating techniques need to be understood for individual species (Merritt *et al.* 2007). The seed dormancy of many species is poorly resolved at present (Merritt *et al.* 2007) and this limits opportunities to devise prescriptions for restoration and management (Maher *et al.* 2008). Including seeds with poorly understood or unknown propagation requirements in seed-mixes is likely to increase both the cost, difficulty, and timeframe of restoration as well as waste significant amounts of valuable seed (Erickson and Halford 2020). For seed-based restoration to be effective dormancy-alleviation treatments must be efficient and repeatable (Merritt *et al.* 2007). To develop such treatments significant understanding of the conditions that alleviate dormancy is required on a species-specific basis. Targeted research into seed dormancy and germination of species is the most direct path to addressing this issue. However, there is limited information on species for which *ex situ* germination cannot be achieved in southwestern Australia.

Appendix 1.1 presents a list of species that cannot at present be germinated from seed reliably and are therefore ideal subjects for future research efforts investigating seed dormancy alleviation and germination stimulation. It has been compiled using previous reports of difficult-to-propagate species (e.g., Ralph, 2003; Maher, 2009; Merritt *et al*. 2007), interrogation of the seed biology literature in Western Australia, and personal communications with experts. Future work is required to define the terminology applied to, and the seed trait criteria defining, intractable species, which will help to further quantity the proportion of species that cannot be germinated on demand.

1.1.5 Dormancy alleviation treatments

There are a significant number of species for which we cannot induce, *on-demand*, a substantial portion of the pure live seed to germinate (*ex situ*) (Appendix 1.1). The discovery of smoke as a germination stimulant (Dixon *et al.* 1995; Flematti *et al.* 2004; Roche *et al.* 1997a; Roche *et al.* 1997b) significantly increased the number of species that were able to be germinated on demand and thus the number of species available for restoration programs. In their foundational study of the response of 94 Western Australian plant species to smoke, Dixon *et al.* (1995) reported 45 species responded positively to aerosol smoke. Prior to this discovery, for example, around two-thirds of the 70–100 species broadcast seeded at Alcoa (a bauxite mine in southwestern Australia–Koch, 2007) were considered difficult to establish and therefore rare or absent from restored sites (Norman *et al.* 2006). Smoke is clearly a powerful ecological signal and has been applied as a germination stimulant with great success. However, many species exhibit no response to smoke under *ex situ* conditions, yet show a clear promotive effect when smoke is applied to habitat soils containing a resident seed bank (Dixon, Roche, and Pate 1995, Roche, Dixon, and Pate 1997, Roche, Koch, and Dixon 1997). Approximately 80% of Jarrah Forest species studied by Norman *et al.*

(2006), and 52% of species studied by Dixon *et al.* (1995), showed no germination response under *ex situ* conditions to smoke, suggesting other factors play a role in dormancy alleviation and germination stimulation of these species. Seed dormancy is known to limit germination response to stimulants such as smoke, while narrowing the hydrothermal conditions that allow germination. Species shown to have no response to smoke may have requirements for dormancy alleviation prior to smoke-stimulated germination or have germination stimuli that are unrelated to smoke.

Where species that were previously considered intractable have been induced to germinate, successful treatment applications typically replicated some phenomenon that seeds would naturally experience in soil (Table 1.2). For example, periods of dry heat replicating summer temperatures, or the passage of fire have been shown to increase germination of species from Anthericaceae, Apiaceae, Cyperaceae, Haemodoraceae, Poaceae and Stylidiaceae (Clarke and French 2005; Tieu *et al.* 2001a). Similar effects have been noted for species such as *Stylidium affine* (Stylidiaceae) and *Conostylis candicans* (Haemodoraceae) but at lower temperatures typically used in afterripening treatments (Turner *et al.* 2009b). The addition of moisture to these treatments (i.e., warm stratification), designed to mimic moist soils post summer rainfall or in the lead up to the germination season, has proven effective in a diverse range of species such as *Marianthus bicolor* (Cyperaceae) , *Acanthocarpus pressii* (Iridaceae) (Turner *et al.* 2006a), *Lomandra preissii* (Asparagaceae) (Merritt *et al.* 2007) and *Hibbertia glaberrima* (Dilleniaceae) (Dalziell *et al.* 2018; Hidayati *et al.* 2012). Where species have proven particularly difficult to germinate afterripening or stratification, seed burial in near-natural or natural conditions has proven effective in producing *in situ* germination or germination upon retrieval in *Lepidosperma scabrum* (Cyperaceae) (Turner 2013), *Orthrosanthus laxus*

(Iridaceae) (Roche *et al.* 1997a), *Trachymene cyanopetala* (Araliaceae) (Dwyer and Erickson 2016), *Clematis pubescens* (Ranunculaceae) (Cromer 2007) and *Persoonia longifolia* (Proteaceae) (Chia *et al.* 2016). The effectiveness of burial treatments suggests a requirement for specific hydrothermal signals not effectively supplied under laboratory conditions, or multiple cycles of conditions over an extended period of time (Hirst *et al.* 2021). To date, very few studies in southwest Australia have investigated what these signals might be (Merritt *et al.* 2007). However, Chia *et al.* (2016) produced significant germination for a notoriously intractable species, *Persoonia longifolia* (Proteaceae), by replicating cycles of specific environmental conditions (namely short pulses of wetting and drying) *ex situ*.

Table 1.2. Examples of dormancy alleviation by conditions that seeds would experience *in situ*

Species	Effective treatment	Natural phenomenon being replicated
Acanthocarpus pressii	Warm Stratification (Turner *et al.* 2006a)	Warm moist soils
Anigozanthos manglesii	Heat (Tieu *et al.* 2001a)	Summer soil temperatures and fire
Austrostipa elegantissima	Afterripening (Turner *et al.* 2009b) Heat (Turner *et al.* 2009b)	Dry periods in soil Summer soil temperatures and fire
Clematis pubescens	Burial (Cromer 2007)	Time in soil, *in situ* temperature and moisture changes
Conostylis candicans	Afterripening (Turner *et al.* 2009b) Heat (Turner *et al.* 2009b)	Dry periods in soil Summer soil temperatures and fire
Hibbertia glaberrima	Warm Stratification (Dalziell *et al.* 2018; Hidayati *et al.* 2012)	Warm moist soils
Lepidosperma scabrum	Burial (Turner 2013)	Time in soil, *in situ* temperature and moisture changes
Lomandra preissii	Warm Stratification (Merritt *et al.* 2007)	Warm moist soils
Loxocarya striatus	Heat + smoke (Tieu *et al.* 2001a)	Fire
Marianthus bicolor	Warm Stratification (Merritt *et al.* 2007)	Warm moist soils
Orthrosanthus laxus	Burial + smoke (Roche *et al.* 1997a)	Time in soil, *in situ* temperature and moisture, fire
Persoonia longifolia	Cycles of warm and cold stratification (Chia *et al.* 2016)	Seasonal and diurnal temperature change
Stylidium affine	Afterripening (Turner *et al.* 2009b) Heat + smoke (Tieu *et al.* 2001a; Turner *et al.* 2009b)	Dry periods in soil Fire
Thysanotus multiflorus	Burial + smoke (Cromer 2007)	Time in soil, *in situ* temperature and moisture, fire
Trachymene cyanopetala	Burial (Dwyer and Erickson 2016)	Time in soil, *in situ* temperature and moisture changes

1.1.6 The Rutaceae

In Western Australia 258 species of Rutaceae are currently described, 83 of which are classified as threatened or Priority flora indicating they are of conservation concern (West Australian Herbarium 1998-2022). A significant presence within understorey vegetation makes the Rutaceae crucial for conservation, and the ever-growing impacts of mining in Australia makes the Rutaceae a target group for inclusion in restoration programs. Both seed collection and germination comprise significant problems for restoration efforts incorporating Rutaceae. In Western Australia, seed production under managed conditions in seed production areas is uncommon (A Cross Personal Communication, 2021) however, the timing and methods of seed collection have been noted to significantly impact seed viability and germination in Eastern Australian *Zieria arborescens* (Rutaceae) (Frith, Offord, and Martyn, 2009) and would likely have a similar effect in WA species. Information is available on the germination requirements of less than 5% of southwest Australian Rutaceae (Table 1.3), and even less is known about species from the Midwest. What little information is available in the literature (Table 1.3) portrays Southwest Australian Rutaceae as a problematic group to grow from seed (Merritt *et al.* 2007). Significant work has been conducted on the seed ecology of Rutaceae from Eastern Australia (Auld *et al.* 2000; Collette and Ooi 2017; Mackenzie *et al.* 2016a; Mackenzie *et al.* 2016b; Offord *et al.* 2004) and while these results support observations of Western Australian Rutaceae emerging post fire, few treatments have produced consistent germination for WA Rutaceae to date (Commander *et al.* 2009a; Cromer 2007; Dixon *et al.* 1995; Paynter *et al.* 1991; Roche *et al.* 1997a). While germination for some species is possible through application of gibberellic acid (Commander *et al.* 2009; Table 1.3), other

species pose a significant barrier to seed-based restoration, as germination has yet to be produced under laboratory conditions.

Table 1.3 Number of southwest Australian Rutaceae that appear in southwest Australian seed biology literature.

Genus[1]	# species[1]	# Species studied	Dormancy type[7]
Boronia Sm.	87	5[2, 6]	PD
Correa Andrews	2	1[2]	PD
Diplolaena R.Br	18	2[2, 3]	PD
Geleznowia Turcz	4	1[2, 4, 5]	PD
Philotheca A.Rich	47	1[2]	PD

[1]Western Australian Herbarium (1998–) [2] Roche; Dixon and Pate (1997) [3] Commander; Merritt; Rokich and Dixon (2009) [4] Paynter and Dixon (1991) [5] Dixon, Roche, and Pate (1995), [6]Cromer, (2007) [7]Baskin and Baskin, (2014)

1.2 Research objectives

1.2.1 General objectives

The aim of this thesis was to explore potential avenues to alleviate dormancy and stimulate germination in a well-known group of intractable species in southwest Australia, the Rutaceae. This was achieved firstly by testing available species for primary dormancy and response to incubation temperatures, stratification regimes, germination stimulants, as well as quantifying emergence under replicated field conditions. Initial results gleaned from this process were used to design experiments investigating the effect of warm stratification on select members of the Rutaceae. Finally, a seed burial trial was conducted to assess how seeds treated with fire cues respond to *in situ* conditions

1.2.2 Specific objectives

1. To establish critical baseline information on the seed characteristics of southwest Australian Rutaceae, assess whether cold or warm stratification can alleviate dormancy, and identify and categorise the germination window that seeds experience under field conditions.
2. To assess the interaction of warm stratification and fire cues on dormancy alleviation and germination stimulation in select Rutaceae, quantify thermal thresholds required for warm stratification in *Rhadinothamnus anceps,* determine how warm stratification effects the optimum temperature, maximum germination proportion and germination speed in *R. anceps*, and investigate the moisture levels required for warm stratification of *R. anceps*.
3. To investigate the temperatures experienced by seeds across the soil profile, determine when seeds maintained under field conditions lose dormancy, identify the response of Rutaceae to *in situ* hydrothermal conditions and fire cues, and determine potential drivers for dormancy alleviation and germination stimulation *in situ.*

2 Chapter 2 The seed morphology, dormancy, and germination of eight southwest Australian Rutaceae

2.1 Abstract

Ecological restoration requires significant understanding of species-specific seed biology, which remains poor for many Australian plants. In Western Australia the Rutaceae are among a group of species unavailable to seed-based restoration due to complexity of seed dormancy. While seeds of many Australian Rutaceae respond to germination cues of heat, smoke, and seasonal temperature, these treatments have failed to produce consistent germination response in Rutaceae from southwestern Australia. We investigated the seed biology of eight species of Rutaceae from southwestern Australia to progress understanding of the requirements for dormancy alleviation and subsequent germination. Germination of all species was significantly improved by the application of 2.89 mM gibberellic acid, while 0.67 µM KAR_1 stimulated germination exclusively in *Rhadinothamnus anceps* at 30°C. Warm stratification (30°C) for 6 weeks alleviated dormancy and allowed seeds of *Diplolaena angustifolia* and *Rhadinothamnus anceps* to germinate at 20°C but was ineffective for *Boronia cymosa*, *B. fastigiata*, *B. ovata*, *Cyanothamnus ramosus*, *Crowea angustifolia* and *Philotheca spicata*. Glasshouse experiments indicated a positive emergence proportion (increase of ~0.2) to aerosol smoke for *Boronia cymosa*, *B. ovata*, *Crowea angustifolia*, *Diplolaena angustifolia* and *Philotheca spicata* sown in late summer. These findings indicate a lack of requirement for KAR_1 in smoke-stimulated germination of study species and provide potential avenues for seed germination which may have implications in propagation techniques for conservation and restoration practice.

2.2 Introduction

Seeds are crucial for the successful implementation of ecological restoration globally and have been widely accepted as the best source material both economically and environmentally for restoration programs (Merritt *et al.* 2007; Merritt and Dixon, 2011). However, seed dormancy is a major barrier to germination on demand with the seeds of most wild species possessing some form of seed dormancy (Baskin and Baskin 2014). For seed-based restoration to be effective, dormancy-alleviating treatments must be efficient and repeatable (Kildisheva *et al.* 2020; Merritt *et al.* 2007). To develop such treatments, significant understanding of the conditions that regulate dormancy is required for each individual species. Without this information species bias in restoration programs can arise (Broadhurst *et al.* 2015b), skewing species compositions towards taxa that are easily germinated rather than those that best reflect the reference ecosystem (McDonald *et al.* 2016). Species bias within restoration places a higher demand on fewer species, which is of particular concern in Australia where more than 90% of seeds deployed in ecological restoration are sourced from remnant native vegetation, while the remaining 10% are sourced from managed seed production areas (Broadhurst *et al.* 2015a; Broadhurst *et al.* 2015b; Nevill and Wardell-Johnson 2016). Consequently, to combat species bias in Australian restoration, a better understanding of dormancy and germination requirements is needed for a broader range of species.

In Western Australia (WA), 24 plant families have been previously identified as having representatives that are difficult to germinate despite being required for ecological restoration projects (Merritt *et al.* 2007). It is unknown how many species from each of these families are currently unavailable to seed-based restoration, and their requirements for dormancy alleviation and germination comprise significant

knowledge gaps within the current literature and practice. Classification of seed dormancy type offers a structured approach that can identify potential factors and methods required for dormancy alleviation and inform the development of treatments to alleviate seed dormancy (Martyn 2009). The Rutaceae in Western Australia are a family that is currently unavailable to seed-based restoration due to complex and poorly understood seed dormancy.

The Rutaceae are an economically- and ecologically-important plant family both globally and within Australia, having been cited as the most chemically versatile of all plant families containing a range of secondary metabolites and a wide range of resistances to biotic and abiotic stresses (Morton and Telmer 2014). In Australia, this family is significantly under-represented in both horticulture and ecological restoration, and this is of particular concern as they make up a significant portion of understorey vegetation in Australia–particularly in the biodiversity hotspot of southwest Western Australia. Additionally, 83 of the 258 currently described Rutaceae in southwestern Australia are of conservation concern (Western Australian Herbarium, 1998 -) and there currently exists no reliable method for their propagation from seed. The germination biology of Australian Rutaceae represents a significant barrier to their broad use, particularly in WA (Cromer 2007; Maher *et al.* 2008; Merritt *et al.* 2007) where few studies have investigated the seed biology of native Rutaceae species. Eastern Australian Rutaceae have received significant attention in recent years (Auld *et al.* 2000; Collette and Ooi 2017; Mackenzie *et al.* 2016a; Mackenzie *et al.* 2016b; Offord *et al.* 2004) and have been found to respond positively to specific seasonal temperatures and fire cues however, the application of similar treatments in WA species (Bell *et al.* 1987; Commander *et al.* 2009a; Dixon *et al.* 1995; Paynter *et al.* 1991; Roche *et al.* 1997a) has yet to be effective for poorly understood reasons.

Available information suggests a single member of WA Rutaceae possesses physiological seed dormancy and its seeds readily imbibe water (Commander *et al.* 2009a) although this is yet to be confirmed for the remaining 257 species. *In situ* observations linking germination events of species in *Boronia* and *Philotheca* to the passage of fire or application of *in situ* smoke have been noted on several occasions in southwest Australia (Norman *et al.* 2006; Roche *et al.* 1997b), but the application of fire-related cues of smoke and heat under laboratory conditions have proven largely inconsistent or ineffective to date in *Boronia*, *Correa*, *Phebalium*, and *Philotheca* (Cromer 2007; Dixon *et al.* 1995; Roche *et al.* 1997a).

To incorporate WA Rutaceae in ecological restoration programs, a better understanding of their seed morphology, seed dormancy classification and germination biology is required. This study aimed to assess seed and embryo morphology in eight species from five species-rich genera from Rutaceae, in conjunction with germination responses to sequential stratification regimes (warm to cool moist conditions) and the application of germination stimulants. This approach was taken to firstly classify seed dormancy, then to use this information to formulate targeted treatments to alleviate dormancy-based blocks to germination on demand. Observations of germination under incubation temperatures replicating natural winter conditions and seedling emergence from sown seed trays were also recorded for seven species as a step towards determining the critical soil-based factors that regulate the timing of *in situ* seedling emergence.

2.3 Methods

2.3.1 Species selection and seed sourcing

We selected representatives of the Rutaceae from *Boronia*, *Crowea*, *Cyanothamnus*, *Diplolaena*, *Philotheca* and *Rhadinothamnus*, with a focus on species within *Boronia* as it is the most species rich genera within the family (Western Australian Herbarium 1998 –). Species targeted for collection from wild populations were *Boronia cymosa*, *Boronia fastigiata*, *Boronia ovata*, *Crowea angustifolia*, *Cyanothamnus ramosus, Diplolaena angustifolia*, *Philotheca spicata*, and *Rhadinothamnus anceps* (Table 2.1). Seeds were collected at the time of natural seed dispersal (late spring to mid-summer) across the greater Perth and Southwest regions by commercial suppliers (Seed Shed, Boddington, Western Australia). Collections were made between November and February in 2018/2019. All seed was stored at 15 % relative humidity (RH) and 15°C at the Western Australia Seed Centre, Kings Park and Botanic Garden, prior to use in experiments (up to 4 months after collection).

2.3.2 Seed and embryo characteristics

Seed mass, viability, imbibition, and embryo morphology were determined for all study species (Fig. 2.1, Table 2.1). Seed mass (g ± s.e.) was determined for four replicates of 25 seeds. Seed length (mm ± s.e.) and seed fill (herein termed viability–% ± s.e.) were determined for four replicates of 25 seeds using light microscopy (Leica 205C camera with analysis in Leica application X, Leica Camera, Wetzlar, Germany), a cut test and X-ray analysis (Autofocus X-ray cabinet, Faxitron, Tucson, USA). Seeds were scored as viable if the endosperm was fully developed, neither shrunken, or retracted from the testa, and showed no signs of internal damage, deformation, or discolouration.

To determine embryo morphology, 30 seeds of each species were imbibed in deionised (DI) water for 24 h. Seeds were then measured longitudinally (µm ± s.e.) with an ocular micrometre, before removing the intact embryo, which was also measured (µm ± s.e.) along the same axis and inspected to determine morphology (Martin 1946).

To assess water permeability of the testa, three replicates of 25 seeds were placed into a Petri dish lined with filter paper irrigated with deionized (DI) water. Seeds were patted dry and weighed after 5 min (time 0) and again after 24 h. Percentage water uptake was determined gravimetrically (Table 2.1), based on the fresh weight of non-imbibed seeds, with the percentage increase in seed mass calculated as:

$[(W_1-W_d)/W_d] \times 100$,

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

2.3.3 Germination biology

To assess the germination response to temperature and germination stimulants, 100% filled seeds were plated in 90-mm Petri dishes containing 0.7% (w/v) water agar only (control) or water agar containing 2.89 mM gibberellic acid (GA_3; Sigma Aldrich Chemicals, Castle Hill, NSW, Australia), or 0.67 µM karrikinolide (KAR_1, as synthesised in Flemmati *et al.* 2005). Seeds were surface sterilised in a 4 % (w/v) sodium hypochlorite (NaOCl) solution supplemented with several drops of Tween 80 (to alleviate surface tension) for 30 minutes under alternating vacuum (-70 kPA) (Turner *et al.* 2018) before plating as four replicates of 25 for each treatment. Plates were incubated at 10°C, 20°C or 30°C under alternating 12/12hr lighting and germination scored weekly as emergence of the radical >2mm for 14 weeks. Primary

seed dormancy was determined as the proportion of viable, non-germinated seeds in control and KAR_1 treatments after four weeks according to the definition by Baskin and Baskin (2004).

Changes in temperature following cold (<15°C) or warm (>15°C) stratification are known to alleviate physiological dormancy and stimulate germination (Baskin and Baskin 2014), and have proven effective in some Australian natives (Merritt *et al.* 2007; Sommerville *et al.* 2013; Turner *et al.* 2006a). To investigate the effectiveness of cold and warm stratification in southwest Australian Rutaceae, seeds which had been sterilised and incubated at either 10°C or 30°C on water agar, GA_3 or KAR_1 as previously described, were moved after six weeks, and incubated for a further eight weeks at 20°C. Germination was scored weekly and defined as emergence of the radicle to >2mm.

2.3.4 Short term afterripening and incubation at alternating winter temperatures

To determine whether warm, dry conditions experienced by seeds in the soil over the summer months (i.e. dry afterripening) alleviate dormancy, seeds were stored at 30°C and 50% RH for three months before sowing on 0.7% (w/v) water agar and incubating at conditions intended to mimic diurnal winter conditions (18/7°C, alternating 12/12 h) indicative of south west Western Australia (Merritt *et al.* 2007). Seeds were first treated with fire cues (heat, smoke, and a combination of both) before being either surface sterilised and incubated at winter conditions as previously described (controls seeds) or placed into afterripening, then surfaced sterilised and incubated as previously described. Heat treatments were applied by placing seeds in foil cups inside a preheated oven at 90°C for 10 minutes (Mackenzie *et al.* 2016a). Aerosol smoke was applied by placing seed lots on trays inside a sealable 60 L plastic propagation tent

before pumping in smoke generated from 100g of oat hay ignited in a bee smoker for 10 minutes (Mackenzie *et al.* 2016a). To avoid pseudo-replication all replicates were kept separate throughout the application of treatments. In the case of combination heat + smoke treatments heat was applied first then smoke. Afterripening conditions were maintained by suspending seeds over a non-saturated solution of lithium chloride (LiCL) calibrated to maintain a constant 50 % RH (364 g L^{-1} to achieve 50 % RH) inside a 270 x 190 x100 mm polycarbonate electrical enclosure box (NHP Fibox, Perth, WA, Australia). The entire enclosure box was then placed within an incubator at 30°C under an alternating 12/12hr lighting cycle. Seeds were removed after three months, before being sterilised and incubated as previously described. Germination was defined as radical emergence >2mm and was scored weekly for 18 weeks.

2.3.5 Patterns of seedling emergence in response to heat, smoke, and environmental conditions

To document the pattern of seedling emergence in response to fire cues, the seeds of seven species (*Boronia fastigiata* was not included due to a lack of seed) were separated into four replicates of 25 seeds each, before being left either untreated (control seeds) or treated with heat, smoke, or a combination of both as previously described. After treatment, seeds were surface sown into replicate punnets containing white quartz sand before sieving sand on top until seeds were covered by a layer 1–2mm deep. Seed of study species are naturally dispersed in summer, and the region experiences a Mediterranean climate with hot dry summers and cool wet winters (Merritt *et al.* 2007). Therefore, punnets were placed in simulated natural conditions at the Kings Park and Botanic Garden on the 10th of February 2020 (the same day seeds were treated) and allowed to receive rainfall until the 08/05/2020. When seasonal rainfall began in May daily watering to maximum soil capacity began, based on soil

and seed observations made by Merritt *et al.* (2007). The experiment ran for 18 weeks until the 11th of September 2020.

2.3.6 Soil conditions

To determine the conditions of temperature and moisture experienced by seeds sown in punnets temperature and moisture data was collected from 10mm below the soil surface using a HOBO Micro Station (H21-USB) equipped with three soil moisture smart sensors (S-SMx0M005) and a single temperature smart sensor (S-TMB-M0xx), which were each placed within different punnets. All data was logged at 10-minute intervals. Temperature data was analysed to determine the minimum and maximum temperature for each day (Fig. 2.6). Soil moisture was averaged across the three sensors and normalised to give a value between 0 (dry)–100% (soil field capacity) across the experimental period (20th of January to the 11th of September 2020, Fig. 2.6).

2.3.7 Statistical analysis

All analyses were conducted in the R statistical environment (R Core Team, 2013) using the *car* (Therneau and Lumley, 2013) package, or with the GERMINATOR software (Joosen *et al.* 2010). Generalised linear models (GLMs; binomial error structure, logit link function) were used to assess the influence of temperature, GA_3 and KAR_1, or heat, smoke, heat + smoke and afterripening, on germination success at either 14 or 18 weeks.

To determine the effect of treatment with fire cues (heat, smoke, heat + smoke) on final emergence under replicated environmental conditions we fitted a GLM (binomial error structure, logit link function) to emergence observed at 18 weeks. All models with interactions were fitted to data separately for each species. Temporal patterns in

emergence were modelled on the number of filled seeds sown and emergence defined as visual observation of seedlings in sand. Parameters estimated using the cumulative emergence curves from GERMINATOR (Joosen *et al.* 2010) were the onset of emergence (i.e. time to 1% of Gmax) and median emergence (i.e. time to 50% of Gmax).

2.4 Results

2.4.1 Seed and embryo characteristics

Seed length and seed mass of the studied Rutaceae varied between species (Table 2.1) with *Diplolaena angustifolia* (Fig. 2.1A) having the largest seeds (3500 ± 7.7µm) and *Philotheca spicata* (Fig. 2.1F) having the smallest (1248 ± 11.7µm).

Figure 2.1 Seed morphology of the eight Rutaceae species assessed in this study. A) *Diplolaena angustifolia*, B) *Cyanothamnus ramosus*, C) *Boronia fastigiata*, D) *Crowea angustifolia*, E) *Boronia cymosa*, F) *Philotheca spicata*, G) *Rhadinothamnus anceps*, H) *Boronia ovata*. Black lines = 1mm.

Following 24 h imbibition, the seed mass of all species increased between 15–31% of initial seed weight (Table 2.1). Embryo length varied proportionately with seed length and all species had an embryo to seed ratio of >0.9. Seed fill at the time of collection and processing ranged from ~50% in *Diplolaena angustifolia* to ~80% in *Rhadinothamnus anceps*.

Table 2.1 Key seed characteristics for eight species of Rutaceae from Southwest Australia. Primary dormancy was determined by incubating seeds at 10°C, 20°C or 30°C on water agar and observing germination over 6 weeks.

Species	Seed length (µm ± s.e.)	Seed weight (mg ±s.e.)	Seed fill (% ± s.e.)	Water uptake (%)	Embryo length (um±s.e.)	Embryo: seed ratio
Boronia cymosa Endl.	1432 (±7.7)	25.3 (±1.1)	68.7 (±0.9)	25.2 (±2.4)	1398 (±45.6)	0.97
Boronia fastigiata Bartl.	1740 (±23.4)	54.8 (±3.4)	68.7(±5.4)	26.0 (±1.3)	1725(±70.4)	0.99
Boronia ovata Lindl.	1268 (±5.7)	15.1 (±0.2)	74.0 (±3.4)	30.2 (±6.6)	1215 (± 26.4)	0.95
Cyanothamnus ramosus Lindl.	2468 (±38.2)	174.9 (±2.0)	78.2 (±1.8)	15.1 (±2.0)	2034(±35.5)	0.82
(syn. Boronia ramosa (Lindl.) Benth.)						
Crowea angustifolia Sm.	2143 (±28.2)	122.1 (±1.1)	91.1 (±2.1)	27.1 (±5.3)	2098 (±26.4)	0.97
Diplolaena angustifolia Hook.	3500 (±7.7)	243.2 (±1.3)	51.7 (±1.8)	19.0 (±2.7)	3321 (±56.5)	0.97
Philotheca spicata (A.Rich.) Paul G.Wilson	1248 (±11.7)	15.0 (±1.3)	62.6 (±5.8)	15.0 (±3.6)	1216 (±19.5)	0.85
Rhadinothamnus anceps (DC.) Paul G.Wilson	1441 (±11.8)	39.1 (±1.0)	81.7 (±1.6)	21.5 (±1.7)	1233(±37.91)	0.97

2.4.2 Germination biology

Germination tests confirmed fresh seeds of all species possessed high levels of primary dormancy, with few seeds (<5%) germinating within 4 weeks of sowing regardless of the incubation temperature used or the natural germination stimulant KAR_1. However, germination was significantly improved with the use of GA_3 across all species (Fig. 2.2). Average germination proportion ranged from 0 in control and KAR_1 treatments, to 0.25–0.95 in seeds treated with GA_3 (Fig. 2). Minimal germination occurred in the presence of KAR_1 or at a constant 30°C (Fig. 2.2) however, germination in the presence of GA_3 was ~0.60 at 30°C for *Diplolaena angustifolia* and *Rhadinothamnus anceps*, the latter of which was also able to germinate (~0.40) at 30°C in the presence of KAR_1.

