The reproductive ecology of the Pepperbark tree (*Warburgia salutaris*) in the Kruger National Park, South Africa

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Declaration

I, Kaylee van den Bosch, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Abstract

Warburgia salutaris, the pepperbark tree, is an endangered tree that is greatly valued in South Africa for its use in traditional medicine. Over-exploitation for medicinal purposes, habitat degradation and loss have all contributed to the species being listed as IUCN Endangered. In the Kruger National Park (KNP), *W. salutaris* fruit production is low and only produced on a few trees and there is a very low proportion of juveniles, suggesting that recruitment is extremely limited in this population. Therefore, in this thesis, the reproductive ecology of the KNP population is studied and compared with a reproductively successful orchard population in Eston (KwaZulu-Natal), to investigate factors that may contribute to limited sexual output in the former.

The aim of the first part of the study was to understand the pollination biology of *W. salutaris*. Potential pollinators were observed and collected for further analysis; nectar volume and sugar concentration were measured; five pollination treatments – autonomous self-, geitonogamous self-, cross-, natural- and natural-bagged pollination (i.e. naturally pollinated flowers were bagged to protect from seed predation) – were set-up to elucidate the breeding system; pollen viability was assessed, and pollen tube growth was compared between pollination treatments. The aim of the second part of the study was to understand the seed ecology of *W. salutaris*. Immature fruit were bagged to assess fruit development in the KNP population and Eston orchard; fruit were analysed to assess the effect of pre-dispersal seed predation and identify seed predators; frugivore activity was observed in Leshiba Game and Nature Reserve (GNR), Limpopo, to identify seed dispersers and assess the mode of dispersal; and seedling emergence trials were conducted to compare seed germination between the natural KNP and Leshiba GNR populations and the Eston orchard.

Results showed that *W. salutaris* flowers are visited by a variety of insects and Lepidoptera (butterflies and moths) are the most likely effective pollinators. Average nectar volume and sucrose concentration was 2.43 μl and 39.74%, respectively. *Warburgia salutaris* is likely self-incompatible as self-pollinated flowers in neither KNP nor Eston resulted in fully developed fruit. In KNP, zero mature fruit were produced from either self- or cross-pollination; however, a small percentage of self (5%) and cross (2%) pollinated flowers were fertilised and then aborted early in development. Similarly, 94% of flowers that began to form fruit in the natural-bagged treatment were aborted. Only two trees in one KNP sub-population produced and sustained fully developed fruit through natural pollination in 2019. In 2020, the same two trees and an additional untagged

tree successfully developed fruit. Pollen viability was high (66–93%) across the five KNP sub-populations and Eston, and percentage pollen tube occurrence for self- and cross-pollination was higher in Eston (self: 60%, cross: 100%) than KNP (self: 33%, cross: 33%), and both self and cross pollen tubes reached the ovules.

Bagged immature fruit were aborted early in development in the KNP population and Eston orchard, and in KNP successfully developed fruit were only present on three of 10 trees in one sub-population. *Ceratitis cosyra*, the marula fly, attacks *W. salutaris* fruit in KNP, Eston, and Leshiba GNR. However, 80% of collected KNP fruit were unaffected by seed predation. Seeds from mature fruit with immature larvae had a far higher germination percentage (77%) than seeds in fruit with fully developed larvae (6%). Therefore, there is a short period where seeds remain viable while infested with *C. cosyra*. Frugivore observations in Leshiba GNR revealed that baboons and vervet monkeys eat *W. salutaris* fruit (except the exocarp) and ingest the seeds. However, their role as effective dispersers of *W. salutaris* is not certain. Frugivore activity was not observed in KNP, but evidence of frugivore activity (i.e. empty fruit exocarps) was found. In the seedling emergence trials KNP had the highest percentage germination (61%), followed by Eston (47%), and Leshiba (33%), and all ungerminated seeds were nonviable.

This study has expanded our knowledge of the reproductive ecology of *W. salutaris* and confirms that sexual reproduction of the species in KNP results in very low fruit and seed production. However, *W. salutaris* has many floral visitors, pollen viability is high, and seeds that are produced germinate successfully. Therefore, the impaired reproductive output in KNP appears to be in the high occurrence of aborted fruit, which was found for both self- and cross hand-pollinations and natural-bagged pollination treatments. For self-pollination this may be late-acting self-incompatibility, i.e. the ovule is aborted after it has been fertilised by self-pollen. However, the even lower success of cross-pollination may be explained by a genetically linked post-fertilisation failure. This is further supported by the higher percentage of pollen tube occurrence compared to fruit production for both self and cross hand-pollinations. The already limited fruit production in KNP is impacted further by pre-dispersal seed predation, lowering the number of seeds that can eventually contribute to seedling recruitment. However, germination of the desiccation-sensitive seeds and seedling establishment may only be possible in seasons with fairly regular and high rainfall. Therefore, it is possible that *W. salutaris* relies on clonal reproduction and resprouting in response to damage to persist in northern KNP. However, long-term reliance on clonal reproduction may impair the ability to reproduce sexually, which will

ultimately be necessary to establish genetically diverse genets that have the potential to adapt to future environmental changes.

Keywords: Breeding system, Germination, Pollination biology, Reproductive ecology, Seed predation

Glossary: Terminology and Abbreviations

Autogamous self-pollination: Pollination within a flower on a tree i.e. pollen from a flower onto the stigma the same flower

Frugivory: Fruit consumption by an animal

Fruit abortion: Fruit which cease development 2–4 weeks after fertilization and no seeds are produced

Dichogamy: The maturation of the male and female structures of a flower at different times, so that self-fertilisation is prevented

Geitonogamous self-pollination: Pollination between flowers on the same individual tree, or ramet i.e. pollen from one flower onto the stigma of a different flower

Late acting self-incompatibility: The ovule is aborted after it has been fertilised by self-pollen

Pistil: Female reproductive structure of a flower i.e. the ovary, style and stigma

Pollen dehiscence: Process by which pollen is released from the anther

Pollen grain number: Number of pollen grains obtained from each flower

Pollen load: Number of pollen grains carried on an insect after visiting a flower

Pollen viability: The pollen's ability to successfully deliver male gametes to the embryo sac

Pollen tube: Tubular structure produced by the male gametophyte of seed plants when it germinates on the stigma

Progamic stage: Stage of reproduction between the pre-pollination and post-zygotic stages

Protogyny: Condition where the female reproductive structures (i.e. the ovary, style and stigma) mature and are receptive before the male reproductive structures (i.e. stamens and pollen)

Recalcitrant seeds: Seeds that are short lived and do not survive drying or freezing.

Reniform: Kidney-shaped

Seed viability: The ability of the embryo to germinate and the degree to which the seed is metabolically active

Seed dispersal: The movement, spread or transport of seeds away from the parent plant

Self-incompatible: Unable to be fertilised by self-pollen

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Chapter one – Rationale and literature review

1.1 Rationale

Warburgia salutaris (Bertol.f.) Chiov. (Pepperbark tree, Canellaceae) is valued in southern Africa for its use in traditional medicine (Botha *et al.*, 2004; Maroyi, 2014). Over the years, the species has been unsustainably harvested, as individuals are stripped of their bark, resulting in increased susceptibility to disease and death (Cunningham, 1993; Botha *et al*., 2004). In South Africa, over-exploitation, habitat degradation, and habitat loss due to urban expansion and agricultural development, have all contributed to its endangered status (under criteria A2acd) on the International Union for Conservation of Nature (IUCN) Red List (Hilton-Taylor *et al*., 1998; Maroyi, 2014).

The vulnerability of *W. salutaris* is complicated further by pre-dispersal seed predation (Johnson *et al*., 1995; Hannweg *et al*., 2015), and the recalcitrant nature of its seeds, which prevents their long-term storage in seed banks (Kioko *et al.*, 2003). In order to facilitate the conservation of *W. salutaris*, the species has been cultivated from cuttings in nurseries since the mid 1980's to supply the local market (Botha *et al*., 2004; Scheepers *et al*., 2011). More recently, tissue culture trials have been successful (Kowalski & Van Staden, 2001; Hannweg *et al*., 2015), and more than 40 000 seedlings have been produced through joint efforts between the Agricultural Research Council (Nelspruit), SANBI (Nelspruit), Sappi, SANParks Skukuza Indigenous Plant Nursery, Fort Hare University and Shaw Research Centre (K. Hannweg, Nov. 2020, pers. comm). The seedlings are available for redistribution to traditional healers and communities that utilise the tree (K. Hannweg, Nov. 2020, pers. comm). Many of these seedlings were grown from healthy *W. salutaris* seed sourced from an orchard in Eston, KwaZulu-Natal (KZN), which sets prolific fruit annually and has successful seedling establishment around the farm (D. Thompson, Jan. 2019, pers. comm.).

The Kruger National Park (KNP) holds one of the largest remaining wild populations of *W. salutaris* in South Africa (Scheepers *et al*., 2011; D. Thompson, Jan. 2019, pers. comm.). However, despite being within a protected area, the trees experience incidents of illegal harvesting (Scheepers *et al.*, 2011). In response, armed guards are employed to monitor and protect the population (since around 2008; SANParks, 2018). In 2009, a survey on the KNP population showed that 61% of the individuals had been damaged by bark harvesting, and the

proportion of individuals in the smallest size class (< 1.5 m) made up 5% of the population (Scheepers *et al.,* 2011). In addition, anecdotal evidence based on field observations over the years suggests that the trees have low fruit production (D. Thompson, Jan. 2019, pers. comm.). Fruit containing viable seeds have only been found on one tree in a small sub-population of five individuals, and fruit containing nonviable seeds have been found on two trees in the largest sub-population (~ 400 individuals) (D. Thompson, Jan. 2019, pers. comm.).

Previous studies on *W. salutaris* have assessed the impact of commercial harvesting on the growth, density and vigour of trees (Botha *et al*., 2004); the medicinal properties of the plant material and cultural importance of the species (Maroyi, 2013; Maroyi 2014; Leonard & Viljoen, 2015); the use of leaves as a more sustainable harvesting option compared to bark (Drewes *et al.,* 2001); and the potential of cryopreservation to store the recalcitrant seeds (Kioko *et al.,* 2003). More recently, the genetic diversity of fragmented populations of *W. salutaris* in Mozambique were analysed and evidence of gene flow between populations was found (Senkoro *et al.,* 2020). Similarly, the genetic diversity of the KNP population has been investigated as inbreeding depression was suspected as a reason for low fruit production; however, while preliminary evidence supports extensive clonal reproduction in the population, overall high genetic diversity was found among sub-populations (D. Thompson, Nov. 2020, pers. comm.). This has brought the reproductive ecology of *W. salutaris* into question – an aspect of the species that has been unstudied until now.

An assessment of sexual reproduction of *W. salutaris* in the natural KNP population and Eston orchard was therefore necessary to investigate other factors that may be contributing to its limited sexual output and recruitment in the KNP. This required an examination of the pollination biology (i.e. pollinator visitation, breeding system, pollen viability, and pollen tube growth), fruit and seed production, pre-dispersal seed predation and seed dispersal within the *W. salutaris* population in KNP. This was repeated in the Eston population with the exception of seed dispersal. Through this study our knowledge of the reproductive ecology of *W. salutaris* has been expanded and contributed to the on-going, broader SANParks Pepperbark tree propagation programme (Hofmeyr *et al.,* 2017).

1.2 Literature review

1.2.1 Plant reproductive ecology

The study of plant reproductive ecology includes aspects of plant reproduction such as life histories; sexual systems; mating mechanisms; and provision, protection, and dispersal of offspring (Wilson, 1983; Lovett Doust & Lovett Doust, 1988). From the perspective of an individual plant, reproductive ecology includes the range of processes that occur from gametogenesis through to seed germination and seedling survival (Smit *et al.*, 1996; Marbaniang & Venugopal, 2019).

Plants display a diverse range of reproductive strategies involving both sexual systems and asexual reproduction (Marshall & Grace, 1992). In sexual systems, flowering and fruiting allow for the transfer of genetic material through generations, while asexual reproduction produces offspring that are genetically identical to the parent plant (Marshall & Grace, 1992). Many plant species rely on both sexual and asexual reproduction to ensure their persistence (Yang & Kim, 2016). Sexual reproduction facilitates dispersal into new environments while asexual reproduction allows for the persistence of advantageous genotypes (Ortega-Baes & Gorostiague, 2013). The various reproductive strategies that plants rely on are influenced by physical factors, such as nutrient availability, light and water; biological factors, such as the nature and availability of pollinators, agents of fruit and seed dispersal; and the effects of pathogens and predators (Marshall & Grace, 1992; Yang & Kim, 2016).

The adaptive significance of the traits associated with pollination, seed dispersal, and seedling establishment are aspects investigated in plant reproductive ecology, which highlights the important interactions that take place between plants and their environment (Barrett & Eckert, 1990). While animals have flexible behavioural responses to their environment, plants ensure their survival through flexible architecture and a capacity for continual growth (Lovett Doust & Lovett Doust, 1988). It is well understood that many flowering plants rely on their environment and other organisms to carry out reproductive processes such as pollination and seed dispersal (Barrett & Eckert, 1990), and a disruption in these relationships could impair the reproductive success of a species (Barrett & Eckert, 1990).

Therefore, reproductive ecology is valuable in the context of conservation of rare or threatened plant species. Williams *et al.* (2013) states the importance of conducting autecological research (e.g. assessing generation time, seed viability and pollination ecology)

on threatened and/or protected species, in conjunction with the understanding of population dynamics and distributions. Understanding the reproductive ecology of a species assists in the understanding of many processes such as recruitment patterns and species survival strategies (Cousins *et al.*, 2013). In addition, it allows us to predict the vulnerability of species to future events that may breakdown the reproductive process. For example, understanding whether a plant relies on generalist or specialist pollinators, the degree of self-compatibility, ability to reproduce through both sexual and asexual reproduction, and/or resprout in response to damage, has implications for its ability to adapt to change, especially in the face of climate change and habitat transformation (Bond, 1994; Kaye, 1999; Bond & Midgley, 2001).

1.2.2 Socio-economic importance of **Warburgia salutaris***: medicinal use and conservation status*

Warburgia salutaris (the pepperbark tree) is both culturally and economically important in South Africa for its use in traditional medicine (Williams *et al*., 2013). It was reported that 72% of Black South Africans depend on traditional health care systems in which medicinal plants are used (Williams *et al*., 2013). This is due to limited access to modern medical facilities in parts of the country, but also for cultural, traditional, and financial reasons (Senkoro *et al.*, 2019).

Warburgia salutaris contains complex compounds in the bark, roots and leaves that give it pharmaceutical value; the most commonly reported being drimane sesquiterpenoids (forms of anti-herbivory compounds) (Kioko *et al.*, 2003). Types of drimane sesquiterpenoids that have been found in *W. salutaris* are: isopolygodial, mukaadial, muzigadial, polygodial, salutarisolide, ugandensidial and warburganal (Maroyi, 2013). These compounds contribute to the antibacterial, anti-fungicidal, anti-microbial and anti-inflammatory properties of the plant material; therefore, it is used to treat multiple ailments (Maroyi, 2014). These include abdominal pains, coughs, colds, inflammation, fever, headaches, bladder infections, skin irritations, sores and ulcers (Maroyi, 2013). Medicinal plants such as *W. salutaris* are used locally but also traded commercially at *muthi* markets (Williams *et al.*, 2013). A recent study (Rasethe, 2019) on medicinal plant trade in Limpopo reported that *W. salutaris* is in high demand, but not always readily available, and is sold at a high price (approximately R30/kg). This high demand often supersedes supply, driving unsustainable harvesting methods of wild populations, which are further affected by urban expansion, habitat transformation and degradation (Cunningham, 1993; Senkoro *et al*., 2019).

In 1998, *W. salutaris* was officially classified as endangered on the International Union for Conservation of Nature (IUCN) Red List, and as of 2009 remains listed under criteria A2acd (Hilton-Taylor *et al*., 1998; Raimondo *et al.,* 2009). This IUCN classification is based on population reduction (A2), as a 50% decline was reported for the South African populations (Williams *et al*., 2008). This population reduction has been "observed, inferred, estimated, or suspected in the past where the causes of reduction may not have ceased OR may not be understood OR may not be reversible" based on (a) "direct observation"; (c) "a decline in area of occupancy (AOO), extent of occurrence (EOO) and/or habitat quality"; and (d) "actual or potential levels of exploitation" (IUCN Standards and Petitions Subcommittee, 2014).

1.2.3. Conservation efforts

Warburgia salutaris has been recognised as a species of conservation concern since 1926 due to over-harvesting of the plant material and the absence of seedlings in the field (Neary, 2018). These observations prompted the first conservation efforts to protect the species. In the mid 1980's, the shoot-tip cutting technique was developed at Durban's Silverglen Medicinal Plant Nursery (Nichols, 2005). This technique was suggested as stems of individuals of *W. salutaris* alongside Lake St. Lucia, KwaZulu-Natal, were found to sprout from their roots after being broken by passing hippopotamuses (Johnson *et al.,* 1995). Nurseries adopted this method, as it has proved to be the easiest method to increase numbers given the lack of viable seed found in the field (Nichols, 2005). Tissue culture trials have also been successful; however, a rigorous procedure is necessary to prevent the oxidation of the phenolics, as this reaction damages the tissue (Kowalski & Van Staden, 2001; Hannweg *et al*., 2015). Various techniques to extend the storage time of seeds have been investigated, for example, seeds of *W. salutaris* have been shown to germinate successfully after rapid dehydration and then cryostorage (for 1 hour) in liquid nitrogen (Kioko *et al*., 2003).

Managing the harvesting and trade of medicinal plant material to support biodiversity conservation remains a controversial issue (Williams *et al*., 2013). *Warburgia salutaris* is on the protected species list (alongside charismatic species such as the baobab and marula trees) under the National Forests Act, 1998 (Act No. 84 of 1998), which prohibits harvesting of plant material for any reason without a permit (National Forests Act 1998, 2019). Various conservation projects have been created that aid the protection of *W. salutaris*, while acknowledging the importance of the species in cultural practices. Sappi and SANParks

Skukuza Indigenous Plant Nursery have been involved in the on-going Pepperbark tree programme. This includes distributing seedlings to communities that rely on the plant, and hosting workshops to teach sustainable planting and harvesting methods, and to learn from traditional healers (Swemmer *et al*., 2017). Similar projects are potentially being extended into Zimbabwe and Mozambique (Senkoro *et al.*, 2019; Maroyi, 2012), and the non-profit organisation, Endangered Wildlife Trust (EWT), is embarking on a project to conserve *W. salutaris* in the Soutpansberg – a known priority area for the species (EWT, 2019).

The Agricultural Research Council (Nelspruit), SANBI (Nelspruit), Fort Hare University and Shaw Research Centre have propagated more than 40 000 *W. salutaris* seedlings (K. Hannweg, Nov. 2020, pers. comm). Many of these seedlings were grown from healthy *W. salutaris* seed sourced from an orchard in Eston, KZN (Hannweg *et al*., 2015). In contrast to the KNP population, the orchard of *W. salutaris* trees in Eston is reported to set prolific fruit annually and has successful seedling establishment around the farm (M. Stainbank, Feb. 2019, pers. comm.). The orchard was established in 1992, originally grown from seed collected from Tembe Elephant Park, KZN (M. Stainbank, March. 2019, pers. comm.). Potential explanations for the high-quality seed in this orchard are reduced pre-dispersal seed predation by fruit flies; the presence of parasitoid wasps; and fruit fly control practices used in the surrounding agricultural areas (Hannweg *et al*., 2015). In addition, high fruit and seed set may be attributed to abundance of pollinators in this area (Hannweg *et al*., 2015). The Eston orchard was included in this study to provide a comparison for identifying factors limiting fruit and seed production in the KNP population.

1.2.4. Floral biology of Warburgia salutaris

Warburgia salutaris is one of 16 species in the evergreen Wild Cinnamon family, Canellaceae (Schmidt *et al*., 2002). The family is distributed across America, Asia and Africa; however, *Warburgia* is the only genus that occurs in Africa. This genus consists of four species: *W. salutaris*, *W. ugandensis* Sprague, *W. elongata* Verdc., and *W. stuhlmannii* Engl. *Warburgia salutaris* occurs in South Africa, Eswatini, Lesotho, Zimbabwe and Mozambique (Burrows *et al*., 2018). Within South Africa, the species is found in a diverse range of habitats; it occurs on dry rocky slopes, in wooded ravines and in wet coastal forests (Coates-Palgrave, 2002; Schmidt *et al.*, 2002).

Warburgia salutaris is considered fast growing and reaches approximately 12 m in height (Botha *et al*., 2004; Burrows *et al*., 2018). The main stem, which can be up to 30 cm in diameter, is covered by dark brown rough bark and the branches are grey with lenticels (Williams *et al*., 2007; Burrows *et al*., 2018). Multi-stem variants are also found, often in response to damage of the branches and roots, i.e. by elephants, kudu (Botha *et al.*, 2004), hippopotamuses (Johnson, 1995), and harvesting by humans (Botha *et al.*, 2004; Scheepers *et al.*, 2011). The leaves are dark glossy green when mature, elliptic to lanceolate and 45–110 x 15–30 mm in size (Burrows *et al*., 2018). The species is recognised by the distinctive peppery smell and taste of the leaves when crushed (Schmidt *et al*., 2002). The flowering season is reported to be from April until June; however, this varies considerably between regions. Around Gauteng, *W. salutaris* flowers as early as February, while in KwaZulu-Natal, near Eston, the tree is reported to flower from July, and in KNP from April. During the flowering season, a multitude of small (5–7 mm diameter), green, inconspicuous flowers are borne in the leaf axils (Schmidt *et al*., 2002) (Fig. 1A & 1B). The floral structure consists of the outer calyx of three green sepals, surrounding two whorls of five green outer petals and five white inner petals (Coates-Palgrave, 2002). The flowers are bisexual; in the centre of the flower are 10 fused filaments, forming a tube, which envelops the ovary and style (Coates-Palgrave, 2002). The style has 3–5 stigmas, which surround a sticky surface and the ovary contains 15–20 ovules (Leonard & Viljoen, 2015). From October through to January (this may differ depending on flowering time), medium-sized (30–50 mm diameter) round green berries develop (Fig. 1C) and turn purple with a white bloom as they mature (Burrows *et al*., 2018). The fruit contain between 2 to ~25 reniform seeds (~10 x 5 mm) with an oily, fleshy endosperm (Salazar & Nixon, 2008).

Figure 1: *Warburgia salutaris* (A) buds, (B) flowers, and (C) fruit at Random Harvest Nursery, Muldersdrift, Johannesburg.

1.2.5. Knowledge gaps in the reproductive ecology of Warburgia salutaris

The reproductive ecology of *W. salutaris* has not yet been investigated. Scheepers *et al.* (2011) recommends a better understanding of the reproductive ecology and regeneration of *W. salutaris* to improve the monitoring programme of the species in the KNP.

1.2.5.1 Pollination

It is mentioned on a nursery website (CJM Growers, 2017) that insects pollinate *W. salutaris*, yet the types of insects, or degree of specialisation, are not specified. Bees have been suggested as insect pollinators of *W. salutaris* (Senkoro *et al.*, 2020) and Muchugi *et al*. (2008) mention that the flowers of *Warburgia ugandensis* (similar in appearance to *W. salutaris*) are suited to small bees. Understanding the pollination of *W. salutaris* will give insight into the pattern of pollen movement within the protected KNP population (Barrett & Harder, 2017). This population consists of several geographically separated sub-populations, and the number of *W. salutaris* individuals in each sub-population is very variable, with some consisting of five or less trees. Therefore, it could be suggested that their small size and relative isolation may influence their chance of pollination, because high-density populations are more likely to draw many pollinators (Kunin, 1993). An understanding of pollinator behaviour together with the breeding system is also necessary, as highly mobile pollinators are more likely to facilitate out-crossing (Barrett & Harder, 2017). For example, it is suggested that high levels of genetic diversity in three separate populations of *W. salutaris* in Mozambique is partly due to pollen-mediated gene flow facilitated by bees (Senkoro *et al*., 2020).

1.2.5.2 Breeding system

The flowers of *W. salutaris* are bisexual; however, it is not known whether *W. salutaris* is self-compatible and can undergo autonomous self-pollination. Results from a genetic diversity study on a population of *W. ugandensis* in Kenya shows that the species has a mixed-mating system, but is predominantly outcrossing (Muchugi *et al*., 2008). Understanding the breeding system of *W. salutaris* will also highlight the species reliance on pollinators and the vulnerability of the smaller sub-populations within the KNP population. Self-incompatible plants are most vulnerable to reduced seed set when opportunities for cross-pollination are low i.e. if there is a lack of pollinator visitation (Kunin 1993). While self-compatibility allows for chances of self-fertilisation despite a lack of pollinator visitation (i.e. reproductive assurance), there is the risk of inbreeding depression. Therefore, it is generally understood that long-lived, large-statured plants have mating systems that avoid selfing, i.e. they are self-incompatible or

dioecious (Scofield & Schultz, 2006). This is because they undergo more mitotic cell divisions than small-statured, annual species and as a result they naturally accumulate more deleterious somatic mutations over time (Krebs & Hancock,1990; Bobiwash *et al.,* 2013).

1.2.5.3 Seed dispersal

The process by which the seeds are released from the pericarp is suggested to be a particularly important factor in the reproductive success of *W. salutaris* (L. Da Luca. March. 2019, pers. comm.). For example, at the Random Harvest Indigenous Nursery in Muldersdrift, Johannesburg, although there are many fruit beneath the *W. salutaris* parent trees from the previous fruiting season, no seedlings have emerged (L. Da Luca. March. 2019, pers. comm.). There is limited information on the frugivores associated with *W. salutaris*. Van Wyk (1984) notes that baboons eat the fruit of *W. salutaris* in KNP and Senkoro *et al.* (2020) mentions that the fruit are dispersed by birds, but the species of birds are not specified. Other animals such as elephants may also disperse the seeds, as reported for *W. ugandensis* in Kenya (Muchugi *et al*., 2008). Once seeds are removed from the fruit, the dispersal distance is determined by the way the frugivore handles the seeds, i.e. dropping seeds at the site, spitting out the seeds, masticating the seeds, or swallowing the seeds (Lambert & Garber, 1998). Apart from seed dispersal, for some plant species, passing through the gut of a frugivore is also advantageous as it can enhance germination rate and success through scarification, pulp removal and fertiliser effect of the faeces (Stringer *et al.,* 2020). However, the effect of gut treatment varies across species and may be detrimental for seed viability (Levey *et al.,* 2002).

1.2.6 Impaired reproductive output of **Warburgia salutaris**

1.2.6.1 Recalcitrant seeds

The recalcitrant nature of *W. salutaris* seeds contributes to the vulnerability of the species. The main factor separating recalcitrant (desiccation-sensitive) from orthodox (desiccation-tolerant) seeds is that recalcitrant seeds are metabolically active when they are shed and remain so, provided they experience minimal water loss (Berjak & Pammenter, 2001). Various intracellular and cellular processes enable orthodox seed tissues to cope with dehydration, namely: the large fluid-filled vacuoles reduce in size by breaking up into many smaller vacuoles, or they are filled with a protein substitute; their embryo cells contain insoluble starch and/or lipids; and the cellular walls have high plasticity (Berjak & Pammenter, 2001). Therefore, under unsuitable conditions *W. salutaris* seeds lose moisture and in turn,

viability (Kioko *et al.*, 2003; Nichols, 2005); however, healthy seeds will germinate within 14 days (Nichols, 2005). Studies have found that even if recalcitrant seeds are stored in a hydrated condition, this is only successful for a short time due to their short lifespan and the proliferation of fungi, which are abundant on recalcitrant seeds (Berjak & Pammenter, 2001; Kioko *et al*., 2003). Therefore, persistence in long-term soil seed banks is not possible for plant species with desiccation-sensitive seeds, but instead seeds should theoretically germinate to form seedling banks (Pammenter & Berjak, 2000). However seedling establishment is not guaranteed, especially under unpredictable environmental conditions such as the semi-arid KNP savannas, where water availability is a major limiting factor (Wilson & Witkowski, 1998).

1.2.6.2 Low seedling establishment

Studies that have examined damage caused by harvesting of *W. salutaris* noted that few young trees were found in the field. Botha *et al.* (2004) found that trees with a basal stem diameter < 1 cm made up less than 10% of a protected population of *W. salutaris* in Limpopo, and in KwaZulu-Natal an absence of seedlings was reported (Hilton-Taylor *et al*., 1998; Scott-Shaw, 1999). In 2006, Scheepers *et al.* (2011) found that *W. salutaris* individuals in the smallest size class (< 1.5 m high) made up 3% of the Kruger National Park population. In response, Thresholds of Potential Concern (TPC's) were developed for managers and researchers to monitor changes in the KNP *W. salutaris* population – this was set at 10% for the percentage of juveniles (Scheepers *et al.*, 2011). In 2008, there were incidents of illegal harvesting in KNP natural populations, which prompted conservation management to deploy armed field rangers to monitor the population daily (Scheepers *et al.*, 2011). In 2009, the proportion of individuals in the smaller size classes was still below the TPC and the proportion of individuals damaged by bark harvesting was above the TPC (set at 60%) (Scheepers *et al.*, 2011). Similarly, a study on two KNP *W. salutaris* sub-populations in 2018 confirmed that there were very few trees in the lowest size class (1–2 m) and no seedlings were recorded (Wilken, 2018).

The lack of recruitment in the KNP population is still of concern. Population structure is influenced by the population's or species' life history strategies and its relationship with the environment. For example, Scheepers *et al*. (2011) cautioned to not assume that the population structure of *W. salutaris* is unhealthy, as some savanna trees, e.g. the baobab tree (Venter & Witkowski, 2010), are very long lived and presumably have sporadic and opportunistic recruitment events. However, when seeds are produced, plants may be limited by suitable

microsites for seedling establishment (Lamont *et al*., 1993; Venter & Witkowski, 2013) and environmental disturbances such as fire, herbivory and drought, can then affect the persistence of saplings (Helm *et al.*, 2011). *Warburgia salutaris* is also able to reproduce vegetatively, and readily resprouts after damage. This accounts for multi-stem variants of the species; in 2006, 59% of the recorded KNP trees were multi-stemmed (Zambatis, 2006). Therefore, the population may be much more reliant on resprouting than seeds for population persistence.

1.2.6.3 Low fruit production

Low fruit and reduced viable seed production have been reported for *W. salutaris* in the KNP and other populations (Hilton-Taylor *et al*., 1998; K. Hannweg, Feb. 2019, pers. comm.). Low fruit: flower ratios may be expected for *W. salutaris,* as under suitable conditions, the trees produce a multitude of flower buds. While it is often suggested this is a result of factors such as pollen limitation, low pollen quality, predation, or resource limitation, this is not always the case, and may be a 'bet hedging' strategy (Ayre & Whelan, 1989). Therefore, the low fruit: flower ratio seen for *W. salutaris* may in part be an adaptive advantage, explained by various hypotheses, including — maximizing flowers to increase pollinator attraction, increasing pollen donation and maximizing fruit production from high quality pollen (Ayre & Whelan, 1989). Regarding the KNP population of *W. salutaris*, an extreme lack of fruit production has been reported (D. Thompson, Jan. 2019, pers. comm.; K. Hannweg, Feb. 2019, pers. comm.). Fruit containing viable seeds have only been found on one tree in a small sub-population of five individuals, and fruit containing nonviable seeds have been found on two trees in the largest sub-population (~ 400 individuals) (K. Hannweg, Feb. 2019, pers. comm.). However, exact quantification of fruit set in this population has not yet been recorded.

1.2.6.4 Pre-dispersal seed predation

The reduced reproductive output in the KNP population has in part been attributed to pre-dispersal seed predation by insect larvae. A study by Hannweg *et al*. (2015) identified *Ceratitis cosyra* Walker (the marula fly) as the only fruit fly affecting *W. salutaris* fruit taken from *W. salutaris* trees in the Skukuza Indigenous Plant Nursery. In emergence trials on this fruit, 400–3000 adult fruit flies were reared per kilogram of fruit, suggesting that significant pre-dispersal seed predation is responsible for the bottleneck in the reproductive output of *W. salutaris* in northern KNP. Several parasitoid wasps from the families Braconidae and Eulophidae were also identified. These wasps utilise the fruit fly larvae inside the *W. salutaris* fruit as hosts for the development of their own eggs (Hannweg *et al*., 2015).

1.2.6.5 Clonal reproduction

Warburgia salutaris is reported to reproduce vegetatively, as root suckering has been observed in the species (Johnson *et al.*, 1995; Hilton-Taylor *et al.*, 1998; Botha *et al.*, 2004), and similar observations were made in the KNP population (Scheepers *et al.*, 2011). The ecological advantages of vegetative reproduction include the ability to "forage" for optimal conditions in patchy environments, reduced mortality risk, and the ability to grow rapidly to compete with other species (Barrett, 2015). Similarly, evolutionary advantages of vegetative reproduction are the reduced costs of sexual reproduction, and the ability of adaptive genotypes to rapidly colonise new environments after a disturbance (Barrett, 2015). However, there are also disadvantages to clonal growth, which include: increased inbreeding depression and pollen discounting where sexual reproduction occurs between ramets of a genet; and increased somatic mutations in highly clonal populations may reduce fertility and the ability to reproduce sexually altogether (Barrett, 2015). For example, it was found in a crossing study on a clonal aquatic plant (*Decodon verticillatus* (L.) Elliott) in North America that sterility was caused by abnormal pollen tube growth (Eckert *et al.*, 1999). In addition, Ally *et al*. (2010) found that pollen viability decreased with the age of a clonal tree as a result of accumulated somatic mutations.

For this reason, it was suspected that the KNP *W. salutaris* population might be clonal; therefore, failed fruit production could be a result of inbreeding depression (D. Thompson, Jan. 2019, pers. comm.). However, results from a recent genetic study on this population found overall high genetic diversity between sub-populations; nonetheless, these contain isolated genets, with clumps of individual ramets, resulting from clonal reproduction (D. Thompson, Nov. 2020, pers. comm.). In response to this study, it was suggested that although genetic diversity is high, there is a possibility that reproduction is compromised by the accumulation of detrimental gene mutations. This possibility can be inferred from the following concepts: First, it is proposed that long-lived plant species, such as trees, can accumulate more deleterious somatic mutations in their lifetime because they undergo more mitotic cell divisions (Krebs & Hancock, 1990; Bobiwash *et al.*, 2013). Second, plant reproductive structures are derived from somatic cell lineages; therefore, somatic mutations have the potential to impact aspects of sexual reproduction (Krebs & Hancock, 1990; Schultz & Scofield, 2009). Third, loci controlling sexual reproductive processes are not subjected to selection when a plant goes through an extended period of vegetative growth, thus, detrimental mutations can accumulate (Krebs & Hancock, 1990; Ally *et al*., 2010). Therefore, the accumulation of deleterious

mutations in reproductive structures may also be a possible underlying driver of the failed reproduction in *W. salutaris*.

1.3 Aim and objectives

The aim of this study is to identify factors at each stage in the reproductive process of *W. salutaris* that may contribute to the lack of reproductive output and subsequent low seedling establishment of the species in the KNP. The reproductive ecology of *W. salutaris* in KNP will be compared with that of the reproductively successful orchard in Eston, KwaZulu-Natal to further highlight where the KNP population is compromised. All objectives apply to both the natural KNP population and Eston orchard, except objective 3 in chapter 3, which only applies to KNP. In addition, due to very low fruit production in KNP, a nearby population situated outside KNP in Leshiba Game and Nature Reserve, Soutpansberg, was selected to supplement data for Objectives 2, 3 and 4 in chapter 3.

Chapter 2 objectives:

1. To identify effective pollinators of *Warburgia salutaris.*
2. To establish whether *Warburgia salutaris* is able to autonomously self-pollinate and whether it is self-compatible if pollinated by geitonogamy, or if it is entirely reliant on vector-facilitated cross-pollination.
3. To assess pollen viability and compare pollen tube growth between hand self-, cross- and natural pollinations.

Chapter 3 objectives:

1. To assess natural fruit development of *Warburgia salutaris*.
2. To investigate the effect of pre-dispersal fruit/seed predation on the reproductive output of *Warburgia salutaris*.
3. To identify species of seed dispersers and assess the mode of seed dispersal.
4. To assess germination success of fruit within selected sub-populations.

1.4 Structure of dissertation

The pollination biology of *W. salutaris* in KNP and the Eston orchard is investigated in chapter 2. The first objective focuses on identifying effective pollinators of *W. salutaris*. This included observations of whether the insect contacts the reproductive structures of the flower,

as well as establishing the site and load of attached pollen on the pollinator after visiting a flower. Nectar measurements were also carried out in Eston to further understand which group of pollinators are most likely attracted to *W. salutaris* flowers. The second objective focuses on the breeding system of *W. salutaris*. Manipulative pollination trials were carried out to assess fruit set following autonomous self-pollination, geitonogamous self-pollination, and cross-pollination, and compared to natural pollination. In addition, naturally pollinated flowers were bagged to assess the effect of pre-dispersal seed predation on natural fruit set. The third objective focuses on the male function of the *W. salutaris* flower. Pollen viability was assessed and pollen tube growth was compared between self-pollination, cross-pollination and natural pollination to clarify the breeding system and establish at what stage the process of fruit production is inhibited. In all instances (except for the nectar measurements), the findings from KNP were compared to those from the Eston orchard.

Aspects of the seed ecology of *W. salutaris* are assessed in chapter 3. Here comparison is made between KNP, the Eston orchard, and Leshiba (a second natural population). The first objective focuses on the development of natural fruit in KNP and Eston. The second objective focuses on the effect of pre-dispersal fruit/seed predation on the reproductive output of *W. salutaris* and the identification of pre-dispersal seed predators. The third objective focuses on the dispersal of *W. salutaris* seeds. This includes frugivore observations in KNP and Leshiba, in which frugivores were identified and the mode of seed dispersal was assessed based on the interaction of the frugivore with the fruit/seeds. The fourth objective focuses on the comparison of germination success between seeds in successfully produced fruit from the three locations.

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Chapter two - Pollination biology of the pepperbark tree, **Warburgia salutaris**

Abstract

In the Kruger National Park (KNP), *Warburgia salutaris* (the pepperbark tree) has minimal fruit and viable seed production and there is subsequent low seedling establishment in the population. The apparent lack of sexual recruitment in KNP raises questions around the reproductive ecology of the species. Therefore, the pollination biology of *W. salutaris* in six sub-populations in KNP was studied to investigate factors that may contribute to its limited reproductive output. Potential pollinators were observed and collected for further analysis; nectar volume and sugar concentration were measured; five pollination treatments – autonomous self-, geitonogamous self-, cross-, natural, and natural-bagged pollination (i.e. naturally pollinated flowers bagged later to protect from seed predation) – were set-up to elucidate the breeding system; pollen viability was assessed; and pollen tube growth for self-, cross- and natural pollination was compared. Results show that *W. salutaris* flowers are visited by a variety of insects, including moths at night. Mean nectar volume was low (2.4 μl) with a high (40%) sucrose concentration. *Warburgia salutaris* is protogynous and likely self-incompatible, as self-pollinated flowers in neither KNP nor Eston orchard resulted in fully developed fruit. The overall percentage fruit sets for hand cross-pollination (2%) and self-pollination (5%) were very low, and all fruit formed aborted early in development. In 2019, only two trees in one KNP sub-population produced fully developed fruit and only through natural pollination. Pollen viability was high (66–93%) across the sub-populations. Percentage pollen tube occurrence for self- and cross-pollination was higher in Eston (self: 60%, cross: 100%) than KNP (self: 33%, cross: 33%), and both self and cross pollen tubes reached the ovules. Pollen tube occurrence was higher than expected (based on low fruit set in the initial pollination trials) in self (33%), cross (33%) and natural (35%) pollinations, and self and cross pollen tubes had an equal percentage occurrence. This study has expanded our knowledge of the pollination biology of *W. salutaris* — lepidopterans are most likely effective pollinators; the species is primarily out-crossing and poor male function is not a reason for the very low fruit set in the KNP population. A genetic or resource induced postzygotic malfunction may be the cause of the observed early fruit abortion, but further studies are needed to confirm this.

Keywords*:* Breeding system, Fruit set, Pollen tubes, Pollen viability, Pollination, Reproductive ecology.

2.1 Introduction

The Kruger National Park (KNP) has one of the largest populations of the endangered pepperbark tree (*Warbrugia salutaris* (Bertol.f.) Chiov.). However, even in this protected area there is concern over the long-term viability of the species. Previous studies in KNP noted that *W. salutaris* has a lack of seedling establishment and low fruit and viable seed set (Botha *et al*., 2004; Hannweg *et al*., 2015). Within this population there is evidence of widespread clonal reproduction; nonetheless, overall high genetic diversity has been found (D. Thompson, Nov. 2020, pers. comm.). This has brought into question the pollination biology of the pepperbark tree – an aspect of the species that has not been investigated nor documented to any extent – unlike its medicinal properties. A clear understanding of the pollination biology of *W. salutaris* will give insight into the low sexual reproduction of the species in the KNP.

Pollination can be inhibited at multiple points in the pollination process. Possible reasons for pollination failure are: the deposition of sterile pollen (as a result of environmental conditions, genetic factors or time taken to reach the stigma); insufficient pollen quantity due to low visitation rates (potentially relating to habitat fragmentation) or inefficient pollinators; deposition of heterospecific pollen which clogs the stigma; self-incompatibility; and inbreeding depression (Ramsey & Vaughton, 2000; Wilcock & Neiland, 2002). Based on the available literature, the pollination biology of *W. salutaris* is under-studied. Bees are suggested as insect pollinators of *W. salutaris* (Senkoro *et al.*, 2020) and *W. ugandensis* Sprague which has flowers similar in appearance to *W. salutaris* (Muchugi *et al.*, 2008). It is also mentioned on a nursery website (CJM Growers, 2017) that insects pollinate *W. salutaris*, yet neither the types of insects, nor degree of specialisation, are specified. In addition, the flowers of *W. salutaris* are bisexual; however, it is not known whether *W. salutaris* is self-compatible and/or can autonomously self-pollinate. Therefore, an understanding of the pollination biology of *W. salutaris* will assist in identifying potential factors contributing to the low reproductive output of the KNP population.

2.1.1 Pollinators

The flowers of *W. salutaris* are small, green, and not visually attractive. According to Faegri and van der Pijl (1979), such flowers are mostly found in plants with abiotic pollination. However, the small subgroup of insect-pollinated flowers without visual attraction are suggested to rely on olfactory cues to attract pollinators. In addition, *W. salutaris* produces

nectar, which functions as a reward for pollinators (Faegri and van der Pijl, 1979). The relatively short cup-shaped corollas of *W. salutaris* flowers (Fig. 1) appear to be accessible by insects with a proboscis, or very small insects that can climb between the petals, e.g. beetles. However, while a flower may be visited by many potential pollinators, the effectiveness of their role as a pollinator needs to be assessed — i.e. pollen must be removed and carried on a part of the insect that will allow pollen deposition on the stigma of the next flower (Lloyd & Barrett, 1996; Barrett & Harder, 2017). An understanding of pollinator behaviour together with the breeding system is also necessary, as highly mobile pollinators have a higher probability of facilitating out-crossing, which is advantageous for self-incompatible species (Barrett & Harder, 2017).

Figure 1: Structure of the *Warburgia salutaris* flower: five green petals surround an inner whorl of five white petals. In the centre is a staminal tube, formed by the fusion of the filaments and anthers, which surrounds the ovary and style. Scale bar: 10 mm.

2.1.2 Breeding system

Warburgia salutaris is hermaphroditic, which is the most common breeding system among plants (Barrett & Harder, 2017). While this allows for reproductive assurance if self-fertilisation is possible, it comes with the cost of inbreeding depression (Goodwillie *et al.*, 2005). Species in the family Canellaceae (in which *Warburgia* is placed) are reported to have mechanisms to prevent self-fertilisation. For example, the wild cinnamon *Canella winterana* (L.) Gaertn. (syn. *C. alba* Murray) shows synchronised dichogamy, whereby all flowers on a plant are protogynous, i.e. functionally female and then become functionally male (Kubitzki, 1993), and reproductive organ movement has been noted for *Cinnamodendron dinisii* Schwacke (Kubitzki, 1993). It has been observed that the androecial tube elongates above and

encloses the stigma when it has become non-functional (Kubitzki, 1993). The arrangement of the reproductive structures in *W. salutaris* does not allow for autonomous contact between the anthers and the stigmatic surface. Therefore, it appears that self-pollen would only be able to be placed on the stigmas if carried by an insect. In addition, long-lived, perennial, woody species generally have traits to avoid self-fertilisation (i.e. are self-incompatible or dioecious) because they undergo more mitotic cell divisions compared to annual species, and therefore accumulate more somatic mutations over time (Krebs & Hancock, 1990; Bobiwash *et al.*, 2013).

2.1.3 Male function: Pollen viability and pollen tube growth

The progamic stage of reproduction is between the pre-pollination and post-zygotic stages (Williams, 2012), essentially the period during which the male gametes are transported by the pollen tube to the female gametes. Studying this stage can give insight into mechanisms of self-incompatibility systems and other post-pollination processes, such as early-acting inbreeding depression (Goodwillie *et al.*, 2005).

2.1.3.1 Pollen viability

Pollination can be inhibited by deposition of nonviable pollen, which can be caused by delayed pollinator visitation, excess moisture, desiccation, or as a result of intrinsic factors that impact pollen production (Faegri & van der Pijl, 1979; Wilcock & Neiland, 2002; Vinod, 2005). Nonviable pollen impacts the proportion of pollen grains that will germinate as well as the quality of the pollen tubes produced (Kalinganire *et al.*, 2000; Vinod, 2005). Studies have found pollen viability can vary among individual trees within the same population (Oni, 1990), and decrease with age in a clonal tree, as a result of accumulated somatic mutations (Ally *et al.*, 2010). Therefore, pollen viability is a useful variable to assess the male function of *W. salutaris* as a potential barrier to reproduction (Vinod, 2005).

2.1.3.2 Pollen tube growth

Self-incompatibility (SI) can be effective at various points in the pollination process. In a sporophytic SI system, self-pollen is recognised immediately on the stigma and secretions of the stigmatic tissue prevent the germination or growth of the pollen, however, in gametophytic SI system the pollen grain will germinate and produce a pollen tube that is then arrested within the style, or if gametes are able to fuse and a zygote forms, it will not develop (Haring *et al.*, 1990). In some cases the degree of self-incompatibility can vary within a species, for example,

in a study on the Easter lily (*Lilium longiflorum* Thunb), variation was found in the selfed seed-set, position of self-pollen tube arrest, and degree of morphologically abnormal pollen tubes in individuals of the same population (Sakazono *et al.*, 2009).

Late acting self-incompatibility may occur, whereby the ovule is aborted after the ovum has been fertilised by sperm in the self-pollen (Krebs & Hancock, 1990; Hao *et al.*, 2012). Late-acting self-incompatibility may be confused with the effect of an accumulation of deleterious mutations, therefore, it is important to distinguish between the two when conducting pollination trials. Potential indicators of deleterious mutations being expressed in field hand-pollination experiments that are relevant to this study are: i) a large variation in aborted seed size — in contrast with a late-acting self-incompatibility response, where embryos would be aborted at a uniform stage in development (Hao *et al.*, 2012) and ii) a positive correlation between self and crossed seed set as the expression of deleterious alleles will reduce both selfed and crossed seed sets (Hokanson & Hancock, 2000; Hao *et al.*, 2012).

The aim of this study was to understand factors relating to the pollination biology of *W. salutaris* that may have consequences for the reproductive success of the species in the Kruger National Park. The first objective was to identify the effective pollinators of *W. salutaris*, the second to establish the breeding system of *W. salutaris* — i.e. to understand if it is self-compatible, or entirely reliant on vector-facilitated cross-pollination. The third objective was to investigate the male function by assessing pollen viability and comparing pollen tube growth between pollination treatments.

2.2 Methods

2.2.1 Study area

The study was conducted in two populations of *W. salutaris*. The first population[1] is in the northern part (altitude: 420–580 m a.s.l.) of the Kruger National Park (KNP), a protected area of 19,485 km^2, situated on the north-eastern border of South Africa (Roocroft & Roocroft, 2012). The landscape here is described generally as Makuleke Sandy Bushveld on sandstone (Mucina & Rutherford 2006). This landscape is highly variable with mountains and plains, forming diverse habitats for plant communities (Mucina & Rutherford 2006). The climate of

[1] Due to the protected status of the species and of this KNP population in particular, localities and data relating to population size and the number of individuals are not presented.

this area is characterised by summer rainfall and dry winters, with an average annual precipitation of ~ 530 mm (Scientific Services, 2018). The highest and lowest mean monthly maximum and minimum temperatures are 32.7 °C in December and 10.8 °C in July, respectively (Climate-Data.org, 2019).

The second study population of *W. salutaris* is an orchard on a farm in Eston, KwaZulu-Natal (altitude: ~ 690 m a.s.l.), located approximately 45 km inland from Durban (M. Stainbank, March. 2019, pers. comm.). There are two sub-populations of trees here: one comprises 90 trees that were planted in 1992 and the other approximately 200 trees planted in 2008. The trees here produce prolific fruit and there is successful seedling establishment around the farm (M. Stainbank, March. 2019, pers. comm.). Therefore, this orchard was chosen as a comparative group of *W. salutaris* for the KNP population. The orchard is surrounded by agricultural land, mainly sugar cane farms, and where natural vegetation does occur, it falls under the Dry Coast Hinterland Grassland vegetation type (Mucina & Rutherford, 2006). The climate of this area is characterised by summer rainfall with some rain in winter (Mucina & Rutherford, 2006). The average annual precipitation is ~ 850 mm (Mucina & Rutherford, 2006). The highest and lowest mean monthly maximum and minimum temperatures are 27.3 °C in February and 5.3 °C in June, respectively (Mucina and Rutherford, 2006).

2.2.2 Study species

Warburgia salutaris is a fast-growing evergreen tree that can reach approximately 12 m in height (Botha *et al.*, 2004; Burrows *et al.*, 2018). Multi-stem variants are also found as the tree readily re-sprouts when damaged by humans, elephants, and fire (Botha *et al.*, 2004). The species is found in a diverse range of habitats; it occurs on dry rocky slopes, in wooded ravines and in wet coastal forests (Coates-Palgrave, 2002; Schmidt *et al.*, 2002). The flowering season is reported to be from April until June; however, this varies considerably between regions. During the flowering season, many small (5–7 mm diameter), green, inconspicuous flowers are borne in the leaf axils (Schmidt *et al.*, 2002). The floral structure consists of the outer calyx of three green sepals, surrounding a whorl of five green petals, and an inner whorl of five white petals (Coates-Palgrave, 2002). The flowers are bisexual; in the centre of the flower are 10 fused stamens, forming a tube, which envelops the ovary and style (Fig. 2) (Coates-Palgrave, 2002). The style has 3–5 stigma lobes, which surround a sticky surface and the ovary contains 15–20 ovules (Leonard & Viljoen, 2015). From October through to January

(this may differ depending on flowering time), medium-sized (30–50 mm diameter) round green berries develop and turn purple with a white bloom as they mature (Burrows *et al.*, 2018). The fruit contains several reniform seeds (~10 x 5 mm) with an oily, fleshy endosperm (Salazar & Nixon, 2008).

Figure 2: *Warburgia salutaris* flower – in the centre of the flower are 10 fused stamens, forming a tube, which envelops the ovary and style, and the sticky stigmatic surface protrudes above. The dehisced anthers can be seen in the exposed part of the androecium. Petals and sepals surround the androecium (some removed for photograph).

2.2.3 Experimental design

Fieldwork in the Kruger National Park took place from the 11th of May – 5th of June 2019. Over the first four days, each sub-population was visited and those with flowering trees were selected for further study. Six sub-populations[2] were selected based on estimated population size (number of individuals), density[3], accessibility, and presence of flowering individuals in May–June 2019 (Table 1). The proportion of trees observed flowering ranged between 2.5 – 100% of the estimated individuals, depending on the population. Flowering was often erratic and imbalanced, occurring on a few localised lower branches of the trees.

Each selected tree was marked with a round, numbered metal tag, which was nailed to the main stem 1 m above the ground. This procedure was repeated at Eston, where fieldwork took place from the 14th of June until the 28th of June 2019. The two populations – older

[2] The sub-populations used were between 900 m to 5.7 km apart.

[3] In the calculation of overall density, the density of each population was weighted by its population size, based on current population density estimates (D. Thompson, Jan. 2019, pers. comm.).

population (~ 90 trees) and younger population (~ 200 trees) – were visited to select flowering trees for further study. Only three trees of *W. salutaris* with accessible flowers, all located in the older population, were marked. Follow-up fieldwork on certain aspects of the pollination biology of *W. salutaris* took place in the 2020 flowering season in KNP (22nd of June –1st of July) and Eston (12th of July – 27th of July). Fieldwork commenced later than intended due to the national lockdown in response to Covid-19.

Table 1: The relative size, density and numbering of flowering individuals[4] (total n = 42) in the six selected sub-populations of *Warburgia salutaris* in the Kruger National Park.