The main effect of temperature was significant for all species (Appendix 2.1), and there was a significant interaction effect between treatment and temperature for *Diplolaena angustifolia* and *Rhadinothamnus anceps* (Appendix 2.1). *Boronia cymosa*, *B. fastigiata*, *B. ovata*, *Cyanothamnus ramosus*, *Crowea angustifolia* and *Philotheca spicata* all failed to germinate to significant proportions (<0.10) at 30°C and all species excluding *Boronia ovata* and *Cyanothamnus ramosus* had the greatest germination at 10°C. Following warm stratification, germination increased significantly in both control and KAR_1 treatments for *Diplolaena angustifolia* (~0.5) and *Rhadinothamnus anceps* (0.75), and there was no observed difference in germination between control and KAR_1 following warm stratification for either of these species (Fig. 2.2).

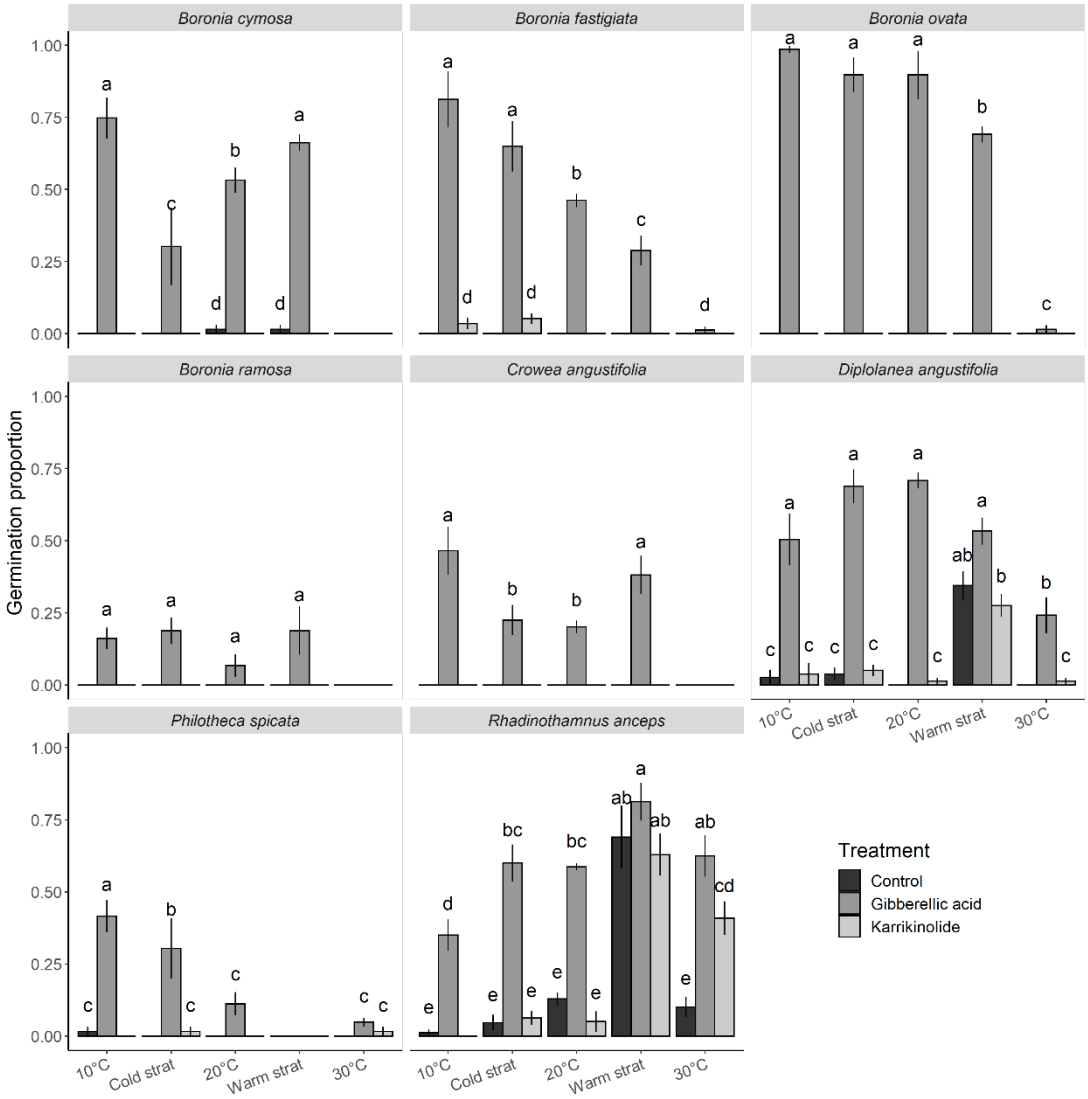

Figure 2.2. Germination proportion (± 95% confidence interval) of seeds incubated at five incubation regimes on water agar or agar containing either 2.89mM GA3 or 0.67µM KAR1. Seeds were incubated for 14 weeks at either 10°C, 20°C or 30°C (control seeds) or for 6 weeks at either 10°C or 30°C before being moved to 20°C for a further 8 weeks (stratification treatments). All seeds were incubated under 12hr diurnal light/dark alterations. Annotated lettering indicates the results of pairwise comparisons among treatments (p <0.05).

2.4.3 Short term afterripening and incubation at alternating winter temperatures

No germination was recorded for fresh, untreated control seeds of study species excluding *R. anceps* (0.08 ± 0.04, p>0.05) (Fig. 3). The application of smoke treatments significantly increased germination of fresh seeds of *D. angustifolia* ($\chi 2$ = 107.09, d.f. = 3, p <0.001) and *R. anceps* ($\chi 2$ = 59.821 d.f. = 3, p <0.001). Combination smoke and heat increased germination of *D. angustifolia* compared to smoke alone, while germination of *R. anceps* decreased with the combination of smoke and heat treatments compared to smoke alone (Fig. 2.3). Germination was relatively unchanged following afterripening of both *D. angustifolia* and *R. anceps* seeds when compared to fresh seeds (Fig. 2.3).

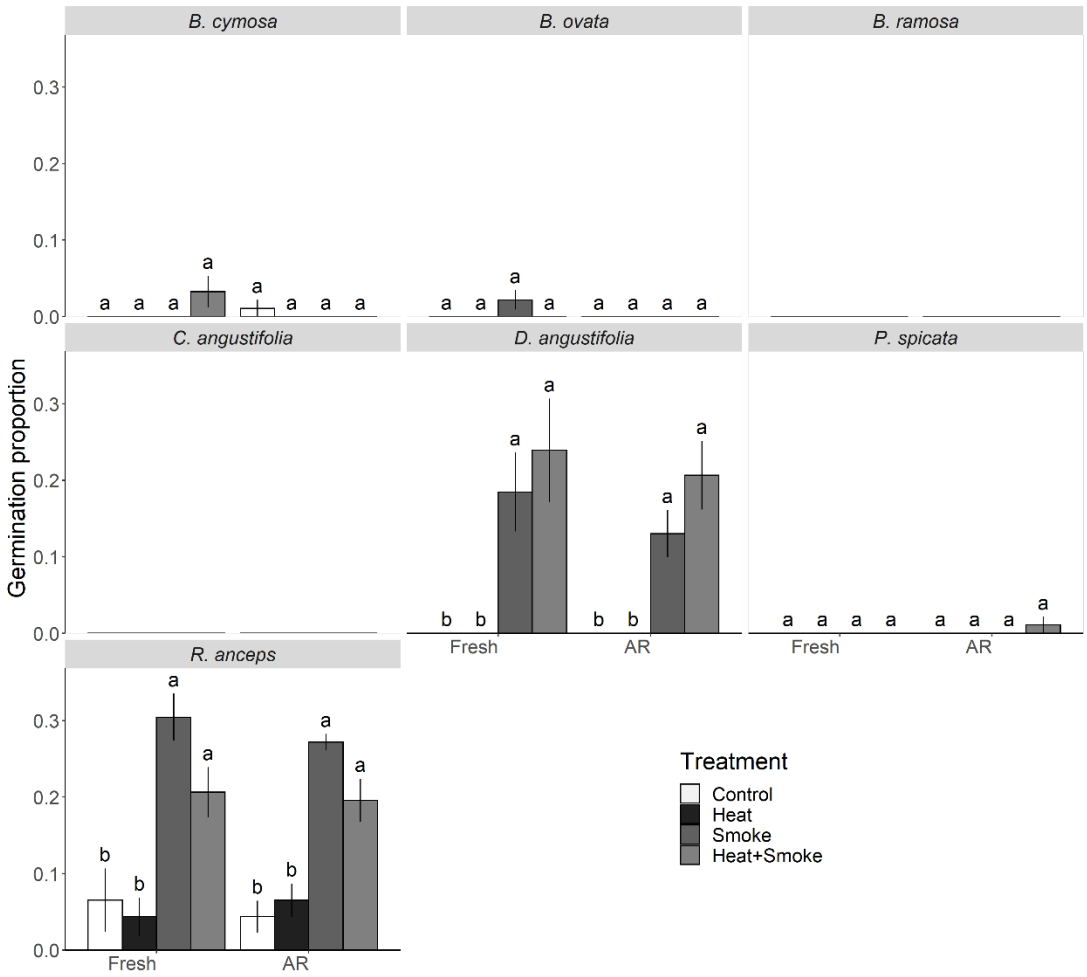

Figure 2.3. Germination proportion (± 95% confidence interval) after 18 weeks for seeds incubated at 18/7°C within 2 months of collection (fresh) or following 4 months of afterripening at 30°C and 50% RH. Annotated lettering indicates the results of pairwise comparisons among treatments (p <0.05).

2.4.4 Patterns of seedling emergence in response to heat, smoke, and environmental conditions

Low final seedling emergence were observed across all species sown into punnets. Smoke or smoke and heat combination treatments were the most effective treatments for all species that germinated to any proportion although the treatment effects were only significant for *B. cymosa*, *B. ovata* and *D. angustifolia* (Fig. 2.4).

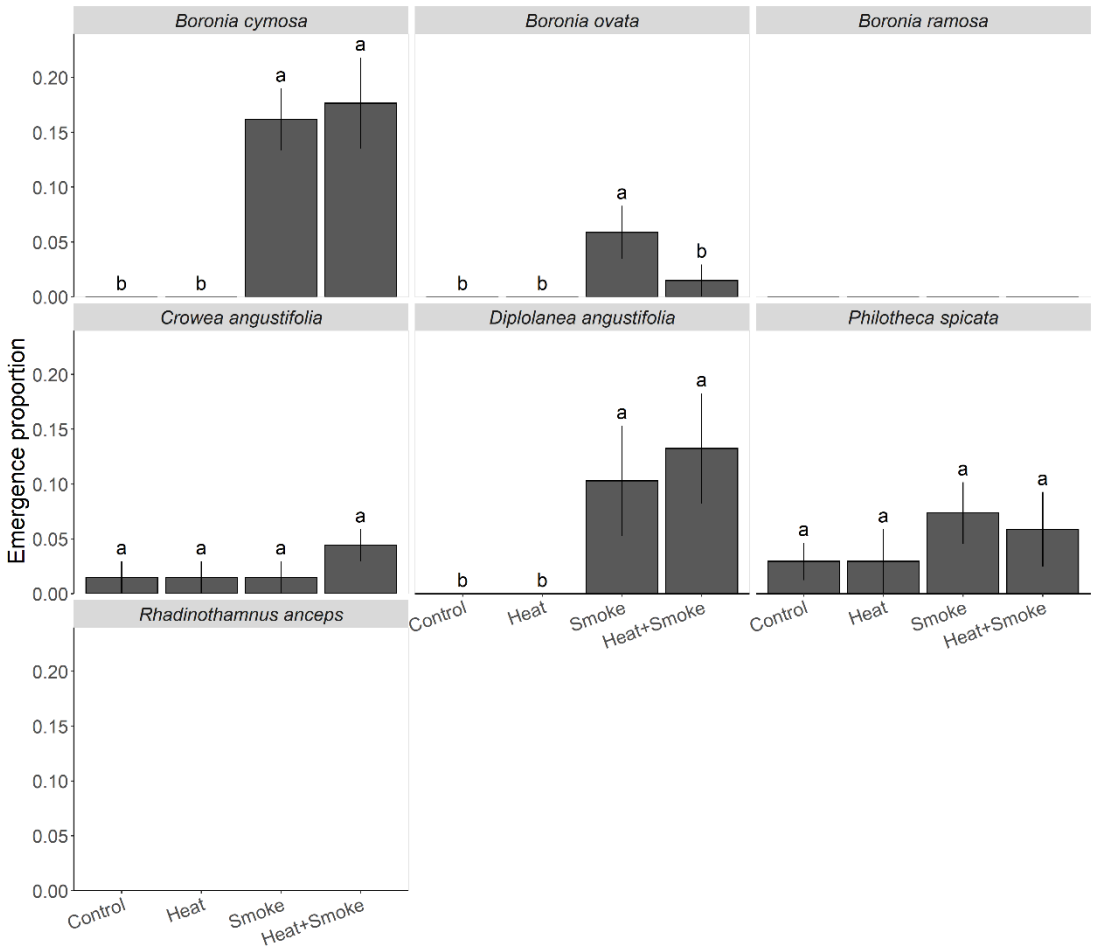

Figure 2.4. Final emergence (± 95% confidence interval) of seeds sown into punnets after 18 weeks of daily watering (from 8 May 2020 to 11 Sept. 2020). Seeds were sown on the 10th of February 2020 and the experiment concluded on the 11 of September 2020. Annotated lettering indicates the results of pairwise comparisons among treatments (p <0.05).

Due to low proportions of emergence across treatments, emergence speed (Fig. 2.5) was calculated from cumulative emergence pooled across treatments that were statistically similar (Fig. 2.4). Therefore, *Boronia cymosa* and *Diplolaena angustifolia* are pooled across smoke and smoke + heat treatments, *Crowea angustifolia* and *Philotheca spicata* are pooled across all treatments and *Boronia ovata* is solely smoke treated seeds. The onset of emergence differed markedly between species, with *D. angustifolia* (3.88 ± 0.26 weeks) and *P. spicata* (2.77 ± 0.92 weeks) emerging within 4 weeks from when daily watering began) and *B. cymosa* (5.45 ± 0.41 weeks), *B.*

ovata (4.81 ± 1.27 weeks) and *C. angustifolia* (8.25 ± 1.02 weeks) taking longer than 4 weeks.

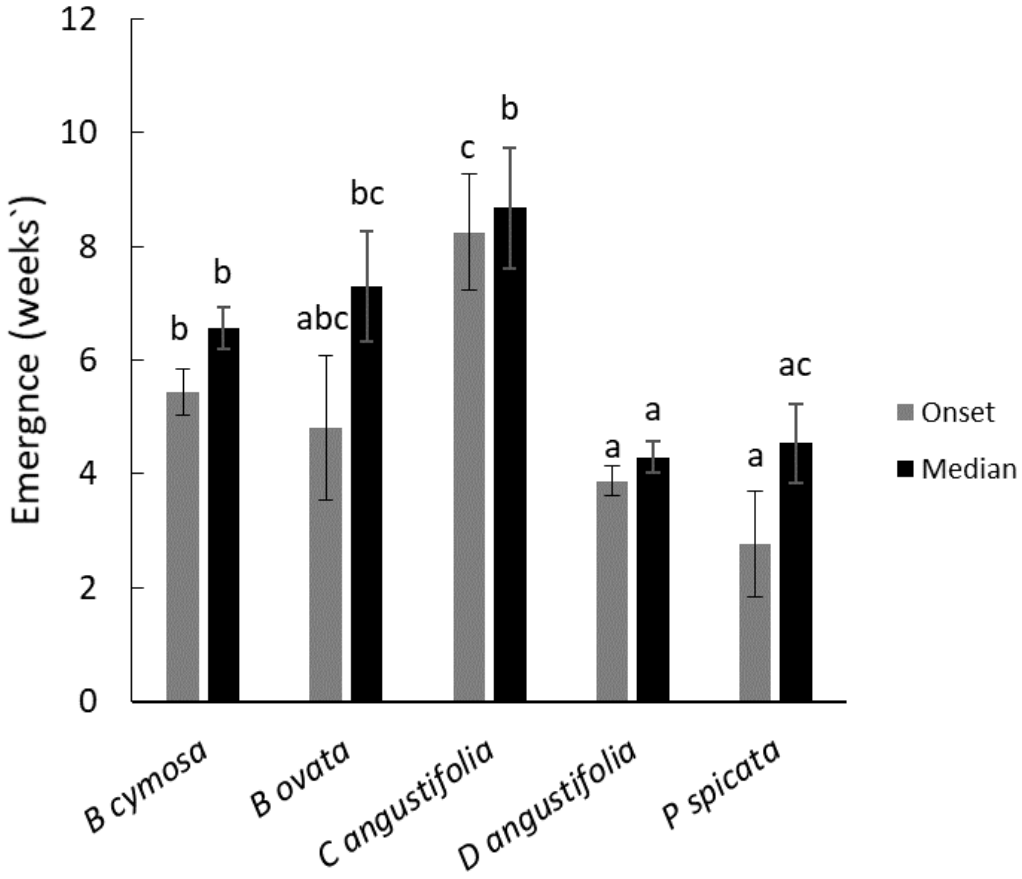

Figure 2.5. Mean (± SE) time (weeks ± s.e.) to onset of seedling emergence (first seedling observed) and median seedling emergence (50%) of Rutaceae exposed to optimum pre-treatment (maximum germination proportion in Figure 4) following the commencement of watering (time 0; 5th of May 2020). Annotated lettering indicates the results of pairwise comparisons among treatments (p <0.05).

2.4.5 Soil conditions

Prior to the onset of winter rainfall, soil moisture content increased above 60% twice during late summer and early autumn (Fig. 2.6). Outside of these two substantial increases in soil moisture, seven rainfall events were recorded before May that increased soil moisture to levels up to 40% (Fig. 2.6). Soil moisture increased when regular watering began on 08/05/2020 and was maintained above 40% until the end of the experiment in September 2020. Following these increases in soil moisture,

germination was observed between 2.8 and 8.3 weeks (Fig. 2.5 & 2.6). During periods of partial or full soil hydration (Table 2.2) seeds experienced a total of 46.9 h at temperatures above 25°C. Most of the time spent below 25°C in hydrated soils was at temperature intervals between 10°C and 20°C. When soils were dry (<20% moisture content) seeds experienced temperatures between 15°C and 25°C the most (1558 h), as well as a significant number of hours at temperatures above 30°C (580 h) which were only rarely seen (3.8 h) while soils were partially or fully hydrated.

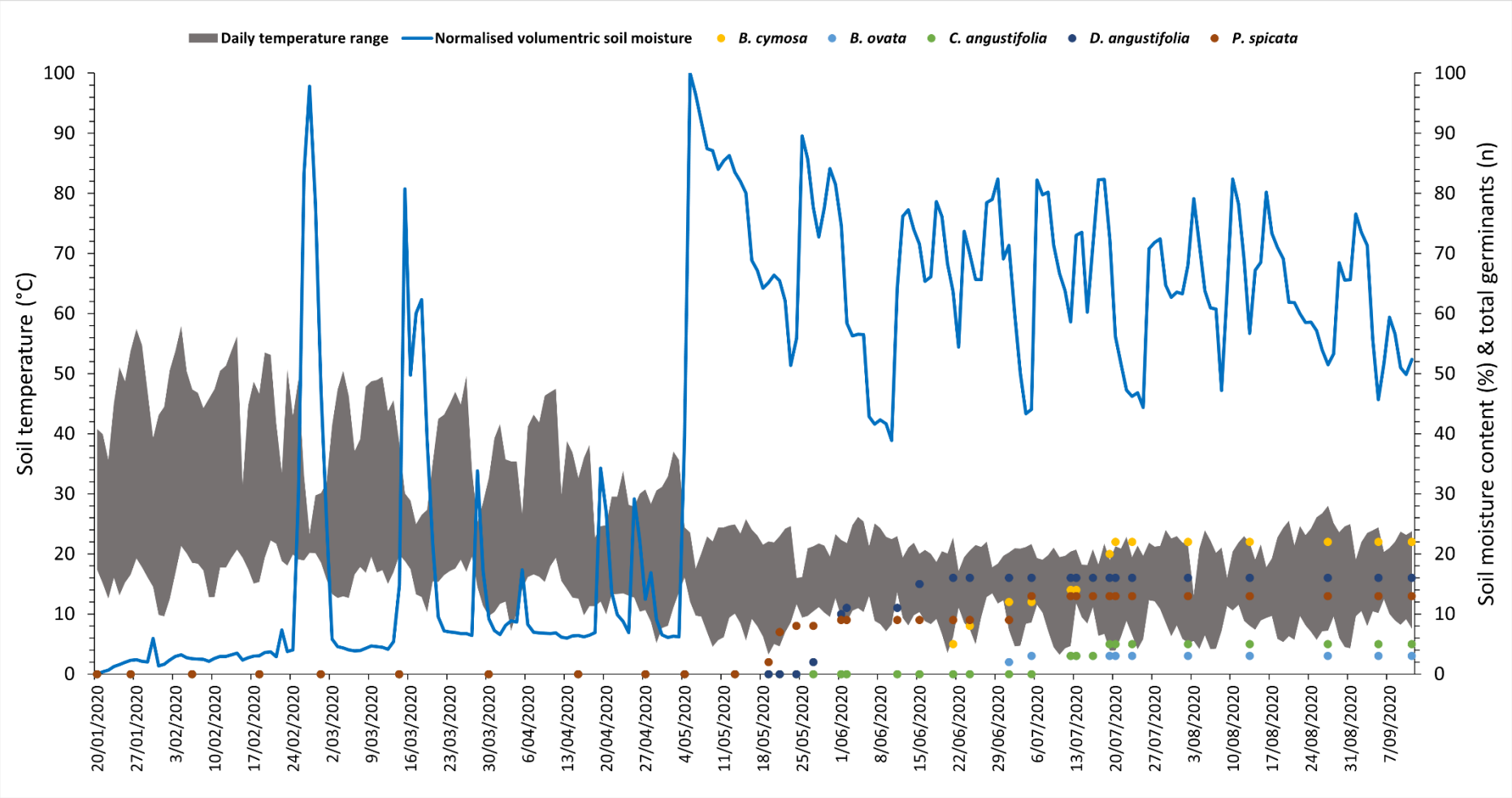

Figure 2.6. Percentage soil moisture [(gH2O/gDW)100]), daily soil temperature range (°C at 10mm depth), and total germination (sum effective treatments) from 20 January 2020 through to 08 September 2020 for punnets containing white quartz sand maintained in the Kings Park Science outside growing area. Points indicate when emergence was noted during routine scoring.

Table 2.2. Number of hours seeds of Rutaceae experienced soil temperature (°C) intervals during periods when soils were dry (<20% moisture content), partially hydrated (20-60% moisture content) or fully hydrated (>60% moisture content) from the 20th of January–30th of July 2020.

	Temperature interval													
Month	0-5	5-10	10-15	15-20	20-25	25-30	30-35	35-40	40-45	45-50	50-55	55-60	60-65	65-70
Soil with <20% moisture content														
Jan	-	0.6	20.4	99.3	43	18	23.9	24.5	21.5	14.8	7.5	2	-	-
Feb	-	0.8	25.8	97	176	73.8	43.8	46.2	66.5	46.6	14.7	1.9	-	-
Mar	-	2.2	46.3	170.8	112	56.2	57.2	48.6	44.3	18.3	0.3	-	-	-
Apr	-	26.3	133.6	208.8	95.3	79.3	44.3	23.1	15	4	-	-	-	-
May	-	7.9	20.6	27.9	8.5	8.4	10.1	1.4	-	-	-	-	-	-
Jun	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total (H)	0	37.7	246.7	603.8	434.8	235.8	179.3	143.8	147.3	83.7	22.4	3.9	0	0
Soil with 20-60% moisture content														
Jan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Feb	-	-	-	4.2	3	7.3	0.1	-	-	-	-	-	-	-
Mar	-	-	19.9	62.8	29.8	15.8	1.9	-	-	-	-	-	-	-
Apr	-	20.7	24.4	22.1	8	-	-	-	-	-	-	-	-	-
May	4.2	26.8	28.8	9.8	7.1	-	-	-	-	-	-	-	-	-
Jun	8.1	29.8	155.4	57.8	25.3	0.1	-	-	-	-	-	-	-	-
Jul	13.5	49.7	259	96.3	42.2	0.2	-	-	-	-	-	-	-	-
Total (H)	4.2	47.4	228.5	156.6	73.2	23	2	0	0	0	0	0	0	0
Soil with >60% moisture content														
Jan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Feb	-	-	-	8.7	66.3	11.9	1.7	-	-	-	-	-	-	-
Mar	-	-	11.3	27	12.8	6.8	0.1	-	-	-	-	-	-	-
Apr	-	-	-	-	-	-	-	-	-	-	-	-	-	-
May	5.8	126.7	254	118.3	76.8	0.9	-	-	-	-	-	-	-	-
Jun	0.5	9.7	152.5	168	29	0.5	-	-	-	-	-	-	-	-
Total (H)	5.8	126.7	265.3	154	184.8	20.1	1.8	0	0	0	0	0	0	0

2.5 Discussion

Seed germination and viability in Australian Rutaceae are highly variable between species (Mackenzie *et al.* 2016a; Martyn 2009; Roche *et al.* 1997a) and our results support these previous findings while presenting potential avenues for the propagation of previously ungerminable southwest Australian Rutaceae. Germination of fresh untreated seeds at a constant 10°C, 20°C or 30°C was negligible for all species. However, the application of warm stratification, GA_3, or KAR_1 improved germination to various degrees across different species (Fig. 2.2). *Rhadinothamnus anceps* was the only species that responded positively to KAR_1, which significantly improved germination at 30°C. An increase in germination proportion was seen in *D. angustifolia* and *R. anceps* following warm stratification however there was no difference between untreated and KAR_1 treated groups. Interestingly, warm stratification appeared to remove the requirement for KAR_1 stimulated germination in *R. anceps* at high temperatures, suggesting warm stratification may play a role in dormancy loss and germination in the inter-fire period. Warm stratification has proven to be effective in several diverse southwest Australian species from different habitats including *Lomandra pressii* (Asparagaceae) (Merritt *et al.* 2007), *Acanthocarpus preissii* (Asparagaceae) *(Turner et al. 2006a)*, *Byblis gigantea* (Cross *et al.* 2013), *Hibbertia spp.* (Hidayati *et al.* 2012) and *Tetratheca* spp. (S. Rajapakshe pers comm.) however, no previous study has demonstrated the potential of warm stratification to bypass the requirement for KAR_1. A lack of germination in control groups of *D. angustifolia* and total lack of germination in *R. anceps* sown into punnets indicates that the requirements for warm stratification observed in the incubators were not provided to seeds under replicated *in situ* conditions. This is further supported by the temperature and moisture data recorded over the course of the experiment as seeds were exposed

to just two days in moist soils nearing temperatures of 30°C (Table 2.2), as opposed to the six weeks at 30°C which promoted germination in incubators (Fig. 2.2).