Sub-population	Size	Density (plants/ha)	Number of flowering individuals
1	Large	119	10
2	Large	37	8
3	Large	53	10
4	Medium	906	5
5	Medium	48	4
6	Small	625	5

2.2.3.1 Effective pollinators: Insect observations and collections, pollen load analysis and nectar measurements

Pollinator observations and collections

In KNP in the 2019 flowering season, an hour of dedicated insect observations were conducted in sub-population 1, 2, 3 and 4 (when weather conditions were warm and dry) from late-morning to midday when insect activity would be highest (i.e. 4 hours in total). Incidental observations of insect activity during hand pollination work were recorded and when possible, insects were captured (n = 51 in total: Appendix 1; Table 1) using a small net, placed in vials with ethyl acetate, and stored in a freezer. In the 2020 flowering season[5], insect observations were conducted over six days (i.e. 9 hours total) on two flowering trees in sub-population 1 and floral visitors were collected (n = 17 in total: Appendix 1; Table 1). The insect specimens

[4] An individual was a single-stemmed (> 5 cm diameter at breast height) or multi-stemmed tree that was not connected to another tagged tree.
[5] Due to COVID19 Lockdown restrictions I was not able to access the flowers during their peak flowering time, therefore, only two trees in sub-population 1 were available for insect observations in late June 2020.

were viewed under a Zeiss Discovery V12 dissecting microscope and photographed with an AxioCam MRc camera attached to it. Field guides and various insect websites (viz. www.abctaxa.be) were used for initial identifications. Entomological experts[6] were then contacted for further identification and confirmation of the insect visitors.

Bushnell Nature View camera traps (models 119456 and 119477, China) were set up for one night in each sub-population to observe whether there was evening insect activity (as physical observations could not be carried out at night in the KNP). One or two cameras were set up in each sub-population, depending on suitability of terrain and ability to reach low-hanging focal flowers. Cameras were placed approximately 1 m away from the flowers and 1–15 flowers were in view, depending on the number of flowers available. The cameras were set to capture three photos every minute from the time the camera was set up mid-morning until collection the next morning. In total, 5704 photos were taken.

In Eston, pollinator observations were conducted to assess insect activity during the day and evening. Daytime observations were conducted for one to two hours between 10:00 and 14:00, to cover the cooler early morning and warmer midday to early afternoon hours, when insect activity would be highest (i.e. 2019: 10 hours total; 2020: 13 hours total). Evening observations took place for an hour from sunset (i.e. from between 17:00 and 17:30) to observe crepuscular insect activity (i.e. 10 hours total in both 2019 and 2020). As with KNP, specimens were sent to entomological experts[6] for identification.

Pollen load

Insects collected were examined for pollen under a portable dissecting microscope in the field on the day of capture. Insects that were found to carry pollen grains in the field were selected for pollen load analysis (Appendix 2; Table 2). Pollen load was analysed using the method outlined in Dafni *et al*. (2005). The insect was placed on a microscope slide and 95% ethanol was slowly dropped over the pollen-carrying parts of the insect (Dafni *et al*., 2005). Once the ethanol had dried, a drop of Calberla's staining solution (method in Appendix 3) was

[6] Dr. James du G. Harrison of the University of the Witwatersrand (beetles), Hermann Staude of LepSoc (moths), Guin Zambatis of the Skukuza Biological Reference Collection, KNP (butterflies); Prof. Michael Kuhlmann at the Zoological Museum of Kiel University, Germany (bees), Dr. John Midgley of KwaZulu-Natal Museum, Pietermaritzburg (flies), Peter Hawkes of AfriBugs, Pretoria (ants), Michael Stiller of Agricultural Research Council (ARC) (thrips) and Beth Grobbelar of ARC (beetles).

placed on the slide and covered with a coverslip and a 1 mm x 1 mm grid. The slide was viewed under a Zeiss Axio Imager M2 microscope and the number of stained pollen grains was counted.

Nectar measurements

Nectar was analysed to assist in the identification of likely pollinators of *W. salutaris.* This was not done in KNP because nectar was initially difficult to draw up into the capillary tube and flowers were prioritised for the pollination trials, pollen viability and pollen tube analyses, and insect observations. Nectar volume and sugar concentration measurements were carried out on 10 flowers each on four trees in Eston, KZN. Flowers were bagged the afternoon before collection and were collected in the early morning to ensure the nectar had neither been drawn up by an insect nor had evaporated. Nectar was extracted using 5 µl capillary tube with a manual aspirator attached to the tube. Nectar volume was measured as a proportion of the nectar in the 5 µl capillary tube and then deposited onto a *Palm Abbe* digital refractometer to calculate nectar sucrose concentration in percentage Brix (1% Bx =1g sucrose/100g solution).

2.2.3.2 Pollination trials

Prior to the pollination trials, flower buds were covered with pale green organza bags[7] (28 cm length x 19cm width) to prevent natural pollination (Fig. 3A). Each bag was placed over a branch with approximately 10 buds. If it was not possible to bag 10 buds, smaller groups of buds on different branches totalling 10 were bagged. The number of flowers used per treatment varied depending on the number of flower buds that opened and were available to use during the fieldwork period (see Appendix 4; Table 3). The following treatments were set up on each selected experimental tree:

1. Autonomous self-pollination: flowers unmanipulated and insect pollinators excluded by bagging the buds.
2. Hand self-pollination*: to test for self-pollination between flowers within the same tree (geitonogamy).
3. Hand cross-pollination: to test for pollination between flowers on genetically different trees.
4. Natural-bagged pollination: flowers left open to pollinators for at least a week and then bagged. The flowers last for approximately a week from opening, therefore, a week was

[7] The cup shape of the flower prevents the spread of pollen onto the bags and other flowers.

sufficient to allow for pollination, however, it is possible that not all flowers were pollinated before bagging.

5. Natural pollination (control): flowers left open to pollinators and to predation of the fruit (i.e. not bagged).

* Note: Self-pollination within the same flower (autogamy) was not initially tested for two reasons: 1) the number of buds available was limited, and 2) anthers were seldom dehisced and providing pollen when stigmas were receptive. However, in Eston a few autogamous self-pollinations were done as it was possible here, and may match what we could expect in KNP.

The bags and branches were labelled with coloured ribbon according to the pollination treatment: autonomous self-pollination (blue), geitonogamous self-pollination (yellow) and cross-pollination (pink) (e.g. Fig. 3A), natural-bagged pollination (orange), and natural-unprotected pollination (red). A fifth green organza bag was left unlabelled and mature flowers from this bag were used as pollen donors, because pollen was often depleted from the flowers left open to insects.

Hand pollinations were conducted as flowers opened. To hand pollinate the flowers, a sewing needle tip was used to remove pollen from a pollen donor and placed on the stigma of the recipient flower (Fig. 3B). 70% ethanol was used to clean the needle between pollinations. In the hand cross-pollinations, pollen was initially taken from a tree within the same population, but not the nearest neighbour, to pollinate the recipient tree. For the most part, however, because of the risk of getting pollen from a clonal tree, donor pollen[8] was taken from a different sub-population. In all the treatments, except for natural pollination, the flowers were left bagged to prevent insect pollination. Self-pollinated flowers were marked using a short piece of red insulation tape, and cross-pollinated flowers with a short piece of white insulation tape, on the leaf creating the axil in which the flower was growing (Fig. 3C).

Although it is standard procedure to emasculate flowers prior to cross-pollination and geitonogamous self-pollination, it was not possible to do this to flowers of *W. salutaris*. The arrangement of the anthers around the gynoecium and within the tightly closed petals made emasculation difficult and damaged the flower when attempted. Emasculation trials were

[8] Donor pollen was transported in a closed vial and used between 3–6 hours in the nearest sub-population.

conducted to test this on 10 flowers of a *W. salutaris* individual near Lanseria, Johannesburg, in March 2019, prior to fieldwork in the Kruger National Park. All flowers died as a result of the damage they incurred during the emasculation process. Lastly, to test the longevity of stigma receptivity, a drop of hydrogen peroxide was place on a few mature flowers with slightly browned stigmas. The stigmas produced bubbles, which indicated they were still receptive to pollen.

Figure 3: Set up of pollination trials: (A) a green organza bag covering flower buds for the cross-pollination treatment (indicated by pink ribbon), (B) K. van den Bosch hand pollinating a flower using magnifying lenses, and (C) a hand cross hand-pollinated flower indicated by the white insulation tape.

2.2.3.3 Male function: Pollen size, viability and pollen tube analysis

Pollen viability

To test for pollen viability, four flowers on each of: five trees in sub-populations 1, 2, 3, and 5; four trees in sub-population 4 and 6 in KNP; and three trees in the Eston orchard were collected (see Appendix 5; Table 4 for breakdown). Each flower was viewed under a dissecting microscope and forceps were used to remove the anther sheath from the flower. The anther sheath was stored in an microcentrifuge tube with 0.25 ml of 0.1% aniline blue solution until laboratory analyses. The microcentrifuge tube was gently vortexed to resuspend the pollen grains, and 40 µl of the solution was placed onto a microscope slide with a 1 mm x 1 mm gridded coverslip and viewed under a Zeiss Axio Imager M2 microscope. Stained (viable) and unstained (nonviable) pollen grains were counted and the percentage of stained pollen grains

was calculated. During the pollen counts it was noted that nonviable pollen appeared to be smaller than viable pollen. Therefore, pollen size (i.e. diameter) of viable (n = 60) and nonviable (n = 60) pollen grains was measured.

Pollen tube analysis

To see whether pollen tubes were forming and reaching the ovule in the three pollination treatments (i.e. self-, cross- and natural pollination), the following was undertaken. In four sub-populations − 1, 2, 3 and 6 – five trees were selected, and a bag was placed over a branch with a minimum of four buds to be used for pollen tube analysis. Two flowers were selected for each treatment: self-pollination, cross-pollination and natural pollination (see Appendix 6; Table 5 for breakdown). For self- and cross-pollination, two flowers per treatment were hand pollinated and collected 24 hours later. For natural pollination, it could not be ascertained if a flower had been naturally pollinated; however, flowers were collected if pollen was visible on the stigma or if the stigma had browned slightly, which indicated the flower had been open for a few days, and therefore, had been potentially pollinated. To further clarify the breeding system, self-pollinations were repeated in KNP and Eston in the latter part of the 2020 flowering season[9], and left for 48 hours to assess if the self-pollen tubes reach the ovules.

Once collected, each flower was viewed under a dissecting microscope, and fine forceps were used to remove the pistil. The pistil was placed in an microcentrifuge tube with 500 µl of ethanol: glacial acetic acid solution (2:1; i.e. Carnoy's solution without chloroform) for 2 hours, and then transferred to 70% ethanol until laboratory analyses (Kalinganire *et al.*, 2000). In preparation for fluorescence analysis, the pistils were rehydrated in 50% ethanol for 20 min, 30% ethanol for 20 min, and finally in distilled water for 20 min. The distilled water was removed from the microcentrifuge tube and replaced with 8 M NaOH and left for 24 hours at room temperature. The NaOH was removed and the pistils were rinsed for 20 minutes, again in distilled water. They were then placed in 0.1% aniline blue in a 0.1 M K_3PO_4 buffer overnight to be stained. Prior to microscope analysis the pistils were removed, cut longitudinally, and placed on a microscope slide with a drop of 50% glycerol to be viewed under an Olympus BX63 fluorescence microscope at the Microscopy and Microanalysis Unit at University of the Witwatersrand.

[9] Due to COVID19 Lockdown restrictions I was not able to access the flowers during their peak flowering time.

2.2.4 Data and statistical analyses

All statistical tests were performed using *R* software, R version 3.3.2 (R Core Team, 2016).

2.2.4.1 Nectar measurements

A Kruskal Wallis test (Shapiro-Wilk normality test; $p < 0.05$) was used to compare nectar volume and nectar sugar concentration using 10 flowers each from four trees in the Eston orchard. A Spearman's rank correlation test (Shapiro-Wilk normality test; $p < 0.05$) was used to analyse the relationship between the nectar volume and nectar sugar concentration.

2.2.4.2 Pollination trials

General linear models (GLM), followed by an ANOVA (test = chi-squared), were used to compare fruit set between each pollination treatment (self-, cross-, natural- and natural-bagged pollination) and across each sub-population (family = binomial, $p < 0.05$). Autonomous self-pollination was excluded from analyses as it consistently resulted in no fruit set. Chi-squared (χ^2) contingency tables and/or Fisher's exact tests (when values in a cell were less than five) were used to compare fruit set between treatments within KNP and within Eston. A simple linear regression ($p < 0.05$) was used to compare the average fruit set for each pollination treatment with population size (number of individuals).

The following indices, adapted from Johnson *et al.* (2009) and Tamura & Kudo (2000), were determined for each individual and averaged across each sub-population. Index of self-incompatibility (ISI) is calculated as percentage fruit set from geitonogamous self-pollination divided by percentage fruit set from cross-pollination. Index of self-incompatibility values range from 0 (fully self-incompatible) to 1 (fully self-compatible).

2.2.4.3 Male function: Pollen viability and pollen tube analysis

General linear models, followed by an ANOVA (test = chi-squared), were used to compare pollen viability between each sub-population, i.e. the six KNP sub-populations and Eston (family = binomial, $p < 0.05$). A Mann-Whitney-U test (Shapiro-Wilk normality test; $p < 0.05$) was used to compare mean pollen viability between KNP and Eston ($p < 0.05$). A Kruskal Wallis test (Shapiro-Wilk normality test; $p < 0.05$) followed by a Kruskal Wallis multiple comparison post hoc test (package "pgirmess"), to compare pollen grain number between sub-populations. Fisher's exact tests were used to compare pollen tube growth (i.e.

growth or no growth) between each pollination treatment (self-, cross-, and natural pollination) and across each sub-population. A two-sample t-test ($p < 0.05$) was used to compare pollen grain size between viable and nonviable pollen.

2.3 Results

2.3.1 Effective pollinators

2.3.1.1 Insect observations and identifications

Warburgia salutaris flowers are visited by a range of insects — including moths (order Lepidoptera), butterflies (order Lepidoptera), flies (order Diptera), beetles (order Coleoptera), bees (order Hymenoptera), ants (order Hymenoptera) and thrips (order Thysanoptera) (Fig. 4; Tables 2 & 3). Mean pollen load carried per insect type is reported in Appendix 2, Table 2.

Figure 4: Floral visitors to *Warburgia salutaris*: (A) butterfly *Axioceres tjoane* in Kruger National Park (KNP), (B) moth *Amata simplex* in KNP, (C) moth *Vietteania intestata* in the Eston orchard, (D) butterfly *Deudorix dinochares* in KNP, (E) bee *Hypotrigona* sp., in KNP and (F) ant *Polyrhachis schistacea* in KNP. Scale bar:10 mm. Photo credits: Mike Goodman for images 4B and 4F.

Table 2: Identifications of insects visiting *Warburgia salutaris* flowers during the day in the Kruger National Park in 2019 and their relative abundance (i.e. rare: < 3 observations; common: 4−10 observations; frequent: > 10 observations over the total observation period in 2019/2020).

Family	ID	Relative abundance
	Lepidoptera (Moths)	
Erebidae	*Amata simplex* Walker	Common
Erebidae	*Pseudonaclia puella puella* Boisduval	Rare
	Lepidoptera (Butterflies)	
Pieridae	*Dixeia doxo parva* Talbot	Common
Lycaenidae	*Hypolycaena phillippus* Fabricius	Common
Nymphalidae	*Axiocerses tjoane* Wallengren	Common
Lycaenidae	*Deudorix dinochares* Grose-Smith	Frequent
Lycaenidae	*Tuxentius melaena melaena* Trimen	Rare
	Diptera (Flies)	
Rhiniidae	Unknown	Frequent
Sciaridae	Unknown	Frequent
	Coleoptera (Beetles)	
Chrysomelidae	*Pseudocolaspis sp.* Laporte	Frequent
	Hymenoptera (Bees)	
Apidae	Allodapini tribe	Common
Apidae	*Compsomelissa sp.* Alfken	Rare
Apidae	*Hypotrigona sp.* Cockerell	Frequent
Apidae	*Meliponula sp.* Cockerell	Rare
	Hymenoptera (Ants)	
Formicidae	*Polyrhachis schistacea* Gerstäcker	Common
Formicidae	*Pheidole* (Westwood), possibly *P. megacephala* Fabricius	Common
Formicidae	*Tetraponera natalensis* Smith F.	Rare

Table 3: Identifications of insects visiting *Warburgia salutaris* flowers in the Eston orchard, KwaZulu-Natal in 2019 and their relative abundance (i.e. rare: < 3 observations; common: 4−10 observations; frequent: > 10 observations over the total observation period in 2019/2020). Moths were observed in the early evening and all other insects visited the flowers during the day

Family	ID	Relative abundance
	Lepidoptera (Moths)	
Noctuidae	*Vietteania intestata* Walker	Frequent
Geometridae	*Eois grataria* Walker	Rare
Geometridae	*Xanthorhoe poseata* Geyer	Common
Tortricidae	*Lozotaenia sp*. Harris	Rare
Geometridae	*Scopula sp.* Schrank	Rare
Erebidae	*Zekelita sp.* Walker	Rare
Erebidae	*Ilemodes astriga* Hampson	Rare
Erebidae	*Dysgonia angularis* Boisduval	Rare
Noctuidae	*Spodoptera exigua* Hübner	Frequent
Geometridae	*Piercia prasinaria* Warren	Rare
	Diptera (Flies)	
Syrphidae	*Graptomyza* Wiedemann	Rare
	Coleoptera (Beetles)	
Nitidulidae	Possibly *Carpophilus* sp. Stephens	Rare
	Hymenoptera (Bees)	
Apidae	*Apis mellifera scutellata* Lepeletier	Frequent
	Hymenoptera (Ants)	
Formicidae	*Camponotus*, possibly *C. niveosetosus* Mayr	Rare
	Thysanoptera (Thrips)	
Thripidae	Possibly *Megalurothrip* sp. Bagnall	Frequent

In the KNP, lepidopterans were frequent floral visitors (2019: 18 visits over 4 hours; 2020: 22 visits over 9 hours). All lepidopterans would insert their proboscis between the inner petal whorl and anthers to access nectar, which would allow them to contact the reproductive structures. The day-flying moth, *Amata simplex*, was found to carry the most pollen grains (n > 1000 on one specimen), situated in a clump on its proboscis (Figs. 5A & 5B). The butterflies, *Deudorix dinochares* (Fig. 5C), *Axioceres tjoane* (Fig. 5E), and *Hypolycaena phillippus* were common visitors and carried 10–30 pollen grains on their proboscises. The butterfly *Dixeia doxo parva* also visited the flowers, but no pollen was found on its proboscis. An undescribed species of beetle in the genus *Pseudocolaspis* (family Eumolpinae) was the most abundant

floral visitor (Fig. 5F); it would crawl around between the inner petals and anthers, however its role as an effective pollinator is uncertain. Pollen grains (n < 10) were seen on the thorax of three beetle specimens, but not detected on the pollen load slide. Flies in the families Sciaridae and Rhiniidae (Fig. 5J) were frequent floral visitors, however they placed their proboscises between the petals and did not contact the anthers. The stingless bee *Hypotrigona sp.* (Fig. 5H) was a frequent visitor in the 2020 flowering season (43 visits over 9 hours), but no pollen was seen on collected specimens. The ant, *Polyrhachis schistacea* (Fig. 5I), was a common floral visitor, however, it did not contact the reproductive structures when feeding on nectar and no pollen was found on the ant when examined under the microscope. Rare visitors included the three species of bees (small brown bee in the tribe Allodapini; *Compsomelissa* sp. & *Meliponula sp.*).

Figure 5: Insects collected in the Kruger National Park visiting *Warburgia salutaris* flowers: (A) moth *Amata simplex*, (B) pollen on proboscis of *A. simplex*, (C) butterfly *Deudorix dinochares*, (D) pollen on proboscis of *D. dinochares*, (E) butterfly *Axioceres tjoane*, (F) beetle *Pseudocolaspis* sp, (G) fly in family Rhiniidae, (H) bee *Hypotrigona* sp. (I) ant *Polyrhachis schistacea*.

At Eston, floral visitation was scarce during the day, but thrips (family Thripidae; Fig. 6A) and bees (*Apis mellifera scutellata;* Fig. 6B; 59 visits over 10 hours) were observed. Thrips were the most abundant visitors, but they did not carry pollen, and the bees carried small (n < 10) quantities of pollen grains on their proboscises, antennae and front legs. During the evening observations, a variety of moths were found to be floral visitors (2019: 64 visits over 10 hours; 2020: 66 visits over 10 hours; Figs. 6C, 5E & 5G). The proboscis would be inserted between the inner petal whorl and anthers to access nectar. Pollen grains were found on the proboscis of five out of the 16 moths, the most pollen being on the *Ilemodes astriga* (n > 1000 pollen grains on one specimen; Fig 5F), the barred carpet moth (*Xanthorhoe poseata*, subfamily Larentiinae; n = 57 pollen grains; Fig. 5D) and *Dysgonia angularis* (n = 39 pollen grains). Pollen was also found on a flower fly (*Graptomyza* sp.) that was caught feeding on nectar (Figs. 5I).

Figure 6: Insects collected in the Eston orchard visiting *Warburgia salutaris* flowers: (A) Thrip, possibly *Megalurothrip* sp., (B) honey bee *Apis mellifera scutellata*, (C) moth *Xanthorhoe poseata*, (D) pollen on proboscis of *X. poseata* (E) moth *Ilemodes astriga*, (F) pollen on proboscis of *I. astriga*, (G) moth *Vietteania intestata*, (H) pollen on proboscis of *V. intestata*, and (I) fly *Graptomyza sp.*

2.3.1.2 Nectar measurements

Warburgia salutaris flowers in KNP were found to have crystallised nectar, suggesting that liquid had evaporated (Fig. 7). As a result, nectar volume/concentration was not quantified as nectar was difficult to draw up into the capillary tube. At Eston, average nectar volume of *W. salutaris* was 2.4 ± 1.56 µl (n = 40) and average sucrose concentration was 39.7 ± 8.6% (n = 40). Nectar sugar concentration and volume had a weak, inverse, non-significant relationship (r = - 0.178, df = 40, p = 0.259) (Fig. 8). There were no significant differences in nectar volume (Kruskal Wallis χ^2 = 6.161, df = 3, p = 0.104) and nectar sucrose concentration (Kruskal Wallis χ^2 = 1.373, df = 3, p = 0.712) between trees in the Eston orchard (Appendix 7; Fig. 1A & 1B).

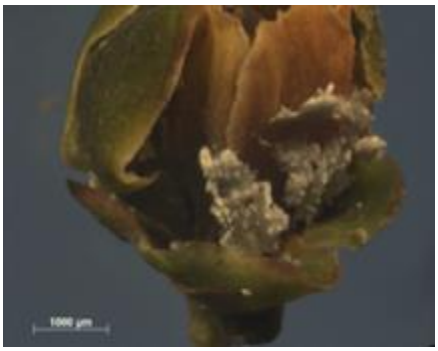

Figure 7: Crystallised nectar between the petals of a *Warburgia salutaris* flower in the Kruger National Park in the 2019 flowering season.

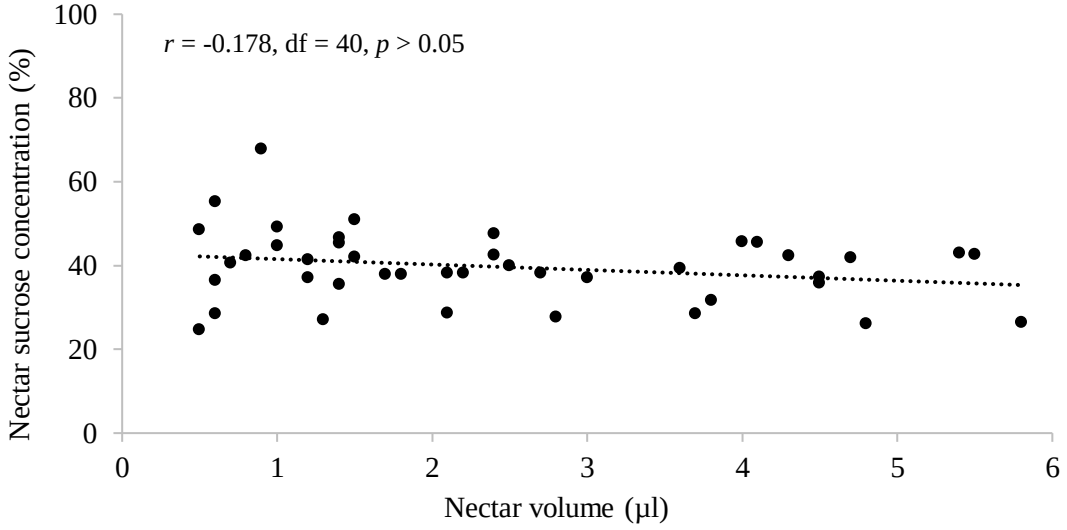

Figure 8: Nectar sucrose concentration (%) against volume (µl) for *Warburgia salutaris* flowers in the Eston orchard. There was a weak, negative relationship between the variables (r = -0.178, df = 40, p > 0.05).

2.3.2 Pollination trials

In KNP, no fully developed fruit were produced for the hand-pollinations, however, a small number were found to have been aborted shortly after fertilisation (Fig. 9), and these numbers were included in percentage fruit set.

Figure 9: (A) A very early aborted fruit, and (B) a comparison of a very early aborted fruit (above) with an unfertilised ovary (below) of *Warburgia salutaris* found in the 2019 Kruger National Park pollination trials.

In total in KNP, 2% (5/245) of hand cross-pollinations and 5% (11/231) of hand self-pollinations resulted in very early aborted fruit (Fig. 10). Autonomous self-pollination was excluded as it consistently resulted in 0% fruit set across all KNP sub-populations and the Eston orchard. Mean ($\bar{x}$) aborted fruit size (i.e. width of widest section of ovary/fruit) was 2. 4 mm (n = 5 crosses) and 2.3 mm (n = 11 selfs). Similarly, 34/36 natural bagged fruit that had started developing were aborted ($\bar{x}$ size = 2.5 mm). Forty-five percent (19/41) of trees had a few immature fruit developing on them (sub-population 1: n = 2; sub-population 2: n = 5; sub-population 3: n = 9; sub-population 4: n = 3), and sub-population 3 was the most productive sub-population with 7/10 trees having produced fruit through natural pollination. However, by the fruiting season in December 2019, only two trees in sub-population 3 out of the 42 tagged KNP trees (i.e. 4.8%) across 4 sub-populations had sustained fully developed fruit. Between KNP and Eston, there was only a significant difference in fruit set for the cross-pollination treatment (Fisher's exact test: Odd's ratio = 0.049, $p < 0.001$; Fig. 10). No fruit were produced for any of the pollination treatments in either sub-population 5 or sub-population 6 (Fig. 11).

The sample size for Eston was far smaller than the KNP, however it produced two important results. First, the cross-pollination treatment resulted in fully developed fruit and had the highest fruit set (6/22; 27%) here, and second, no fruit was produced for the self-pollination treatment. The natural-bagged treatment resulted in the second highest fruit set (7/27; 26%) in Eston (Fig. 10). Although according to Figure 10, natural fruit production in Eston appears equally as low as KNP, this is not a true representation of overall fruit production in this orchard.

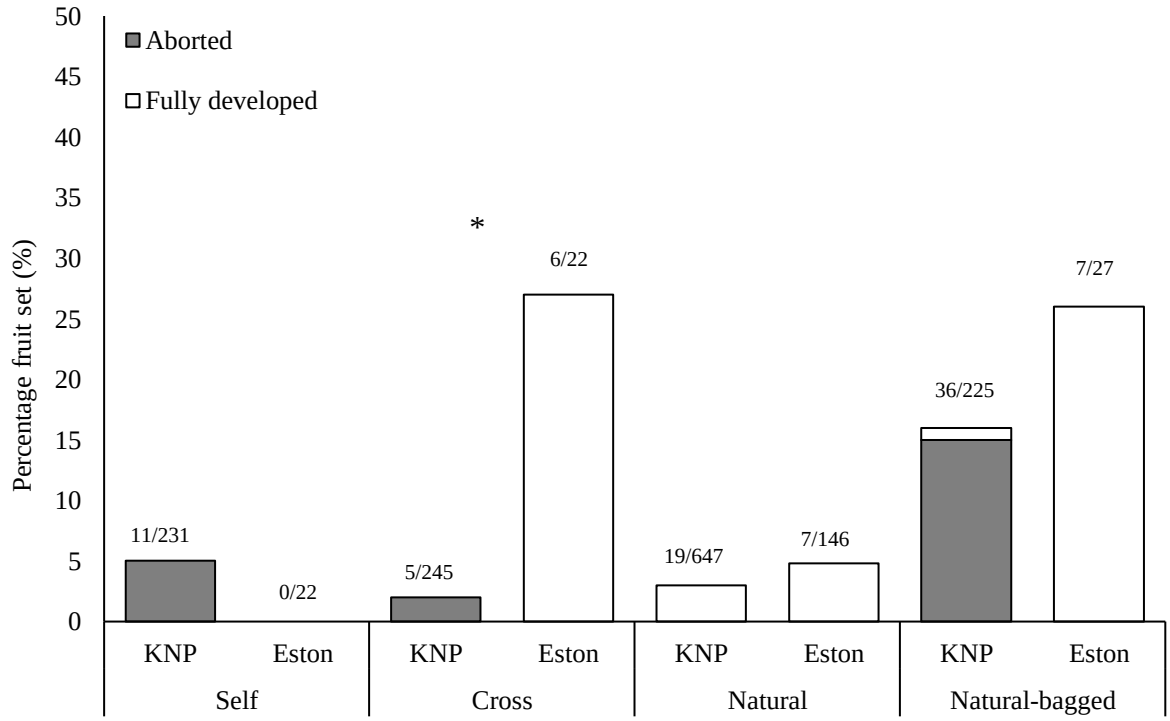

Figure 10: Percentage fruit set (including aborted and fully developed fruit) between the four pollination treatments in *Warburgia salutaris* in the Kruger National Park (KNP) and Eston orchard. Autonomous self-pollination was excluded as it consistently resulted in no fruit. Asterisk (*) indicates significant difference in fruit set for cross-pollination between KNP and Eston (Fisher's exact test, $p < 0.001$).

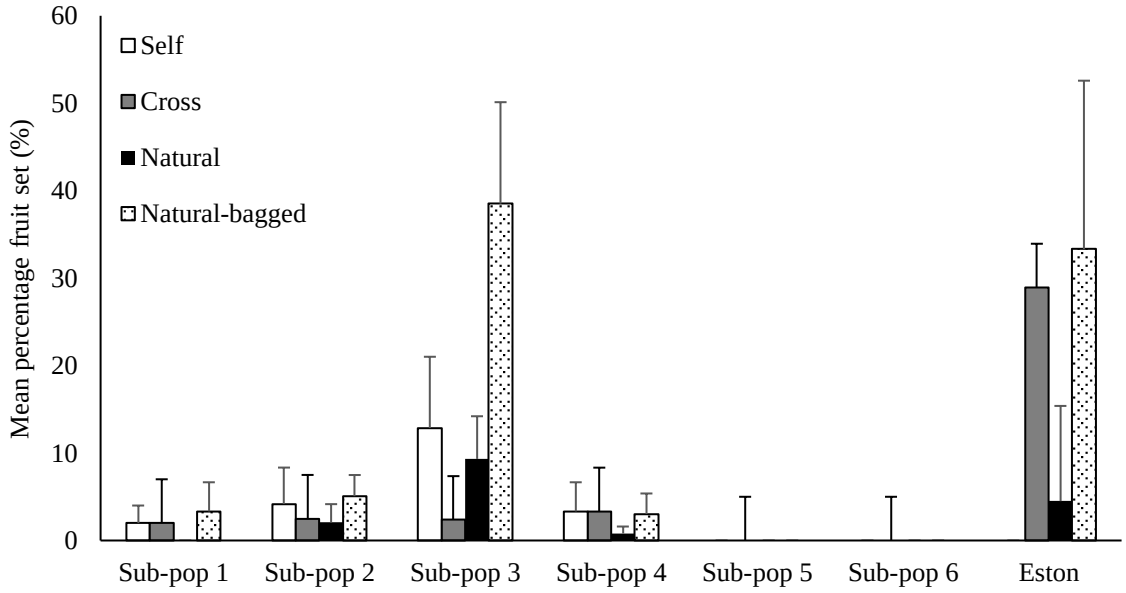

Figure 11: Mean (+ SE) percentage fruit set for each pollination treatment (excluding autonomous self-pollination) across the six Kruger National Park sub-populations and Eston orchard.

There was no significant difference between fruit set total for self-pollination (GLM ANOVA: $\chi^2_{6, 232} = 12.032$, $p = 0.061$; Fig. 12A). However, there was a significant difference in fruit set total for cross-pollination (GLM ANOVA: $\chi^2_{6, 251} = 15.714$, $p = 0.015$; Fig. 12B), natural-pollination (GLM ANOVA: $\chi^2_{6, 672} = 32.445$, $p < 0.001$; Fig. 12C) and natural-bagged pollination (GLM ANOVA: $\chi^2_{6, 225 =} 60.435$, $p < 0.001$; Fig. 12 D).

Within KNP only, there were no significant differences in fruit set total for self-pollination (GLM ANOVA: $\chi^2_{3, 189} = 7.2042$, $p = 0.06567$; Fig. 12), or cross pollination (GLM ANOVA: $\chi^2_{5, 239} = 2.4924$, $p = 0.7776$; Fig. 12). However there were significant differences in fruit set total for natural pollination (GLM ANOVA: $\chi^2_{5, 641} = 31.565$, $p < 0.001$; Fig. 12) and natural-bagged pollination (GLM ANOVA: $\chi^2_{5, 218} = 58.392$, $p < 0.001$; Fig. 12). Within KNP only, fruit set for the natural and natural-bagged treatments in the large sub-population 3 was still significantly higher than the other sub-populations, and there was still no significant difference in fruit set for self-pollination between the sub-populations. However, there was no significant difference in fruit set across the sub-populations for cross-pollination when Eston was excluded.

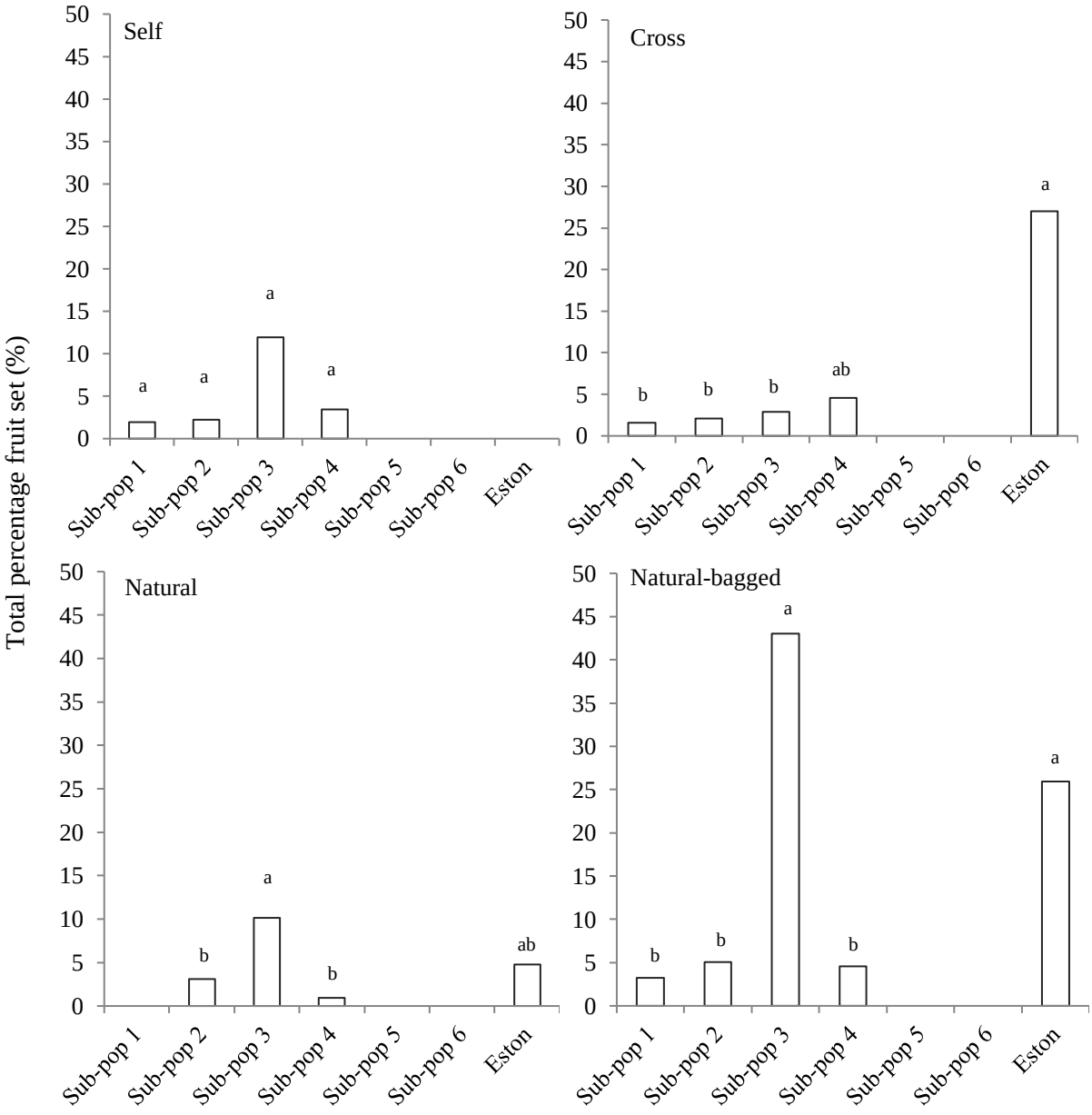

Figure 12: There were significant differences in fruit set total for cross-pollination (GLM: $\chi^2_{6, 251} = 15.714$, $p < 0.05$), natural-bagged (GLM: $\chi^2_{6, 225} = 60.435$, $p < 0.001$) and natural pollination (GLM: $\chi^2_{6, 672} = 32.445$, $p < 0.001$) between the Eston orchard and Kruger National Park sub-populations, but not for self-pollination. Lower case letters indicate significant differences between sub-populations (GLM ANOVA, $p < 0.05$).

Comparison between treatments within KNP and Eston

In the KNP, natural-bagged pollination resulted in significantly higher fruit set than natural- (2x2 $\chi^2_1 = 31.535$, $p < 0.001$), cross- (2x2 $\chi^2_1 = 27.142$, $p < 0.001$) and self-pollination (2x2 $\chi^2_1 = 14.506$, $p < 0.001$) (Fig. 13A). In Eston, fruit set from natural-bagged pollination was significantly higher than natural pollination (Fisher's exact test: odd's ratio = 0.142, $p < 0.01$) and cross-pollination (Fisher's exact test: odd's ratio = 8.768, $p < 0.05$; Fig. 13B). In Eston, zero fruit resulted from self-pollination, and in KNP, there was no significant difference in fruit set between self-pollination and cross-pollination (2x2 $\chi^2_1 = 1.937$, $p = 0.164$; Fig. 13).

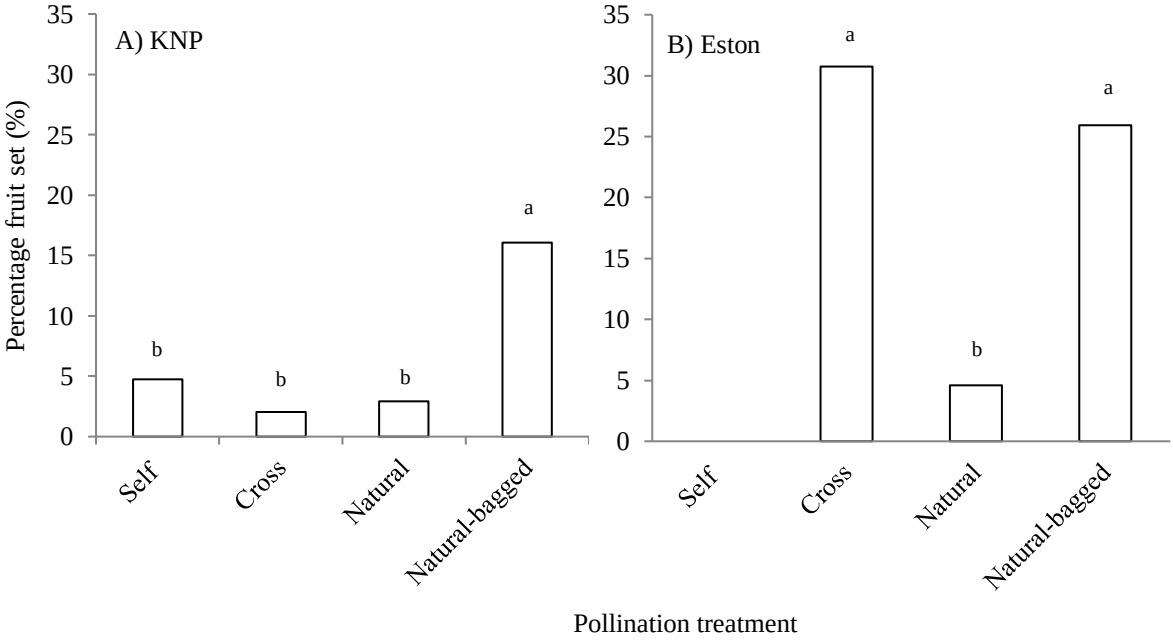

Figure 13: (A) In the Kruger National Park (KNP), the natural-bagged pollination treatment was significantly higher than natural-, cross- and self-pollination (χ^2 test, $p < 0.05$). (B) In the Eston orchard, the natural-bagged and cross-pollination treatments were significantly higher than natural pollination (Fisher's exact test, $p < 0.05$). Lower case letters indicate significant differences between treatments.

Index of self-incompatibility (ISI)

Sub-population 1, 2 and 3 were the only sub-populations that produced fruit for self- and cross-pollination. All three sub-populations had ISI values of 1, i.e. suggesting they are fully self-compatible. However, given that none of the self- or cross-pollinations resulted in fully developed fruit, we cannot confirm *W. salutaris* is self-compatible. However, in Eston,

all trees had ISI values of 0, i.e. are self-incompatible, as only cross-pollination resulted in fully developed fruit.

Population size and fruit set

Population size (i.e. estimated number of individuals in each sub-population) had no significant effect on percentage fruit set from self (R^2 = -0.181, p = 0.785), cross (R^2 = -0.158, p = 0.688), natural (R^2 = -0.197, p = 0.887) or natural-bagged (R^2 = -0.197, p = 0.920) pollination treatments (Fig. 14).

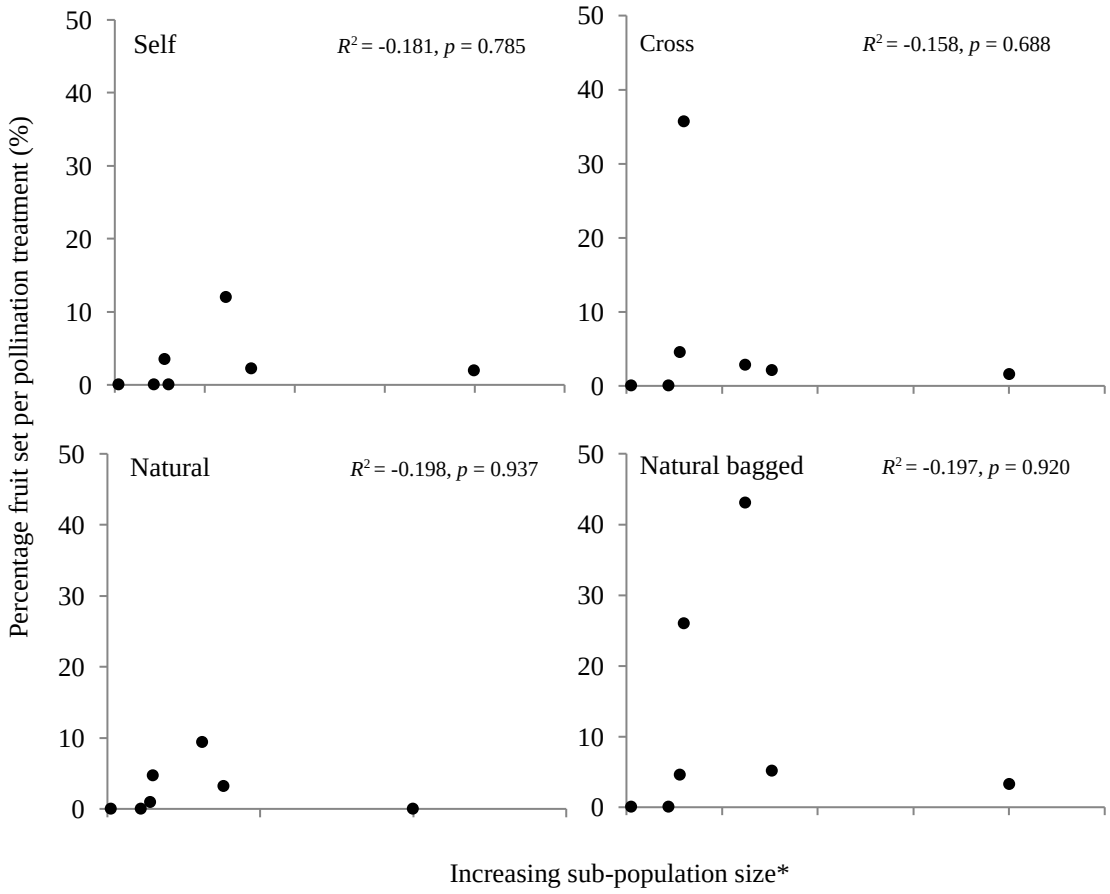

Figure 14: Mean percentage fruit set for each pollination treatment per sub-population in relation to increasing sub-population size (x-axis is an interval scale). There were no significant relationships for any of the regression analyses (p > 0.05). *Due to the protected status of the species, values of estimated number of individuals were excluded from the figure.

2.3.3. Male function: Pollen viability and pollen tube analysis

Observations of the development of *W. salutaris* from bud to mature flower shows that it is protogynous. When anthesis occurs, the inner whorl of petals is tightly bound to the ovary, covering the undehisced anthers, and the plump, sticky stigma protrudes above (Fig. 15A). After approximately 24 hours, the inner whorl of petals opens slightly and reveals the dehisced anthers (Fig. 15B). The stigma does not become non-functional at this stage, but instead remains sticky and receptive.

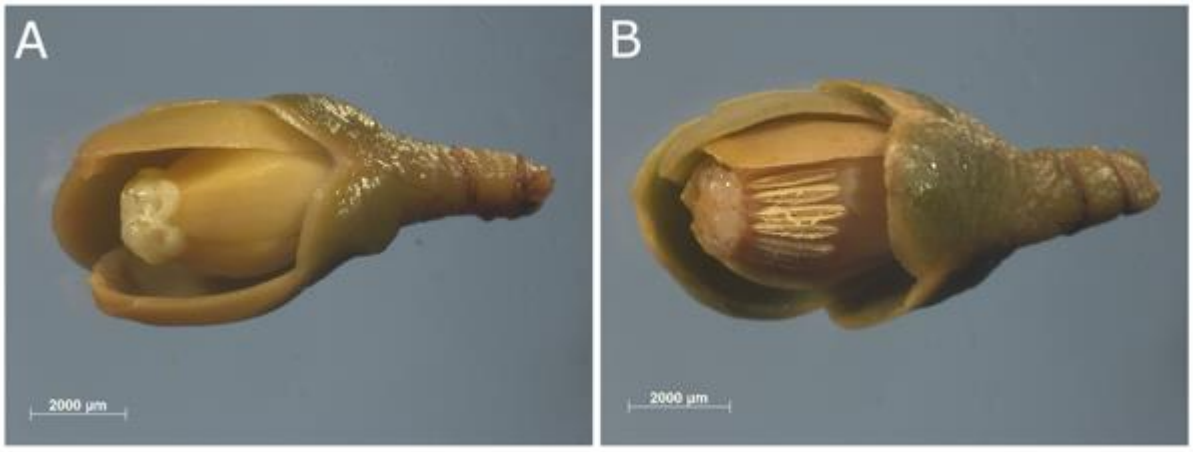

Figure 15: (A) a receptive stigma and inner whorl of petals covering stamens, and (B) exposed dehisced anthers (three petals removed) of a *Warburgia salutaris* flower.

2.3.3.1 Pollen viability

Viable pollen (33.04 ± 3.8 µm diameter, n = 60) was larger and darker than nonviable pollen (~ 26.36 ± 3.9 µm diameter, n = 60) (t = 9.457, df = 118, $p < 0.001$; Fig. 16). Percentage pollen viability for each sub-population was relatively high (Fig. 17). Sub-population 6 had the highest pollen viability (93%), while sub-population 4 had the lowest (66%) (Fig. 17).

There were significant differences in pollen viability across KNP sub-populations and Eston (GLM ANOVA: $\chi^2_{6, 111} = 477.7$, $p < 0.001$; Fig. 17). Overall, average pollen viability was significantly higher in the KNP sub-populations combined than in the Eston population (Mann-Whitney U test: W = 954, $p < 0.05$; Appendix 8; Fig. 2).

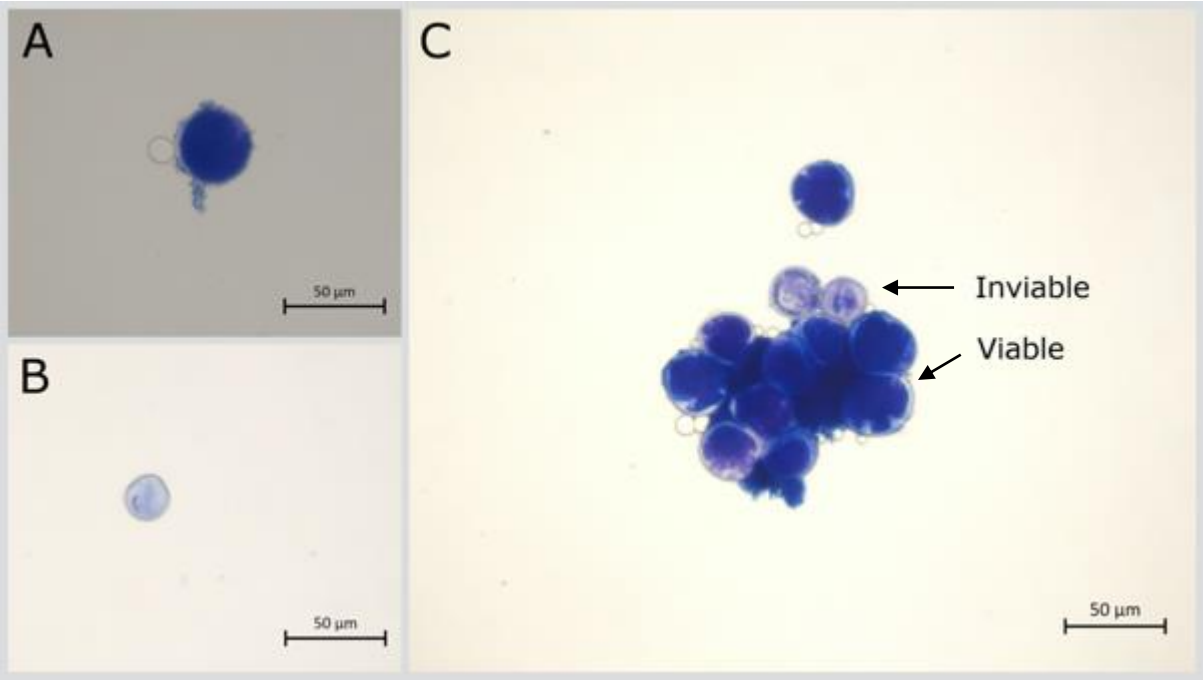

Figure 16: (A) A viable pollen grain, (B) a nonviable pollen grain, and (C) nonviable pollen grains amongst viable pollen grains (indicated with arrows).

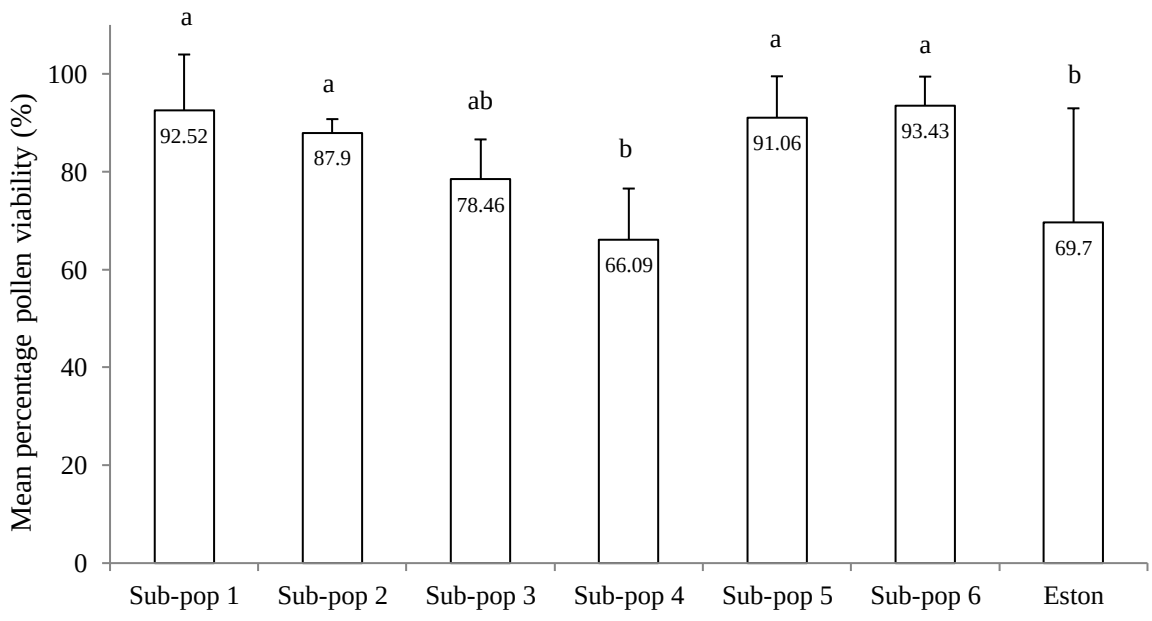

Figure 17: Mean (+SE) percentage pollen viability for the six Kruger National Park sub-populations and Eston orchard (values indicated on graph). Lower case letters indicate significant differences between sub-populations (Kruskal Wallis multiple comparison test, $p < 0.05$).