Little is currently known about the exact requirements for dormancy alleviation by warm stratification in Australian Rutaceae. Our results indicate that temperatures of 30°C for 6 weeks were sufficient to release dormancy in a portion of seeds, allowing them to germinate when incubated at 20°C. Rainfall over the warmer months leading into the cooler winter months, which is the typical germination season in southwestern Australia, is likely to provide similar conditions to the warm stratification treatments applied here (Merritt *et al.* 2007; Turner *et al.* 2006a). However, the level of soil moisture required, and the minimum water potential at which stratification can occur is yet to be investigated. If warm stratification was to play a role in seed germination during the inter-fire period, then a requirement for substantial rainfall leading up to the germination window would be a sound ecological strategy to ensure seedling survival and species persistence. Further investigation is required to determine the critical temperatures, water potentials and minimum durations that are needed to promote dormancy loss via warm stratification for the development of nursery production methods for these species.

A lack of KAR_1 (a germination stimulant found in smoke, Flematti *et al.* 2004) response in most study species does not neatly align with observations of WA Rutaceae emerging from soil following fire (Roche *et al.* 1997; Norman *et al.* 2006), the positive response to KAR_1 observed in *Diplolaena grandiflora* (Commander *et al.* 2009a) or germination in response to fire cues in Eastern Australian *Boronia* spp. (Mackenzie *et al.* 2016a). Our results do however show that more indicative fire cues, particularly aerosol smoke, can have a positive impact on germination (Fig. 2.4). This suggests that smoke-based stimulants other than KAR_1 are likely responsible for increases in

germination previously observed in many/most Rutaceae species (Mackenzie *et al.* 2016a; Norman *et al.* 2006; Roche *et al.* 1997b). Moreover, similar observations have been made in several other smoke responsive species including members of the Haemodoraceae (Downes *et al.* 2013) such as *Anigozanthos manglesii* (Flematti *et al.* 2011), and the Cyperaceae *Lepidosperma scabrum* (Turner 2013). Smoke is a complex mix of more than several thousand chemicals (Burger *et al.* 2018) including cyanide which is regarded as an important signalling molecule in seed germination, that is present in smoke as glyceronitrile, and has been shown to stimulate germination in species where KAR_1 does not (Downes *et al.* 2013; Flematti *et al.* 2011). While KAR_1 may be effective in certain members of the Rutaceae under specific conditions, the results presented here advocate for the use of aerosol smoke or smoke water, rather than KAR_1, in species that have proven intractable under commonly applied propagation techniques.

While germination in the presence of smoke or KAR_1 was low for all species tested here, the final germination observed in seeds treated with GA_3 was significantly higher than that achieved for WA Rutaceae to date (Bell *et al.* 1987; Commander *et al.* 2009a; Dixon *et al.* 1995; Paynter *et al.* 1991; Roche *et al.* 1997a). Gibberellic acid is known to stimulate germination in a diverse range of species (Bell *et al.* 1995; Erickson 2015; Just *et al.* 2019; Turner *et al.* 2009a), and combined with developed, linear embryos and unrestricted imbibition (Table 2.1), confirms the presence of non-deep possibly or intermediate physiological seed dormancy (Baskin and Baskin 2014) in the eight study species of Rutaceae. Gibberellic acid has also been shown to promote germination in Eastern Australian Rutaceae (Martyn 2009) and a few WA Rutaceae (*D. grandiflora;* Commander *et al.*, 2009, *B. ramosa* & *B. fastigiata*; Bell, Plummer and Taylor, 1993) although its wide spread application to other WA species of this difficult-to-propagate

group has not been seen to date. Application of GA_3 to a wider range of WA Rutaceae is likely to improve the ability of *ex situ* restoration seed banks to assess viability through germination, the growing of plants for the recovery of threatened Rutaceae species (Martyn 2009) and the use of common native Rutaceae in restoration programs via seed based propagation. Confirmation of physiological dormancy in a range of WA Rutaceae also helps to identify likely factors for dormany alleviation, as seeds with this form of physiological dormancy have been shown to respond readily to seasonal temperature changes, wet/dry cycling, stratification and dry afterripening (Merritt *et al.* 2007), which is further supported by the results presented here (Fig. 2.2, 2.3, 2.4, 2.6).

The increase in emergence seen for seeds sown into punnets (Fig. 2.4, 2.5, 2.6) is comparable with results obtained on previously studied Rutaceae (Roche *et al.* 1997a). Following the commencement of watering and high levels of sustained soil moisture (Table 2.3, Fig. 2.6), the onset of emergence was observed after 2.8-8.3 weeks (Fig. 2.5). Seeds that germinate within 4 weeks can be considered non-dormant (Baskin and Baskin 2004) and a portion of seeds of *Diplolaena angustifolia* and *Philotheca spicata* were seen to emerge within four weeks following elevated soil moisture. As well, fresh, and after-ripened seeds of *D. angustifolia* germinated within 4 weeks in incubators, suggesting a small portion of fresh seeds are non-dormant upon dispersal, and that these seeds emerge rapidly once smoke and sufficient soil moisture are available during the cooler winter months. *Philotheca spicata* was not seen to germinate to any significant proportion in fresh or after-ripened seeds (Fig. 2.3), and the rapid emergence observed in punnets (Fig. 2.5) was likely due to dormancy loss during the three-month period seeds were exposed to *in situ* soil conditions in punnets. A lack of germination following three months of laboratory-

based afterripening indicates periods of soil hydration, in combination or separately to periods of dry afterripening, drove a small proportion of dormancy loss in *P. spicata* seeds (Fig. 2.4, 2.6). Similar processes over the three months seeds were exposed to rainfall may also explain an increase in emergence after four weeks of elevated soil moisture in *B. cymosa*, *B. ovata*, *C. angustifolia* and *P. spicata* however, the delay in the onset of germination for these seeds may have been due to a requirement for a period of warm stratification that acted separately to, or in combination with, afterripening and wet/dry cycling. These results highlight the need to explore combinations of treatments that have been shown to alleviate physiological dormancy (Chia *et al.* 2016; Hidayati *et al.* 2012; Merritt *et al.* 2007; Turner *et al.* 2006a), rather than simply the main effects of individual treatments. Certainly, longer periods in soil does result in high proportions of viable seed germinating in many taxa that are otherwise intractable using fresh seed (Roche *et al.* 1997a). Thus, a particular focus on the use of long-term seed burial and retrieval studies matched by understanding the physiological changes in seeds will improve understanding of the ways in which Rutaceae seeds are regulated by and respond to *in situ* soil conditions as a pathway to improving germination on demand.

2.6 Conclusions

This study has provided evidence that eight species of Western Australian Rutaceae possess physiological seed dormancy, and that at least some of these species germinate readily following a period of warm stratification. A general lack of seed germination response to KAR_1 indicates the need for alternative germination stimulants present in smoke, and this insight should be beneficial for future propagation efforts incorporating the Rutaceae. Further investigation into the interaction between germination stimulants other than KAR_1 and dormancy alleviation

by stratification should elucidate optimum lab based treatments for germination. Finally, the time course of germination reported here, coupled with observations of near-natural temperature and moisture conditions leading to dormancy loss, may allow for the development of nursery-based protocols for the on-demand germination of WA Rutaceae.

3 Chapter 3 Warm stratification facilitates dormancy loss in southwest Australian Rutaceae

3.1 Abstract

The ability of organisms to respond to, and persist through, stochastic environmental conditions is critical to species survival. 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 timing of seed germination can vary between years, and the germination strategies of different species to favourable conditions has significant consequences for recruitment. The timing of seed germination is often dictated by the response of seed dormancy status to environmental thresholds of temperature and moisture. However, the mechanisms by which seed dormancy is alleviated, and the conditions under which seeds are cued to germinate, remain unresolved for many species which can make these species intractable under nursery conditions. To understand how the germination of species responds to stochastic environments, we investigated the effect of warm stratification (> 15°C) on dormancy loss and germination in five species of intractable southwest Australian Rutaceae. Warm stratification above 25°C for 4–8 weeks was effective in alleviating dormancy, as determined by germination success, of *Diplolaena dampieri* and *Rhadinothamnus anceps*. Germination success in these two species increased markedly with increasing warm stratification duration up to 8 weeks, from 0.22 to 0.57 in *Diplolaena dampieri* and 0.12 to 0.53 in *Rhadinothamnus anceps*. During warm stratification the temperature windows that support germination was seen to progressively widen. *Diplolaena dampieri* and *Rhadinothamnus anceps* had opposite directional shifts in temperature base and ceiling temperatures required for germination, allowing for confirmation for the first time of type 1 and type 2 non-

deep physiological dormancy (PD) respectively in these species. In the context of the southwest Australian environment, type 1 and type 2 non-deep PD appear to dictate risk-taking and risk-avoiding ecological strategies. Classification of type of non-deep PD offers a structured approach to predict seed germination response to environmental conditions, which is particularly important in the context of climate change where it may aid in species management and conservation in the face of warming temperatures. While three study species failed to germinate, warm stratification was effective for two species and should be trailed in a greater number of known intractable taxa. Finally, the interaction between dormancy alleviation and the subsequent shifts in the temperatures required for germination needs to be understood for a greater number of species to implement effective management and restoration programs.

3.2 Introduction

Recruitment from soil seed banks is mediated by the loss of seed dormancy and the conditions that support germination (Gulden *et al.* 2004; Ooi 2010; Salazar *et al.* 2011). Germinating seeds and newly established seedlings are highly vulnerable to environmental changes, and conditions required for germination, emergence and seeding establishment are usually far more restrictive than long-term requirements for persistence of mature plants (Hardegree *et al.* 2018). Seed dormancy is highly prevalent (ca. 90% of species) in Mediterranean-climate ecosystems (Baskin and Baskin 2014), where it ensures germination occurs following the onset of seasonal conditions favourable for emergence and development of seedlings (Baskin and Baskin, 2014).

Bet-hedging is an important ecological strategy that ensures organism survival under stochastic environmental conditions (Simons 2011). Seed populations may exhibit bet-

hedging strategies through the delay of germination imposed by seed dormancy, spreading germination temporally to reduce year-to-year variation in genotype fitness (Cross *et al.* 2015). Worldwide, physiological dormancy (PD) (Baskin and Baskin 2014) is the most common class of seed dormancy (Finch-Savage and Leubner-Metzger 2006). PD is characterised by seeds that possess fully developed embryos, unrestricted imbibition and an inability to germinate within four weeks under suitable temperature and moisture (Baskin and Baskin, 2014). Seeds in the soil seed bank experience climate-driven seasonal variations in temperature and moisture, which are regulators of seed dormancy alleviation (Finch-Savage and Leubner-Metzger, 2006; Thompson and Ooi, 2010) and ensure germination occurs in periods when water is not limiting for seedling establishment (Baskin and Baskin 2014; Merritt *et al.* 2007). Variation in dormancy within seed populations enables bet-hedging strategies that avoid complete seed bank depletion, as some seeds remain dormant even when soil conditions are suitable to support the early stages of germination (Cross *et al.* 2015; Pake and Venable, 1996).

A period of warm to hot dry conditions, which in Mediterranean-climate ecosystems often occurs between cooler germination seasons (Merritt *et al.* 2007), can facilitate dormancy loss through a process termed afterripening (Turner *et al.* 2009b). Dormancy loss can also occur under moist soil conditions (termed stratification) that occur between, in the lead up to, and during, the germination season (Merritt *et al.* 2007). Stratification between temperatures of 0–10°C (cold stratification) typically alleviates dormancy in summer annuals, and time above 15°C (warm stratification) is generally associated with alleviating dormancy in winter annuals (Baskin and Baskin 2014). During alleviation of non-deep PD, there is a shift in the base or ceiling temperatures that will support germination, 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 (Baskin and Baskin, 2014).

In type 1 non-deep PD there is an increase in the ceiling temperature, which has been demonstrated in *Arabidopsis thaliana* (Baskin and Baskin, 1983) and *Capsella bursa-pastoris* (Baskin and Baskin, 1989; Soltani *et al.* 2017). In contrast, in type 2 non-deep PD there is a decrease in the base temperatures, as shown for *Aesculus hippocastanum* (Steadman and Pritchard, 2004), *Ambrosia artemisiifolia* (Baskin and Baskin, 1980), and *Leptochloa panicea* (Baskin *et al.* 1999; Soltani *et al.* 2017). As temperature thresholds for germination widen with dormancy loss, the risk of germinating into unfavourable conditions increases (Baskin and Baskin, 2014). To ensure germination does not occur under marginal conditions the physiological thresholds required for germination following dormancy alleviation can be very exact and unlikely to occur at a time when soil moisture is limiting (Adondakis and Venable, 2004; Simons and Johnston, 2006). If the thresholds for germination are not met, some seeds can re-enter dormancy (i.e., secondary dormancy) (Baker *et al.* 2005a; Collette and Ooi 2020a; Finch-Savage and Footitt, 2017; Finch-Savage and Leubner-Metzger, 2006), bet-hedging on more favourable conditions in the future. Hydrothermal thresholds for germination have received significant attention (Cochrane 2017; Cochrane 2018; Gummerson 1986; Kebreab and Murdoch, 2000; Luna *et al.* 2012; Rajapakshe *et al.* 2020; Turner *et al.* 2018) and it is well established that increasing water stress narrows temperature thresholds while reducing germination rate (Kebreab and Murdoch, 2000; Rajapakshe *et al.* 2020). However, how these thresholds change with dormancy loss, the specific hydrological threshold under which

stratification no longer occurs, and how these germination functional traits will be impacted by climate change remains unknown for many species.

In southwestern Australia, the majority of species germinate in late autumn and early winter following a warmer period of elevated soil moisture upon the onset of rainfall in early autumn (Merritt *et al.* 2007). Therefore, it comes as no surprise that species previously thought of as intractable from this region, such as *Acanthocarpus preissii* (Turner *et al.* 2006a), *Lomandra preissii* (Merritt *et al.* 2007), *Persoonia longifolia* (Chia *et al.* 2016; Norman and Koch 2006), and species of *Hibbertia* (Hidayati *et al.* 2012), have shown significant positive germination results in response to warm stratification. However, it appears that no studies have reported the effect of stratification on the shift in thermal requirements for germination in southwest Australian species, having instead reported the effect on germination at single constant (Cross *et al.* 2013) or alternating (Chia *et al.* 2016; Merritt *et al.* 2007; Turner *et al.* 2006a) incubation temperatures. Seasonal shifts in temperature thresholds for germination gives seeds the ability to avoid conditions marginal to seedling survival (Long *et al.* 2015). For example, lower maximum or optimum temperature thresholds in winter annuals allow seeds to germinate at a time when temperatures are cooler and moisture is more available (Huang *et al.* 2016). The onset of the germination window (i.e., the period over which environmental conditions are conducive to seed germination and seedling establishment) can be variable between seasons and how germination functional traits (hydrothermal thresholds, germination speed, etc) vary in response to local climate has significant effects on recruitment dynamics (Saatkamp *et al.* 2019). While it is yet to be confirmed for species in southwestern Australia, we expect germination functional traits of these winter germinating species to follow similar patterns to those seen in winter annuals, such as those in the Sonoran Desert which have been shown

to vary their response to water availability and temperature within seasons (Huang *et al.* 2016; Ten Brink *et al.* 2020). Investigation of warm stratification requirements, which have proven effective in other known intractable species in southwestern Australia, may yield insights into the dormancy alleviation requirements of other species for which germination cannot currently be achieved.

Efforts to understand the requirements for dormancy alleviation and germination in species of southwest Australian Rutaceae have yet to be successful (Dixon, Roche, and Pate 1995, Roche, Dixon, and Pate 1997, Rokich et al. 2002, Norman et al. 2006, Merritt et al. 2007, Maher, Standish, and Hallett 2008). It has been established that many species within the Rutaceae produce seeds that possess physiological dormancy (Baskin and Baskin 2014; Collette and Ooi 2020a; Collette and Ooi 2017; Commander *et al.* 2009a; Mackenzie *et al.* 2016a; Martyn 2009) although the depth and type of PD is unknown for most Australian Rutaceae species. Many species of Rutaceae are among a large proportion of southwest Australian flora that cannot be reliably germinated under laboratory conditions or for the purpose of seed-based restoration (Merritt *et al.* 2007). In eastern Australia, some species of Rutaceae achieve maximum germination only in the presence of smoke and/or heat (Collette and Ooi 2017; Mackenzie *et al.* 2016a). However, the application of these treatments is yet to be shown to be universally effective in fresh seed of Western Australian (WA) Rutaceae. Field observations of Rutaceae emerging following wildfire and smoke application to habitat soils containing Rutaceous seed in Western Australian ecosystems suggest heat and/or smoke play a significant role in seed dormancy alleviation (Norman *et al.* 2006; Roche *et al.* 1997a), despite the lack of success reported by previous studies employing these treatments (Dixon *et al.* 1995; Roche *et al.* 1997a), indicating that dormancy alleviation may also require pre-treatments of

warm stratification and afterripening in line with what seeds experience *in situ* within the soil seed bank (Merritt *et al.* 2007).

We conducted a series of *ex situ* experiments to understand mechanisms of dormancy alleviation in the Rutaceae. The primary aim was to investigate the germination response of seeds to warm stratification and fire cues. The specific aims were to determine: (1) the class and type of seed dormancy present in study species; (2) investigate the interaction between water stress, warm stratification, and fire cues on seed dormancy loss and germination; and (3) determine the effect of warm stratification temperature and duration on the temperatures over which seed germination occurred. We hypothesise that increasing durations of warm stratification will reduce seed dormancy thereby widening the germination window by decreasing the optimum temperature for germination. It is expected that as dormancy is lost, seeds will become responsive to smoke and/or heat, and that increasing water stress during warm stratification will significantly limit dormancy loss and germination under optimum conditions. Identification of the thresholds required for dormancy loss will provide insights into how seedling recruitment is mediated by environmental conditions of temperature and moisture.

3.3 Methods

3.3.1 Seed source

Southwestern Australia experiences a Mediterranean climate with cool wet winters and hot dry summers (Fig. 3.1), with most native species dispersing seed between November and February (Merritt *et al.* 2007). Mature seeds of *Boronia fastigiata*, *Crowea angustifolia*, *Diplolaena dampieri*, *Philotheca spicata* and *Rhadinothamnus anceps* (Table 3.1) were collected in late spring (November of 2020) from wild

populations at various sites across southwest Western Australia. Seeds were 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.

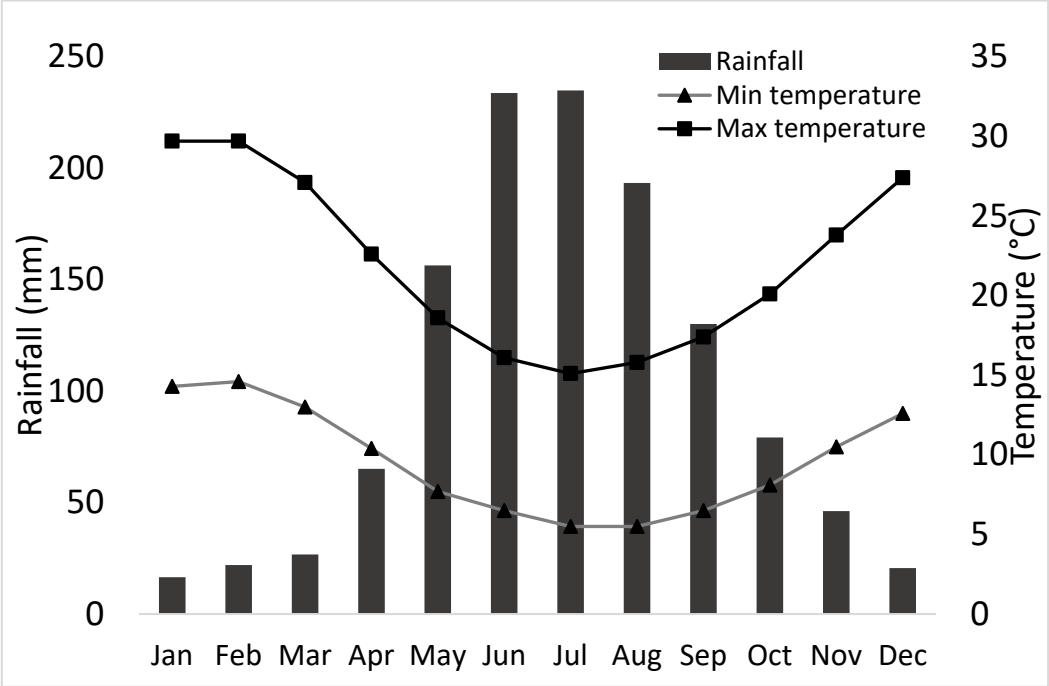

Figure 3.1. Mean rainfall and temperature from 1935–2021 (n = 84) from the Australian Bureau of Meteorology weather station (site: 009538) at Dwellingup, Western Australia.

3.3.2 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, table 1) where damaged or unfilled seeds were removed (experimental seed fill Table 3.1). Seed weight (mg) was determined for four replicates of 25 filled seeds and the mean divided to determine single seed weight.

To assist in classifying seed dormancy class, embryo morphology and water uptake was documented for all species. Three replicate samples of 20 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 deionized (DI) water. Seeds were weighed prior to imbibition and again after 24h 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:

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

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

3.3.3 Response to warm stratification following the application of fire cues

To determine the response of the five study species to fire cues known to promote germination of related species (Mackenzie *et al.* 2016a) seeds were treated with a 10 min application of aerosol smoke, heat or a combination of both smoke and heat. Three replicates of 20 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.* 2016a). Seeds treated with smoke were first 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 being exposed to 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 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 incubating 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 three replicates of 20 seeds for each treatment were moved after 4, 8 or 12 weeks from 30°C 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 four weeks to determine the proportion of primary dormancy (Table 3.1) as per Baskin and Baskin (2004), and again after 4 weeks incubation at the transferal temperature.

3.3.4 Identification of the optimal germination temperature following different stratification regimes

Due to limited seed supply in other species, *Rhadinothamnus 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. 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 southwest 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 25°C, 20°C, 15°C, 10°C and 5°C, those stratified at 25°C were moved to 20°C, 15°C, 10°C and 5°C and those stratified at 20°C were moved to 15°C, 10°C and 5°C. Seeds acting as controls were surface sterilised and plated as previously described before incubation at 30°C, 25°C, 20°C, 15°C, 10°C and 5°C for the entire duration of the experiment. Germination was defined as previously described and scored every two days for 60 d.

3.3.5 Interaction of warm stratification and moisture stress

To examine the interaction of warm stratification and water stress on seed germination, *Rhadinothamnus anceps* seeds were surface sterilized as previously described then placed onto germination papers in 90 mm Petri dishes irrigated with differing concentrations of polyethylene glycol 8,000 (PEG) solution (10 mm per Petri dish) following Michel (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 loss 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 and scored every two days until no new germination had been observed for three weeks (25 days scoring total).

3.3.6 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 four 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.

3.3.6.1 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;

$$germination = \frac{Gmax}{1 + \frac{time^b}{t50}}$$

Where *Gmax* is the upper limit for germination with the lower limit for germination rate assumed to be 0, *t50* is the time required for germination to reach 50% from *Gmax*, and *b* is the slope of the germination function at *t50*. A full model was created for the number of germinated seeds over the number of seeds incubated for all temperature and stratification regimes. The *anova* function was used to assess the explanatory power of stratification duration and germination temperature and water stress as

factors influencing *t50* and *Gmax*, versus a global model without stratification duration, or germination temperature. Due to poor germination proportion following stratification at temperatures below 30°C (<0.20), 20 and 25°C stratification regimes were removed from the analysis.

3.3.6.2 Thermal performance

We estimated model fits for final germination and germination rate (1/t50) for thermal response data using Beta, Yan and Hunt and Broken-Stick thermal performance functions described by Asbury and Angilletta Jr (2010), Yan and Hunt (1999) and Yeager and Ultsch (1989) respectively. The *AICcmodavg* package (Mazerolle, 2013) was used to assess the explanatory power of each model and final model selection chosen accordingly (Supplementary Table 1). The final model for both germination proportion and thermal performance was that described by Yan and Hunt (1999) for the temperature response of maximum rate of growth in plants;

$$\text{rmax, gmax} = Rmax\left(\frac{Tmax-T}{Tmax-Topt}\right)\left(\frac{T}{Topt}\right)^{\frac{Topt}{Tmax-Topt}}$$

where rmax is the maximum germination rate (rmax) or maximum germination proportion (gmax) at any temperature (T), Topt is the optimum temperature for germination at the peak of the performance function, Tmax is the limit of thermal tolerance and Rmax is the asymptotic maximum germination rate at Topt.

3.4 Results

3.4.1 Seed characteristics and viability

Study species had an individual seed weight between 0.8 and 2.9 mg with seed fill >70% upon receival from seed collectors (Table 3.1). Seeds of all five species possessed fully developed, linear embryos (Martin 1946) and significantly increased

in mass after 24 h of imbibition indicating water movement into seeds. High primary dormancy (>86%), the possession of developed linear embryos, coupled with the increase in seed mass when exposed to moisture suggests seeds of all five study species possess a level of physiological dormancy (Baskin and Baskin 2004).

Table 3.1. Seed traits of the five Rutaceae species examined in this study. Seed viability was determined by x-ray analysis when the seeds were obtained (initial) and again following removal of unfilled seeds (experimental).

Species	Collection date	Collection location in Western Australia	Individual seed weight (mg)	Initial seed fill (%)	Experiment seed fill(%)	Water uptake (%)	Primary dormancy (%)
Boronia fastigiata	1/12/2020	Boddington	55(±3)	83(±2)	98(±1)	26(±1)	100
Crowea angustifolia	1/12/2020	Northcliffe	122(±1)	91(±1)	98(±1)	21(±2)	100
Diplolaena dampieri	24/11/2020	Myalup	243(±1)	72(±2)	97(±1)	19(±3)	88
Philotheca spicata	24/11/2020	Boddington	15(±1)	93(±1)	98(±1)	15(±4)	100
Rhadinothamnus anceps	1/12/2020	Torbay	39(±1)	94(±1)	98(±1)	21(±2)	86

3.4.1.1 Response to warm stratification following the application of thermal (fire) cues

Seed germination was recorded for only two species, *D. dampieri* and *R. anceps* (Fig. 3.2, 3.3). Control seeds 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 eight weeks (*D. dampieri*; log-odds ratio = 12.89, probability ratio = 0.93, Z = 11.07, p <0.001 & *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 (Fig. 2, 3). Germination temperature was also significant for both species however, the effect of decreasing germination temperature was positive for *D. dampieri* (log-odds ratio=1.15, probability ratio= 0.54, Z= 9.44, p <0.001) and negative for *R. anceps* (log-odds

ratio=0.89, probability ratio= 0.47, Z= -8.99, p <0.001). Fire cues had no significant effect on seed germination of study species (Fig. 3). 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) and this was reflected in the negative germination response of combination heat and smoke treatments, which was only significant for *D. dampieri* (log-odds ratio = 0.30, probability ratio = 0.23, Z = -6.85, p <0.001).