2.3.3.2 Pollen number

There were significant differences in the pollen grain number per flower between sub-populations (Kruskal Wallis $\chi^2_5 = 29.269$, $p < 0.001$). The average number of pollen grains was significantly lower in sub-population 2 (n = 16) and sub-population 5 (n = 17) (Kruskal Wallis $\chi^2_6 = 32.17$, $p < 0.001$, Fig. 18). The significant relationships remained the same when Eston was excluded (Kruskal Wallis $\chi^2_5 = 29.269$, $p < 0.001$), as when Eston was included. Overall, average pollen number was not significantly different between the KNP sub-populations combined and Eston (Mann-Whitney U test: W = 630.5, $p = 0.729$) (Appendix 9, Fig. 3).

In sub-population 5, the anthers never dehisced, even in mature flowers. Pollen was manually removed by slicing open the anthers. This may be the reason for low average total pollen here (Fig. 18). It is interesting to note that although the anthers in sub-population 5 did not dehisce, the sub-population had among the highest average percentage pollen viability.

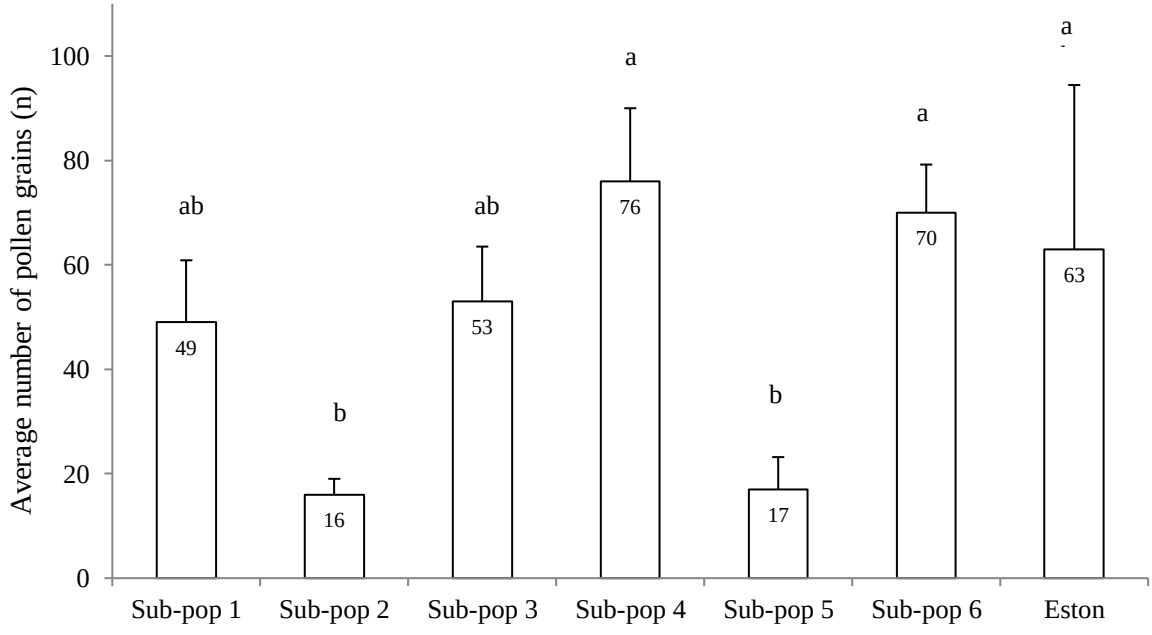

Figure 18: Mean (+SE) number of pollen grains for the six Kruger National Park sub-populations and Eston orchard (values indicated on graph). Lower case letters indicate significant differences between sub-populations (Kruskal Wallis multiple comparison test, $p < 0.05$).

2.3.3.3 Pollen tube production

Pollen tubes were produced in self-, cross- and natural pollinations (Fig. 19A, 19B & 19C). In KNP, pollen tubes were found in 33% (13/40) of self-pollinated flowers, 33% (13/40) of cross-pollinated flowers, and 35% (14/40) of naturally pollinated flowers (Fig. 20). In Eston, pollen tubes were found in 100% (4/4) cross pollinated flowers, 67% (2/3) self-pollinated flowers, and 33% (2/6) naturally pollinated flowers (Fig. 20). Pollen tube production for cross-pollination was significantly higher in Eston than KNP (Fisher's exact test: odd's ratio = 0, $p < 0.05$)(Fig. 20).

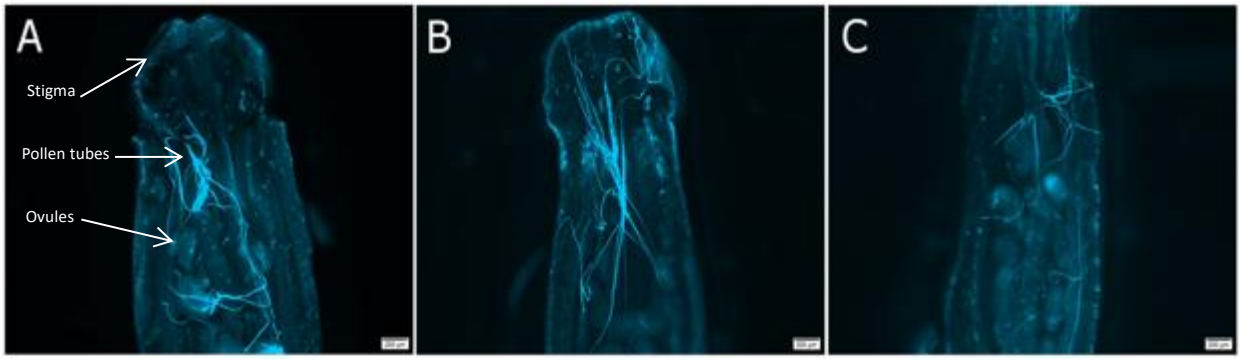

Figure 19: Pollen tubes resulting from (A) natural pollination, (B) cross-pollination, and (C) self-pollination. Viewed under an Olympus BX63 fluorescence microscope.

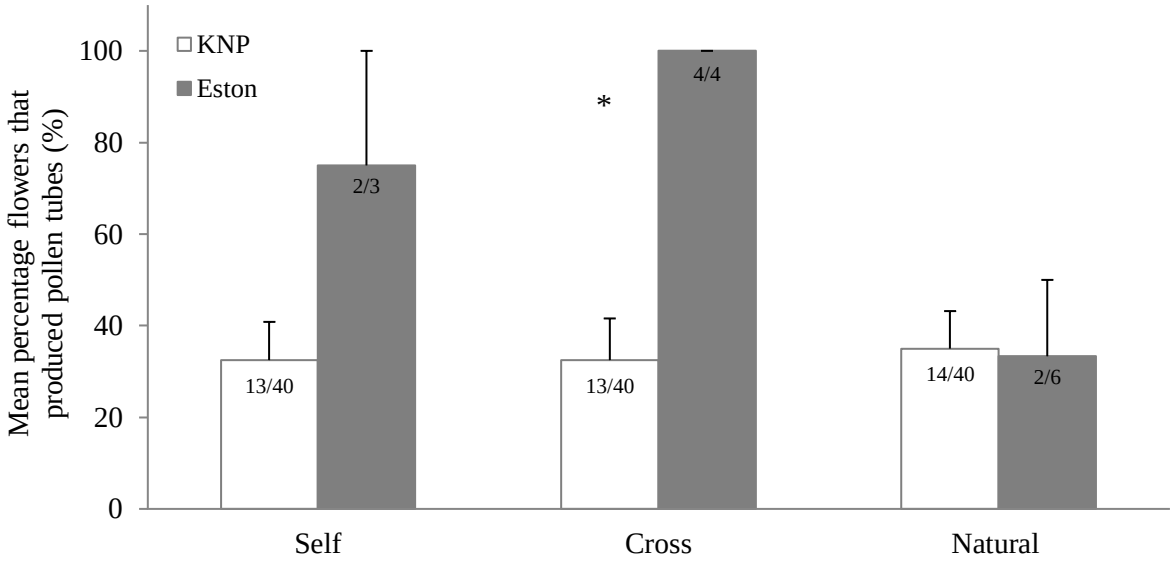

Figure 20: Mean (+ SE) percentage flowers that produced pollen tubes for each pollination treatment in the Kruger National Park (KNP) and Eston orchard. Asterisk (*) indicates significant difference in pollen tube production between KNP and Eston for cross-pollination (Fisher's exact test, $p < 0.05$).

Across the sub-populations, there were significant differences in pollen tube production for self-pollination (Fig 21A), and cross-pollination (Fig 21B), but not for natural pollination (Appendix 10, Fig. 4). Overall, sub-population 3 had the highest percentage pollen tube occurrence in KNP (Fig. 22).

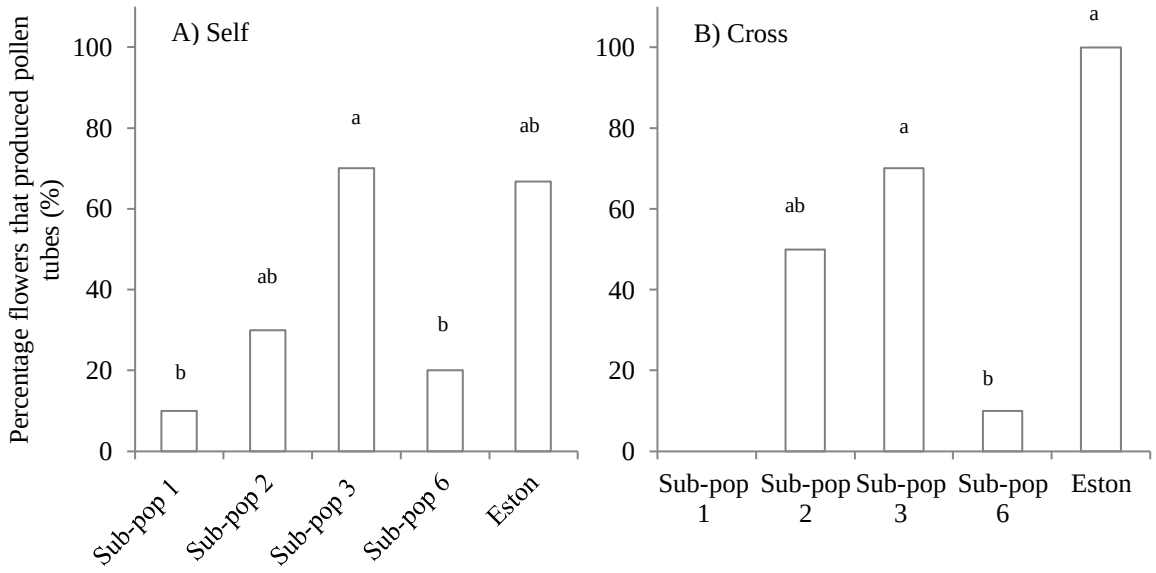

Figure 21: Percentage flowers with pollen tubes for (A) self-pollination and (B) cross-pollination in four Kruger National Park sub-populations and Eston orchard. Lower case letters indicate significant differences between sub-populations (Fisher's exact test, $p < 0.05$).

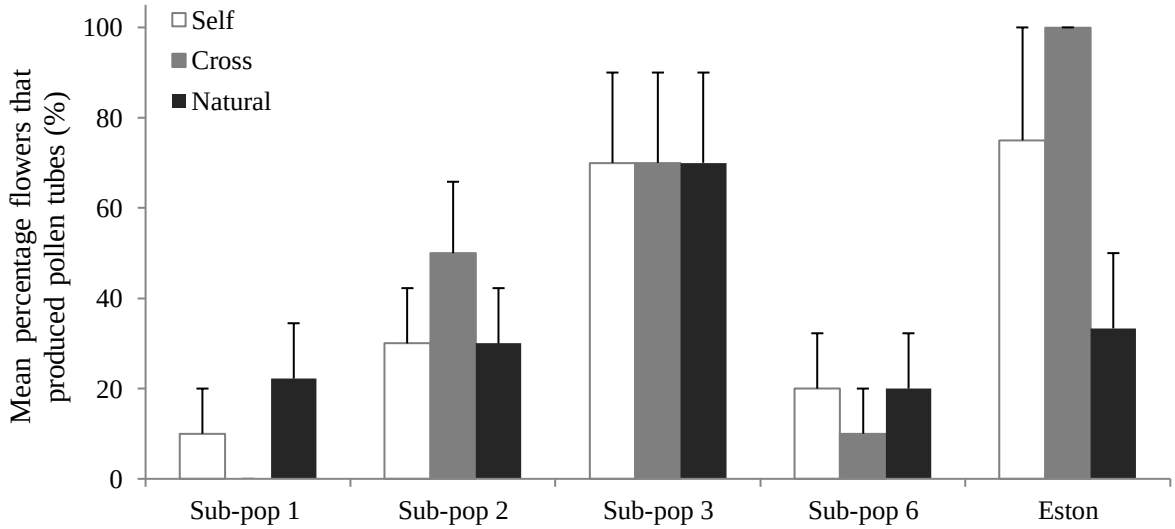

Figure 22: Mean (+ SE) percentage flowers with pollen tubes for each of the pollination treatments in the four Kruger National Park sub-populations and Eston orchard.

When pollen tubes were examined after 48 hours' post hand pollination, in Eston, 40% of self-pollen tubes reached the ovules, whereas in KNP only 7% reached the ovules (Fig. 23).

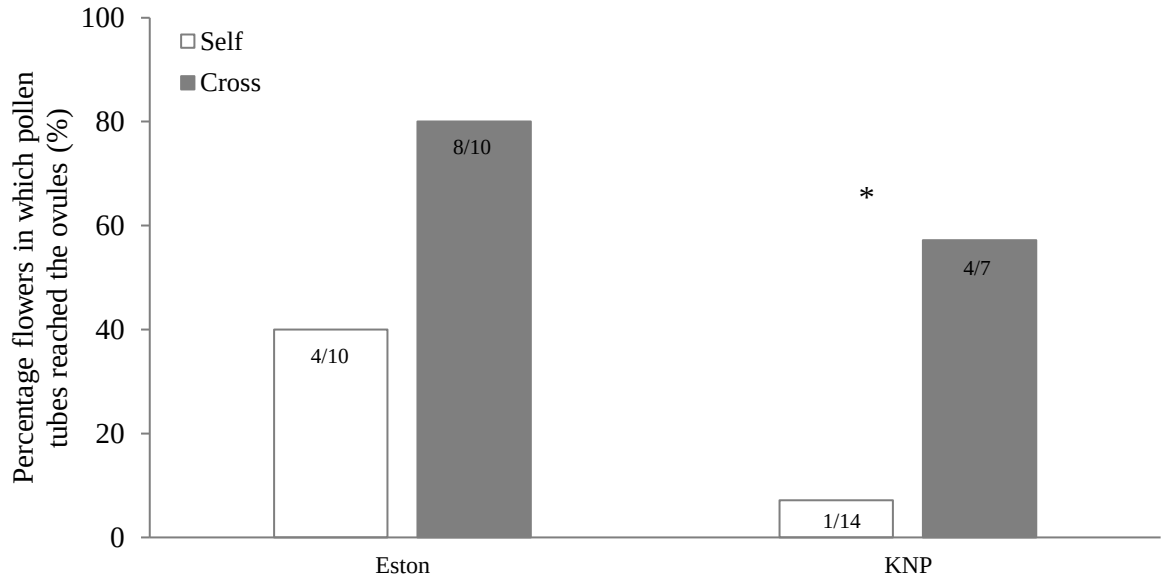

Figure 23: Percentage flowers in which pollen tubes from self- and cross-pollination reached the ovules after 48 hours' post hand pollination in the Kruger National Park (KNP) and Eston orchard. Asterisk (*) indicates significant difference between cross and self-pollination in KNP (Fisher's exact test, $p < 0.05$).

2.4 Discussion

2.4.1 Pollinators

In the Kruger National Park (KNP), *Warburgia salutaris* attracts a variety of insect floral visitors. Insects are divided into functional groups based on their morphology and behaviour, and each functional group may vary in their ability to pollinate a flower (Faegri & van der Pijl, 1979). The beetles (*Pseudocolaspis* sp.) were the most abundant floral visitors and could climb inside the flower and move over the reproductive structures. However, their role as effective pollinators is uncertain, firstly, because *W. salutaris* appears to be self-incompatible, therefore, the beetle's low vagility and limited ability to carry pollen makes them ineffective pollinators. Secondly, beetles in this genus are known to be pollen feeders (Steiner, 1998). Therefore, they may impair pollination by depositing self-pollen and contribute to pollen limitation by feeding on the pollen (Faegri & van der Pijl, 1979). The stingless bees (*Hypotrigona* sp.) and lepidopteran visitors were both common floral visitors, especially in the

2020 flowering season. However, only the lepidopterans were found to carry pollen on their proboscises following a floral visit (e.g. > 1000 pollen grains for the day flying moth, *Amata simplex*). In comparison to beetles, the lepidopterans have a higher vagility, and therefore can carry cross-pollen to conspecific flowers. In addition, their long proboscis was observed being inserted between the inner petals and reproductive structures to access the nectar (if nectar was not evaporated/crystallised), facilitating the scraping up of pollen when the proboscis is removed. The ants (*Polyrhachis schistacea*) and flies (families Rhiniidae and Sciaridae) appeared to be the least compatible pollinators as they would either access nectar from the outside of the flower or between the two petal whorls and no pollen was carried by them.

In the Eston orchard, thrips (possibly *Megalurothrips* sp.) were the most abundant floral visitors, and up to 20 thrips and their larvae could be found in a single flower. However, their ability to pollinate *W. salutaris* flowers remains unclear. Thrips are less likely to move between trees and they did not carry pollen when examined, therefore they are not considered suitable pollinators for self-incompatible plants (Eliyahu *et al.*, 2015). Honeybees (*Apis mellifera scutellata*) were also common floral visitors in the 2020 flowering season, however, they carried limited pollen on their body. In the early evening, *W. salutaris* flowers were visited by a variety of settling moths at a higher rate than diurnal pollinators and carried pollen on the proboscis.

Pollinators visit flowers with preferential nectar concentrations to match the energetic requirements of the insect and the insect's mechanical ability to ingest the nectar (Daniel *et al.*,1989; Josens & Farina, 2001). An increase in nectar sugar concentration results in an increase in the viscosity of the nectar fluid, and this results in a decreased rate of flow through an insect's proboscis (Josens & Farina, 2001). Therefore, the optimal nectar concentration for butterflies (20–25%; Kim *et al.,* 2011) is generally lower than that of honeybees (~ 35%; Kim *et al.,* 2011) and stingless bees (~ 40% for *Hypotrigona* sp.*;* Kajobe, 2007). Similarly, settling moths visit flowers with a moderately concentrated nectar (~ 20–34%) (Makholela & Manning 2006). Overall, a relatively high average sucrose concentration (39.7 ± 8.6%) was found for *W. salutaris*, and the range of nectar concentrations (24.7–67.9%) overlaps the optimal range for butterflies, honeybees, stingless bees and moths. Nectar concentration can fluctuate with changes in temperature and humidity, and as a result can fluctuate during the day (Rathcke, 1992). Therefore, the crystallised nectar found in KNP flowers may be a result of higher temperatures causing the water in the nectar to evaporate. Flowers can alter the timing of peak

nectar production, and this may coincide with the activity of their main effective pollinator (Young, 2002). However, in this study nectar was only measured in the morning from flowers that had been bagged the day before. Assessing nectar production over time may assist in understanding the relative importance of diurnal and nocturnal pollinators (Young, 2002).

The pollinator observations revealed that *W. salutaris* has an abundance of previously unreported floral visitors. Despite the limited pollen load found on the insects (with the exception of *A. simplex*), the lepidopterans (moths and butterflies) are the most likely effective pollinators of *W. salutaris.* They are able to insert their proboscis between the enclosed petals to access nectar and their relatively higher vagility enables them to carry cross pollen between trees. Furthermore, moths were the most abundant visitors in the Eston orchard, and were also captured on the camera trap photographs at night in the KNP. The solitary bees and honeybees were frequent visitors in KNP and Eston, respectively, in the 2020 flowering season. Therefore their role as pollinators cannot be disregarded, however the lack of pollen found on the specimens in this study suggests they are not effective. Pollen load may have also been low because the anthers in some flowers were not yet dehisced at the time of the visit, pollen may have already been removed, and lastly, pollen may have been consumed by pollen feeders.

2.4.2 Breeding system

Pollination trials were conducted to establish the breeding system of *W. salutaris*. It was confirmed that *W. salutaris* cannot autonomously self-pollinate, and percentage fruit set from self-pollination (5%) and cross-pollination (2%) treatments was very low in KNP sub-populations. In addition, neither resulted in fully developed fruit; therefore, we couldn't safely confirm that *W. salutaris* here is self-incompatible. In Eston, although the sample size was much smaller, the results were clearer: no fruit resulted from self-pollination and successful cross pollinations resulted in fully developed fruit. These results were the same for additional pollination trials carried out on *W. ugandensis* in Eston, where only cross-pollinations resulted in fully developed fruit. Furthermore, the self-pollinations left for 48 hours showed that geitonogamous self-pollen tubes can reach the ovules. In contrast, the additional autogamous self-pollinations did not produce pollen tubes or fruit in Eston. Therefore, it appears that geitonogamous self-pollen tubes can occasionally fertilise the ovules, but the fruit are aborted shortly after fertilisation. Some plants have mixed-mating strategies for reproductive assurance, for example, delayed selfing, whereby self-pollination occurs if the flower was not

cross-pollinated (Goodwillie *et al*., 2005; Charlesworth, 2006). However, the results from this study show that *W. salutaris* is primarily out-crossing, as there was no evidence of a fully developed self-pollinated fruit. In KNP, where flowering is limited and pollinators are restricted to flowers on the same tree, this can result in higher levels of self-pollination and ovule discounting. In contrast, in Eston, pollinators were observed moving between trees across the orchard, facilitating outcrossing.

2.4.3 Male function: Pollen viability and pollen tube analysis

The low percentage fruit set in KNP was assessed further by looking at the male function. Male sterility can manifest in the form of malformed stamens, non-dehiscent anthers, and production of nonviable pollen (Vinod, 2005). In this study the average percentage pollen viability in KNP (84%) was higher than in Eston (70%). In addition, there were no individuals in KNP with significantly low pollen viability. These results suggest that pollen viability is not a factor contributing to the low reproductive output in the KNP population. However, regarding pollen number, it was often difficult to find flowers with sufficient pollen for hand pollinations and pollen viability analysis. This is most likely from the high occurrence of pollen feeding beetles found within the KNP *W. salutaris* flowers. There was also a lack of anther dehiscence in sub-population 5, where, no fruit for natural- or hand pollinations were found. The cause of this is not known, however, non-dehiscence of anthers is a form of male sterility that is likely the result of mutations in nuclear and/or cytoplasmic genes (Vinod, 2005).

Pollen tube formation was assessed to further clarify the breeding system and to establish at what stage the process of fruit production is inhibited (Goodwillie *et al.*, 2005). The higher percentage occurrence of pollen tubes relative to the percentage fruit set indicates that there may be a postzygotic mechanism influencing reproductive output (Krebs & Hancock,1990; Hao *et al.*, 2012; Goodwillie *et al.*, 2005). Two likely mechanisms are the accumulation of deleterious mutations, or late-acting self-incompatibility (Krebs & Hancock,1990; Hao *et al.*, 2012). It is suggested that variation in aborted seed size and a positive correlation between self and cross seed set distinguishes the expression of deleterious mutations from late-acting self-incompatibility, however this was not possible in this study as fruit were aborted at a very early stage in development. For the hand self-pollinations this may be late-acting self-incompatibility, however, the even lower success of cross-pollinations may suggest that it is the result of expressed deleterious mutations after the ovule is fertilised. The

KNP *W. salutaris* population is possibly much older than the Eston orchard (which was established in 1992). Therefore, the level of deleterious genes that accumulate over time would be higher in KNP than Eston, which may provide an additional explanation for lower fruit set in KNP.

2.4.4 Sexual versus asexual reproduction

The abortion of fruit in the cross- (2%) and natural-bagged pollination treatments and very low fruit set for natural (4%) pollination suggests that there is a barrier to reproduction in the KNP population. Some evidence of initial fruit development was found on 46% of trees in KNP during the flowering season in 2019; however, during the return field visit, no fruit was found on any tree except two trees in sub-population 3. In 2020, fruit was produced on the same two trees and an additional tree in the same sub-population. In Eston, although results from the natural pollination treatment suggest fruit set is equally as low as KNP, this figure (4.8%) is not a true representation of fruit set in the orchard. Most fruit appeared to be produced higher in the canopy which was too high to access.

Fruit abscission is a known phenomenon in healthy trees and may be attributed to resource partitioning. For example, the selective abortion of lower quality fruit (e.g. that have low seed set due to fertilisation by few pollen grains) may occur so that resources are directed to mature high quality fruit (Stephenson, 1981). However, this does not seem to be the main explanation in KNP as there is an extreme lack of fruit production overall. A more likely explanation for the early loss of fruit may be for preferential resource allocation to storage organs for vegetative growth and asexual reproduction (Stephenson, 1981) – which recent genetic analyses suggest is widespread within the KNP population (D. Thompson, Nov. 2020, pers. comm.). Furthermore, it was determined that *W. salutaris* is self-incompatible, therefore, fruit abortion in general may also be the result of self-pollination between neighbouring clonal ramets of *W. salutaris*.