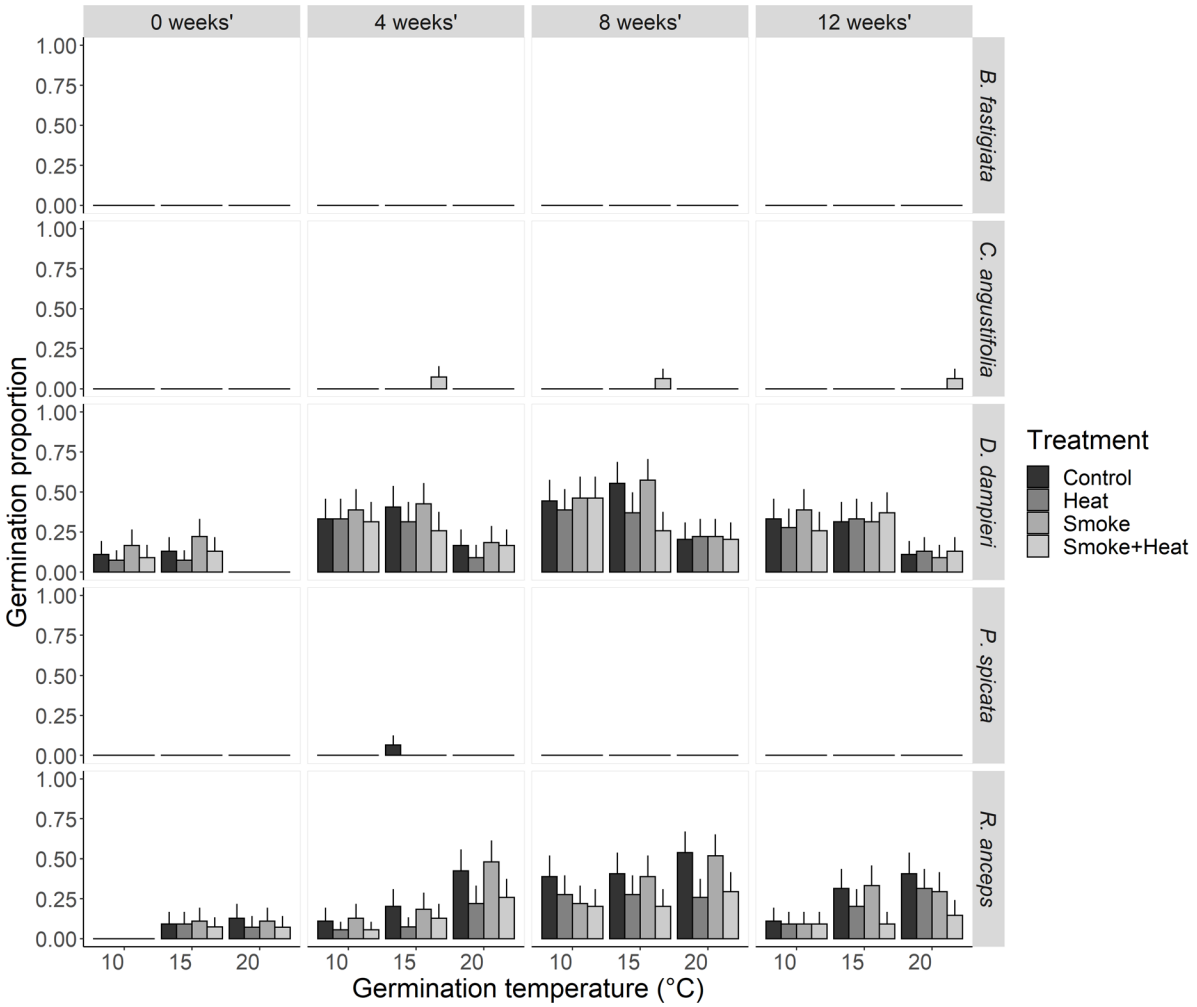

Figure 3.2 Germination proportion (± 95% confidence interval) for five species of Rutaceae from Southwest Western Australia subjected to combinations of fire cues and warm stratification (4, 8 & 12 weeks) at 30°C prior to incubation at 10, 15 or 20°C in alternating 12/12hour light.

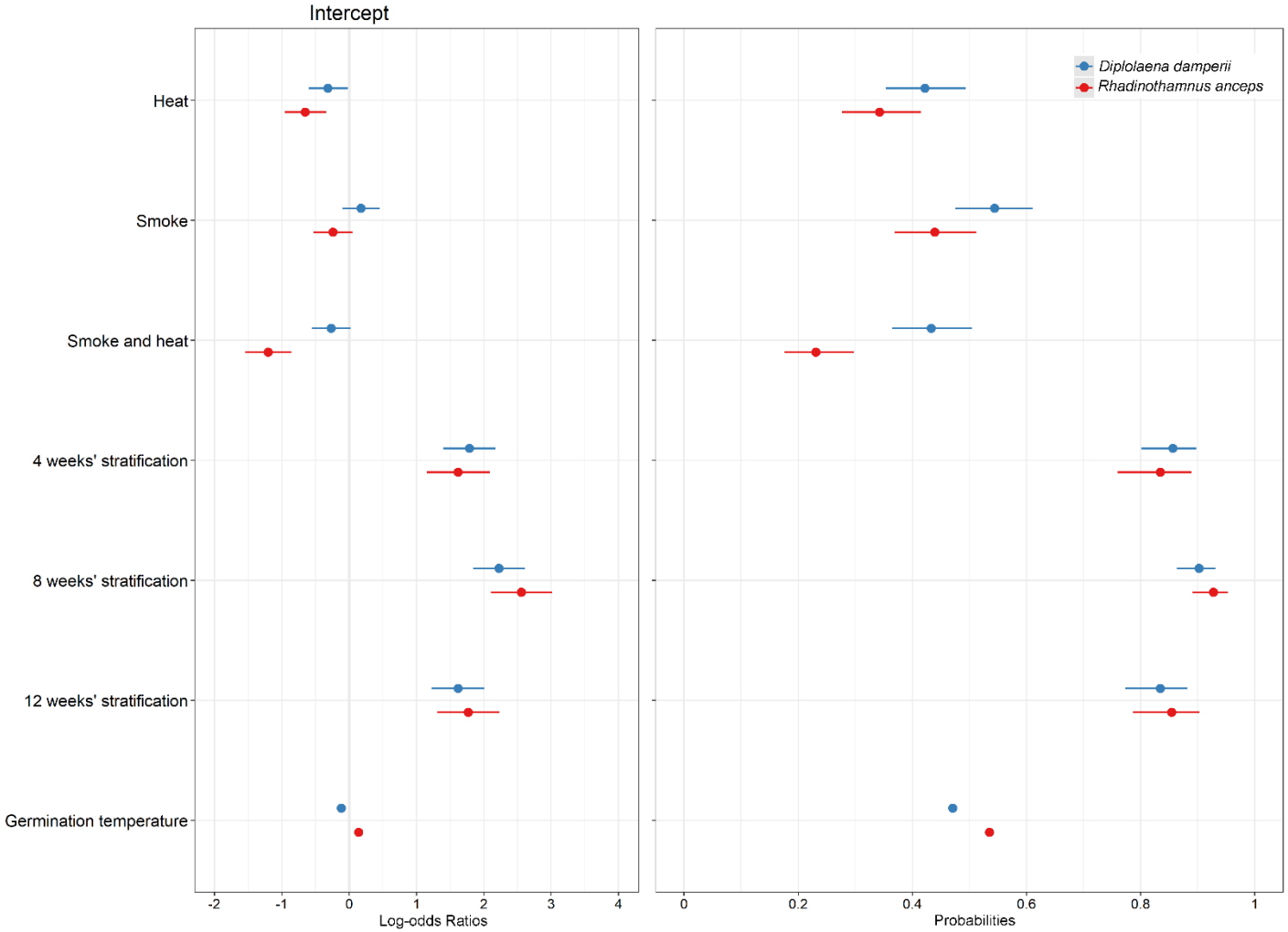

Figure 3.3 Summary Forest plot from GLM showing odds ratios and converted probability ratio estimates (+95% confidence intervals) for germination response of *Diplolaena dampieri* (blue) and *Rhadinothamnus anceps* (red) to smoke and heat cues or increasing durations of warm stratification. The intercept for the regression was determined as untreated seeds incubated under identical conditions.

3.4.1.2 Identification of the optimal germination temperature following different stratification regimes in Rhadinothamnus anceps

Germination proportion in *Rhadinothamnus anceps* was higher over a wider range of temperatures following warm stratification (Fig. 3.4), compared to unstratified (control) seeds. Control groups that received no stratification did not germinate at either 5°C or 10°C and had consistently higher final germination percentages when incubated at 25°C (0 weeks stratification; Fig 3.4, $p < 0.05$). Following stratification between 1 and 8 weeks the magnitude and speed of germination was seen to increase while the range of temperature that seeds responded to significantly widened, with a decrease in the base temperature able to support germination (Fig. 3.4). Maximum germination proportion (0.48 ± 0.02) was achieved at 25°C after 2 weeks stratification at 30°C, though this was very similar when compared to the germination proportion observed at 20°C (0.46 ± 0.01) ($p = 0.43$). Stratification for greater than two weeks increased germination at temperatures <25°C but decreased germination at 25°C, while stratification for greater than 8 weeks reduced the overall germination proportion at all temperatures. Stratification at temperatures below 30°C produced far less germination than stratification at 30°C. Models applied to seeds stratified at 20–25°C were unable to converge and have been excluded from analysis (supplementary Fig. 3.3).

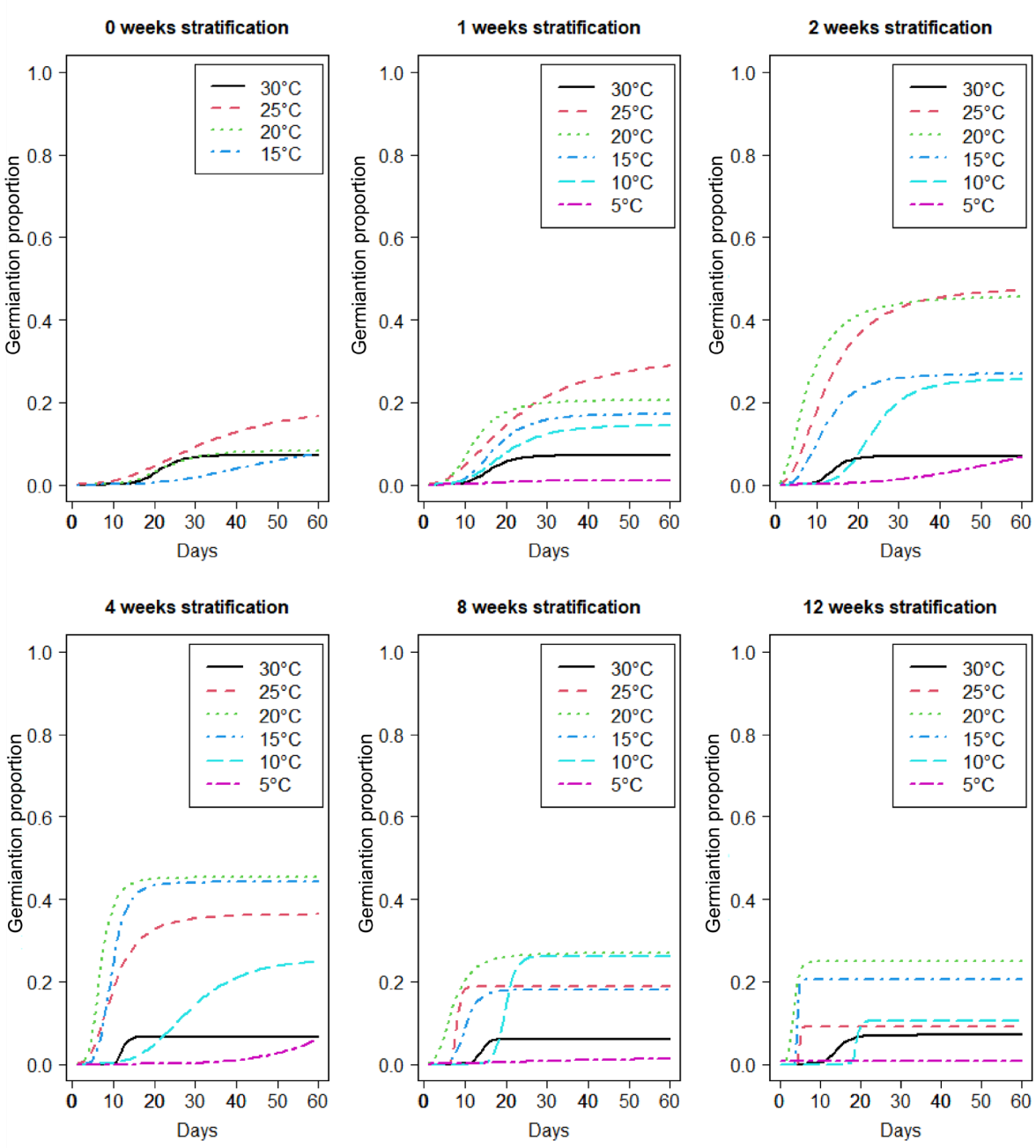

Figure 3.4 Mean cumulative germination proportion for seeds of *Rhadinothamnus anceps* following increasing durations (from 1 to 12 weeks) of warm stratification at 30°C. Following warm stratification seeds were then incubated at one of six different temperatures (5–30°C) in alternating 12/12hr light/dark for a further 60 days.

3.4.1.3 Thermal performance

Assessment of AIC found that Yan and Hunt were the best fit for germination proportion data, while beta was selected as the best fit for thermal performance (1/t50) (Supplementary Table 3.1). However, a visual inspection of the model (Supplementary Fig. 3.2) showed poor model fit and the second most powerful model (Yan and Hunt) was thus selected (Supplementary Table 3.1). The Yan and Hunt log logistic curve was able to be fitted across all temperatures tested following stratification at 30°C (Fig. 3.5). When fitted to maximum germination proportion the optimum temperature for germination (Topt) was 25.5 ± 1.7°C in control groups and decreased significantly ($p<0.05$ in all cases) with increasing duration of stratification (Table 3.2) between 2 and 8 weeks, where the minimum optimum temperature for germination was estimated to be 18.6 ± 1.4°C (Table 2). When compared to control seeds (0 weeks stratification) the maximum germination proportion (rmax) 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 stratification, with maximum germination (0.48 ± 0.03) achieved following 2 and 4 weeks (Table 3.2). For all stratification durations the Tmax was within the range 30.8–31.4°C (Table 3.2).

Table 3.2. Parameter estimates (± s.e.) from Yan and Hunt model fits for the final germination proportion and germination rate (1/t50) of seeds of *Rhadinothamnus anceps* warm stratified at 30°C for 1-12 weeks. gmax = maximum germination proportion, rmax = maximum germination rate, Topt = optimum temperature, Tmax ceiling temperature.

Trait	Parameter	Stratification duration					
		0	1	2	4	8	12
Final Germination	gmax	0.15±0.05	0.25±0.04	0.48±0.03	0.48±0.03	0.25±0.03	0.20±0.03
	Topt	25.46±1.71	22.01±1.35	21.24±0.76	19.72±0.7	18.65±1.44	19.56±1.59
	Tmax	31.38±1.7	31.45±1.21	30.8±0.53	30.64±0.55	31.44±1.44	31.24±1.56
Germination Rate	rmax	0.05±0.02	0.07±0.02	0.1±0.02	0.12±0.02	0.12±0.02	0.22±0.02
	Topt	26.55±4.62	20.87± 3.68	21.25±2.44	21.76±2	21.43±2.04	21.66±1
	Tmax	35.13±2.66	35.92±4.92	34.52±1.69	34.41±1.36	34.41±1.41	34.08±0.66

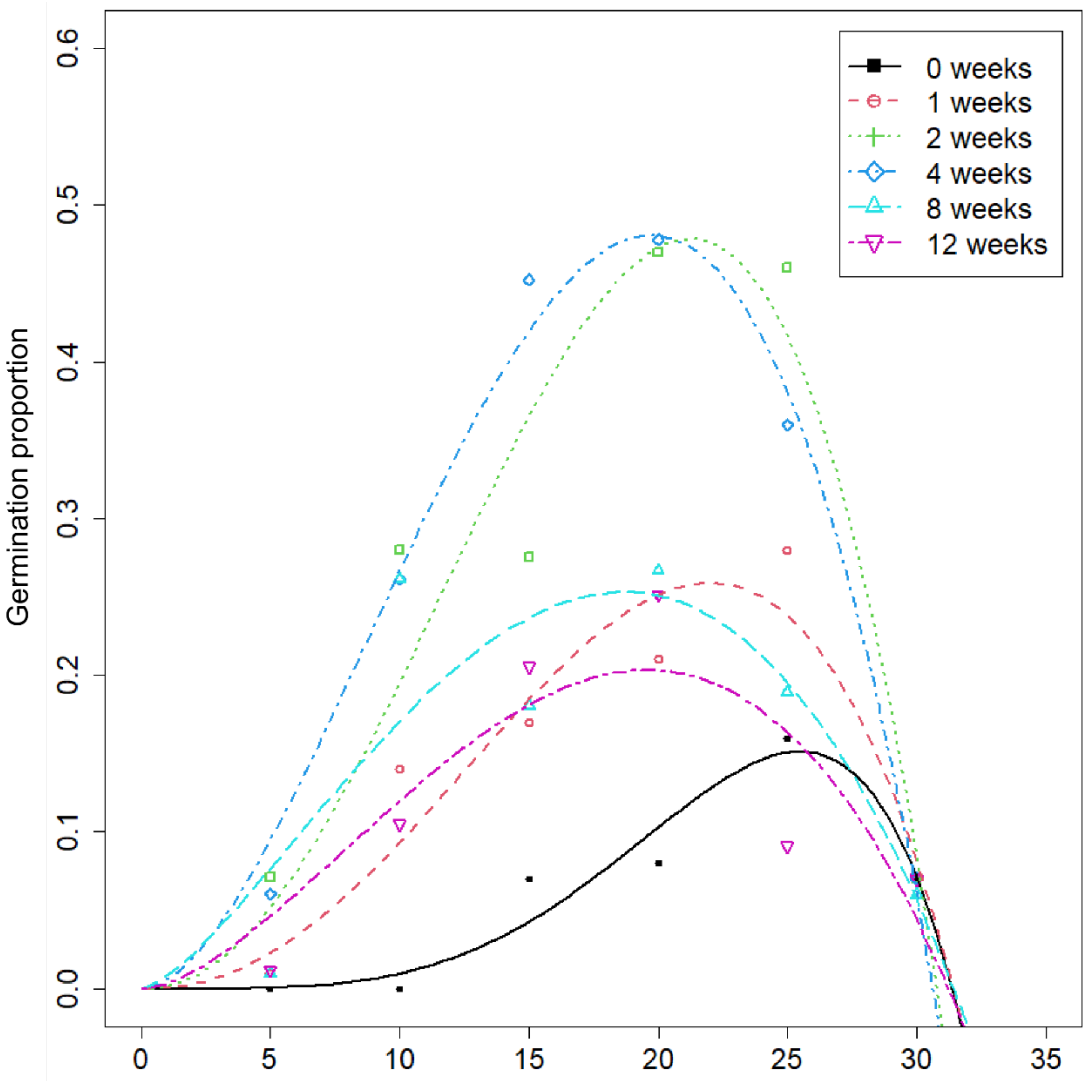

Figure 3.5 Maximum germination proportion for *Rhadinothamnus anceps*. Symbols represent the average maximum gemination proportion estimate for seeds at 5–30°C incubation temperatures following increasing duration of stratification. Lines represent the permutations of the Yand and Hunt thermal performance curve fittered to the maximum germiantion proportion estiamte (rmax) for each stratification duration.

The log logistic model fitted to the inverse of t50 (Fig. 3.6) showed an increase in the speed of germination (rmax) with increasing stratification duration (Table 3.2) between 1 and 12 weeks which was significant after 2 weeks ($p < 0.05$ in all cases). The optimum temperature (Topt, range; 20.9–26.6°C) and maximum temperature for germination (Tmax, range; 34.1–35.9°C) was not significantly affected by increasing duration of stratification.

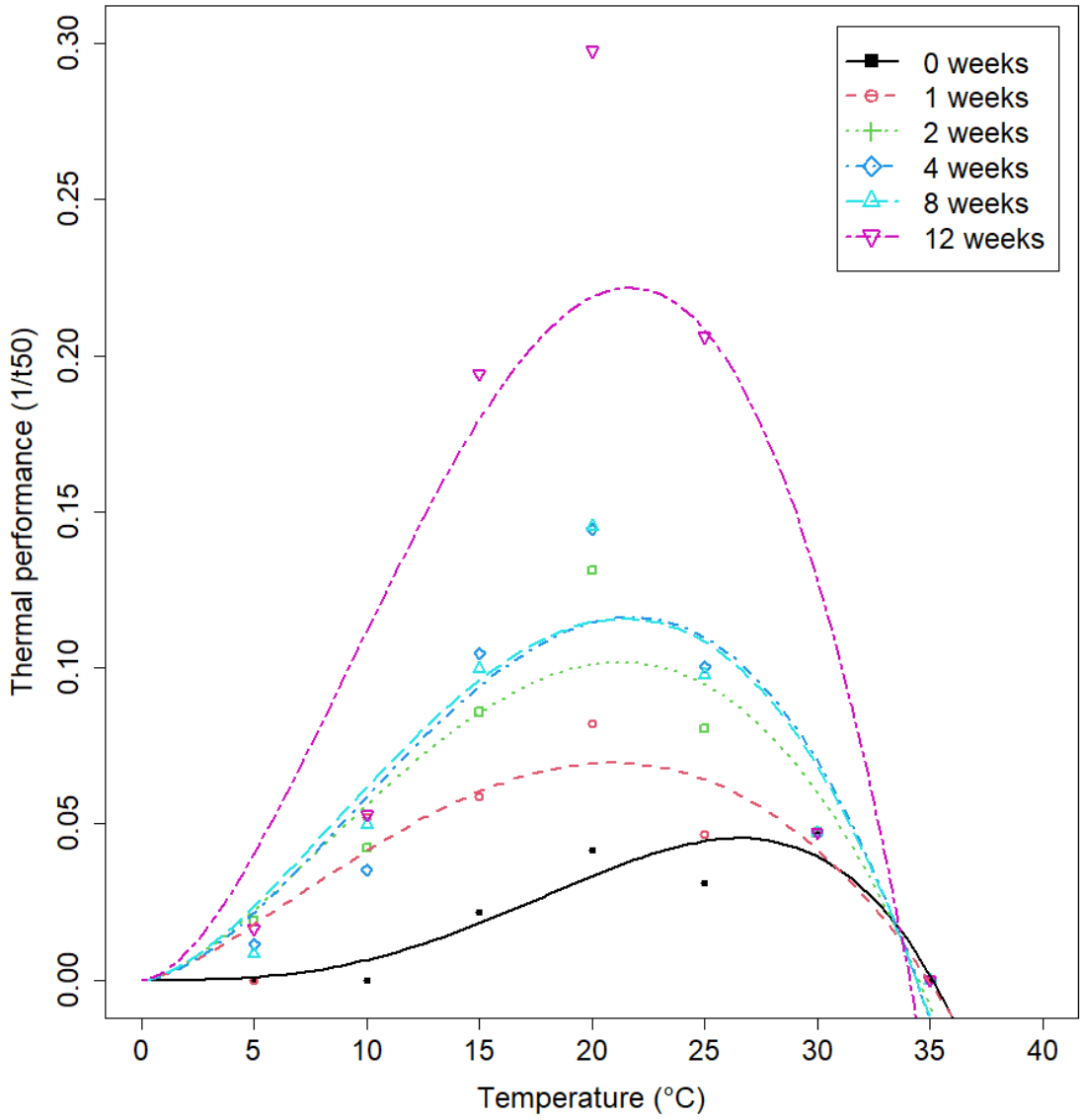

Figure 3.6 Thermal performance for *Rhadinothamnus anceps*. Symbols represent the average 1/t50 estimate for seeds at 5-30°C incubation temperatures following increasing duration of stratification. Lines represent the permutations of the Yan and Hunt thermal performance curve fittered to the maximum germiantion proportion estiamte (rmax) for each stratification duration.

3.4.1.4 Interaction of warm stratification and water stress

Increasing water stress during warm stratification had no effect on maximum germination proportion or t50 at 20°C ($p = 0.81$, $F = 0.63$). The maximum germination proportion (± s.e.) achieved was between 0.39 ± 0.03 and 0.44 ± 0.03 and the time to achieve 50% germination (t50) between 4.3 ± 0.5 and 4.9 ± 0.4 days (Fig. 3.7).

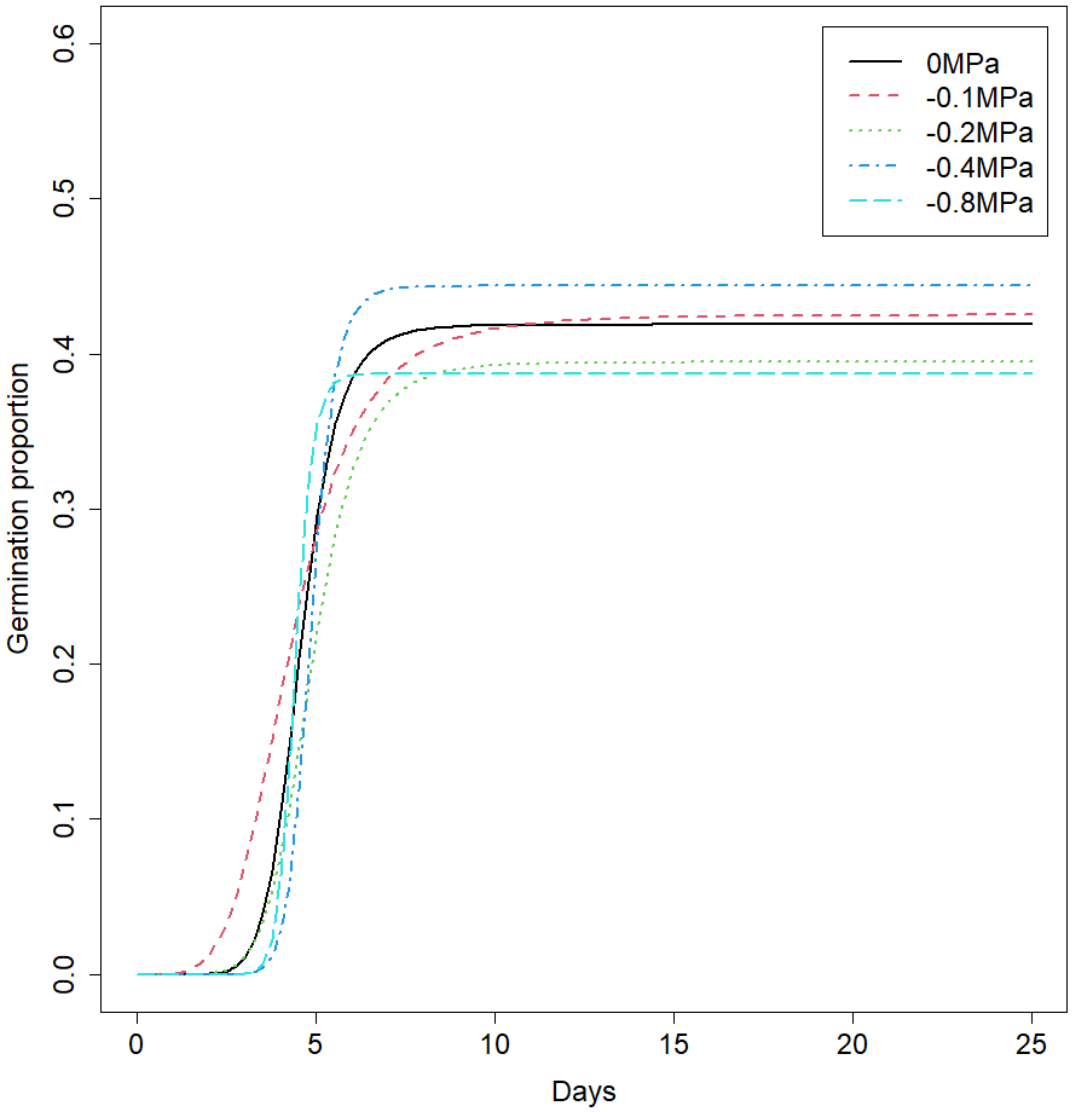

Figure 3.7 Mean cumulative germination proportion for seeds of *Rhadinothamnus anceps* incubated in alternating 12/12hr light/dark at 20°C following the application of 4 weeks stratification at 30°C in combination with a range (0 to 0.8 MPa) of water stresses.

3.5 Discussion

Two out of five species of Rutaceae were observed to germinate to high proportions after several weeks of warm stratification followed by incubation at cooler temperatures. These results add to reports of warm stratification alleviating dormancy in previously intractable Mediterranean-type climate taxa (Chia *et al.* 2016; Hidayati *et al.* 2012; Merritt *et al.* 2007; Turner *et al.* 2006a). The thermal thresholds of both

Diplolaena dampieri and *Rhadinothamnus 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 (to -0.8 MPa) had no interactive effect on dormancy alleviation by warm stratification or final germination. Prior to this study it was unknown how seeds of *D. dampieri* or *R. anceps* alter their requirements for germination during dormancy alleviation and the results presented here provide potential evidence for bet-hedging traits expressed in the delay of germination of southwest Australian species.

Freshly matured seeds of all five study species were dormant according to Baskin and Baskin (2004), with 0-0.14 germinating after 4 weeks incubation in alternating light at 10°C, 15°C or 20°C. Developed linear embryos and the unrestricted uptake of water suggests seeds of all five species possess PD, and the positive response to warm stratification for *Diplolaena dampieri* and *Rhadinothamnus anceps* indicates that seeds of these species possess non-deep PD (Baskin and Baskin 2014). Unexpectedly, the base and ceiling temperatures required for germination differed in these two species following dormancy alleviation (Fig. 3.2, 3.5). Following warm stratification *Diplolaena dampieri* was able to germinate at warmer conditions (Fig. 3.2, 3.3) which is typical of seeds with type 1 non-deep PD, whereas, *R. anceps* exhibited a decrease in the base temperature for germination (Fig. 2, 3, 4) which is often seen in seeds with type 2 non-deep PD (Soltani *et al.* 2017). Seeds released into the soil-seed bank in temperate areas of Australia experience a period of warm stratification followed by a decrease in soil temperature prior to increases in soil moisture characteristic of the germination window during the cooler winter months (Merritt *et al.* 2007). An increase in the ceiling temperature threshold for germination during this period of warm stratification, as seen in *D. dampieri*, suggests seeds are seeking to

germinate while temperatures remain warm during mid to late autumn, rather than delay germination until the onset of cold winter conditions. Oppositely, a decrease in the base 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.

The contrasting types of non-deep PD that occur in *D. dampieri* and *R. anceps* appear to be alternative recruitment strategies responding to variation in the onset of the growing season. Both Turner *et al.* (2006a) and Merritt *et al.* (2007) have shown southwest Australian soils possess intermittent moisture from April to May, and consistently high moisture from June to September. By seeking relatively warmer temperatures characteristic of the early germination window in April to May *D. dampieri* may profit from a longer growing period, possibly yielding establishment and reproductive advantages (Donohue *et al.* 2010; Narita 1998; Ross and Harper 1972; Stratton 1992; Ten Brink *et al.* 2020) and a competitive benefit over later emerging individuals (Ten Brink *et al.* 2020; Verdú and Traveset 2005). However, the early stages of the germination window have a greater drought mortality risk (Donohue 2014; Mercer *et al.* 2011; Ten Brink *et al.* 2020; Thomson *et al.* 2017) as the soil moisture that drove dormancy release may not be consistently present post-germination (Harrison *et al.* 2018). By seeking cooler temperatures more characteristic of the germination window *R. anceps* appears to be risk-avoiding early season drought mortality in favour of more predictable and consistent rainfall and therefore soil moisture later in the season. In the context of temperate southwest Australia, type 1 non-deep PD appears to be a ‘high-risk high-reward` strategy while type 2 non-deep PD is a `risk-avoidance` strategy. How species possessing different types of non-deep

PD respond to stochastic environmental conditions is an area of seed ecology that requires further research, particularly under current predictions for climate change.

While *D. dampieri* and *R. anceps* had a significant positive response to warm stratification, there was no response to the application of either smoke or heat. This finding does not neatly align with previous records for Australian Rutaceae as significant germination responses have been noted following the application of heat, smoke or KAR_1 (Commander *et al.* 2009a; Mackenzie *et al.* 2016a), or the passage of fire (Norman *et al.* 2006; Roche *et al.* 1997a). It does however demonstrate the inconsistency of smoke response within the Rutaceae and suggests that dormancy alleviation may reduce the requirement for smoke stimulated germination. While this has yet to be demonstrated with confidence there are examples to be found. *Lomandra preissii* stratified at 33/18°C for 8 weeks did not require smoke for germination as was the case for control seeds (Merritt *et al.* 2007), indicating the removal of smoke as a germination requirement for a portion of seeds. Similarly, Tieu *et al.* (2001b) found that smoke response in known smoke-responsive species was not absolute but was a dynamic response dependant on seed age and afterripening. The role of smoke as a germination stimulant, rather than a dormancy alleviator, has been made clear over recent years (Merritt *et al.* 2007) however the interaction of germination stimulation and dormancy alleviation is an area that requires more investigation in intractable species. In contrast to the present study's findings, Eastern Australian Rutaceae such as *Asterolasia buxifolia* (Collette and Ooi 2020a; Collette and Ooi 2017) and members of *Boronia* (Mackenzie *et al.* 2016a; Mackenzie *et al.* 2016b; Martyn 2009) have been found to respond much more readily to germination cues of smoke and heat. While certain members display limited germination regardless of treatment (Mackenzie *et al.* 2016a; Martyn 2009) and seasonal dormancy cycling (Collette and Ooi 2020a), others

appear to germinate over a range of seasonal temperatures (Mackenzie *et al.* 2016a). It is likely that limited response to germination stimulants observed in Western Australian Rutaceae is driven by Mediterranean rainfall patterns which may have facilitated a requirement for afterripening or wet/dry cycling prior to germination stimulation. Comparatively, greater rainfall throughout the year in much of Eastern Australia (Collette and Ooi 2020b) and higher amounts of summer rainfall may have reduced the requirement for such pre-treatments in particular members of the Rutaceae, allowing dormancy to be overcome by periods of stratification at seasonal temperatures (Mackenzie *et al.* 2016a).

3.6 Conclusions

Two members of the Rutaceae possess alternate types of non-deep physiological dormancy and have dormancy alleviated by warm stratification. Observed shifts in thermal thresholds that support germination in these species have increased our understanding of recruitment dynamics in southwestern Australia and allowed for the identification of different recruitment strategies that exist between *D. dampieri* and *R. anceps*. Implications of these findings are three-fold; firstly, warm stratification should be applied to a greater number of taxa in southwestern Australia as it is likely to increase the proportion of species able to be germinated on-demand. Secondly, identification of the type of non-deep physiological dormancy present in Australian taxa offers a potential method for predicting seedling recruitment timing and response to environmental change. Finally, understanding the thermal thresholds required for dormancy alleviation by warm stratification should be pursued as a matter of urgency to understand and potentially to manage the effects of climate change in southwest Australian vegetation.

4 Chapter 4 Seed ageing under laboratory and burial conditions facilitates dormancy loss in four southwest Australian Rutaceae

4.1 Abstract

Timing of germination and successful establishment of seedlings relies upon environmental conditions experienced by seeds in the soil seedbank. If the conditions present in the soil are unsuitable for dormancy alleviation and germination, then there is little chance for seedling establishment. The ecological requirements for seed dormancy alleviation and stimulation of germination remain unknown for many species in highly diverse flora of the biodiversity hotspot of southwestern Australia, which limits ability to include them in restoration seeding mixes. This study investigated the germination response of seeds to smoke and heat treatments in combination with soil burial and afterripening in four species of southwest Australian Rutaceae. Data loggers were deployed in remnant Banksia woodland for 6 months over summer, autumn, and winter to measure soil temperature (0-10cm, 1 cm increments) and moisture (1 cm, 5cm and 10cm) *in situ*, alongside experimental seed burial trials of *Boronia fastigiata*, *Crowea angustifolia*, *Philotheca spicata* and *Rhadinothamnus anceps*. Seed moisture status was recorded weekly for retrieved seeds that had been buried at 1 cm in the soil in summer until the onset of the germination season in May when seeds were fully hydrated. Buried seeds of *Boronia fastigiata*, *Crowea angustifolia*, *Philotheca spicata* and *Rhadinothamnus anceps* became non-dormant and germinated within incubators from seed retrieved in April and May (2-3 months after burial) and germinated to significant proportions *in situ* in July. Significant effects of burial location, exposure to summer rainfall, and pre-treatment with smoke and/or heat were detected among

study species. Exclusion of moisture from buried seeds demonstrated that afterripening and/or wet-dry cycling are the major drivers of dormancy alleviation for study species. While this is a step towards developing germination protocols for species currently unavailable to restoration, significantly more research is required to ensure seeds deployed in restoration have the greatest potential for consistent and reliable germination and subsequent establishment.

4.2 Introduction

Ecological restoration relies on the ability to re-establish taxonomically diverse plant communities from seed, tissue culture or cuttings (Turner 2013; Turner *et al.* 2021b). Establishment of plants through direct seeding, or nursery production of plants from seed, is regarded as best practice for returning diverse species (Merritt and Dixon 2011). In Australia, 90% of seed used in ecological restoration activities is sourced from wild plants (Broadhurst *et al.* 2015a), and this continues to place immense pressure on wild populations (Nevill *et al.* 2018) which currently supply almost all the many thousands of tons of seed required for current and future restoration projects in Australia. As restoration demands for seed needs continue to increase in response to ongoing land degradation, land clearing and climate change, there is a fear that wild populations will be unable to maintain supply, and current and future restoration programs will be unable to meet their targets for species return.

Scarcity of seed is rapidly becoming a significant issue (Hancock *et al.* 2020), especially as concerns around climate change and seed provenance raise discussion about the suitability of seed collection locations (Broadhurst *et al.* 2015b). As demand for seed in ecological restoration continues to increase, and the need to source seed from greater distances grows due to necessity of collection or climate sourced provenancing, it is likely that seeds will be deployed at significant distances from the

collection location (Breed *et al.* 2013). Seed provenance and the maternal environment is known to affect the depth of seed dormancy established during seed development (Fredrick *et al.* 2017; Gray *et al.* 2019a). How seeds with high levels of adaptation to the maternal environment will respond to new environments is not well understood, but it is clear that hydrothermal thresholds play a significant role in the processes of dormancy alleviation and germination (Rajapakshe *et al.* 2020). Seed wastage resulting from poor understanding of the conditions required for seed dormancy alleviation and germination compounds the scarcity of native seeds for ecological restoration projects (Merritt and Dixon 2011).

In Australia, ~80% of species produce seeds with some form of dormancy (Collette and Ooi 2020b) with methods to alleviate dormancy poorly understood for key families such as the Cyperaceae, Rutaceae, Restionaceae and Ericaceae (Merritt *et al.* 2007). While the conditions required to alleviate seed dormancy are known for many species, facilitating relatively easy establishment of these species from seed, in biodiverse regions such as Western Australia there are a large number of species—hundreds, and perhaps even thousands— for which poor understanding of seed dormancy alleviation and germination requirements continues to impede reliable germination for activities such as ecological restoration (Gibson-Roy *et al.* 2021; Maher *et al.* 2008; Merritt and Turner 2017; Merritt *et al.* 2007; Roche *et al.* 1997a; Rokich and Dixon 2007). This has resulted in an underrepresentation of key groups of species in restoration projects (Merritt *et al.* 2007). To meet the current demand for seed to support ecological restoration needs, both in Australia and globally, it is critical that we maximise the number of species able to be deployed.

Knowledge of seed traits such as dormancy cycling and tolerance of seed to elevated soil temperatures, and environmental traits such as temperature and moisture

throughout the soil profile, can aid restoration efforts and management strategies for threatened species under a warming and drying climate (Saatkamp *et al.* 2019). Parallel to the inability to germinate certain species in Western Australia is a lack of information regarding seed traits and the environmental conditions that influence them. One such group is the Rutaceae, which comprises well known understorey taxa such as *Boronia*, *Crowea* and *Philotheca*, commonly found throughout the southwest of Western Australia as dominant elements of many vegetation communities (West Australian Herbarium 1998-2022). Currently, no methodology exists to reliably alleviate dormancy in the seeds of Rutaceae for use in restoration programs (Merritt *et al.* 2007), and the return of these species in ecological restoration has largely relied on seedling from respread topsoil (Koch *et al.* 1996; Rokich *et al.* 2000). Few published studies have investigated seed ecology and the specific processes regulating seed dormancy alleviation and the stimulation of germination in Rutaceae species; accounts of Rutaceae emerging naturally from the soil seed bank in Western Australia following fire or anthropogenic disturbance (fire breaks, roadside clearing, etc) suggests that these phenomena play a role in recruitment of some species (Norman *et al.* 2006; Roche *et al.* 1997b). *In situ* aerosol smoke application (Roche *et al.* 1997a; Rokich *et al.* 2002) and the *ex situ* treatment of seeds with smoke water and the smoke-derived chemical karrikinolide (KAR_1) have been tested with mixed effects on Western Australian Rutaceae (Commander *et al.* 2009a), but studies from Eastern Australia report positive effects of seasonal temperature, heat, and smoke on seed germination of species from *Boronia* and *Asterolasia* (Collette and Ooi 2017; Mackenzie *et al.* 2016a). While no consistent treatment effect has been noted for WA Rutaceae, smoke stimulates germination in at least some species of *Boronia*, *Diplolaena* and *Geleznowia* (Roche *et al.* 1997a). However, there remains few insights into the specific

environmental conditions required for dormancy alleviation and germination. Therefore, there is a need to better develop dormancy alleviation and germination protocols able to produce plants *ex situ,* and to understand whether species will germinate when included in broadcast seed mixes. The clearest path to developing such protocols is to understand the conditions experienced by seeds in soil from dispersal until the onset of germination and how these conditions influence and regulate seed dormancy status.

To improve understanding of the seed ecology of southwest Australian Rutaceae, this study investigated natural temperature and moisture regimes experienced by seeds of four species from four genera in soil to inform experimental studies of dormancy alleviation and germination stimulation in response to temperature and moisture cues. The specific aims were to (1) identify when seeds in the field lose dormancy through regular germination testing of retrieved seeds; (2) investigate the germination response of seeds in which dormancy has been alleviated to conditions of temperature and moisture, in combination with fire cues of heat and smoke; (3) evaluate natural temperature and moisture regimes experienced by seeds in soil over multiple seasons; and (4) determine experimental approaches for the alleviation of seed dormancy and stimulation of germination. By burying seeds under field conditions in remnant bushland and excluding rainfall from a portion we tested the hypothesis that changes in seed moisture status drive dormancy alleviation. Lab-based storage experiments run in parallel with burial treatments tested the hypothesis that *in situ* changes in temperature during dry periods, rather than constant temperatures often used in lab-based afterripening treatments, are required for dormancy alleviation of study species. These insights, gleaned from experiments in a single location, on seeds of species

from different collection locations, will also help to understand how variation in collection location and deployment site may affect future restoration success.

4.3 Methods

4.3.1 Species selection and study site

Mature seeds of *Boronia fastigiata*, *Philotheca spicata*, *Crowea angustifolia* and *Rhadinothamnus anceps* (Table 4.1) were collected in November 2020 by a commercial seed supplier (Seed Shed, Boddington, Western Australia) from wild populations in southwestern Australia. Collections were processed to pure filled seeds (>97% filled seeds) using a vacuum aspirator (SELECTA BV Gravity Seed Separator, the Netherlands), with seed fill confirmed using X-ray imaging (MultiFocus X-ray cabinet, Faxitron, Tucson, USA, n = 100) (Table 4.2). Seeds were stored at 15°C and 15% relative humidity (RH) at the Western Australia Seed Centre, Kings Park and Botanic Garden prior to experimental use. All field experiments were conducted in remnant Banksia woodland (hereafter referred to as the study site), at Curtin University, Western Australia.

Table 4.1 Location of study site and collection locality of study species in Western Australia.

Species	Locality	Lat	Long
Study site	Bentley, Perth	-32.0111	115.8897
Boronia fastigiata	Boddington	-32.7895	116.4652
Crowea angustifolia	Northcliffe	-34.6231	116.1375
Philotheca spicata	Boddington	-32.7895	116.4652
Rhadinothamnus anceps	Torbay	-35.0198	117.6486

To evaluate the temperature and moisture conditions naturally experienced by seeds at the study site, data loggers with temperature and moisture sensors were deployed at three locations from January 2021 until July 2021. To measure soil temperature, 33

TC Temperature Loggers (DS1921G, Thermocron, NSW, Australia.) were buried at three random locations nearby seed burial plots within the study site on 27/01/21. Each location was assigned 11 temperature loggers which were buried at 10mm increments from the soil surface to a depth of 100mm, ensuring that no logger was directly above or below another. Loggers were set to record temperatures once per hour. To measure soil moisture a single HOBO USB Micro Station Data Logger (IC-H21-USB) equipped with three EC-5 Soil Moisture Smart Sensors (S-SMC-M005) was deployed centrally within the study site on the 26/02/21. Soil moisture sensors were buried at a depth of 10mm, 50mm and 100mm and were set to log soil moisture once per hour. Daily weather data recorded at the Perth weather station (station number: 9225; Lat: 31.92°S; Long: 115.87°E) for the duration of this study were obtained from the Australian Bureau of Meteorology (Bureau of Meteorology 2021). Soil moisture data were first normalised (expressed as a percentage of soil field capacity) using the minimum and maximum recorded values of *Banksia* woodland soil when dry and at field capacity (m^3/m^3) as the lower and upper limits (Rokich 2000).

4.3.2 Germination response to *in situ* and *ex situ* conditions following the application of fire cues

To determine the germination response of seeds to actual and replicated field conditions, the interaction of seed burial, afterripening, and fire cues on seed germination success was examined (Fig. 4.1). Seeds were untreated (controls) or treated with either heat, smoke, or a combination of smoke and heat (smoke + heat). Heat treatments were applied for 10 minutes by placing seeds on a plastic tray inside an oven preheated to 90°C (Mackenzie *et al.* 2016a). Smoke treatments were applied by placing seeds on plastic trays inside a 60 L plastic propagation tent and pumping in smoke, generated by combusting 100 g of oat hay inside a bee smoker, for 10

minutes (Mackenzie *et al.* 2016a). For combination smoke + heat treatments, heat treatments were applied prior to smoke exposure.

Following application of fire cues, seeds were divided into 4 treatments including burial, hermetically sealed burial, afterripening, or controlled storage (4 replicates of 25 seeds per treatment). Seeds from controlled storage treatments were kept at 15°C and 15% relative humidity (RH) at the Western Australia Seed Centre. Afterripened seeds were suspended over a non-saturated solution of lithium chloride (364 g L^{-1} to achieve 50% RH) (Just *et al.* 2019) inside a 270 x 190 x 100 mm polycarbonate electrical enclosure box (NHP Fibox, Perth, WA, Australia) and kept at a constant 30°C until retrieval for germination assessment. Seeds undergoing burial, afterripening or controlled storage were heat sealed inside 5 x 3 cm nylon mesh bags along with 1 g of white quartz sand. Seeds undergoing hermetically sealed burial were placed inside 5 x 3 cm water resistant mylar foil bags at ambient RH (~50% RH). To determine if dormancy is alleviated and seeds become germinable in the six months following seed dispersal, three replicate nylon and mylar bags (+ seeds) were buried at a depth of 10 mm in four replicate sites at the study site in January 2021 using a randomised block design (Fig. 4.1). To determine whether germination occurred *in situ* following the onset of seasonal rainfall, bags were retrieved and carefully inspected for germination in April (5/4/2021), May (5/5/2021), and June (18/6/2021). Germinated seeds were counted and removed from bags, and an additional 25 seeds were taken from each treatment at each replicate site, air dried overnight, and analysed using x-ray (Autofocus X-ray cabinet, Faxitron, Tucson, USA) to establish the proportion of filled seeds remaining. To determine if dormancy had been alleviated but seeds had not germinated, non-germinated seeds from laboratory and field-based treatments were subsequently sown onto Petri dishes. Prior to sowing, seeds from controlled storage,

afterripening and burial in mesh bags were surface sterilised in 0.3% w/v chlorine (1 x Milton® tablet per 100 ml water–active ingredient Sodium Dichloroisocyanurate) for 30 minutes. Mylar and mesh bags were then opened and laid onto a Petri dish containing 0.7% (w/v) water agar and incubated under a 12/12-hour light/dark regime at either 20/5°C, 20/10°C or 30/10°C, with germination monitored weekly for 12 weeks. Temperature regimes were selected based on field data obtained from the site in the previous year and represent cool, warm, and hot conditions experienced over the winter months when seeds typically germanite.

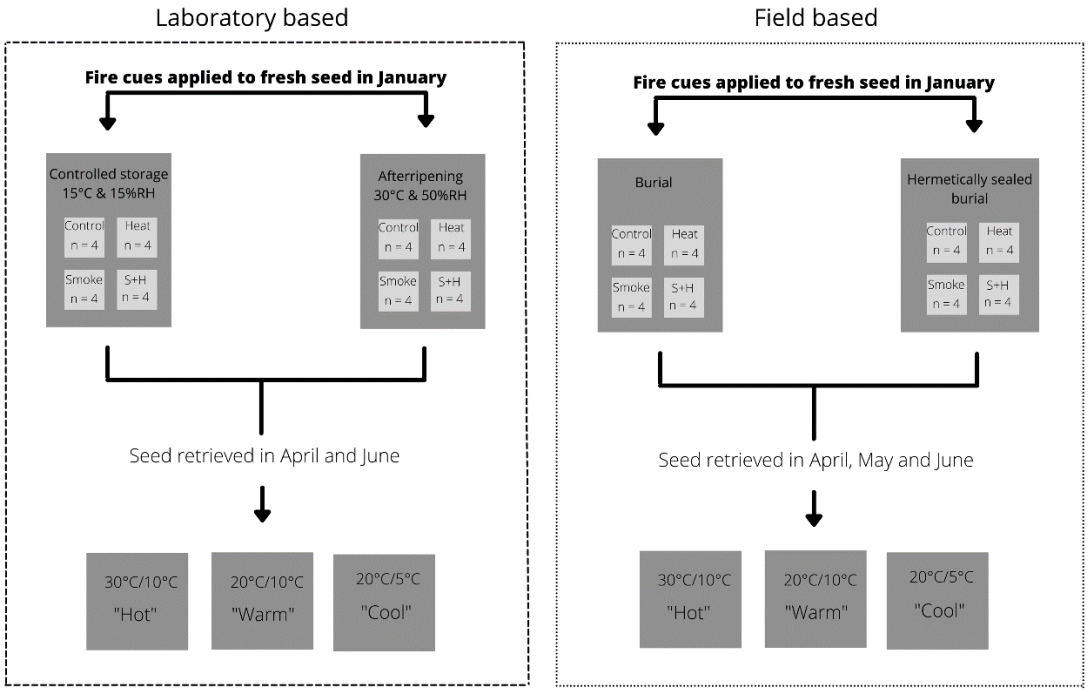

Figure 4.1. Schematic diagram of laboratory- and field-based experiments. Fresh seeds were exposed to fire-derived cues of heat and/or smoke in January 2021 before being assigned to treatment conditions in the laboratory or field. Seeds in the laboratory were placed into controlled storage (15°C and 15% RH) or afterripening (30°C and 50% RH). Seeds in the field were buried in remnant *Banksia* woodland at a depth of 10mm. Seeds were then retrieved in April (5/4/2021), May (5/5/2021), or June (18/6/2021), with germination scored and germinated seeds removed before sowing remaining seeds onto water agar and incubating at "cool" (20/5°C), "warm" (20/10°C), or "hot" (30/10°C)" alternating temperature regimes for 12 weeks.

4.3.3 *In situ* seed water activity and germination response to smoke water or karrikinolide following burial

To determine how seed moisture status fluctuates over time in soil in response to changing soil conditions, the relative humidity of seeds was measured with a Hygropalm HP23-AW-A Water Activity Meter (Rotronic, New York) (Supplementary material 4.2). For each species enough seed to fill the chamber (approximately 1000) were sealed into four nylon mesh bags and buried at a depth of 10mm on 15/02/21. Each of the four bags was retrieved weekly, opened and seed mixed before adding seeds to the chamber in the field. Seeds were allowed to equilibrate within the chamber for 20 minutes before measurements were made. Seeds were then resealed inside each of the four bags and reburied. Measurements were taken weekly for 10 weeks, after which seeds were removed and used to determine whether seeds respond to chemicals in smoke other than KAR_1. Seeds were retrieved on 05/05/21 (onset of the winter rainfall season), and immediately soaked for 24 hours in either 10% (v/v) smoke water (Dixon *et al.* 1995) or 0.67 µM karrikinolide (KAR_1, as synthesised in Flemmati *et al.* 2005). Four replicates of 25 seeds for each study species were then surface sterilised as previously described before plating on 0.7% (w/v) water agar and incubating at alternating 20/10°C under a 12/12-hour light/dark regime. Germination was scored every 2 days for 30 days, with germination defined as radicle emergence >2 mm.

4.3.4 Statistical analyses

Germination proportion for each treatment were calculated based on the portion of filled seeds determined from x ray analysis. All figures present germination proportion ± 95% confidence interval (CI). All statistical analysis were conducted in the R statistical environment (R Core Team, 2013). Binomial generalised linear modelling

(GLM) with a logit link function was used to analyse the main and interaction effects of treatment (untreated, smoke, heat, smoke + heat), retrieval month (April, May, June) and burial site on germination success in the field (Fig. 4.2) and of treatment (untreated, smoke, heat, smoke + heat), retrieval month (April, May, June) and incubation temperature (20/5°C, 20/10°C or 30/10°C) on germination success under laboratory conditions (Table 4.3). The effect of either water (control), smoke water, or KAR_1, on germination success of seeds retrieved in May (Fig. 4.3) was also analysed using the same method. Species were analysed separately and the *anova* function from the *car* package (Fox *et al.* 2007) was used to generate significance values between factors. To determine differences in daily temperature range between sites and depth a generalised linear mixed model (GLMM) was fitted to daily temperature range using the *lme4* package (Bates 2010). Month was included as a random effect. Data were checked for normality and homogeneity of variance and no transformation was required. Pairwise comparisons based upon model predictions were made between depths using the least-squares means method in the *lsmeans* package (Lenth and Lenth 2018).

4.4 Results

4.4.1 Germination response to *in situ* and *ex situ* conditions following the application of fire cues

No *in situ* germination was recorded for any study species following retrieval in April or May, but significant germination (0.1-0.7) was recorded for seeds in bags retrieved in June (Fig. 4.2). There was an effect of burial site on the *in situ* germination of *Boronia fastigiata* ($\chi2 = 53.956$, d.f. = 3, $p < 0.001$), *Crowea angustifolia* ($\chi2 = 164.981$, d.f. = 3, $p < 0.0001$) and *Rhadinothamnus anceps* ($\chi2 = 40.964$, d.f. = 3, $p < 0.0001$),

but not *Philotheca spicata* ($\chi 2 = 5.41$, d.f. = 3, $p = 0.14$). There was no consistent positive effect of a particular site on germination proportion with site 3 having the greatest germination proportion for *Boronia fastigiata*, site 1 for *Crowea angustifolia* and site 2 for *Philotheca spicata*. All species germinated to the lowest proportion in site 4 (Fig. 4.2).

Table 4.2. Seed fill and seed dormancy characteristics of Rutaceae species used in this study. Primary dormancy was determined as the percentage of ungerminated seeds in controlled storage treatments in April. Dormancy type follows the definition of Baskin and Baskin, (2014).

Species	Collection seed fill (%)	Processed seed fill (%)	Primary dormancy (%) *	Dormancy type**
Boronia fastigiata	83	97	100	PD
Crowea angustifolia	91	98	94	PD
Philotheca spicata	93	98	95	PD
Rhadinothamnus anceps	94	97	90	PD

Treatment with aerosol smoke alone produced the highest germination proportion for *Boronia fastigiata* (0.33 ± 0.12) and *Philotheca spicata* (0.36 ± 0.11), while combination heat and smoke were most effective for *Crowea angustifolia* (0.72 ± 0.11) (Fig. 4.2). Smoke exposure did not promote seed germination of *Rhadinothamnus anceps,* but rather germination was improved by heat treatment when seeds were buried in site 3 (Fig. 4.2).