A plant's reliance on sexual and asexual reproduction can be influenced by multiple factors, including genetics, plant size, environmental conditions (i.e. soil moisture, light intensity and nutrient availability), disturbance, and population density (Yang & Kim, 2016). Seedling establishment and survival is episodic for some species, especially under unsuitable environmental conditions, therefore, sexual reproduction may be of lower importance in long-

lived plants (Ortega-Baes & Gorostiague, 2013; Yang & Kim, 2016). Recent genetic analyses (in which the two fruiting trees were included), suggest that the fruiting trees are genetically distinct from the surrounding trees in sub-population 3. Therefore, they were produced from seed, unlike many of the surrounding clonal ramets, and it is possible that they are a comparatively successful genotype.

2.4.5 Impact of disturbance

Warburgia salutaris is able to resprout through vigorous coppicing when damaged, and as a result many of the trees in KNP are multi-stemmed (Hilton-Taylor *et al*., 1998; Botha *et al*., 2004). Similarly to clonal reproduction, the ability to resprout requires the plant to have stored reserves (e.g. starch) and meristems to enable regrowth (Pate *et al.,* 1990). This allocation of resources to storage for resprouting is traded off with growth or sexual reproduction (Kruger *et al.,* 1997). For example, a study on regeneration in forests on the south east coast of South Africa found that areas with many multi-stemmed species also had a lack of seedlings (Kruger *et al.,* 1997). Evidence of resprouting and a lack of seedlings is also reported for *W. salutaris* and was observed in KNP. In contrast, the *W. salutaris* trees that produced mature fruit are large and have limited coppicing, suggesting they have been exposed to low levels of disturbance (i.e. herbivory, fire and damage by elephants), and this may be an additional reason for their successful reproduction (Helm *et al*., 2011).

Evidence of elephant damage during the study, and coppicing in response to past damage was noted in sub-populations 1, 2 and 3. During the study, five tagged trees, along the ridge of sub-population 1 and 3, were impacted by elephants – three were completely pulled down and two were partially broken. The evergreen trees are particularly susceptible to elephant damage during the winter months, when they stand out amongst the drier vegetation on the slopes (S. Chauke, May 2019, pers. comm.). The impact of elephant damage was assessed in two sub-populations in the KNP *W. salutaris* population in 2018 (Wilken, 2018). Results showed that growth has not been hindered by the damage, but it is not known what effect it may have on reproduction (Wilken, 2018). Based on observations made during this current study, it was noted that in some cases *W. salutaris* appears to respond to branch breakage with an over-production of flower buds on damaged branches. There is literature describing stress-induced flowering in response to light intensity, temperature, poor nutrition, drought, low oxygen, crowding, and root removal (Takeno, 2016). However, there is little

information to explain flowering in response to herbivory and branch breakage (Takeno, 2016). While many flowers were produced on some broken branches, no fruit had matured on these branches on the return visit.

The area where *W. salutaris* occurs in KNP was last exposed to fire in 2007 and 2004. The cause of fire in these years is not specified, however fires in 2001, 1999 and 1996 were classified as arson. Botha *et al.* (2004) reported that *W. salutaris* trees exposed to regular fires were more susceptible to disease and had a higher percentage mortality, especially young plants and saplings. While no information exists on the effects of fire on reproduction of *W. salutaris*, the long-term effects of fire on reproduction has been described for other woody species in savannas (Hoffmann, 1998). Fire can negatively impact sexual reproduction in plants by destroying the reproductive structures of adults and damaging and/or killing juveniles which have not yet reached a fire-tolerant size (Hoffmann, 1998). Trees exposed to frequent fire have higher production of root suckers and in some cases also have a lower reproductive output (Hoffmann, 1998).

2.4.6 Climate effects

The climate in northern KNP is hotter and receives a lower mean annual rainfall (~ 530 mm) than the Eston orchard in KZN (~ 850 mm). Furthermore, the northern eastern part of South Africa has a higher coefficient of variation of precipitation (30–35%) than the eastern coast of KZN, near Eston (< 25%; Schulze, 1997). Total annual rainfall in KNP, measured at the Skukuza camp weather station from 1977 to 2017 also reflects this high variability (Dube & Nhamo, 2020). Flowering and fruiting may vary with interannual rainfall and temperature changes (Craparo *et al.,* 2020), however these relationships have not been monitored for *W. salutaris*. In periods of low rainfall, drought stress can impact aspects of reproduction such as nectar concentration and pollen viability (Descamps *et al*., 2018), seedling survival (Venter & Witkowski, 2013), and driving plants to direct resources from sexual reproduction to vegetative growth and asexual reproduction (Helm *et al.,* 2011).

2.4.7 Future work and recommendations

This study has revealed important and previously undocumented aspects of *W. salutaris*' reproductive biology. However, it has also posed more questions that need to be answered and which should help guide future studies on the species.

Our understanding of the pollination biology of *W. salutaris* can be explored further. Direct nocturnal pollinator observations should be carried out in KNP to quantify floral visitation rate by moths in this population and identify the species of moth as these might differ from those observed in Eston, KZN. Pollinator exclusion experiments could be set up to quantify the relative contribution of nocturnal and diurnal pollinators to *W. salutaris* fruit set (e.g. Balmford *et al.*, 2006; Payne *et al.*, 2016). The importance of nocturnal pollinators can also be further established by measuring nectar production at different times of the day and night to see when nectar volume is at its peak (Young, 2002). In addition, floral scent analysis and floral spectral reflectance analysis would also be valuable to isolate important pollinators of *W. salutaris* (Walsh *et al.*, 2019). Pollinator efficiency in KNP can be assessed by looking at pollen deposition on stigmas of naturally pollinated flowers.

There was an obvious difference in reproductive success between the sub-populations in the KNP. Sub-populations 5 and 6 did not produce fully developed fruit for natural pollination or the hand pollinations. In sub-population 3 some natural fruit were produced and there was evidence that some of the hand pollinations fertilised the ovules, however development ceased prior to maturation. For follow up studies, pollen tube data should be compared between the sub-populations that produced natural fruit and the sub-populations that did not produce any fruit. Aspects of stigma receptivity (i.e. timing and duration) could also be assessed as a potential barrier to reproduction (Kalinganire *et al.*, 2000). The finding that anthers in sub-population 5 did not dehisce (possibly as a consequence of male sterility) should be revisited as this may be more widespread in the KNP population.

This is the first study to quantify fruit set within the KNP population. It will be important to continue monitoring fruit set in each sub-population to better understand the patterns of reproduction and establish whether fruit set is getting progressively lower or remaining stable. These data can then be compared with climate data, such as temperature and rainfall, to identify climate-related links that may give insight into the low fruit set in KNP. Further assessments should be done to confirm whether the juveniles or saplings in the population are in fact new genets (established from seed), or ramets produced through clonal reproduction. Finally, cross-pollinations between different genets (identified in the new genetics findings) within sub-populations should be conducted to see if fruit is successfully produced from genets that are in close proximity (e.g. between the fruiting trees and surrounding unrelated genets).

The inclusion of the Eston orchard served as a control to compare with KNP. However, repeating aspects of this study in other large natural populations of *W. salutaris* (e.g. Soutpansberg, areas of the Mpumalanga escarpment, Northern KZN) would be useful to see whether they also have limited sexual output and recruitment and investigate whether reasons for this are similar to the KNP population.

2.5 Conclusion

This study has revealed that *W. salutaris* produces low volumes of relatively concentrated nectar. This reward attracts a variety of floral visitors, but Lepidoptera are most likely effective pollinators. The flowers rely on insects to facilitate cross-pollination as the flowers are self-incompatible – no fully developed fruit from self-pollination were observed, despite pollen tubes being able to reach the ovules in some cases. Male function was not impaired as pollen viability was high overall, however, insect pollen load was limited in KNP and Eston, and in KNP very little pollen was available for hand pollinations. Only three trees in one KNP sub-population sustained fully developed fruit, and even when hand pollinated and protected from seed predation, the fruit that were initially fertilised were aborted early in development. Pollen tube occurrence was higher than expected (based on low fruit set in the initial pollination trials), suggesting that a genetic and/or resource induced postzygotic malfunction is negatively affecting sexual reproduction in *W. salutaris*. Therefore, it is possible that *W. salutaris* relies on clonal reproduction and resprouting to respond to disturbance and persist under unsuitable environmental conditions for sexual recruitment in northern KNP. However, reliance on long-term clonal reproduction may impact the success of future sexual reproduction. From a conservation perspective, sexual recruitment will be necessary to establish new genetically diverse genets of *W. salutaris* in order to maximise evolutionary potential of the endangered species in the Kruger National Park.

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2.7 Appendices

Appendix 1:

Table 1: Number of specimens per insect type collected in the 2019 and 2020 flowering seasons in the Kruger National Park (KNP) and Eston orchard. Total number of floral visits recorded over total number of observation hours for the highly mobile insects are given in brackets. Total number of specimens per insect type and overall percentage contribution is given in the last column.

Insect type	KNP 2019	KNP 2020	Eston 2019	Eston 2020	**Total**
Beetles	17	3	3	0	**23 (21%**
Butterflies	7 (13/4 hrs)	6 (21/9 hrs)	0	0	**13 (12%)**
Moths	3 (5/4 hrs)	1(1/9 hrs)	6 (64/10 hrs)	10 (66/10 hrs)	**20 (18%)**
Bees	4 (6/4 hrs)	6 (43/9 hrs)	1 (1/10 hrs)	5 (59/13 hrs)	**17 (15%)**
Flies	6 (18/4 hrs)	1 (1/9 hrs)	2 (2/10 hrs)	0	**8 (7%)**
Thrips	0	0	10	6	**16 (14%)**
Ants	14	0	0	0	**14 (13%)**
Total	**51**	**17**	**22**	**21**	**111 (100%)**

Appendix 2:

Table 2: Number of specimens per insect type that carried pollen in the 2019 and 2020 flowering seasons in the Kruger National Park (KNP) and Eston orchard. Mean + SE pollen load (n) carried by each insect type is given in the last column.

Insect type	KNP 2019	KNP 2020	Eston 2019	Eston 2020	**Total**	**Mean + SE pollen load (n)**
Beetles	4	1	2	0	**7 (24%)**	**2 ± 0.54**
Butterflies	3	1	0	0	**4 (14%)**	**10 ± 5.07**
Moths	2	1	2	6	**11 (38%)**	**96 ± 84.09**
Bees	3	0	0	3	**6 (21%)**	**3 ± 0.98**
Flies	1	0	0	0	**1 (3%)**	**1 ± 0.08**
Thrips	0	0	0	0	**0 (0%)**	**1** ± 0.07
Ants	0	0	0	0	**0 (0%)**	**0**
Total	**13**	**3**	**4**	**9**	**29 (100%)**	**28 ± 15.22**

Appendix 3: Calberla's staining solution ingredients (Dafni *et al*., 1995):

Ingredients

- 5 ml glycerine
- 10 ml 95% ethanol
- 15 ml distilled water
- Saturated aqueous basic fuchsin
- Glycerol

Method

Mix a small amount of fuchsin and glycerol together (1:1). Mix the glycerine, 95% ethanol, distilled water and add 6 drops of the fuchsin: glycerol solution.

Appendix 4:

Table 3: Total number of flowers used per pollination treatment per sub-population of *Warburgia salutaris* in the Kruger National Park (KNP), Limpopo and Eston, KwaZulu-Natal.

Sub-population	Number of flowering trees	Autonomous self-pollination	Cross-pollination	Self-pollination	Natural pollination	Natural-bagged pollination
Sub-population 1	10	83	52	52	116	31
Sub-population 2	8	73	48	45	160	50
Sub-population 3	10	101	67	67	128	72
Sub-population 4	5	48	22	29	107	16
Sub-population 5	4	30	14	14	48	14
Sub-population 6	5	66	27	24	88	26
KNP total	**42**	**401**	**245**	**231**	**647**	**225**
Eston	3	20	13	8	32	8
KNP & Eston Total	**45**	**421**	**258**	**239**	**679**	**217**

Appendix 5:

Table 4: Total number of *Warburgia salutaris* flowers used per sub-population to analyse pollen viability in the Kruger National Park (KNP), Limpopo and Eston, KwaZulu-Natal.

Sub-population	Flowers per tree (n) x Trees (n)	Total per sub-population (n)
Sub-population 1	4 x 5	20
Sub-population 2	4 x 5	20
Sub-population 3	4 x 5	20
Sub-population 4	4 x 4	16
Sub-population 5	4 x 4	16
Sub-population 6	4 x 5	20
Eston	4 x 3	12
Total	**124**	**124**

Appendix 6:

Table 5: Total number of *Warburgia salutaris* flowers used per sub-population to analyse pollen tube growth and compare between cross-, self- and natural pollination in the Kruger National Park (KNP), Limpopo and Eston, KwaZulu-Natal.

	Cross-pollination	Self-pollination	Natural pollination	
Sub-population	Flowers per tree (n) x Trees (n)	Flowers per tree (n) x Trees (n)	Flowers per tree (n) x Trees (n)	Total per sub-population (n)
Sub-population 1	2 x 5	2 x 5	2 x 5	30
Sub-population 2	2 x 5	2 x 5	2 x 5	30
Sub-population 3	2 x 5	2 x 5	2 x 5	30
Sub-population 6	2 x 5	2 x 5	2 x 5	30
Eston	2 x 3	2 x 3	2 x 3	18
Total	**46**	**46**	**46**	**138**

Appendix 7:

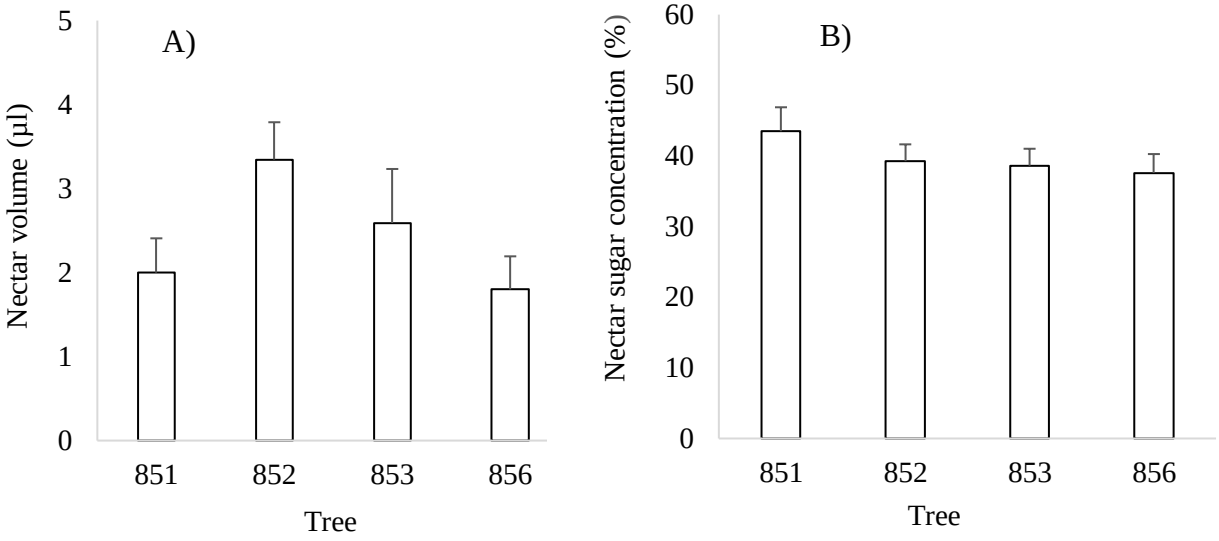

Figure 1: A) Mean (+ SE) nectar volume (µl) for each *Warburgia salutaris* tree measured in Eston, KwaZulu-Natal (χ^2 = 6.1609, df = 3, p = 0.104) and B) mean (+ SE) nectar sucrose concentration (%) for each tree measured in Eston, KwaZulu-Natal (χ^2 = 1.3734, df = 3, p = 0.712).

Appendix 8:

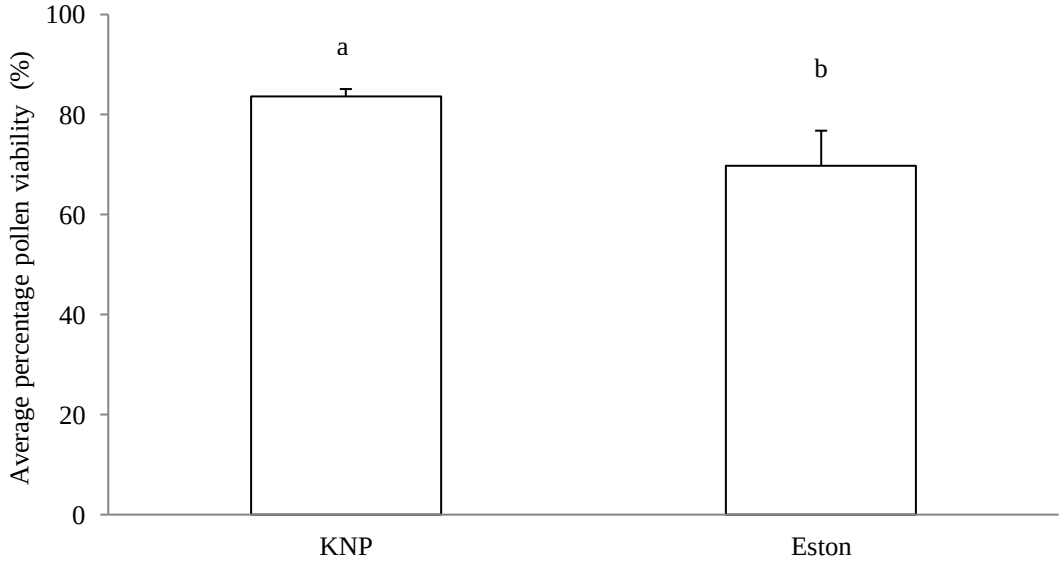

Figure 2: Mean (+ SE) percentage pollen viability of *Warburgia salutaris* in the KNP sub-populations combined and Eston (Mann-Whitney U test: W = 954, p = 0.006).

Appendix 9 :

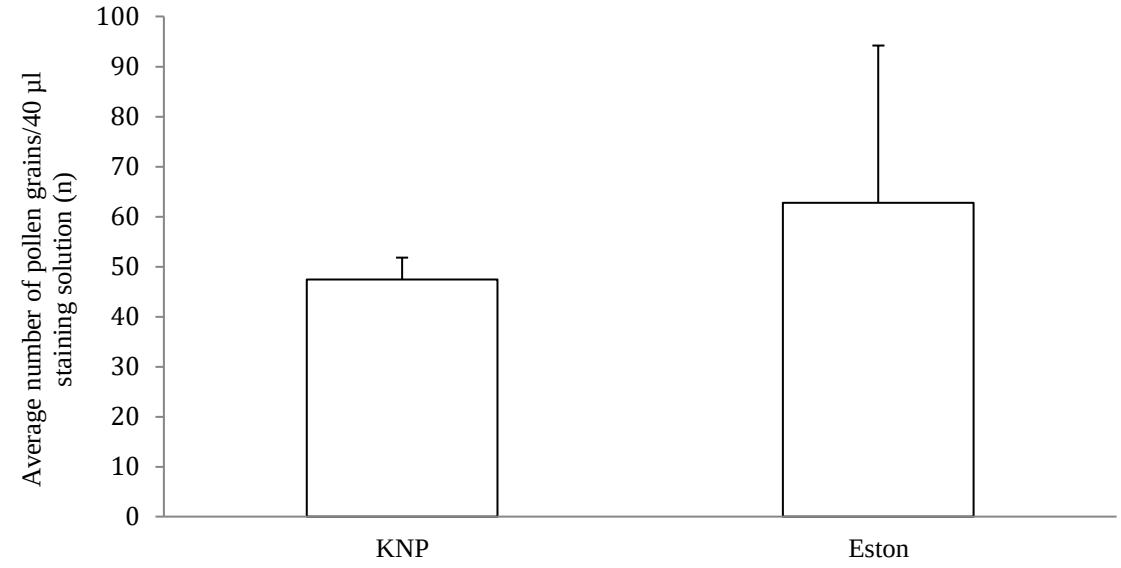

Figure 3: Mean (+ SE) number of pollen grains in the KNP sub-populations combined and Eston (Mann-Whitney U test: W = 630.5, $p = 0.729$).

Appendix 10:

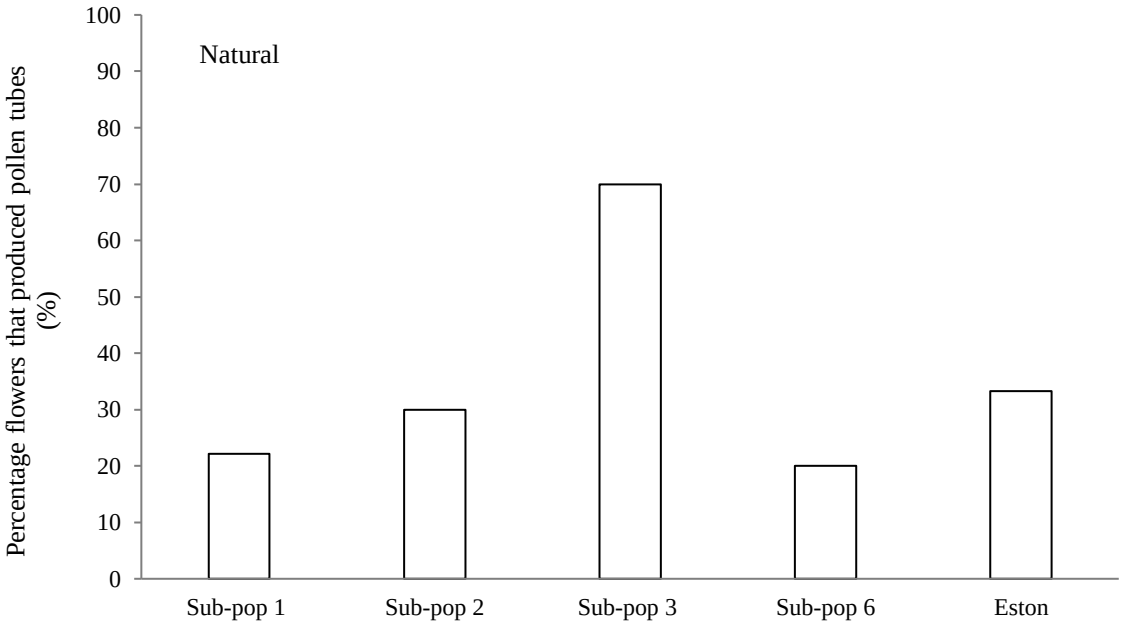

Figure 4: Total percentage flowers that produced pollen tubes for natural pollination in four KNP sub-populations and Eston. There were no significant differences between populations ($p > 0.05$).

Chapter three - Seed dispersal, predation, and germination in the pepperbark tree, **Warburgia salutaris**

Abstract

There is concern for the long-term viability of the protected *Warburgia salutaris* population in the Kruger National Park (KNP), as the few fruit produced are heavily impacted by pre-dispersal seed predation and there is a lack of seedling establishment. Consequently, because so few healthy fruit are matured, little is known about the sexual recruitment of *W. salutaris* in KNP. Therefore, aspects of the seed ecology of *W. salutaris* were studied to better understand the limited sexual reproductive output of the species in this population. Immature fruit in the KNP population and Eston orchard (KZN) were bagged to compare fruit development. Frugivore observations were carried out to identify frugivores and the mode of seed dispersal. Fruit were examined to assess the effect of pre-dispersal seed predation and confirm identity of seed predators, and seedling emergence trials were conducted to investigate the impact of seed-predation on seed germinability. Seedling emergence trials were conducted to compare germination of the desiccation-sensitive seeds between the natural KNP and Leshiba Game and Nature Reserve (GNR) populations and a successfully fruiting Eston orchard. Only 21% (14/64) of bagged fruit matured and all mature fruit were on 3/10 tagged trees that had begun developing fruit in the KNP. Frugivore observations at Leshiba GNR revealed that baboons and vervet monkeys eat *W. salutaris* fruit and ingest the seeds, presumably dispersing them a distance from the parent tree. Although frugivore activity was not observed in KNP in 2019, evidence was found that some fruit had been eaten. *Ceratitis cosyra*, the marula fly, attacks *W. salutaris* fruit in KNP, Leshiba GNR and Eston. Seeds from fruit with immature larvae had a far higher germination percentage (77%) than seeds in fruit with fully developed larvae (6%), although the highest percentage seed germinability was found in seeds protected from the fly. Therefore, there is a short period during larval development where seeds remain viable despite being infested with *C. cosyra*. *Warburgia salutaris* seeds from KNP had the highest percentage germination (61%), followed by Eston (47%), and Leshiba GNR (33%), and all ungerminated seeds decomposed, affirming a lack of seed dormancy and that *W. salutaris* cannot rely on soil seed banks. This study has confirmed that sexual recruitment in KNP is constrained by the extremely limited fruit production, which is partly due to a high level of fruit abortion (even when protected from seed predators). Fruit abortion and subsequent lack of recruitment is

mostly likely a result of a combination of genetic (e.g. clonal reproduction) and ecological factors (e.g. a water-limited environment).

Keywords: Frugivory, Germination, Pre-dispersal seed predation, Reproductive ecology, Seed dispersal

3. 1 Introduction

Warburgia salutaris (Bertol.f.) Chiov. (pepperbark tree) is a highly valued medicinal plant in southern Africa (Maroyi, 2014). In unprotected areas, unsustainable harvesting of the *W. salutaris* for traditional medicine and habitat loss have contributed to the species' Endangered status (Hilton-Taylor *et al*., 1998; Raimondo *et al.,* 2009). However, even in the protected population of *W. salutaris* in the Kruger National Park (KNP), there is an apparent lack of seedling establishment (Botha *et al*., 2004; Scheepers *et al*., 2011). A population survey, carried out in 2009, showed that juveniles (< 1.5 m in height) made up only 5% of the population and there was evidence of root suckers connecting trees (Scheepers *et al.,* 2011). Similarly, Wilken (2018) found very few trees in the lowest size class (1–2 m) in two KNP sub-populations and no seedlings were recorded. This has prompted the need to investigate aspects of the seed ecology of *W. salutaris* in order to understand the lack of sexual recruitment in this population.

Seeds play a critical role in the reproductive success of plant communities, as they enable dispersal into new habitats and, in some cases, are able to persist in seed banks across seasons until suitable environmental conditions allow them to germinate (Murdoch & Ellis, 2000; Yang & Kim, 2016). Regarding *W. salutaris*, we lack an understanding of the fate of the seeds once a fruit is successfully produced. This is in part due to the very low fruit production of the species in KNP – pollination trials conducted in the KNP 2019 flowering season revealed that only 4% of naturally pollinated flowers resulted in the development of fruit (Chapter 2).