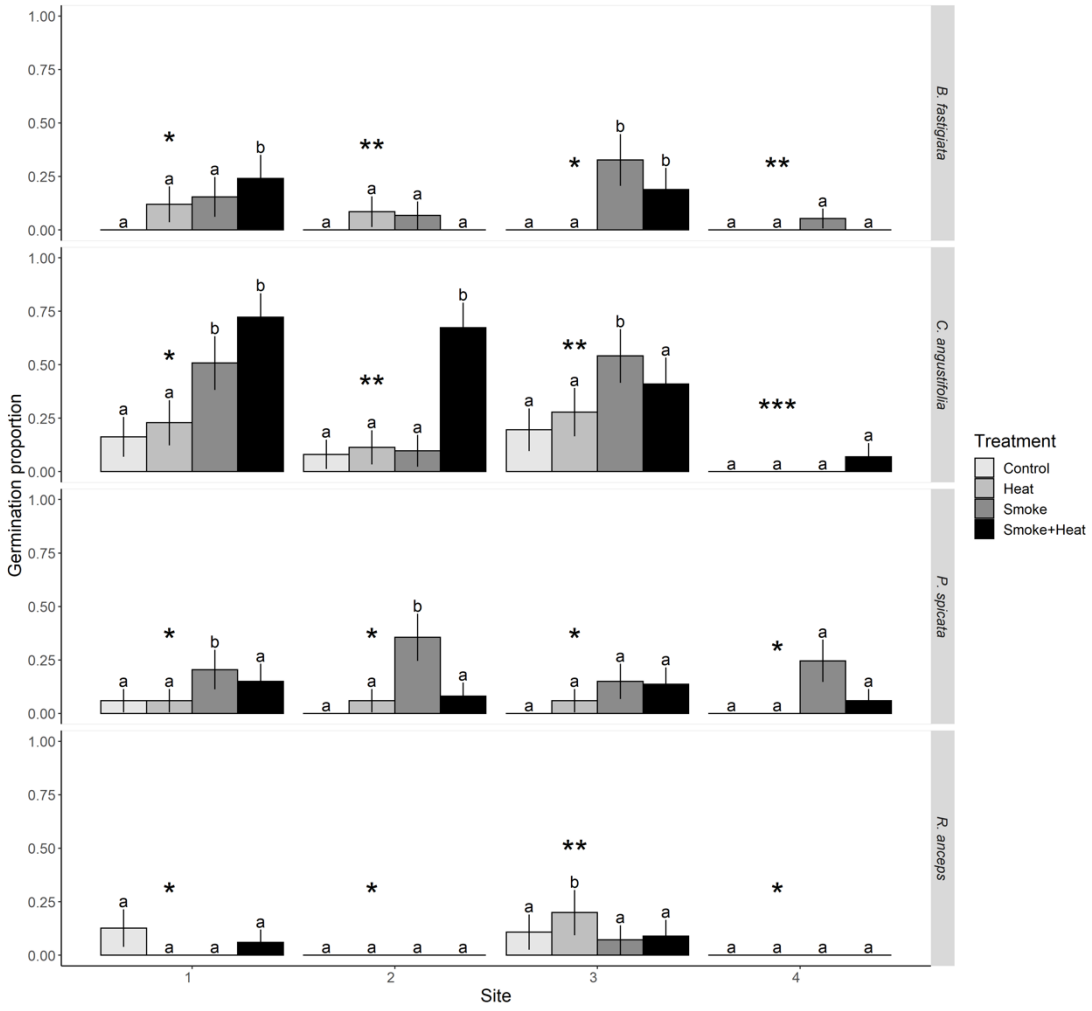

Figure 4.2. *In situ* germination success (± 95% confidence interval) of four species of Rutaceae recorded six monhts after burial in January 2021. Plots with different letters denotes significant differences among treatments within sites (p <0.05), while asteriscs denote significant differences among sites (p <0.05). No germination was noted for seeds retrieved on the 5/4/2021 or 5/5/2021.

Seed germination of all four study species following 12 weeks of incubation was significantly affected (Table 4.3) by incubation temperature, burial or storage conditions, and the application of fire cues (Fig. 4.3). Highest germination proportion for all species were achieved with the application of smoke or smoke + heat (Fig. 4.3). *Boronia fastigiata* (0.47 ± 0.13) and *Philotheca spicata* (0.22 ± 0.09) had the greatest germination proportion following pre-treatment with aerosol smoke and retrieval from

hermetically sealed bags, although the effect was two-fold in *Boronia fastigiata* when compared to *Philotheca spicata* (Table 4.3).

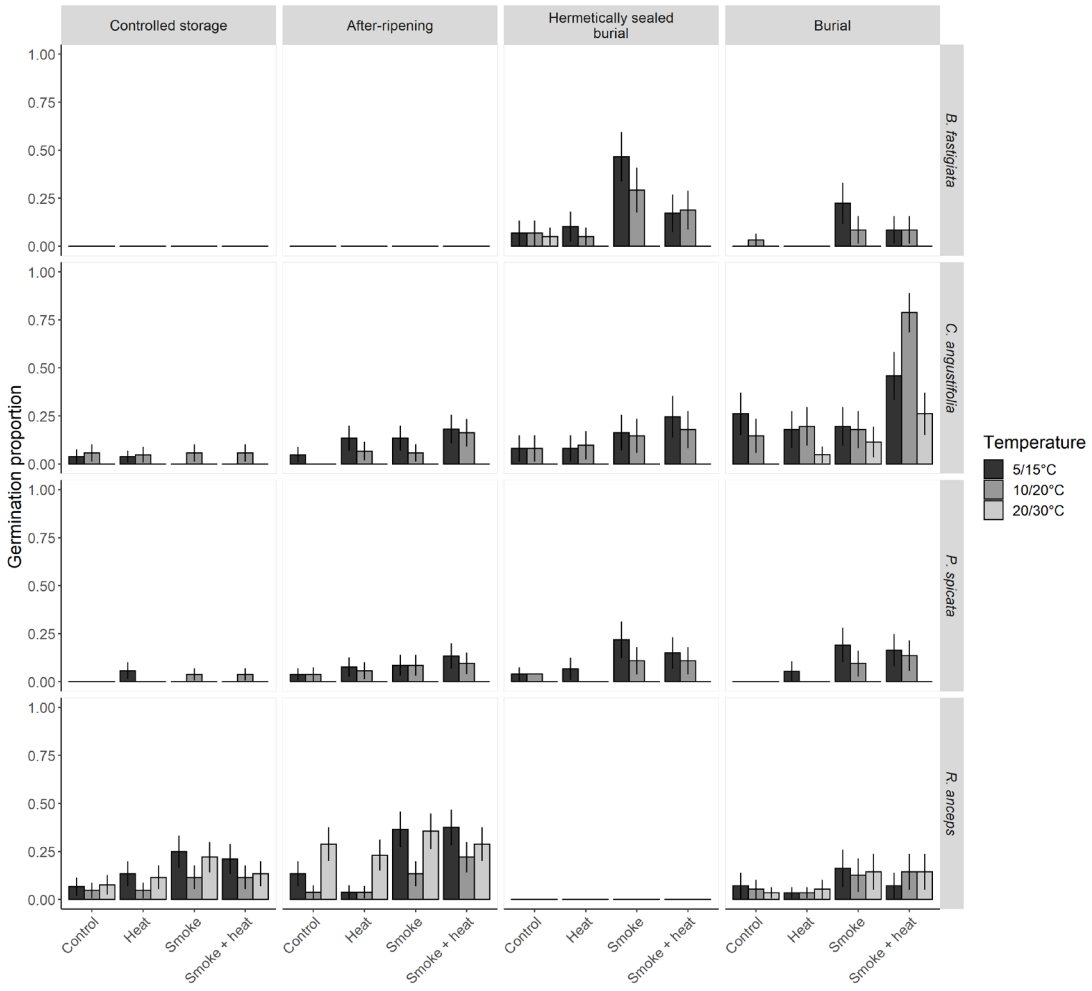

Figure 4.3. Germination proportion (± 95% confidence interval) in four species of Western Australian Rutaceae following 12 weeks of incubation after retrieval from controlled storage (15°C & 15% RH), afterripening (30°C & 50% RH), hermetically sealed burial (burial in water resistant bags) and burial (burial in mesh bags). Seeds were buried in remnant banksia woodland in Janurary 2021 and retreived in April 2021. See Table 4.3 for statistical differences.

Increasing incubation temperature negatively affected germination in most species with seeds germinating to greater proportions at cooler temperatures following the application of smoke (Fig. 4.3). The exception to this was observed in *Rhadinothamnus anceps* seeds with a significant positive interaction effect between

incubation temperature and pre-treatment with high germination observed at the warmest temperature regime ($\chi 2 = 19.91$, d.f. = 3, $p = 0.0002$).

A significant interaction effect was present between germination temperature and treatment on seed germination ($\chi 2 = 13.90$, d.f. = 3, $p = 0.003$), and between treatment and pre-treatment ($\chi 2 = 30.55$, d.f. = 9, $p = 0.0003$) for *Crowea angustifolia*. Seeds pre-treated with a combination of heat and smoke germinated to significantly greater proportions than those treated with either smoke or heat alone (0.19 to 0.78) following burial.

Table 4.3. GLM summary for the effect of temperature (5/15°C, 10/20°C or 20/30°C), treatment (controlled strorage, afterripening, burial, hermetically sealed burial) and pre-treatment (smoke, heat or smoke + heat) on the binomial outcome of germination success *in situ* following retrieval of *Boronia fastigata*, *Crowea angustifolia*, *Philotheca spicata* and *Rhadinothamnus anceps* in April.

Species	Variable	D.f.	χ2	Sig
Boronia fastigiata	Temperature	2	40.53	***
	Treatment	5	21.34	***
	Pre-treatment	4	44.34	***
	Temp: Treatment	3	0.11	
	Temp: Pre-treatment	3	5.89	
	Treatment: Pre-treatment	9	0.30	
Crowea angustifolia	Temp	1	66.10	***
	Treatment	3	178.40	***
	Pre-treatment	3	92.75	***
	Temp: Treatment	3	13.90	**
	Temp: Pre-treatment	3	5.80	
	Treatment: Pre-treatment	9	30.55	***
Philotheca spicata	Temp	1	61.77	***
	Treatment	4	15.33	**
	Pre-treatment	4	25.47	***
	Temp: Treatment	3	1.72	
	Temp: Pre-treatment	3	2.55	
	Treatment: Pre-treatment	9	16.43	.
Rhadinothamnus anceps	Temp	1	0.83	
	Treatment	3	62.46	***
	Pre-treatment	3	73.54	***
	Temp: Treatment	3	7.54	.
	Temp: Pre-treatment	3	19.91	***
	Treatment: Pre-treatment	9	15.89	.

Symbols denote significance at p value: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Seeds of all four study species germinated in incubators when retrieved in April, however no germination was seen in the field for April (Table 4). No field germination was recorded for May and incubator germination for all species was lowest in May, for *Boronia fastigiata* and *Rhadinothamnus anceps* this meant no germination whatsoever in May, while *Crowea angustifolia* and *Philotheca spicata* germinated to 0.27±0.11 and 0.09±0.07 respectively. In June, substantial germination was recorded in the field, but ungerminated filled seeds that were incubated did not germinate (Table 4.4).

Table 4.4 Germination proportion (±95% confidence interval) from burial treatments which produced the greatest germination in incubators or the field for seeds retrieved in April, May, and June. *Boronia fastigiata*, *Philotheca spicata* and *Rhadinothamnus anceps* were incubated at 5/15°C and pre-treated with aerosol smoke. *Crowea angustifolia* was incubated at 20/10°C and pre-treated with smoke + heat.

Species	Month	Incubator		Field	
		Germination	95% CI	Germination	95% CI
Boronia fastigiata	April	0.22	0.11	0.00	0.00
Boronia fastigiata	May	0.00	0.00	0.00	0.00
Boronia fastigiata	June	0.00	0.00	0.33	0.12
Crowea angustifolia	April	0.79	0.10	0.00	0.00
Crowea angustifolia	May	0.28	0.11	0.00	0.00
Crowea angustifolia	June	0.00	0.00	0.72	0.11
Philotheca spicata	April	0.19	0.09	0.00	0.00
Philotheca spicata	May	0.10	0.07	0.00	0.00
Philotheca spicata	June	0.00	0.00	0.36	0.11
Rhadinothamnus anceps	April	0.16	0.10	0.00	0.00
Rhadinothamnus anceps	May	0.00	0.00	0.00	0.00
Rhadinothamnus anceps	June	0.00	0.00	0.20	0.11

4.4.2 *In situ* seed water activity and germination response to smoke water or karrikinolide following burial

When retrieved in May, seeds of *Boronia fastigiata, Crowea angustifolia, Philotheca spicata* and *Rhadinothamnus anceps* treated with smoke water germinated to significantly greater proportions than those in control or KAR_1 treatments ($p < 0.05$, Fig. 4.4). *Boronia fastigiata* and *Rhadinothamnus anceps* were the only two species that did not germinate in the absence of smoke water. Both *Crowea angustifolia* and

Philotheca spicata germinated to low proportions in the absence of smoke water, but germination was significantly enhanced by the application of smoke water (Fig. 4.4).

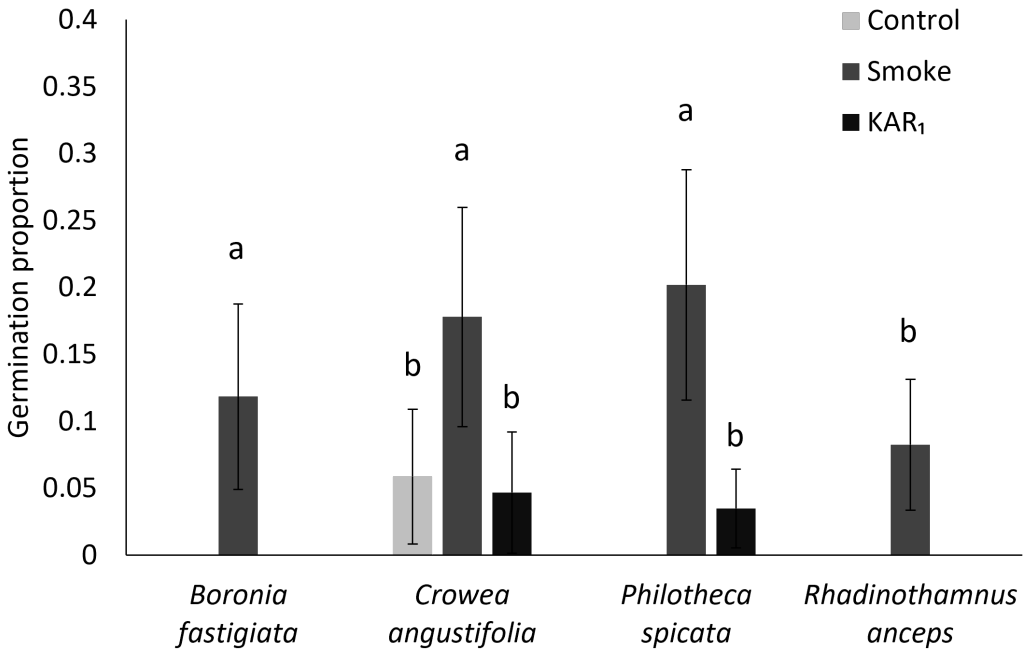

Figure 4.4. Germination proportion (±95% confidence interval) of Rutaceae seeds buried in January 2021 and retrieved in May 2021. Seeds were treated for 24 hours in either 10% (v/v) smoke water (Kings Park and Botanic Garden, Perth) or 0.67µM KAR_1 (Flemmati *et al.* 2005) before incubation at 20/10 °C alternating temperature regime. Bars with differing or no letters are significantly different ($p<0.05$).

Seed moisture status changed with fluctuations in soil moisture, which occurred during, or immediately after rainfall events (Fig. 4.5, 1 cm panel). The range of temperatures within the soil narrowed as depth increased, while the magnitude of soil moisture was lowest at 5 cm (Fig. 4.5). The duration of elevated soil moisture following a rainfall event was greater at 5 cm and 10 cm, and lowest at 1 cm (Fig. 4.5). All recorded rainfall events, excluding one event on the 12th of March, produced an increase in normalised soil moisture content > 15%, which increased seed water activity (equilibration relative humidity) to levels > 30%. A localised rainfall event not recorded at the BOM weather station produced an increase in soil moisture on the 3rd of April.

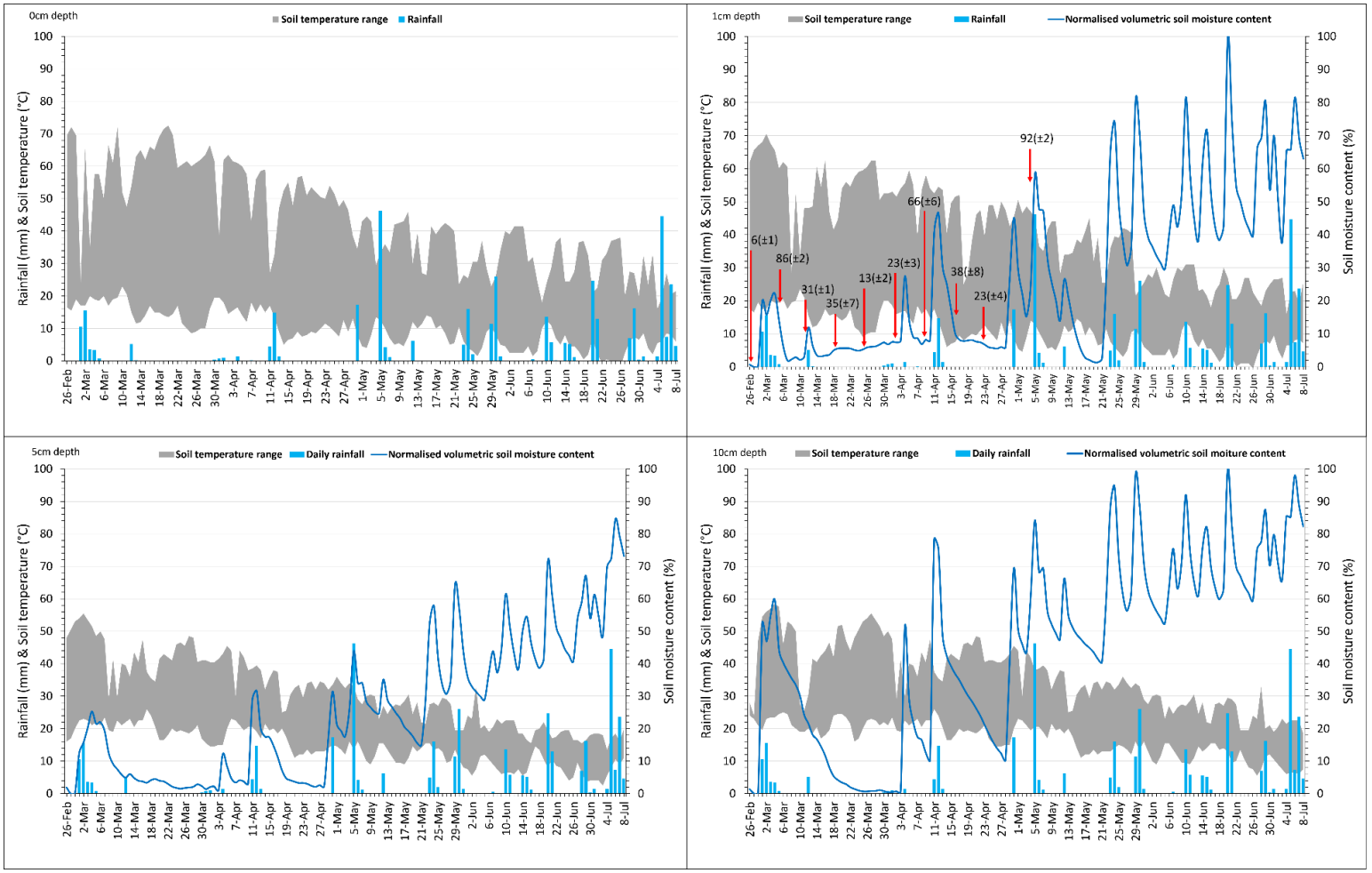

Figure 4.5 Normalised soil moisture (n = 1) and mean temperature (n = 3) measured at 0, 1, 5 and 10cm (top left of each panel) throughout the soil profile in remnant *Banksia* woodland. Rainfall data was sourced from BOM (2021). Temperature data for 0cm is Thermochron (DS1921G) readings taken on the soil surface. Red arrows and annotations denote mean seed relative humidity (% ± standard deviation) across the four study species.

4.4.3 Soil conditions

Both site ($\chi 2 = 31.4$, d.f. = 2, $p < 0.001$) and depth ($\chi 2 = 941.68$, d.f. = 10, $p < 0.001$) had a significant influence on the daily temperature ranges recorded throughout the sampling period (January to July 2021) (Fig. 4.6, Table 4.6). Sites two and three were found to have no significant difference between each other ($p = 0.69$), however both were significantly different to site one ($p < 0.0001$ in both cases). For all three sites, temperature on the soil surface was significantly different to any other depth ($p < 0.0001$). Starting at 1 cm, every 1 cm increment within the soil profile was statistically like those directly adjacent, but those within 2 cm of each other were significantly different ($p < 0.05$, Supplementary material Table 1), down to 7cm where temperature profiles between 2 cm increments became similar. Mean daily temperature ranges recorded for each month at each site are presented in Supplementary material (Fig. S1).

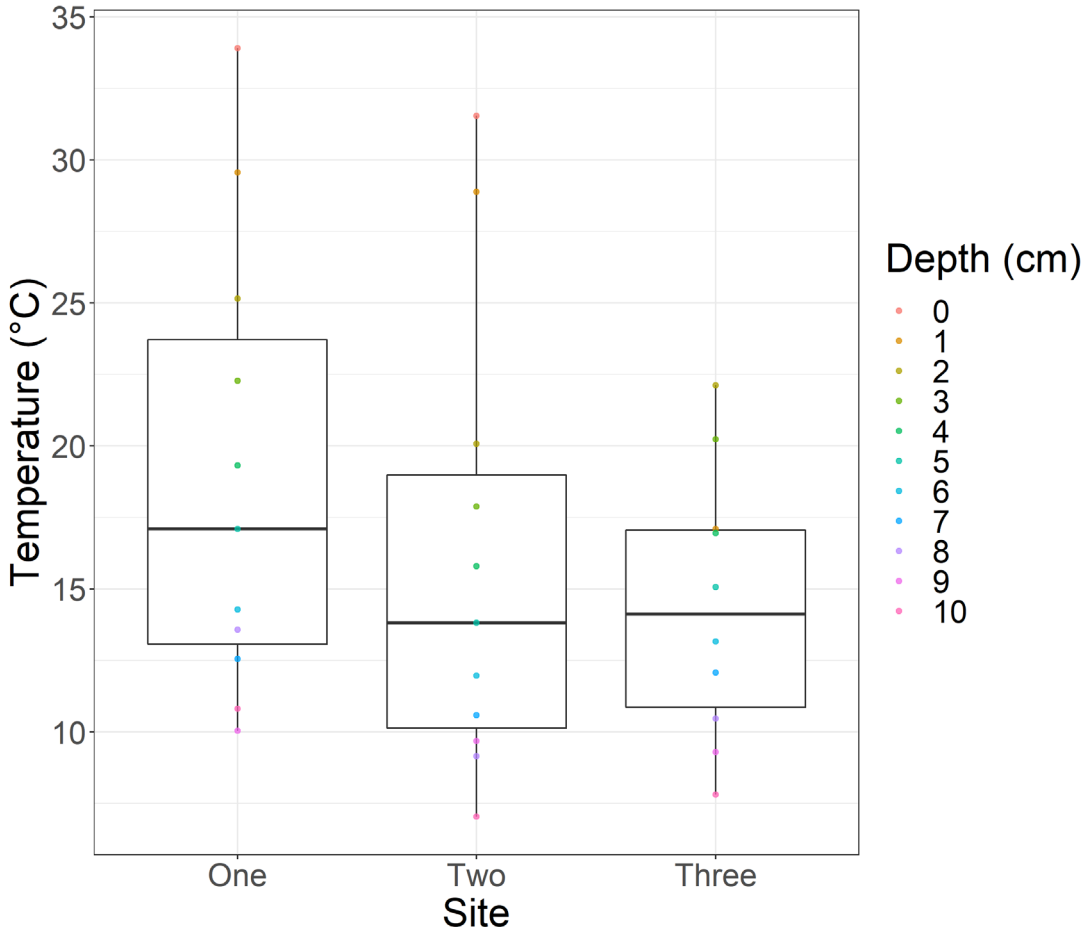

Figure 4.6. Mean daily temperature range recorded in *Banksia* woodland soil in the top 10 cm of the soil profile recorded at 1 cm increments in three replicate sites (one, two and three) from 25/01/2021 until 08/07/2021. Mean daily temperature range for each month (January–July) are presented in supplementary material (Fig. S1).

Table 4.5 Summary of sample periods within which seeds at 1 cm were exposed to temperature and moisture ranges. Conditions experienced equate to experimental conditions: WS, Warm Stratification; CS, Cold stratification; AR, Afterripening. Stratification is defined here as normalised soil moisture content >15%, cold is defined as temperatures <10°C and warm is defined as temperatures >15°C. Stratification was arbitrarily determined as normalised soil moisture content >15% as significant rainfall events coincided with increases in soil moisture >15%. Cold and warm stratification temperatures follow those defined by Baskin and Baskin (2014). Germination (1–4mm radicle protrusion) was noted *in situ* in retrieved seed burial bags for all four species on 18/06/21 and was not observed during previous retrevials on 05/04/21 and 05/05/21.

Dates	Temperature range (°C)	Normalised soil moisture content range (%)	Days	Treatment
26/02/21–28/02/21	16–67	0–1	3	AR
01/03/21–04/03/21	17.5–70.5	15–22	3	WS
05/03/21–03/04/21	9–62.5	2–14	29	AR
04/04/21–05/04/21	15–59.5	15–27	1	WS
06/04/21–10/04/21	13–58	8–9	4	AR
11/04/21–15/04/21	6–55	17–41	4	WS/CS
16/04/21–28/04/21	7.5–52	5–10	12	AR
29/04/21–08/05/21	5.5–50.5	22–58	9	WS/CS
09/05/21–11/05/21	5–44	15–25	2	WS/CS
12/05/21–14/05/21	10–34.5	20-26	2	WS
15/05/21–21/05/21	6.5–45	2–8	6	AR
22/05/21–28/05/21	3–42	26–74	6	WS/CS
29/05/21–18/06/21	1.5–31	29–81	20	WS/CS

4.5 Discussion

This study provides the first account of *in situ* timing of dormancy alleviation for southwest Australian Rutaceae. Dormant seeds buried in January had begun to lose dormancy upon retrieval in April (Fig. 4.3) and May (Table 4.4) and germinated to significant proportions in soil in June (Fig. 4.2). However, a significant portion of seed failed to germinate, suggesting additional dormancy alleviation cues are required following seed dispersal and subsequent years in soil. Interestingly, seeds retrieved in May germinated to the lowest proportions observed (Table 4.4). Seeds with physiological dormancy are known to cycle between dormant, conditionally dormant and non-dormant states in response to seasonal fluctuations in soil moisture and temperature (Finch-Savage and Footitt 2017; Finch-Savage and Leubner-Metzger 2006), and this has been demonstrated in eastern Australian Rutaceae (Collette and Ooi 2020a). Dormancy cycling has also been observed in other Australian species (Baker *et al.* 2005a; Merritt *et al.* 2007) but no study to date has observed fluctuations of dormancy status within a single season. While it is unclear whether seeds from the May retrieval (Table 4.4) had begun to enter dormancy or had become conditionally dormant, it appears that a period of drought between the April and May (Fig. 4.5, 1 cm panel) led to the observed decrease in germination (Table 4.4). Typical of dormant or conditionally dormant seeds is a narrow temperature (Baskin and Baskin 2004) and moisture (Baskin and Baskin 2014; Lewandrowski *et al.* 2017; Meyer *et al.* 1997) threshold required for promoting germination. If a lack of consistent soil moisture in late April and early May caused seeds to become (conditionally) dormant then it is likely that incubation temperatures tested here (Fig. 4.3) were outside of the narrow thresholds required for supporting the germination of seeds from the May retrievals. Conditions experienced by seeds in the soil prior to significant germination observed

in June (Fig 4.6) were apparently more conducive to germination which may have been due to dormancy alleviation between May and June, or because *in situ* conditions of temperature and moisture were within the narrow germination thresholds for seeds with conditional dormancy, thus kickstarting the germination process. Germination of all four study species observed following the April and June retrievals was double that observed in May, highlighting how variation in climatic conditions can promote germination when conditions become favourable (e.g., onset of reliable winter rainfall, retrieval, and incubation of buried seeds under favourable conditions, or the supply of irrigation). The potential of Rutaceae to enter conditional dormancy, their susceptibility to dry periods within the germination season, and their slow germination response are potential reasons as to why they are difficult to reliably propagate from seed. Careful management of hydrothermal conditions may be required for an extended period when propagating these species, particularly in nursery settings where seeds may experience seasonal fluctuations that can limit their response to pre-treatments such as afterripening, soil storage, heat, or smoke.