Seed fate can be influenced by multiple interacting factors, including, the seed's inherent viability and germination rate, influence of pre- and post-dispersal seed predation, pathogens, environmental conditions (e.g. drought), the mode of seed dispersal, and availability of suitable microhabitats (Kozlowski, 1972). The lack of viable seed reported for *W. salutaris* in the KNP, has been attributed in part to pre-dispersal seed predation by insect larvae

(Hannweg *et al*., 2015). Yet, there is still no evidence of seedling recruitment in the population which suggests seedling establishment may be further negatively impacted by other factors. These include the recalcitrant (i.e. desiccation-sensitive) nature of the seeds (Pammenter & Berjak, 2000), ineffective frugivores (Stringer *et al*., 2020), and a lack of microhabitats for seedling establishment (Lamont *et al.*, 1993). An understanding of interactions between factors relating to the seed ecology – viz., fruit production, seed dispersal, seed predation, and germination – is necessary to understand the low sexual reproductive output of *W. salutaris* in the KNP.

3.1.1 Pre-dispersal seed predation

Pre-dispersal seed predation reduces the number of viable seeds available to eventually germinate and is considered a selective pressure on various reproductive traits including flowering and fruiting phenology, number and size of fruit and seeds, and seed packing strategies (Crawley, 2000; DeSoto *et al.*, 2016). *Warburgia salutaris* is reported to be heavily infested by seed predators, mainly fruit flies (Hannweg *et al*., 2015). However, it has not been documented how pre-dispersal seed predators develop within the fruit of *W. salutaris* – i.e. whether the eggs of the flies are laid within the ovules or in the mesocarp tissue. Larvae of endophytic seed predators live and pupate within specific spatial compartments i.e. only utilising the resources in an individual seed while external feeders may consume several seeds from the outside (Ramirez & Traveset, 2010; DeSoto *et al.*, 2016). Endophytic seed predators are considered more specialised seed predators as their body size is limited by seed size of the host resource (Ramirez & Traveset, 2010). These two options may have different implications in terms of seed viability. For example, oviposition within the ovule results in a completely nonviable seed, as seen in wasp (*Eurytoma aloineae* Burks) predation of *Aloe pretoriensis* Pole Evans seeds (van den Bosch *et al*., 2019). However, oviposition within the fruit pulp allows a period where seeds are able to develop before predation by the insect larvae (Ramirez & Traveset, 2010). Pre-dispersal seed predation may also have indirect effects on dispersal success, for example, fruit/seed predation may reduce seed dispersal distance by frugivorous birds if they turn brown and soft, and fall passively to the ground before bird dispersal can occur (Borowicz & Juliano, 1986).

3.1.2 Seed dispersal

Plants exhibit a range of dispersal mechanisms, both abiotic (i.e. by water, wind, gravity, and ballistic dispersal) and biotic (i.e. by animals, including, endozoochory and epizoochory) (Willson & Traveset, 2000). Seed dispersal reduces density-dependent mortality by moving seeds away from the parent plant and enabling species to colonise distant or vacant local sites (Howe & Mirti, 2004). Dispersal distances and effectiveness of seed dispersal are not easy to monitor in the field, however, these variables can be inferred from identifying the mode of seed dispersal and (in the case of frugivory) the behaviour of the seed disperser (Gross-Camp & Kaplin, 2011).

The removal of seeds from the pericarp is suggested to be a particularly important factor in the reproductive success of *W. salutaris,* and similarly in a study on *Warburgia ugandensis* Sprague (Muchugi *et al*., 2008). There is limited information on the frugivores associated with *W. salutaris*. Senkoro *et al.* (2020) mentions fruit are dispersed by birds and Van Wyk (1984) notes that baboons eat the fruit of *W. salutaris* in the KNP. The fruits of other species in the family Canellaceae are reportedly red, and for this reason birds are suggested to disperse their fruit (Kubitzki, 1993). In contrast, the fruit of the genus *Warburgia* are green and are the second largest fruit (3–6 cm) amongst the genera of the Canellaceae, with *Cinnamosma macrocarpa* H. Perrier in Madagascar having the largest (6–9 cm, also round and green; Kubitzki, 1993). While fruit dispersal syndromes have been suggested, clear distinctions are not found across all plant families, particularly for distinguishing between bird and monkey dispersal (Pizo, 2002). As a result, the bird-monkey syndrome and a ruminant-rodent-elephant syndrome are suggested (Gautier-Hion *et al*., 1985). *Warburgia salutaris* appears to fall mostly under the bird-monkey syndrome, which include berries and drupes with a succulent pulp or arillate seeds, and no protective seed cover (e.g. a lignified seed coat), however, fruit in this category are also described as brightly coloured, but *W. salutaris* fruit are a dull green colour (Gautier-Hion *et al*., 1985).

Frugivores can process the fruit in various ways, which ultimately influences the effectiveness of seed dispersal. This includes dropping seeds at the site, spitting out the seeds and only eating the fruit pulp, masticating the seeds, and swallowing seeds (Lambert & Garber, 1998). Seed ingestion can increase percentage germination and germination rate in some species through: chemical/mechanical scarification of a lignified seed coat (which *W. salutaris* does not have), separation of seeds from the pulp, and nutrient provision by surrounding faecal matter after egestion (Levey *et al.*, 2002). Gut retention time may also influence the effect of

gut treatment, and this differs greatly between frugivores, for example, the frugivorous Knysna turaco (*Tauraco corythaix* Wagler) has a gut transit time of 12–28 minutes (Wilson & Downs, 2012). In contrast, a study on primate digestion shows that vervet monkeys have an estimated transit time of 31 hours (Lambert, 1998), and the olive baboon gut retention time is estimated to be longer than 24 hours (Kunz & Linsenmair, 2008). However, because *W. salutaris* seeds are soft and susceptible to desiccation, it is possible that gut treatment, especially for long periods of time, is not beneficial for recalcitrant species.

3.1.3 Seed viability and germination

Germination success can be influenced by various factors including; inherently low vigour (e.g. if produced through self-pollination versus cross-pollination; Jersakova & Johnson, 2006), unsuitable soil or environmental conditions (Murdoch & Ellis, 2000), post-dispersal seed predation (Willson & Traveset, 2000), pathogens e.g. proliferation of seed-associated fungi (Berjak & Pammenter, 2001), and parasites (Willson & Traveset, 2000). The recalcitrance of the *W. salutaris* seeds contributes to the vulnerability of the species (Kioko *et al.*, 2003). The seeds are shed at a high moisture content, and if under unsuitable recruitment conditions the seeds lose moisture and in turn, viability (Pritchard *et al.*, 2004; Nichols, 2005). Therefore, there is a short period wherein suitable conditions need to coincide for *W. salutaris* seeds to be removed from the fruit, placed in an ideal microsite, and germinate before drying out. A study on seed recalcitrance in African dryland species showed that desiccation-sensitive seeds (e.g. of *Trichilia emetica* Vahl) germinated faster than desiccation-tolerant species (e.g. *Sclerocarya birrea* (Sond.) J.O. Kokwaro) (Pritchard *et al.*, 2004). Therefore, under suitable conditions, healthy seeds should theoretically rapidly germinate readily to form seedling banks (Pammenter & Berjak, 2000) – which *W. salutaris* in KNP appears to lack as well. Seedling establishment is a major obstacle in the process of plant reproduction, where the seedling is particularly vulnerable to disturbances such as fire, drought, floods, herbivory and shade intolerance (Kitajima & Fenner, 2000). The ability to form seedling banks is probably only found in desiccation-sensitive species in moist tropical forest environments (Sautu *et al.*, 2006). Considering the KNP is a semi-arid environment, water availability is most likely a major limiting factor for seedling establishment (Wilson & Witkowski, 1998).

The time for seeds to germinate has important implications for plant reproductive success (Thompson, 2000). A delay in germination can either be a result of seed dormancy, which prevents seeds from germinating under unsuitable conditions that would result in lower

seedling survival (Murdoch & Ellis, 2000), or seed quiescence, where environmental conditions are not conducive to germination, i.e. as a result of desiccation or cold temperatures (Murdoch & Ellis, 2000). Seed banks formed by dormant or quiescent seeds may be transient (i.e. persist for < 1 year; e.g. *Kumara plicatilis* (L.) G.D.Rowley; Cousins *et al.*, 2014), short-term persistent (i.e. 1–5 years; e.g. *Vachellia nilotica* (L.) P.J.H.Hurter & Mabb. Hayne*;* Wilson & Witkowski, 1998) or remain for more than 5 years as long-term persistent seed banks (e.g. *Acacia saligna* (Labill.)H.L.Wendl*;* Holmes & Newton, 2004). While dormancy is not associated with recalcitrant seeds because they are shed in a metabolically active state (Berjak & Pammenter, 2001), some temperate plant species are reported to have dormant recalcitrant seeds (e.g. *Acer pseudoplatanus* L.; Hong & Ellis, 1990). However, this has not been reported from *W. salutaris*.

Given the limited sexual reproduction reported for *W. salutaris* in the field, little is known about the fate of seeds in fruit that are occasionally produced. Therefore, the aim of this study was to investigate aspects of the fruit and seed ecology that may help explain the impaired sexual reproductive biology of *W. salutaris* in the KNP. There are four objectives associated with this aim. The first objective was to assess fruit development in KNP and Eston, the second objective was to investigate the effect of pre-dispersal seed predation and identify the fruit/seed predators, the third objective was to identify species of seed dispersers and investigate the process of seed dispersal, and the fourth objective was to compare the germination success of seeds between the natural and cultivated populations.

3.2 Method

3.2.1 Study area

The study was conducted across three populations of *Warburgia salutaris*. The first, and focal, population is situated in the northern part (420–580 m, a.s.l) of the Kruger National Park (KNP) (Roocroft & Roocroft, 2012). The landscape here is described generally as Northern Sandveld on Waterberg sandstone (Gertenbach, 1983), and the climate is characterised by summer rainfall and dry winters, with the average annual precipitation being ~ 530 mm (Scientific Services, 2018). However, interannual rainfall is highly variable ,as seen over the last 15 years (Appendix 1; Figure 1). The highest and lowest mean monthly maximum and minimum temperatures are 32.7 °C in December and 10.8 °C in July, respectively (Climate-Data.org, 2019). The topography of the area is highly variable as it falls within the

eastern limit of the Soutpansberg mountains, and *W. salutaris* is found along the south-east facing slopes of the ridges (Gertenbach, 1983). A second natural population in Leshiba Game and Nature Reserve in Limpopo (~ 1300 m, a.s.l) was selected to supplement data from the KNP, as a result of the low fruit set in KNP in the 2019 fruiting season. This reserve is situated in the western section of the Soutpansberg Mountains near Makhado (Louis Trichardt) at a slightly lower, but similar latitude, for example, the Punda Maria camp in KNP. The vegetation type here is described generally as Soutpansberg Mountain Bushveld. This area receives summer rainfall (~ 540 mm per year), and temperatures can range between 12 °C in winter months and 40 °C in summer months (Kabanda, 2020).

The third population is a *W. salutaris* orchard on a sugar cane farm in Eston, KwaZulu-Natal (~ 690 m, a.s.l) (M. Stainbank, March. 2019, pers. comm.). The farm forms part of a conservancy, therefore, between the agricultural lands there are areas of natural vegetation mainly falling under the Dry Coast Hinterland Grassland vegetation type (Mucina & Rutherford, 2006), which serve as habitat for wildlife such as warthog, bushbuck and vervet monkeys (M. Stainbank, March. 2019, pers. comm). The climate of this area is characterised mainly by summer rainfall and some rain in winter (Mucina & Rutherford, 2006), with average annual precipitation of ~ 850 mm (Mucina & Rutherford, 2006). The mean monthly maximum and minimum temperatures are lower than at Punda Maria; being 27.3 °C in February and 5.3 °C in June, respectively (Mucina and Rutherford, 2006). Here the *W. salutaris* trees have successful fruit production, and there is evidence of seed dispersal and subsequent seedling establishment around the farm (M. Stainbank, March. 2019, pers. comm.). For these reasons, Eston was included as a successfully recruiting *W. salutaris* population with which to compare and interpret the reproductive ecology of the wild population, and in particular the KNP population.

3.2.2 Study species

Warburgia salutaris is a fast-growing evergreen tree that can reach approximately 12 m in height under optimal conditions (Botha *et al*., 2004; Burrows *et al*., 2018). Multi-stem variants are also found as the tree readily re-sprouts when damaged, through bark stripping by humans and/or branch breakage by elephants, or other natural disturbances such as fire, for example (Botha *et al.,* 2004). Across the species' distribution it is found in a diverse range of habitats; from dry rocky slopes, to wooded ravines, and wet coastal forests (Coates-Palgrave, 2002; Schmidt *et al.,* 2002). The flowering season is typically from April until June; however,

this varies considerably between regions. During the flowering season, a multitude of very small (5–7 mm diameter), non-showy, green flowers are produced in the leaf axils (Schmidt *et al*., 2002). The flower is bisexual; it has 10 fused stamens forming a staminal tube that wraps around the ovary and style (Coates-Palgrave, 2002). From October through to January (this varies depending on flowering time), medium-sized (30–50 mm diameter) round green berries develop (Fig. 1) and turn purple with a white bloom as they mature (Burrows *et al*., 2018). The fruit contains several (2 to approximately 25) recalcitrant seeds (~ 10 x 5 mm) with an oily, fleshy endosperm (Salazar & Nixon, 2008). As the seeds are vulnerable to desiccation, they need to be planted while they still have a high moisture content for successful germination (Kioko *et al.,* 2003).

Figure 1: Fruit of *Warburgia salutaris* at Random Harvest Nursery, Muldersdrift, July 2020. Scale bar: 50 mm.

3.2.3 Experimental design

In 2019, fieldwork for this section of the study took place from the 26th of November until the 6th of December. In the first phase of the study, six sub-populations in KNP were chosen: sub-population 1 (n = 10 trees), sub-population 2 (n = 8 trees), sub-population 3 (n = 10 trees), sub-population 4 (n = 5 trees), sub-population 5 (n = 4 trees), and sub-population 6 (n = 5 trees), totalling 42 trees. During the fruiting season, fruit were only found on two trees (4.8% of tagged trees) in sub-population 3; therefore, this part of the study is restricted to this sub-population. As a result, a second natural population, Leshiba Game and Nature Reserve (GNR) in the Soutpansberg, was selected to supplement data in the KNP. In 2020, immature fruit were bagged in June, in KNP, and July, in Eston. A return trip in the 2020 fruiting season

took place from the 16th until the 18th of November in KNP and the 29th until the 30th of October in Eston.

3.2.3.1 Assessment of natural fruit set

Fieldwork in the 2020 flowering season commenced later than intended (22nd June), as a result of the national lockdown in response to Covid-19. Most trees had finished flowering by this time, although some had small immature fruit, of which some had aborted (i.e. were purple) and were still attached to the branch. To assess the cause of this, the small purple aborted fruit (n = 116) were collected from seven trees in KNP and examined under a Zeiss Discovery V12 dissecting microscope for evidence of fruit predation or disease. In addition, immature fruit (n = 64) across 10 trees in KNP and across three trees Eston (n = 29) were bagged to see if they would mature fully or if development of these fruit also ceased during the maturation process.

3.2.3.2 Pre-dispersal seed predation

Fruit collected from KNP (n = 54 in December 2019), Leshiba GNR (n = 101 in November 2019), and Eston (n = 27 in October 2020) were examined for evidence of fruit and/or seed predation. The mean number of larvae per fruit was calculated for each population. A number of larvae and pupae from each population were placed in emergence containers to identify seed predators of the fruit. Emerged insects were transferred into a freezer and then to a vial containing 70% ethanol. The insects were examined under a Zeiss Discovery V12 dissecting microscope and photographed with the attached AxioCam MRc camera. As with the insect visitors, field guides and published literature were used to provide preliminary identifications and compare these with the previously identified seed predators.

To compare the effect of the stage of seed predator development on seed germinability, the following mature fruit were collected from Random Harvest Nursery in (Johannesburg) in September 2020: A) 10 fruit with small, immature *Ceratitis cosyra* Walker larvae (Fig. 2A) that had not eaten into the seed coat, B) nine fruit with large *C. cosyra* larvae (Fig. 2B) where holes were noted in the seed coat, and C) 8 healthy fruit which were used as a control (Table 1).

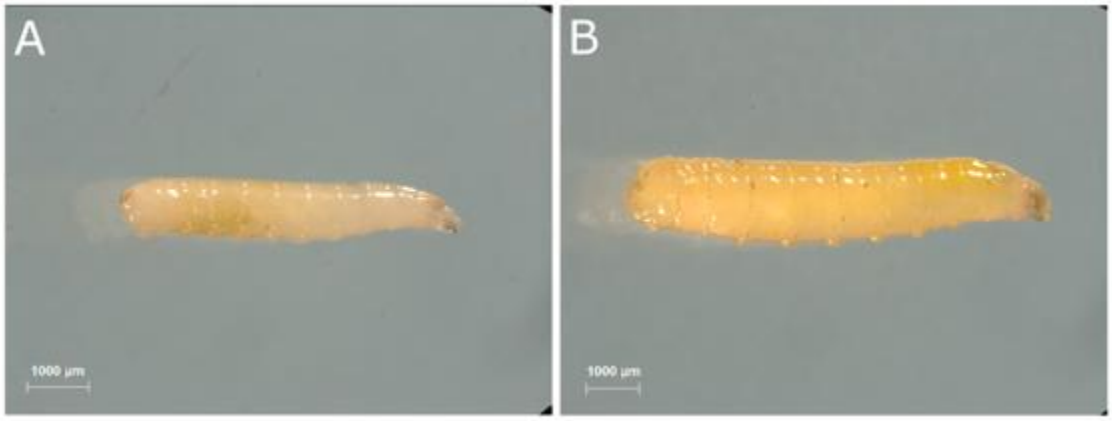

Figure 2: A) Small immature *Ceratitis cosyra* larva that had not eaten into the seed coat of the *Warburgia salutaris* seeds, and B) large fully developed *C. cosyra* larva that had eaten through the seed coat.

Table 1: Number of *Warburgia salutaris* fruit collected from Random Harvest Nursery (in September 2020) for each fruit type (i.e. healthy, infested with immature larvae, and infested with fully developed larvae) and number of seeds planted for each in the germination trials to assess the impact of seed predation on seed germinability.

Fruit type	Number of fruit	Number of seeds planted
Healthy	7	50
Infested with immature larvae	10	70
Infested with fully developed larvae	9	67

The seeds were removed from the fruit and a maximum of 30 seeds were placed in each 28 cm x 32 cm surface area, 10 cm depth seedling tray. The seeds were sown in a mixture of river sand and sieved compost (2:1) and lightly covered with river sand (Kioko *et al.* 2003; Nichols, 2005). Trays were kept outside in dappled sunlight and were watered once a day from 10th September 2020 until end of germination. Last day of germination (16th of November 2020) was established when there had been no further seedling emergence for more than two weeks. The seeds were planted in soil; therefore, a seed was considered germinated once the hypocotyl emerged from the soil. The number of seedlings that emerged was counted daily.

3.2.3.3 Seed dispersal

Frugivore observations were carried out to identify effective seed dispersers of *W. salutaris* fruit. The majority of observations took place in Leshiba GNR, as there was concern that the very limited fruit production in the KNP would limit the chances of detecting frugivore activity in this population.

In Leshiba GNR, three camera traps were set up for 24 hours a day over 5 days (i.e. 120 hours in total) at two sites: one with a single fruiting tree (Site A), and one with five fruiting trees (Site B). At site A, three camera traps were set up; two facing toward fruit lying on the ground, and one facing up into the tree canopy to see if fruit was removed before falling to the ground, and if any nocturnal activity (i.e. from bush babies) took place. At site B, two camera traps were set up, both facing fruit on the ground. All camera traps were set on motion detection, and once triggered, would take three photos followed by a 30 s to 1 min video. Each morning and afternoon the images and videos were transferred from the memory card to a hard drive, and any necessary adjustments to the camera settings were made. Optimal settings were a one-minute video, followed by a 10 second interval, with medium LED brightness and medium sensitivity to motion. This included adjusting the video length, the number of photos captured once triggered, position of the camera, LED brightness, and the level of sensitivity to motion. The process was repeated in KNP for two full days; however, only one site in sub-population 3 was suitable for frugivore observations as only two trees had fruit. Three cameras were set up; two facing toward fruit lying on the ground, and one facing up into the tree canopy.

Analysis of the camera trap footage included: (1) identifying the species of each fruit-eating visitor, and (2) describing the frugivore behaviour, i.e. noting if seeds were ingested along with the fruit flesh, or whether they were dropped at the site of the tree. Once frugivores were identified, their seed dispersal effectiveness was inferred from knowledge of their behaviour, average home range size, and gut passage rate.

3.2.3.4 Seedling emergence trials

Mature fruit collected from *W. salutaris* populations in KNP (n = 22) and Leshiba GNR (n = 35) were used in the germination trials. Seeds from Eston were sent up at the time of the germination trials as the timing of the field visit to Eston in October 2019 did not coincide with the field visit to KNP and Leshiba GNR (November/December 2019). The seeds had been

removed from the fruit and stored in a container at 3 °C a week prior to planting. For KNP and Leshiba GNR, seeds were removed from the fresh fruit and pooled for each site. A maximum of 30 seeds were planted in each 28 cm x 32 cm surface area, 10 cm depth seedling tray. Ninety seeds in total were planted for fruit from Eston, 74 seeds from KNP, and 46 seeds from Leshiba GNR (Table 2).

Table 2: Number of *Warburgia salutaris* seeds from three populations (KNP, Leshiba GNR and Eston) planted (total n = 210 seeds) in each seedling tray to compare seed germination success.

Tray	Eston A	Eston B	Eston C	KNP A	KNP B	KNP C	Leshiba A	Leshiba B
Number of seeds (n)	30	30	30	30	30	14	30	16

As with the seedling emergence trials in objective 1, the seeds were sown in a mixture of river sand and sieved compost (2:1) and lightly covered with river sand (Kioko *et al*. 2003; Nichols, 2005). Trays were kept outside in dappled sunlight and were watered once a day from 20 January 2020 until end of germination (i.e. the date after which there had been no seedling emergence for > 2 weeks) on the 4th of April 2020. The number of germinated seeds that emerged was counted daily and seedlings were removed once they had reached 5 cm in height (above soil level) and transplanted to individual pots (± 10 cm depth x 12 cm diameter) filled with potting soil and compost and were well watered once daily. Germinated seedlings from the Kruger National Park population were returned to the Skukuza Indigenous Plant Nursery in KNP for inclusion, for the first time, as stock material in the broader propagation programme. Seeds that did not germinate were excavated from the seedling trays to determine seed fate. It was recorded whether the seeds were 1) intact and firm, 2) partially decomposed, or 3) missing.

3.2.4 Data and statistical analyses

All statistical tests were performed using *R* software, R version 3.3.2 (R Core Team, 2016). A 2x2 Chi-squared contingency table was used to compare maturation and abortion of bagged immature *W. salutaris* fruit between KNP and Eston. A 2x2 Chi-squared contingency

table was used to compare germination success of seeds from *W. salu*taris fruit infested with immature and large *C. cosyra* larvae.

3.2.4.4 Seedling emergence trials

General linear models (GLM), followed by an ANOVA (test = chi-squared), were used to compare germination success between 1) the three populations and 2) eight seedling trays (family = binomial, $p < 0.05$). A Kruskal Wallis test (Shapiro-Wilk normality test; $p < 0.05$) followed by a Kruskal Wallis multiple comparison post hoc test (package "pgirmess") was used to analyse significant differences in time to germination between 1) the three populations and 2) eight seedling trays.

3.3 Results

3.3.1 Assessment of natural fruit set

Microscope examination of the small, purple, aborted fruit (Fig. 3A) showed that 9% (11/116) of the fruit had evidence of holes in the ovary (Fig. 3B), which may have been the cause of abortion in these cases. However, the majority were undamaged and the potential cause of abortion was not obvious. Aborted fruit size (measured as widest section of ovary/fruit) ranged from 1– 2.8 mm ($\bar{x}$ = 1.7 mm, n = 116).

Figure 3: A) range of sizes of aborted *Warburgia salutaris* fruit B) hole at the base of the ovary (circled) in aborted *W. salutaris* fruit. Mean aborted fruit size = 1.7 mm (range: 1– 2.8 mm, n = 116).

In Eston, 26% (8/29) of bagged immature fruit formed fully developed fruit, and in KNP 21% (14/64) fruit developed to maturity. The proportion of fully developed fruit in Eston and KNP is not significantly different ($\chi^2_1 = 0$, $p > 0.05$) (Fig. 4). All fully developed fruit

were on 3/10 trees in KNP – two tagged trees and an additional untagged tree. Aborted fruit size (measured as widest section of ovary/fruit) ranged from 1.8–11 mm ($\bar{x}$ = 3 mm, n = 39). Missing fruit in KNP were in bags that were pulled off the trees, possibly by elephants. Therefore, it is not known whether the fruit would have matured, however, given that they were on trees which did not have any fully developed fruit, it is possible they were also aborted. However, including the missing fruit under aborted fruit does not change the significance of the Chi-squared test ($\chi^2_1 = 0.114$, $p = 0.736$).

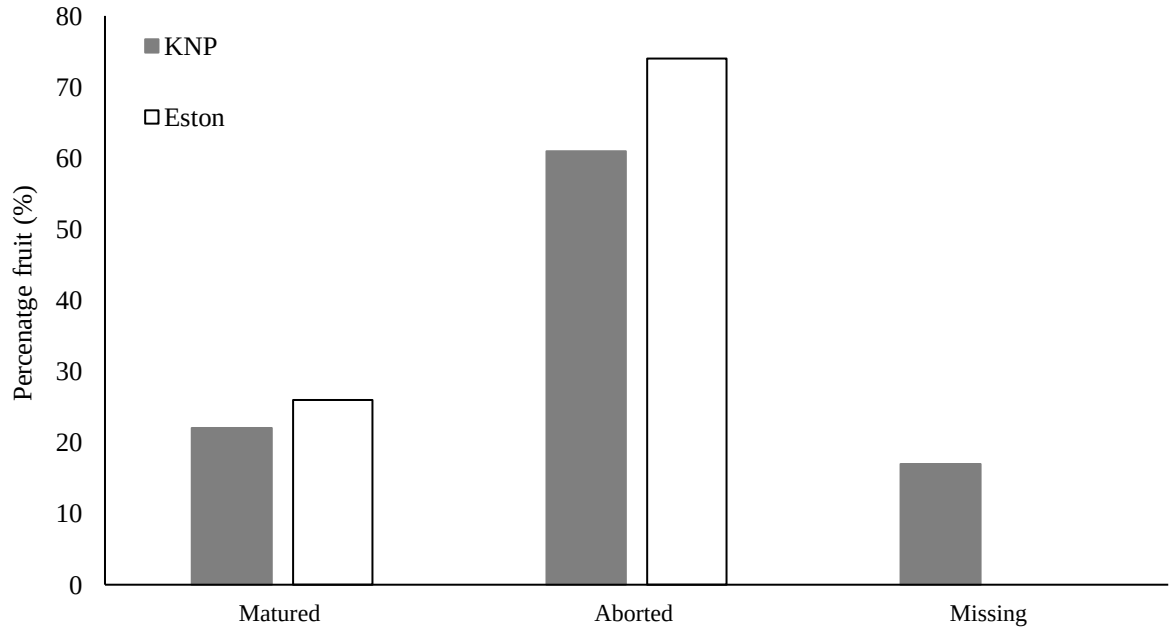

Figure 4: Percentage (%) of immature bagged fruit that matured, aborted and were missing in the Kruger National Park (KNP) (n = 64 bagged) and Eston orchard (n = 29 bagged) on the return visit to each site in the 2020 fruiting season. The proportion of fully developed and aborted fruit in Eston and KNP is not significantly different (χ^2, $p > 0.05$).