All four study species were smoke responsive; however, this response was tied to other treatments such as such as burial, dry heat and afterripening (Figs. 4.2, 4.3). The application of smoke is effective in a range of Australian species (Downes *et al.* 2015; Hodges *et al.* 2019; Rokich and Dixon 2007; Turner 2013) but has been inconsistent in promoting germination of WA Rutaceae likely due to the poor resolution of innate physiological seed dormancy (Dixon *et al.* 1995; Roche *et al.* 1997a). As well, it appears that all four study species respond to chemicals in smoke other than KAR_1 (Fig. 4.4) which is unsurprising as other Australian taxa are responsive to cyanide present in smoke as glyceronitrile (Downes *et al.* 2013; Flematti *et al.* 2011). Seeds treated with smoke water also germinated to greater proportions than those retrieved

in May in other experiments, and this may have been caused by variation in dormancy alleviation due to seeds being in different burial locations, the timing of smoke application, or differences in method of smoke treatment. While this variation in germination between smoke treatments cannot be explained in the present study, future research could identify the optimum timing and method of smoke application for Rutaceae and should focus on protocols that maximise the application of compounds other than KAR_1. Indeed, smoke is known to stimulate germination following the alleviation of seed dormancy, and it is possible that early summer application of smoke treatments in the present study limited the effectiveness once dormancy was alleviated. In combination with smoke treatments, the application of short periods of dry heat should also be explored as 10 minutes at 90°C significantly improved smoke stimulated germination of *Crowea angustifolia* (Fig. 4.2, 4.3). An additive effect of smoke and heat has been demonstrated in eastern Australian Rutaceae (Mackenzie *et al.* 2016a) as well as several other WA species such as *Anigozanthos manglesii*, *Stylidium affine* and *Actinotus leucocephalus* (Norman *et al.* 2006; Tieu *et al.* 2001a) though no published study has investigated the effect of dry heat pulsing and smoke application on dormancy alleviation and germination in any WA Rutaceae taxa. Seed dormancy has also been shown to restrict responsiveness to the germination environment and reduce sensitivity to smoke (Baker *et al.* 2005a; Finch-Savage and Footitt 2017), and a lack of dormancy alleviation underscored the limited smoke stimulated germination in previous studies on native Rutaceae for fresh seed (Cromer 2007; Norman *et al.* 2006; Roche *et al.* 1997a) yet provided high germination in field treated soil seed banks (Dixon *et al.* 1995).

The exact conditions that alleviate dormancy over time in soil are unknown for most species, although the results presented here unsurprisingly suggest that different

members of the Rutaceae respond to different environmental cues. *Boronia fastigiata* had a significant germination response to burial in water resistant bags, however no germination was seen for seeds stored under controlled conditions (15°C) or afterripened (at 30°C) in the laboratory for an equal period (Fig. 4.3). The present study could only test the effect of storage under cool dry (15°C and 15% RH) or warm dry (30°C and 50% RH) conditions for up to 6 months. Greater durations of storage are a known requirement for afterripening response in some Australian species (Turner *et al.* 2009b) and it is possible that increased storage durations would produce greater germination among study species. Parallel to the need to test longer afterripening durations is a need to test a greater range of storage conditions that reflect those experienced by seeds in soil. As seeds of *B. fastigiata* were sealed to exclude moisture, dormancy had to have been alleviated by temperatures experienced by seeds in a dry environment while in the soil over the warmer months (Fig. 4.5), and the temperature regimes applied in controlled storage or afterripening (Fig. 4.3) were clearly insufficient for dormancy alleviation of this species. In comparison, *Crowea angustifolia* and *Philotheca spicata* had similar germination proportion between burial or controlled storage treatments (Fig. 4.3). Consequently, dormancy alleviation in these species may be driven by fluctuations in temperature and/or moisture. These results highlight the significance of burial experiments as a tool to understand seed ecology and discern the critical factors that drive dormancy alleviation and germination, but careful monitoring of soil conditions as well as *in situ* germination timing is required if lab-based treatments for dormancy alleviation are to be developed based on field observations.

Significant information on the temperatures required for dormancy alleviation and germination of Western Australian species is available (Merritt *et al.* 2007; Turner *et*

al. 2006a) however, the exact temperatures required for many Australian species remains unknown. In this study, detailed empirical data on soil conditions during dormancy alleviation and germination of Rutaceae has been obtained which has significantly improved our overall understanding of the interplay between these factors. Interestingly, the variation in germination between burial sites suggests that the heterogeneity of microclimates may play a significant role in the recruitment and niche partitioning of Rutaceae species. *Philotheca spicata* was the only species that germinated to similar proportions regardless of burial location, whereas *B. fastigiata* and *C. angustifolia* each had a greater germination response within a particular site (Fig. 4.2). *Rhadinothamnus anceps* germinated to the lowest proportions *in situ* (0–0.19) of the study species, and germination may have been limited by the disparity between seed collection and deployment locations. While our data does not show the exact thermal conditions at each burial location, temperature records across the study site (Fig. 4.5, 4.6) nevertheless indicate significant vertical and horizontal variation in temperature at small scale with all three replicates located within the same 400 m^2 *Banksia* woodland fragment. While spatial variation in temperature may have promoted or limited dormancy alleviation and germination at different sites, it is also probable that seed hydration over the experimental period drove the observed variation in final germination that was observed. *Banksia* woodland soils are known to be hydrophobic and comprised principally of sands (Ritchie *et al.* 2020) and structural variation in the soil matrix and overlying vegetation throughout the site may have significantly influenced the moisture status of seeds at different burial sites as weather conditions changed over time. Whether variations in temperature and/or moisture drove the spatial variation in dormancy alleviation and germination (Fig. 4.2) these results highlight the need to accurately understand microsite variation in hydrothermal

conditions across a site to ensure seeds have the best chance of germination and survival when used for horticulture or restoration. The variation in germination proportion seen within the study site suggests further complications for deployment of restoration seeds to sites outside of their typical range. However, seeds sourced from sites throughout southwestern Australia and buried in a single Perth Metro location did germinate to high probabilities (up to 80% of seeds sown) suitable for their use in restoration. How these individuals may have emerged, established, and survived following initial germination was unable to be investigated here. Likewise, variation in germination response between deployment (Study site–*Banksia* woodland fragment) and collection locations (Table 4.1) was not a focus of the present study. As seed scarcity and climate change continue to drive new practices in seed sourcing and deployment it is imperative that seed ecology, and particularly the hydrothermal requirements for dormancy alleviation, germination, and seedling establishment, are clearly understood to ensure seedling survival and ongoing plant recruitment in restoration sites.

4.6 Conclusions

In the present study, *Boronia fastigiata* and *Philotheca spicata* responded positively to burial while stored in a dry state, while *Crowea angustifolia* and *Rhadinothamnus anceps* required brief periods of wetting leading up to the winter wet season. Significantly more research is required to develop *ex situ* germination protocols for southwest Australian Rutaceae, with this study showing (Table 4.6) a series of potential treatment applications based upon conditions of temperature and moisture that improve *in situ* germination of study species. ‘Move-along’ experiments that mimic natural thermal conditions have proven effective in alleviating dormancy of historically problematic species though do require considerable time and relatively complex

experimental approaches before successful (Baskin and Baskin 2003; Chia *et al.* 2016; Turner *et al.* 2009a). Using this approach, laboratory-based experiments utilising combinations of hydrothermal conditions (detailed in Table 4.6) may help to shed light on potential avenues for on-demand germination of Australian Rutaceae.

5 Chapter 5

General discussion

5.1 Summary of findings

Seed dormancy is a critical part of plant ecology that allows species to persist and recruit in ecosystems with extreme and stochastic environmental conditions at various times of the year that optimise the recruitment potential for a species. In this thesis I have shown that understanding the complexity of seed dormancy and its interplay with environmental conditions can lead to development of protocols able to germinate seeds of taxa previously unavailable to restoration. The findings within this thesis highlight seed germination protocols that were effective in several members of the Rutaceae (Chapters 2 & 4) and identifies groups of taxa that require further investigation (Chapter 1). Classification of types of non-deep physiological dormancy (Chapter 3) provided insights into the recruitment ecology of study species that have implications for conservation, seed-based restoration, and species management under climate change.

In Chapter 2, I investigated and categorised a range of seed traits to determine the dormancy class of study species. Based on these results I was able to identify avenues for seed-based propagation of intractable Rutaceae. Study species, which accounted for ~20% of the taxonomic diversity at the genus level, were found to have small (2–5 mm long) seeds that were highly viable when fresh (50 to 81% seed fill) and water permeable. As well, each species was found to possess a fully developed embryo that had a physiological inhibiting mechanism suppressing germination when exposed to optimal germination conditions. Considering the prolonged time required for germination (>4 weeks) and the response to GA_3, it is highly likely that either non-deep or intermediate physiological dormancy is present across the Rutaceae. Both warm

stratification and afterripening (via soil burial) overcame dormancy to a lesser or greater degree. Importantly, dry afterripening under laboratory conditions over the first 5 months post dispersal had no impact on dormancy loss of study species, but an equal period in dry soil under glasshouse conditions produced emergence in five of the seven species studied when exposed to optimal germination conditions. These results suggest that variable temperature (8–72°C; Chapter 2 & 4) or moisture (0–60% of field capacity–Chapter 2 & 4) in the lead up to the germination season (commencing ~18 May to ~24 June; Chapter 2 & 4), rather than constant temperatures and moisture (30°C, 50% relative humidity; Chapter 2 & 4) used in afterripening treatments, drive dormancy loss which then allows germination to be stimulated by smoke when soil moisture becomes non-limiting in late May (Chapter 2 & 4). As well, unlike many other native species (i.e., *Astroloma xerophyllum*, Turner *et al.* 2009; *Lepidosperma scabrum*, Turner 2013; *Stylidium affine*, Turner, Merritt, *et al.* 2009; Appendix 2, Roche, Dixon, and Pate 1997), dormancy was found to be significantly alleviated within six months of seed dispersal. The low emergence proportions of seeds sown in the glass house, when compared to higher proportions under laboratory conditions, suggests emergence bottlenecks exist for study species, and that careful management of sowing conditions and watering regimes is required during and post-dormancy loss to support and enhance seedling emergence. Based on these initial results, on-demand germination of southwest Australian Rutaceae can be achieved for several of these taxa through the application of gibberellic acid (*Boronia cymosa*, *B. fastigiata*, *B. ovata, Crowea angustifolia*, *Cyanothamnus ramosus*, *Diplolaena angustifolia, D. dampieri*, *Philotheca spicata*, *Rhadinothamnus anceps*) or warm stratification (*Diplolaena angustifolia, D. dampieri*, *Rhadinothamnus anceps*) which has never been

demonstrated for any Western Australian species of Rutaceae previously, validating the dormancy classification approach adopted during the first part of this study.

Building upon these insights further, in Chapter 3 I investigated the germination response of five species to variations in stratification and incubation temperatures and the duration of exposure to these conditions. Warm stratification was found to occur under water stresses as low as -0.8 Mpa and was most effective at 30°C, rather than 20°C or 25°C. Where warm stratification alleviated physiological dormancy, the window of temperatures able to support germination widened in both *Diplolaena dampieri* and *Rhadinothamnus anceps* though these germination windows opened in different directions allowing the type of non-deep physiological seed dormancy to be assigned (Baskin & Baskin 2000). Interestingly, the ceiling temperature for germination for the seeds of *D. dampieri* increased (from 15°C to 20°C; Chapter 3, Fig. 2) as dormancy was lost, whereas for *R. anceps* seeds a decrease in the base temperature required for germination (from 15°C to 10°C; Chapter 3, Fig. 2, and from 25°C to 18°C; Chapter 3, Table 2) was observed as seed became relatively less dormant. These shifts in temperature requirements provided evidence for type 1 and type 2 non-deep physiological dormancy (PD) in *D. dampieri* and *R. anceps* respectively. Following from this finding I hypothesised that in the context of the southwestern Australian climate, type 1 non-deep PD constitutes a risk-taking strategy, allowing species to germinate earlier in the season and gain access to resource and competitive advantages at the risk of drought mortality as the climate transitions into a winter rainfall pattern. In comparison, type 2 non-deep PD restricts seed germination to cooler temperatures more likely to occur further into the germination season, allowing species to risk-avoid unpredictable conditions in the early germination season. This work highlights the need to better understand the type of non-deep physiological

dormancy present in southwest Australian species as a tool to better manage species as well as the importance of understanding the underlying ecological drivers governing germination, seedling recruitment and species persistence.

In Chapter 4, I investigated the change in dormancy status of four study species overtime by periodically (monthly for three months) undertaking a germination test on seeds stored under warm dry laboratory conditions or buried in the field in various ways. By controlling storage temperature and humidity in the lab and limiting seed exposure to changes in soil moisture in the field, I tested the hypothesis that fluctuating hydrothermal conditions in the field, rather than constant warm dry conditions often used in lab-based afterripening treatments, drive dormancy loss in study species. *Philotheca spicata* and *Rhadinothamnus anceps* showed slightly improved germination over the duration (~6 months) of the experiment however, there was no clear preference for a particular treatment, although *R. anceps* did not germinate to any proportion in hermetically sealed bags which excluded moisture, hinting at a requirement for dormancy release by warm stratification as demonstrated in Chapters 2 and 3. *Boronia fastigiata* germinated to the greatest proportions observed here when retrieved from hermetically sealed bags which excluded all soil moisture during soil burial. This finding is significant as it suggests this species requires periods of warm to hot dry conditions for the alleviation of dormancy, rather than fluctuations in seed moisture status, and that constant 30°C afterripening temperatures often used in seed germination studies are insufficient for this species. During burial soil temperatures were observed to rise to 72°C during summer, well above the 30°C temperatures used in the laboratory. Contrastingly, *Crowea angustifolia* showed a significant preference for burial in mesh bags, and a requirement for heat shock (90°C for 10 mins). Therefore, it is likely that this species requires the direct passage of fire, in combination

with wet/dry cycling in the lead up to the germination season for the alleviation of dormancy. The surprisingly varied response of the four study species to *in situ* burial under dry (hermetically sealed bags) or natural (mesh bags) conditions highlights species specific requirements for dormancy alleviation in the field that may be challenging to replicate in a laboratory environment particularly if simple, broadly applicable treatments are to be applied across many different Rutaceae species.

5.2 Implications

The requirement for vast quantities of seeds to supply current and future restoration programs across Australia is set to place immense pressure on wild plant populations (Commander 2021; Hancock *et al.* 2020; Van Moort 2021). Significant work is required to ensure that seed supply can meet demand, and, in this thesis, I have demonstrated how a better understanding of seed biology can help to design germination protocols that are effective for species previously unavailable to seed-based restoration, while enabling more efficient use of seed. By increasing the diversity of species available to restoration it may be possible to reduce the pressure applied to those species with more easily germinated seeds.

In Chapter 2 I identified physiological dormancy as a barrier to on-demand germination in eight members of the Rutaceae and demonstrated that gibberellic acid and warm stratification can overcome this barrier. The application of gibberellic acid employed here used high concentrations of lab-grade gibberellic acid in a controlled and sterile environment. While this method is not ideal for large scale restoration programs, there exists commercially available products (e.g., ProGibb, Sumitomo Chemical Company) that could be used as a pre-treatment prior to sowing under nursery or field conditions. This may be a useful technique to establish problematic species however, it bypasses an understanding of seed ecology and the natural cues of dormancy alleviation that

are required to establish healthy restoration sites able to maintain ongoing recruitment. While it is likely that the application of gibberellic acid will be effective for a greater range of physiologically dormant species, application of warm stratification through careful timing of seed sowing and watering, or using temperature-controlled incubators and horticultural heat mats/beds (Turner *et al.* 2021a), is a method requiring an understanding of species ecology that may produce a more resilient result. Further, there are a range of species such as *Acanthocarpus preissii* (Turner *et al.* 2006a), *Lomandra preissii* (Merritt *et al.* 2007), and *Persoonia longifolia* (Chia *et al.* 2016; Norman and Koch 2006) where applications of warm stratification have proven effective where gibberellic acid has not. Similarly, smoke and particularly butenolides present in smoke, (i.e., Karrikins; Flematti *et al.* 2004) are additional treatments based upon seed ecology that can be applied to large quantities of seed and is often effective in producing germination of certain species, notably many understorey taxa that form persistent soil seedbanks and which can be difficult to germinate (Dixon *et al.* 1995; Roche *et al.* 1997a). In Chapter 4 I showed that KAR_1 was ineffective in study species, and that smoke, rather than KAR_1, was required to produce germination. This result while unexpected is nevertheless unsurprising as a range of smoke responsive native species have been shown to be stimulated by chemicals in smoke other than KAR_1, (i.e., *Andersonia latiflora, Anigozanthos manglesii, Conostylis candicans* and *Ficinia nodosa*; Flematti *et al.* 2011). While this was not explored in the current thesis beyond confirmation that smoke stimulates germination of non-dormant seeds though KAR_1 does not, it does pose some intriguing research questions to be explored in future as to the nature of the stimulant found in smoke that promotes germination in species of Rutaceae, and whether this observation is universal across all Rutaceae that are smoke responsive.

In chapter 3 I further explored the role of warm stratification in the dormancy alleviation and germination of five Rutaceae and demonstrated its effectiveness in *Diplolaena dampieri* and *Rhadinothamnus anceps*. Warm stratification has proven effective in dormancy alleviation of a diverse range of Western Australian species, including *Acanthocarpus preissii* (Asparagaceae) (Turner *et al.* 2006a), *Lomandra preissii* (Asparagaceae) (Merritt *et al.* 2007), *Persoonia longifolia* (Proteaceae) (Chia *et al.* 2016; Norman and Koch 2006), *Byblis gigantea* (Byblidaceae) (Cross *et al.* 2013), *Glossostigma trichodes* (Phrymaceae) (Tuckett *et al.* 2010), various species of *Hibbertia* (Dilleniaceae) (Hidayati *et al.* 2012), as well as *Marianthus erubescens* (Pittosporaceae) and *Patersonia occidentalis* (Iridaceae) (Fontaine 2013). For each of these species, dormancy alleviation and germination were found to increase with increasing duration of warm stratification up to a point usually around 6 to 8 weeks in duration. In Chapter 3 I also demonstrated increasing dormancy loss with increasing stratification time, however, there was a negative impact of stratification beyond 8 weeks on germination of both *Diplolaena dampieri* and *Rhadinothamnus anceps* as seeds either perished under the extended warm moist conditions, or re-entered dormancy (Finch-Savage and Leubner-Metzger 2006). The ability of warm stratification to alleviate physiological dormancy is still yet to be widely explored in Western Australian species and its effectiveness thus far warrants its further investigation in a wider range of taxa. Of particular importance is the shift in optimum temperatures required for germination following warm stratification observed in *Diplolaena dampieri* and *Rhadinothamnus anceps*. That these species shifted their thermal requirements for germination during dormancy loss is not a newly discovered phenomena (Baskin and Baskin 1989; Baskin and Baskin 1980; Soltani *et al.* 2017; Steadman and Pritchard 2004) however, it is a process that has been poorly

investigated in southwest Australian species. Therefore, careful consideration must be given when reporting optimum germination temperatures for species with physiological dormancy. Here I found a ~7°C decrease in the optimum temperature (decreased from 25.46°C to 18.65°C) for germination of *R. anceps* after eight weeks of warm stratification, and a lack of understanding of this phenomenon could lead to failed germination attempts in the future. Further, a shift in requirements for germination in response to warm moist conditions in the lead up to the germination season has significant implications for species management under climate change as small shifts in rainfall patterns or soil temperatures at critical times may have profound consequences on germination timing and seedling emergence (Cochrane 2016; Cochrane *et al.* 2015; Wu *et al.* 2019).

The evidence for climate warming is now unequivocal with far ranging impacts now expected based on our current levels of CO_2 emissions (Huang *et al.* 2018). Globally, climate change is predicted to bring changed rainfall patterns and increasing temperatures and evaporation (Chen *et al.* 2020). In southwestern Australia predictions are consistent with global trends and it is expected that the region will experience consistent drying and warming as the century progresses (Asseng and Pannell 2013; Chen *et al.* 2020; Yates *et al.* 2010). Seeds of *R. anceps* warm stratified at either 20°C or 25°C for 1–12 weeks showed far less improvement in germination compared to stratification at 30°C (supplementary Fig. 3.3), indicating that dormancy alleviation by warm stratification for a portion of the seed cohort of this species occurs above 25°C. Higher stratification temperatures (i.e., 30°C) are more likely in years with earlier seasonal rainfall, as the hydrological threshold for stratification is met before seasonal decreases in temperature. Variation in the thermal threshold for stratification among individual seeds of *R. anceps* allows dormancy loss in a greater number of

seeds in such years, while giving a portion of seeds the ability to remain dormant if rainfall does not occur earlier in the season. In years without earlier rainfall seeds possessing lower thermal thresholds for stratification may still become non-dormant and germinate. Under a warming climate it is likely that the conditions conducive to warm stratification will become more frequent when soil moisture is available, though whether soil moisture persists for enough time while the soil temperature is appropriate for warm stratification remains to be seen. Increasing durations of warm stratification caused a progressive reduction in the optimum temperature under which *R. anceps* will germinate. If climate change was to provide greater frequency of warm stratification to soil-stored seeds, it is likely that this shift in optimum temperature would limit germination until temperatures sufficiently decreased or seeds re-entered PD. What effect increased durations of warm stratification would have on optimum germination temperature of seeds with type 1 non-deep PD (i.e., *D. dampieri*) was unable to be investigated in depth in the present thesis due to a lack of seed. However, the increase in ceiling temperatures for germination observed in *D. dampieri* suggests seeds would be unable to risk-avoid by lowering the thermal threshold for germination and would instead germinate at warmer temperatures. Critical to the response of warm stratification mediated dormancy loss under a warming climate is the hydrological threshold below which stratification cannot occur. Our results showed that water stress of -0.8 MPa had no effect on dormancy loss by warm stratification. While increasing temperatures may extend the window in which moist seeds undergo warm stratification, increasing frequency of drought will limit not only the ability to seeds to germinate, but the processes that drive dormancy alleviation by warm stratification.

5.3 Future directions

In Chapter 1 of this thesis, I identified taxa in Western Australia that are currently unavailable to seed based restoration due to complex dormancy alleviation requirements. This list comprises species with physiological seed dormancy that have produced poor results in the published literature and would benefit from future research using the techniques and processes detailed throughout this thesis. The results in Chapters 2, 3 and 4 make it clear that no single treatment will work consistently across the Rutaceae, and this will likely be the same for each family detailed in Supplementary material 1. Further study is required to elucidate the hydrothermal conditions required to alleviate dormancy and support germination on a species-by-species basis, and careful attention should be paid to the conditions of temperature and moisture that seeds are likely to experience in soil. This thesis (Chapter 2 & 4) provides detail on the temperature and moisture conditions experienced by seeds in soil during dormancy loss and germination. These records, while insightful, should be used in conjunction with previously published reports that describe the soil conditions over all seasons and how these impact seed moisture content (Merritt *et al.* 2007; Turner *et al.* 2006a) to determine lab and field-based treatments for further studies.

Further, there is a critical need to establish the hydrothermal thresholds for stratification of southwest Australian species, as this is a step towards understanding the mechanisms of dormancy alleviation and understanding limitations to species recruitment that occur *in situ* and form barriers to successful return of species in restoration. Similarly, the role of dry conditions, and the rapid fluctuation between dry and imbibed seed states that occur over the year (often termed wet/dry cycling) need to be investigated in conjunction with stratification to better understand the processes

that drive dormancy alleviation. While lab-based experiments are critical to the development of dormancy alleviation and germination protocols, it is also important to incorporate field- and nursery-based experiments into future efforts. Ultimately, the goal of overcoming complex seed dormancy for the purposes of using seeds in restoration activities is to produce plants from broadcast seed in a field or nursery setting. While laboratory and field-based experiments can elucidate and quantify the conditions required for dormancy alleviation and germination, translating these phenomena into treatments usable at scale is rarely the focus of seed research. To ensure restoration can meet current and future requirements for biodiverse return of species it is imperative that understanding of seed biology is translated into treatments able to be effectively employed by industry.

5.4 Conclusion

The findings of this thesis highlight the complexity of seed dormancy and the importance of understanding seed ecology to achieve greater diversity in the species we can return in ecological restoration and establish in horticulture. Depending on the species of Rutaceae in question, methods for on-demand germination are available; however, significantly more work is required to scale up the methodology by which these species can be implemented in restoration seeding or incorporated in nursery production. Greater investigation into warm stratification, wet/dry cycling and the effect of naturally indicative hydrothermal conditions is required on a greater range of species possessing seeds with intractable dormancy. The success of these treatments here and elsewhere (Chia *et al.* 2016; Cross *et al.* 2013; Dalziell *et al.* 2018; Hidayati *et al.* 2012; Merritt *et al.* 2007; Turner *et al.* 2006a) suggest species previously unavailable due to an inability to produce germination on-demand will yield positive

germination results when subjected to careful application of temperature and moisture regimes across multiple seasons.

Notably, this thesis has identified that a shift in thermal requirements for germination occurs during dormancy alleviation, a finding that sheds light on the complexity and difficulty of inducing germination in seeds with physiological dormancy. Investigation of the hydrothermal thresholds of physiological dormancy alleviation, and the impacts this has on requirements for germination will uncover key aspects of the environment that mediate successful seedling recruitment. This will have significant implications for how and when we introduce broadcast seed to restoration sites, how we manage species under a warming climate, and will help to develop protocols for *ex situ* dormancy alleviation and plant production.

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Appendix

Appendix 1 Supplementary data for Chapter 1

Appendix 1.1 Families and genera shown to have at least one species with low (<30%) germination percentages in response to dormancy breaking and germination stimulating treatments or having been cited as difficult-to-germinate. Species with known complex biology (e.g., Orchidaceae) or physical seed dormancy have been excluded.

Family	Genera	Reference
Anarthriaceae	*Lyginia*	(Maher *et al.* 2008)
Apiaceae	*Xanthosia*	(Cromer 2007; Maher *et al.* 2008)
Araliaceae	*Astrotricha*	(Erickson 2015)
Araliaceae	*Trachymene*	(Cromer 2007; Dwyer and Erickson 2016; Erickson 2015; Erickson *et al.* 2018; Hidayati *et al.* 2019)
Asparagaceae	*Acanthocarpus*	(Commander *et al.* 2009b; Cromer 2007; Turner *et al.* 2006a)
Asparagaceae	*Laxmannia*	(Cromer 2007; Roche *et al.* 1997a)
Asparagaceae	*Lomandra*	(Cromer 2007; Merritt *et al.* 2007; Norman *et al.* 2006; Rokich *et al.* 2002)
Asparagaceae	*Sowerbaea*	(Cromer 2007; Maher *et al.* 2008; Roche *et al.* 1997a; Tieu *et al.* 2001a)
Asparagaceae	*Thysanotus*	(Bell *et al.* 1995; Cromer 2007; Maher *et al.* 2008; Norman *et al.* 2006; Rokich *et al.* 2002)
Asteraceae	*Polycalymma*	(Roche *et al.* 1997a)
Asteraceae	*Quinetia*	(Cromer 2007; Maher *et al.* 2008)
Asteraceae	*Podolepis*	(Bunker 1994; Clarke *et al.* 2000; Dwyer and Erickson 2016; Morgan 1998; Norman *et al.* 2006)
Asteraceae	*Trichocline*	(Cromer 2007; Norman *et al.* 2006; Roche *et al.* 1997a; Roche *et al.* 1997b)
Boraginaceae	*Trichodesma*	(Erickson 2015; Erickson *et al.* 2018) (Jurado and Westoby 1992)
Brassicaceae	*Lepidium*	(Erickson 2015; Erickson *et al.* 2018)
Campanulaceae	*Lobelia*	(Cromer 2007)
Campanulaceae	*Wahlenbergia*	(Cromer 2007)
Campanulaceae	*Isotoma*	(Norman *et al.* 2006)
Celastraceae	*Stackhousia*	(Cromer 2007)
Celastraceae	*Tripterococcus*	(Cromer 2007; Norman *et al.* 2006)
Chenopodiaceae	*Atriplex*	S. Turner Pers. Comm (2021)
Chenopodiaceae	*Dysphania*	(Commander *et al.* 2017; Erickson 2015)
Chenopodiaceae	*Maireana*	(Cromer 2007; Merino-Martín *et al.* 2017)
Colchicaceae	*Burchardia*	(Cromer 2007; Norman *et al.* 2006)
Colchicaceae	*Wurmbea*	(Cochrane *et al.* 2002; Cromer 2007)
Cyperaceae[1]	*Baumea*	(Cromer 2007; Maher *et al.* 2008)

Cyperaceae [1]	*Cyathochaeta*	(Norman *et al.* 2006)
Cyperaceae [1]	*Eleocharis*	(Cochrane *et al.* 2002)
Cyperaceae [1]	*Gahnia*	(Roche *et al.* 1997a)
Cyperaceae [1]	*Lepidosperma*	(Cromer 2007; Maher *et al.* 2008; Roche *et al.* 1997b; Turner 2013)
Cyperaceae [1]	*Mesomelaena*	(Cromer 2007; Maher *et al.* 2008)
Cyperaceae [1]	*Schoenus*	(Cromer 2007)
Cyperaceae[1]	*Tetraria*	(Cromer 2007; Maher *et al.* 2008; Roche *et al.* 1997a)
Dasypogonaceae	*Calectasia*	S. Turner Pers. Comm
Dasypogonaceae	*Dasypogon*	(Cromer 2007; Maher *et al.* 2008)
Dilleniaceae	*Hibbertia*	(Dalziell *et al.* 2018; Hidayati *et al.* 2012; Maher *et al.* 2008; Norman *et al.* 2006; Rokich *et al.* 2002)
Elaeocarpaceae	*Tetratheca*	(Cromer 2007; Norman *et al.* 2006)
Ericaceae[2]	*Andersonia R.Br.*	(Bell *et al.* 1995; Cochrane *et al.* 2002; Cromer 2007; Dixon *et al.* 1995; Flematti *et al.* 2011; Just 2018; Norman *et al.* 2006; Roche *et al.* 1997a)
Ericaceae[2]	*Astroloma R.Br.*	(Bell *et al.* 1993; Chia *et al.* 2016; Cromer 2007; Dixon *et al.* 1995; Just 2018; Koch 2007b; Rokich *et al.* 2002; Turner *et al.* 2009a)
Ericaceae[2]	*Brachyloma*	(Dixon *et al.* 1995)
Ericaceae[2]	*Conostephium*	(Just 2018)
Ericaceae[2]	*Leucopogon*	(Allan *et al.* 2004; Bell *et al.* 1995; Commander 2008; Cromer 2007; Just 2018; Maher *et al.* 2008; Merritt *et al.* 2007; Norman and Koch 2008; Norman *et al.* 2006; Roche *et al.* 1997a)
Goodeniaceae	*Brunonia*	(Merino-Martín *et al.* 2017; Roche *et al.* 1997a)
Goodeniaceae	*Damperia*	(Maher *et al.* 2008)
Goodeniaceae	*Goodenia*	(Cromer 2007; Erickson 2015)
Goodeniaceae	*Lechenaultia*	(Cromer 2007; Maher *et al.* 2008; Norman *et al.* 2006; Rokich *et al.* 2002)
Goodeniaceae	*Scaevola*	(Cromer 2007; Maher *et al.* 2008)
Gyrostemonaceae	*Codonocarpus*	(Baker *et al.* 2005b)
Gyrostemonaceae	*Gyrostemon*	(Baker *et al.* 2005b; Downes *et al.* 2013)
Gyrostemonaceae	*Tersonia*	(Baker *et al.* 2005b; Bell *et al.* 1993)
Haemodoraceae	*Anigozanthos*	(Light *et al.* 2014; Maher *et al.* 2008) (Tieu *et al.* 2001a)
Haemodoraceae	*Conostylis*	(Cromer 2007; Maher *et al.* 2008; Norman *et al.* 2006; Roche *et al.* 1997a; Rokich *et al.* 2002) (Turner *et al.* 2009b) (Downes *et al.* 2015) (Turner *et al.* 2009b)
Haemodoraceae	*Macropidia*	(Roche *et al.* 1997a; Tieu *et al.* 1999)

Haloragaceae	*Glischrocaryon*	(Cromer 2007; Norman *et al.* 2006)
Haloragaceae	*Gonocarpus*	(Cromer 2007; Norman *et al.* 2006)
Hemerocallidaceae	*Agrostocrinum*	(Cromer 2007) (Norman *et al.* 2006)
Hemerocallidaceae	*Tricoryne*	(Cromer 2007; Norman *et al.* 2006; Roche *et al.* 1997a)
Hemerocallidaceae	*Johnsonia*	(Cromer 2007)
Hemerocallidaceae	*Stypandra*	(Cromer 2007)
Hemerocallidaceae	*Dianella*	(Cromer 2007; Hodges *et al.* 2019; Maher *et al.* 2008; Norman *et al.* 2006)
Hemerocallidaceae	*Corynotheca*	S. Turner Pers. Comm (2021)
Hemerocallidaceae	*Caesia*	(Maher *et al.* 2008)
Iridaceae	*Orthrosanthus*	(Norman *et al.* 2006) (Cromer 2007) (Cochrane *et al.* 2002)
Iridaceae	*Patersonia*	(Bell *et al.* 1995; Cromer 2007; Maher *et al.* 2008; Norman *et al.* 2006)
Lamiaceae	*Hemiandra*	(Cromer 2007) (Cochrane *et al.* 2002)
Lamiaceae	*Hemigenia*	(Cromer 2007; Norman *et al.* 2006)
Myrtaceae[5]	*Calytrix*	(Cromer 2007)
Myrtaceae[5]	*Chamelaucium*	(Cromer 2007)
Myrtaceae[5]	*Hypocalymma*	(Bell *et al.* 1995; Cromer 2007; Norman *et al.* 2006)
Myrtaceae[5]	*Scholtzia*	(Maher *et al.* 2008)
Myrtaceae[5]	*Verticordia*	(Cochrane *et al.* 2002; Cromer 2007; Maher *et al.* 2008; Rokich *et al.* 2002) (Cochrane *et al.* 2002)
Pittosporaceae	*Billardiera*	(Cromer 2007)
Pittosporaceae	*Marianthus*	(Bell *et al.* 1995; Cromer 2007) Merritt *et al.* 2007, Cromer 2007)
Pittosporaceae	*Billardiera* (syn. *Sollya*)	(Bell *et al.* 1987; Cromer 2007; Norman *et al.* 2006) (Roche *et al.* 1997a; Roche *et al.* 1997b)
Poaceae	*Amphipogon*	(Maher *et al.* 2008) (Cromer 2007)
Poaceae	*Austrostipa*	(Cromer 2007; Maher *et al.* 2008; Turner *et al.* 2009b)
Poaceae	*Cynodon*	(Erickson *et al.* 2018)
Poaceae	*Enneapogon*	(Erickson 2015)
Poaceae	*Eragrostis*	(Erickson 2015)
Poaceae	*Neurachne*	(Norman *et al.* 2006) (Gray *et al.* 2019b) (Cromer 2007)
Poaceae	*Paraneurachne*	(Erickson 2015)
Poaceae	*Tetrarrhena*	(Cromer 2007; Norman *et al.* 2006)
Polygalaceae	*Comesperma*	(Cromer 2007; Rokich *et al.* 2002)
Proteaceae	*Adenanthos*	(Cromer 2007; Maher *et al.* 2008; Rokich *et al.* 2002; Tieu *et al.* 1999; Tieu *et al.* 2001a)
Proteaceae	*Conospermum*	(Cromer 2007; Krauss *et al.* 2006)
Proteaceae	*Grevillea*	(Baker *et al.* 2005b; Cromer 2007)

Proteaceae	*Isopogon*	(Cromer 2007)
Proteaceae	*Persoonia*	(Cromer 2007; Maher *et al.* 2008) (Chia *et al.* 2016) (Mullins *et al.* 2002)
Proteaceae	*Stirlingia*	(Cromer 2007)
Proteaceae	*Synaphea*	(Cromer 2007)
Ranunculaceae	*Clematis*	(Cromer 2007; Norman *et al.* 2006)
Restionaceae[3]	*Alexgeorgea*	(Maher *et al.* 2008; Rokich *et al.* 2002)
Restionaceae[3]	*Desmocladus*	(Maher *et al.* 2008; Rokich *et al.* 2002)
Restionaceae[3]	*Dielsia*	(Maher *et al.* 2008)
Restionaceae[3]	*Hypolaena*	(Maher *et al.* 2008)
Restionaceae[3]	*Lepidobolus*	(Bell *et al.* 1993)
Restionaceae[3]	*Loxocarya*	(Cromer 2007; Koch 2007b; Rokich *et al.* 2000; Tieu *et al.* 2001a)
Rubiaceae	*Opercularia*	(Cromer 2007; Maher *et al.* 2008; Roche *et al.* 1997b; Rokich *et al.* 2002)
Rutaceae[4]	*Boronia*	(Cromer 2007; Dixon *et al.* 1995)
Rutaceae[4]	*Correa*	(Roche *et al.* 1997a)
Rutaceae[4]	*Crowea*	(Cromer 2007)
Rutaceae[4]	*Philotheca*	(Maher *et al.* 2008; Norman *et al.* 2006)
Santalaceae	*Leptomeria*	(Cromer 2007; Norman *et al.* 2006)
Scrophulariaceae	*Eremophila*	(Richmond and Ghisalberti 1994)
Scrophulariaceae	*Myoporum*	S. Turner unpublished data
Scrophulariaceae	*Verbascum*	(Cromer 2007)
Solanaceae	*Symonanthus*	S. Turner unpublished data (2021)
Solanaceae	*Solanum*	(Commander *et al.* 2008; Erickson 2015)
Thymelaeaceae	*Pimelea*	(Cromer 2007)
Violaceae	*Hybanthus*	(Cromer 2007; Norman *et al.* 2006)
Xanthorrhoeaceae	*Chamaescilla*	(Allan *et al.* 2004)

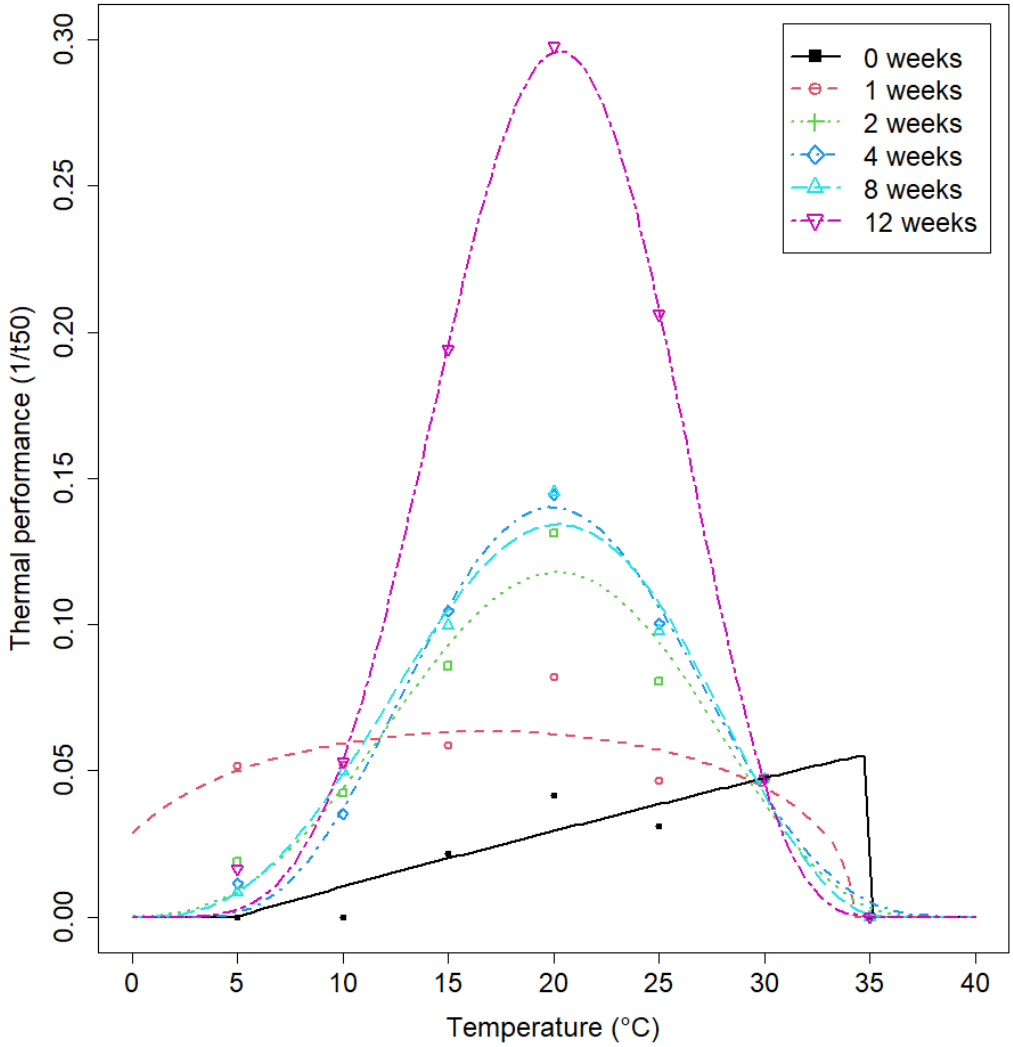
[1]Majority of nutlet Cyperaceae are difficult to germinate. [2]All of the Restionaceae are difficult, partially due to deep dormancy though small seeded and some large seeded species are smoke responsive. [3]All of the woody fruit Ericaceae can be considered difficult due to dormancy imposed by the endocarp and endosperm. [4] Most Rutaceae are considered difficult to germinate [5] Difficult to germinate taxa appear to be from subfamily Myrtoideae & tribe Chamelaucieae i.e., *Actinodium, Aluta, Astartea, Baeckea, Calytrix, Chamelaucium, Darwinia, Hypocalymma, Pileanthus, Scholtzia, Thryptomene, Verticordia* (S. Turner Pers. Comm, 2021).

Appendix 2 Supplementary data for Chapter 2

Appendix 2.2 Output of binary logistic regression assessing the main and interaction effects of treatment (control, heat, smoke, or smoke + heat) and temperature (10°C, 20°C or 30°C constant or 6 weeks cold or warm stratification) on germination success in 8 species of southwest Australian Rutaceae.

Species	Variable	$\chi 2$	Df	sig
B. cymosa	Treatment	393.72	2	***
	Temperature	127.42	4	***
	Treatment: Temperature	2.96	8	
B. fastigiata	Treatment	403.35	2	***
	Temperature	157.57	4	***
	Treatment: Temperature	3.62	8	
B. ovata	Treatment	105.822	2	***
	Temperature	28.063	4	***
	Treatment: Temperature	0	8	
B. ramosa	Treatment	256.578	2	***
	Temperature	74.379	4	***
	Treatment: Temperature	0	8	
C. angustifolia	Treatment	256.578	2	***
	Temperature	74.379	4	***
	Treatment: Temperature	0	8	
D. angustifolia	Treatment	336.17	2	***
	Temperature	87.11	4	***
	Treatment: Temperature	80.16	8	***
P. spicata	Treatment	112.674	2	***
	Temperature	60.916	4	***
	Treatment: Temperature	6.948	8	
R. anceps	Treatment	218.168	2	***
	Temperature	278.119	4	***
	Treatment: Temperature	54.475	8	***

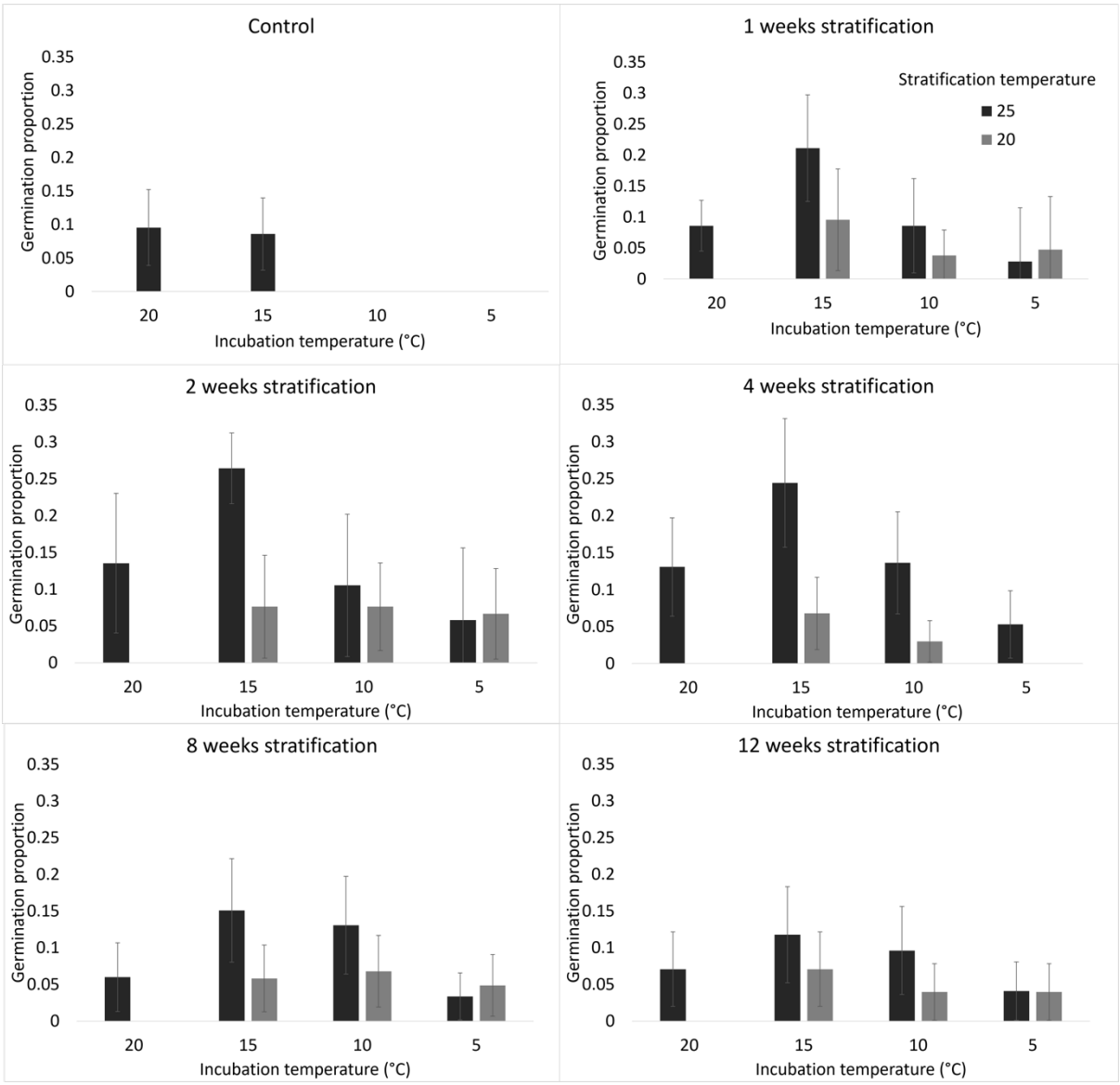
Significance codes: 0 '***'



Appendix 3.3 Thermal performance for *Rhadinothamnus anceps*. Symbols represent the average 1/t50 estimate for seeds at 5-30°C incubation temperatures following increasing duration of stratificaiton. Lines represent the permutations of the beta thermal performance curve fittered to the maximum germination proportion estimate (rmax) for each stratification duration.

Appendix 3.4 AIC rankings obtained from the *AICcmodavg* package (Mazerolle, 2013) for each model applied to each parameter.

Parameter	Model	df	AIC
Germination proportion	Beta	31	-154.6
Germination proportion	Broken stick	25	-113.5
Germination proportion	Yan and hunt	19	-166.4
1/t50	Beta	31	-233.6
1/t50	Broken stick	25	-142.4
1/t50	Yan and hunt	19	-153.6



Appendix 3.5 Germiantion proportion (±95% confidence interval) of *Rhadinothamnus anceps* seeds at 5, 10, 15 or 20°C following warm stratification at either 20 or 25°C for 0, 1, 2, 4, 8 and 12 weeks.

Appendix 4 Supplementary data for Chapter 4

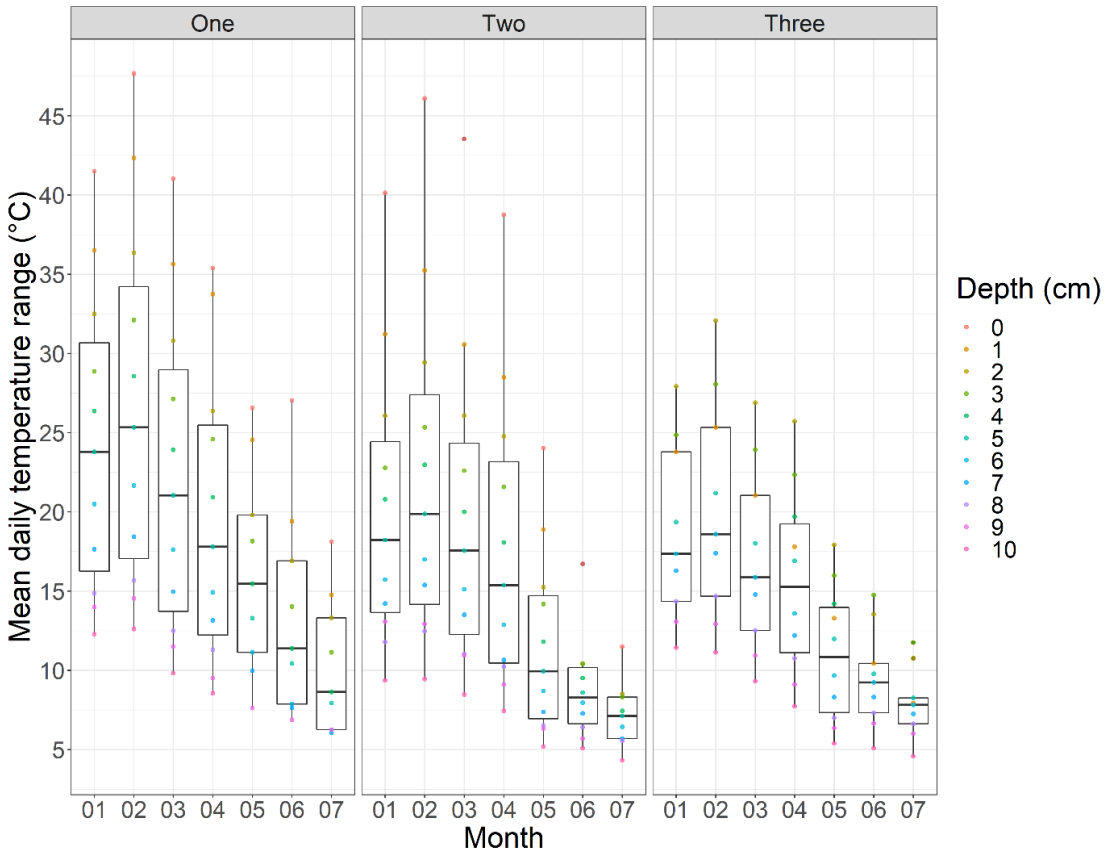
Appendix 4.6 Seed releative humidity recorded at specific dates, times and ambient temperatures for four species of Rutaceae buired at 1 cm in remnant Banksia woodland at Curtin Universtiy, Perth, Western Australia.

	Species					
Date	*Boronia fastigiata*	*Crowea angustifolia*	*Philotheca spicata*	*Rhadinothamnus anceps*	Temperature (°C)	Time
26/02/2021	7.5	6.9	5.6	6.03	29.48	1430
5/03/2021	86.15	84.7	88.5	84.8	26.63	1030
11/03/2021	30.91	32.52	31.81	30.11	27.6	1040
18/03/2021	28.13	44.8	32.4	34.74	30.05	1030
25/03/2021	11.5	15.94	12.36	12.82	29.38	1300
1/04/2021	18.66	24.63	22.7	25.5	38.03	1322
8/04/2021	65.22	59.76	73.42	64.77	29.27	1120
16/04/2021	44.92	30.23	31.74	44.41	26.45	1530
23/04/2021	17.92	25.55	25.4	22.99	29.28	1300
4/05/2021	89.95	93.92	92.96	90.32	23.67	1240

Appendix 4.7 Model statistics assessing differences in soil temperature ranges at 1 cm increments from the soil surface (0cm) to a depth of 10cm.

Contrast	estimate	SE	d.f.	t.ratio	p.value
0-1	8.461	1.13	191	7.486	<.0001
0-2	9.958	1.104	191	9.018	<.0001
0-3	12.271	1.104	191	11.113	<.0001
0-4	14.99	1.161	191	12.914	<.0001
0-5	17.082	1.104	191	15.47	<.0001
0-6	19.269	1.104	191	17.451	<.0001
0-7	20.668	1.104	191	18.718	<.0001
0-8	22.451	1.146	191	19.592	<.0001
0-9	23.194	1.117	191	20.772	<.0001
0-10	24.918	1.146	191	21.745	<.0001
1-2	1.497	1.004	191	1.491	0.9211
1-3	3.809	1.004	191	3.796	0.0089
1-4	6.529	1.082	191	6.034	<.0001
1-5	8.621	1.004	191	8.59	<.0001
1-6	10.807	1.004	191	10.769	<.0001
1-7	12.206	1.004	191	12.163	<.0001
1-8	13.989	1.044	191	13.396	<.0001
1-9	14.733	1.015	191	14.52	<.0001
1-10	16.456	1.044	191	15.759	<.0001
2-3	2.313	0.977	191	2.367	0.3933
2-4	5.032	1.056	191	4.764	0.0002
2-5	7.124	0.977	191	7.292	<.0001
2-6	9.311	0.977	191	9.53	<.0001
2-7	10.709	0.977	191	10.962	<.0001
2-8	12.493	1.019	191	12.258	<.0001
2-9	13.236	0.99	191	13.375	<.0001
2-10	14.96	1.019	191	14.678	<.0001
3-4	2.72	1.056	191	2.575	0.2696
3-5	4.811	0.977	191	4.925	0.0001
3-6	6.998	0.977	191	7.163	<.0001
3-7	8.397	0.977	191	8.595	<.0001
3-8	10.18	1.019	191	9.988	<.0001
3-9	10.924	0.99	191	11.038	<.0001
3-10	12.647	1.019	191	12.409	<.0001
4-5	2.092	1.056	191	1.98	0.6628
4-6	4.278	1.056	191	4.05	0.0035
4-7	5.677	1.056	191	5.374	<.0001
4-8	7.46	1.098	191	6.792	<.0001
4-9	8.204	1.069	191	7.678	<.0001
4-10	9.927	1.098	191	9.038	<.0001
5-6	2.187	0.977	191	2.238	0.4809
5-7	3.585	0.977	191	3.67	0.0137
5-8	5.368	1.019	191	5.268	<.0001
5-9	6.112	0.99	191	6.176	<.0001

5-10	7.835	1.019	191	7.688	<.0001
6-7	1.399	0.977	191	1.432	0.9389
6-8	3.182	1.019	191	3.122	0.0735
6-9	3.926	0.99	191	3.967	0.0048
6-10	5.649	1.019	191	5.543	<.0001
7-8	1.783	1.019	191	1.75	0.8076
7-9	2.527	0.99	191	2.553	0.2811
7-10	4.25	1.019	191	4.17	0.0022
8-9	0.744	1.031	191	0.721	0.9997
8-10	2.467	1.055	191	2.338	0.4128



Appendix 4.8 Model statistics assessing differences in soil temperature ranges at 1 cm increments from the soil surface (0cm) to a depth of 10cm.