3.3.2 Pre-dispersal seed predation

Fruit impacted by seed predation are identified by holes in the exocarp (Fig. 5A) and brown decaying fruit pulp and seeds (Fig. 5B). Emergence trials revealed the larvae found in the fruit from Kruger, Eston and Leshiba GNR, to be of the marula fly, *Ceratitis cosyra* (Fig. 6A & 6B). Two additional seed predators were found in Eston: a small fly (*Zaprionus sp.* Coquillett) and unidentified Coleopteran larvae. The mean number of seed predators per fruit was highest in KNP (n = 15), and lower in Leshiba GNR (n = 7) and Eston (n = 6) (Table 3).

The percentage fruit infested is not directly comparable between KNP (20%) and Eston (59%), as infested fruit were selected specifically for identification of seed predators in the 2020 fruiting season in Eston. However, percentage fruit infested in KNP is lower than expected based on previous seed predator studies (Hannweg *et al.*, 2015).

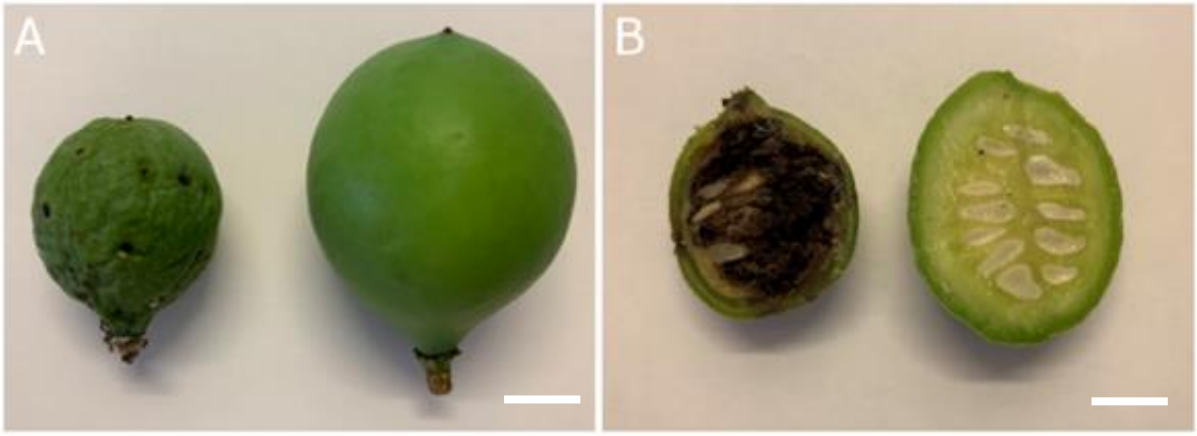

Figure 5: (A) A fruit of *Warburgia salutaris* impacted by seed predators (left) versus a healthy fruit (right). (B) A bisected fruit impacted by seed predators (left) versus a bisected healthy fruit (right). Scale bar: 10 mm.

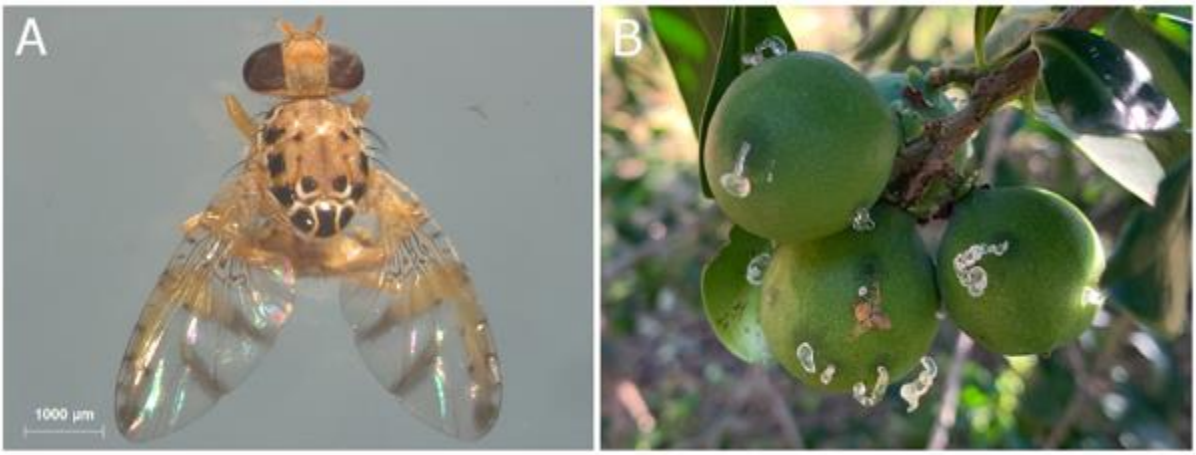

Figure 6: (A) Marula fly (*Ceratitis cosyra*) were found in fruit from KNP, Leshiba GNR and Eston, and (B) *C. cosyra* on a fruit impacted by seed predation at the Random Harvest Nursery, Johannesburg.

Table 3: Number of *Warburgia salutaris* fruit collected from KNP, Leshiba GNR (in November/December 2019) and Eston (in September 2020), number of fruit impacted by seed predation, total number of larvae that emerged from the fruit, and average number of larvae/fruit. * Infested fruit were specifically selected for identification of seed predators.

Population	Fruit collected (n)	Fruit impacted by seed predation	Total larvae (n)	Average number of larvae/fruit
KNP	54	20% (n = 11)	170 (all *C. cosyra*)	15
Leshiba GNR	101	15% (n = 15)	108 (all *C. cosyra*)	7
Eston	27	*59% (n = 16)	113 (*C. cosyra*: n = 75; *Zaprionus sp*.: n = 24; Coleopteran larvae: n = 14)	6

The fly oviposits the eggs in the fruit pulp and not directly into the developing seeds. However, the seeds are damaged as the larvae begin to eat through the seeds, as evidenced by the hollow seeds found in heavily infested fruit. Germination trials on seeds from fruit impacted by seed predation showed that seeds from fruit with immature larvae had a lower percentage germination success (77%) than seeds from healthy fruit (84%), but a significantly higher percentage germination success than seeds in fruit with fully developed larvae (6%; $\chi^2_1 = 68.148$, $p < 0.001$; Table 4).

Table 4: Number of *Warburgia salutaris* seeds planted from Random Harvest Nursery for each fruit type (i.e. healthy, infested with immature larvae, and infested with fully developed larvae) and percentage emergence (%) of seeds from each fruit type.

Fruit type	Number of seeds planted (n)	Percentage germination (%)
Healthy	50	84
Infested with immature larvae	70	77
Infested with fully developed larvae	67	6

3.3.3 Seed dispersal

At Leshiba GNR, baboons (*Papio ursinus* Kerr) and vervet monkeys (*Chlorocebus aethiops pygerythrus* F. Cuvier) were the only frugivores captured in the camera trap footage (Fig. 7A and Fig. 7B, respectively). Primate activity was not restricted to a particular time of day, and was captured at various times at each site, between 05:30 until 17:30. In total, 94 observations of baboons and 3 observations of vervet monkeys were recorded over the 5 days. Activity was spread throughout the day, with peaks in the early morning, midday and highest in mid-afternoon (Fig. 8). In addition, 92 instances of fruit feeding over the 5 days were captured, and a maximum of six fruit were recorded being eaten by an individual in a single feeding bout, which was captured on videos between 30 seconds to 1 minute. Both adults and juveniles ate the fruit. No nocturnal frugivore activity was seen on the camera traps, and no birds were observed, or photographed/filmed eating any fruit. Regarding feeding behaviour, both the baboons and monkeys would pick up the fruit from the ground (not the branches), squeeze the seeds and fruit pulp into their mouth, and then drop the fruit exocarps at the site. This was confirmed by collecting the fruit exocarps from where they were dropped as observed in the camera trap videos (Fig. 7C). Therefore, the seeds are possibly ingested and dispersed away from the parent tree if they are not digested.

Figure 7: Camera trap images in Leshiba GNR of (A) a baboon picking up a *Warburgia salutaris* fruit, (B) a vervet monkey eating a *W. salutaris* fruit, and (C) discarded fruit exocarps.

In the KNP, no primate activity was seen on the camera traps. A small number of fruit that appeared to have been eaten into, as seen in Leshiba GNR (Fig. 7C), were found below the tree in sub-population 3. It was noted that fruit had been removed either during the night or early morning, but this footage was not captured as shadow movement appeared to trigger the

camera, and as a result the memory card was full by 20:30. In addition, on both nights, the camera footage showed nocturnal rodent activity on the ground beneath the tree, however, no interaction between the fruit and rodent(s) was captured.

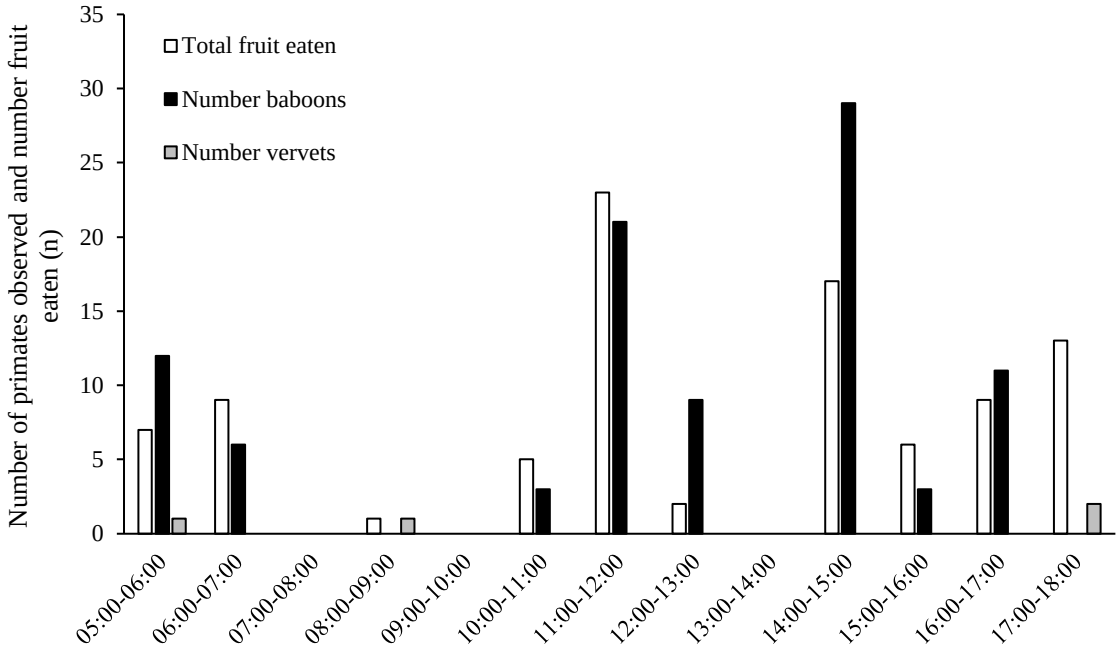

Figure 8: Accumulated number of *Warburgia salutaris* fruit eaten, number of baboons, and number of vervet monkeys captured on the camera trap footage over 5 days to show peaks in frugivore activity at Leshiba GNR throughout the day from 05:00 until 18:00.

3.3.4 Seedling emergence trials

Warburgia salutaris seeds undergo epigeal germination, where the hypocotyl elongates and arches upwards pulling the cotyledons above the soil (Fig. 9). The mean number of days for the first seedling to emerge was 35 days from planting (KNP: 35 days; Eston: 36 days; Leshiba GNR: 35 days; Table 5), and the mean number of days for all seedlings to emergence across the three populations was 48 days. The Eston seedlings took longer to emerge (51 days) than KNP (47 days) seeds (Kruskal-Wallis $\chi^2_2 = 7.528$, $p = 0.023$). The average time spread of emergence (i.e. between 1st and last emergence) across the three populations was 31 days

(Table 5). Eston had the longest time spread of emergence (n = 40 days), followed by KNP (n = 33 days), and Leshiba GNR (n = 21 days) (Fig. 9, Table 5).

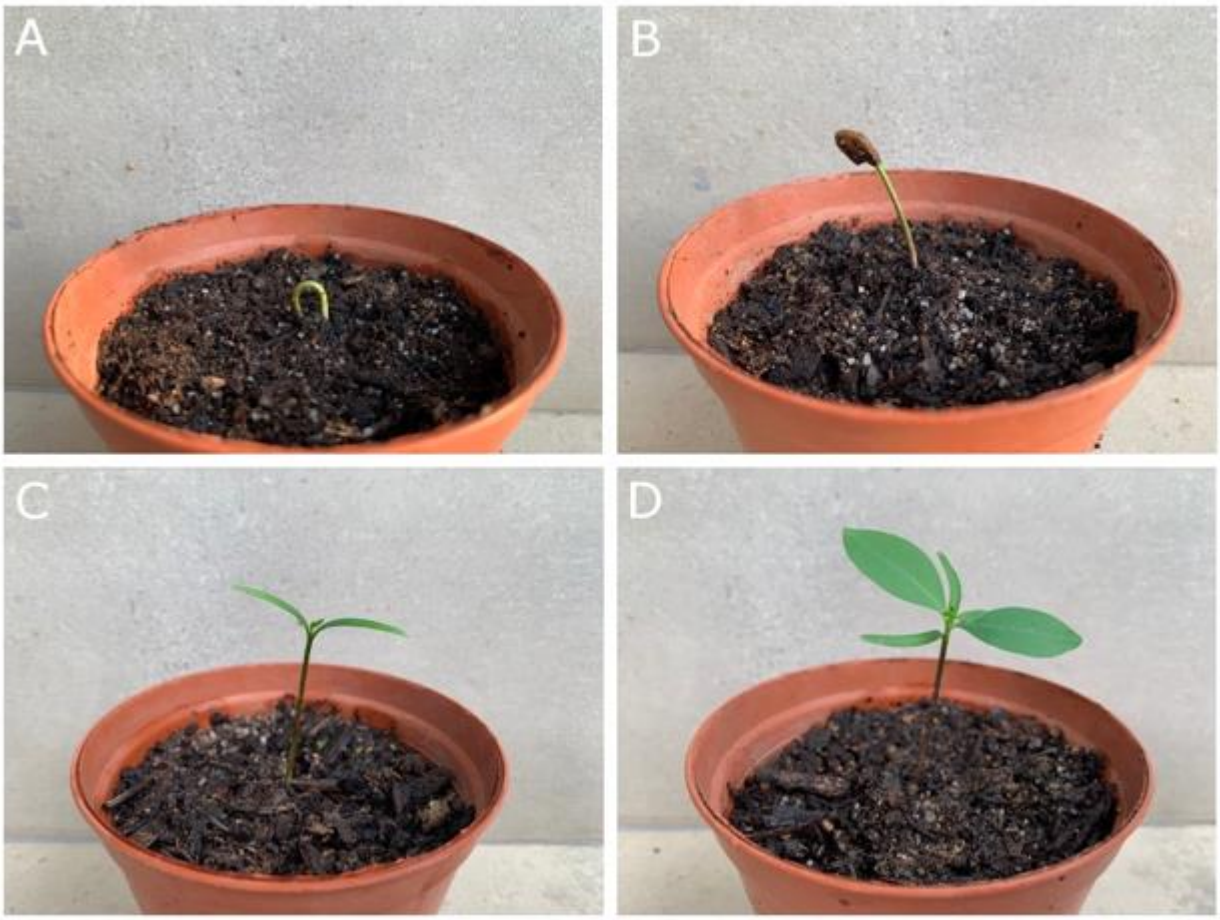

Figure 9: Process of *Warburgia salutaris* seedling emergence: (A) the hypocotyl arches just above soil (~ 4 weeks after planting), (B) the cotyledons emerge above the soil surface (~ 6 weeks after planting), (C) the hypocotyl is upright (~ 8 weeks after planting), and (D) leaves are produced although cotyledons (i.e. the reserves for seedling growth) remain attached (~ 10 weeks after planting).

Table 5: First day of emergence, last day of emergence (i.e. the date after which there had been no seedling emergence for > 2 weeks), and time spread of emergence (days), mean ± SE emergence time (days), and final emergence percentage (%) between *Warburgia salutaris* seeds from KNP, Eston and Leshiba GNR.

Population	First day	Last day	Time spread of emergence (days)	Mean ± SE emergence time (days)	Final emergence percentage (%)
KNP	35	68	33	47 ± 1.2	61%
Leshiba GNR	35	56	21	45 ± 1.9	33%
Eston	36	76	40	51 ± 1.5	47%

In total, the KNP seed had the highest final emergence percentage (61%), followed by Eston (47%), and Leshiba GNR (33%) (Fig. 10, Table 5). There was a significant difference in the percentage emergence between the three populations (GLM ANOVA: $\chi^2_{2,\ 207} = 9.39$, $p < 0.01$; ($\chi^2_{\ 2} = 9.26$, $p < 0.01$). Percentage emergence was significantly lower at Leshiba GNR than KNP ($\chi^2_{\ 1} = 13.18$, $p < 0.001$) and Eston ($\chi^2_{\ 1} = 7.93$, $p < 0.01$). There was a significant difference in the percentage emergence between the eight seedling trays (GLM ANOVA: $\chi^2_{7,\ 202} = 26.33$, $p < 0.001$) (Fig. 11; Appendix 2; Table 1). Percentage emergence in tray "Leshiba GNR A" (13%) was significantly lower than all other trays (GLM ANOVA: Z = -3.18, $p < 0.01$) (Fig. 11).

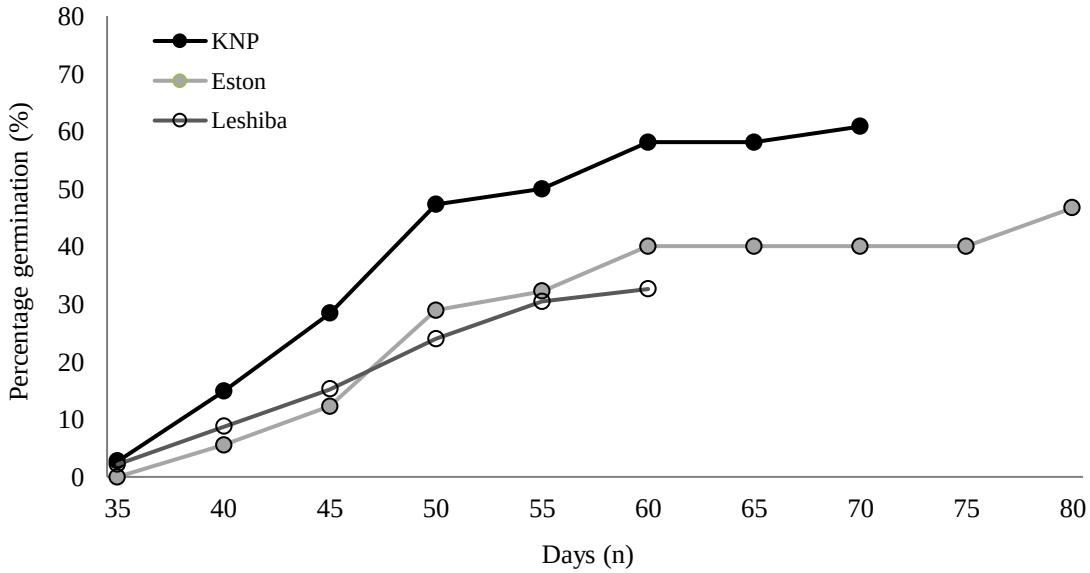

Figure 10: Emergence rate of *Warburgia salutaris* seeds from Kruger National Park (KNP), Eston and Leshiba GNR over time.

The percentage of recovered ungerminated seeds across the populations was 43% (n = 32) for KNP, 29% (n = 26) for Eston, and 43% (n = 20) for Leshiba GNR (Table 6). All recovered seeds were soft and partially decomposed (Fig. 12); these were considered nonviable, although they was not tested for viability using tetrazolium staining. In addition, in KNP and Eston seedling trays there were a few instances of seeds that had germinated (KNP: n = 4; Eston: n = 3), but had not managed to emerge above the soil. Therefore, the total germination including these partially germinated seeds for KNP and Eston is 66% and 50%, respectively (Table 6). Unrecovered seeds had most likely completely decomposed. This result is consistent with literature, which reports that recalcitrant seeds cannot be stored in soil seed banks and must germinate readily to ensure reproductive success (Kioko *et al.,* 2003).

Table 6: Assessment of *Warburgia salutaris* seeds that did not germinate. All recovered seeds were either partially decomposed or had undergone germination and not survived, and unrecovered seeds were considered fully decomposed.

Population	Recovered seeds (%)	Unrecovered seeds (%)	Partial germination (n)	Total germination (%)
KNP	22 (n = 16)	12 (n = 9)	4	66
Leshiba GNR	43 (n = 20)	24 (n = 11)	0	33
Eston	30 (n = 27)	14 (n = 13)	3	56

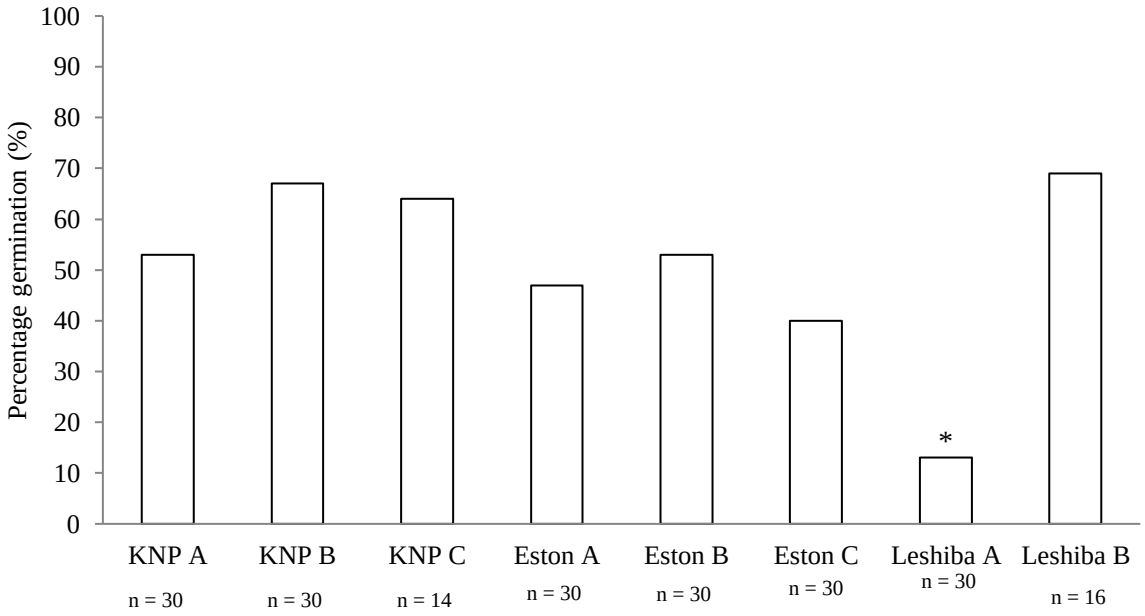

Figure 11: Percentage emergence of *Warburgia salutaris* seeds in each seedling tray. Sample size is indicated below each bar. Average percentage emergence for each population, KNP, Eston and Leshiba GNR, was 61%, 47% and 41%, respectively. Asterisk (*) indicates significantly lower germination percentage for Leshiba A (GLM χ^2, $p < 0.001$).

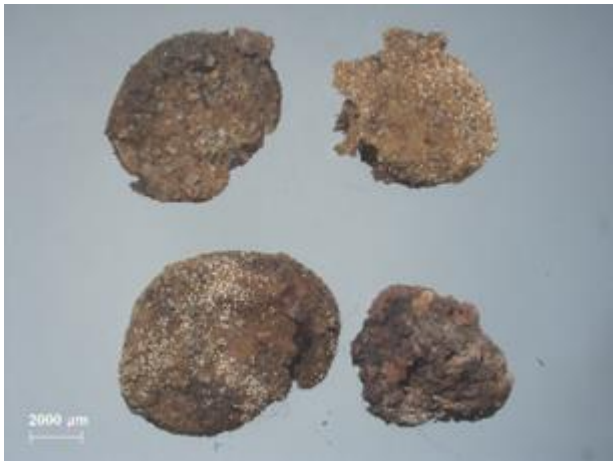

Figure 12: Ungerminated and partly decomposed seeds of *Warburgia salutaris* recovered from the soil of the seedling trays.

3.4 Discussion

3.4.1 Fruit production versus vegetative reproduction

This study has confirmed that fruit production of *W. salutaris* in KNP is extremely low. During the field visit in December 2019, mature fruit were found on only two tagged trees in sub-population 3. In 2020, three trees produced fruit – the same two trees that had fruit in 2019 and an additional untagged tree (also in sub-population 3). In addition, seeing the trees later than planned in the 2020 flowering season was beneficial because very small purple aborted fruit (which had been fertilised earlier in the flowering season) were found still attached to the trees. The majority (91%) of these aborted fruit were undamaged and the cause of abortion could not be identified. Furthermore, 67% of bagged immature fruit (i.e. protected from fruit flies) were aborted and another 17% which had most likely also aborted, were missing (i.e. the bags had been pulled off by animals, possibly elephants).

Reasons for fruit abortion include damage (i.e. by seed predators), underlying genetic abnormalities, and limited resources (e.g. inorganic nutrients, water and carbohydrates; Stephenson, 1981). In KNP, genetic abnormalities and/or resource limitation is the most likely explanation for the abortion of fruit and subsequent low fruit production. Under conditions where resources are limited, the early loss of fruit allows resources to be allocated to storage organs and vegetative growth (Stephenson, 1981). Recent genetic (microsatellite) analyses of *W. salutaris* in KNP suggest that clonal reproduction is widespread within the KNP sub-populations (D. Thompson, Nov. 2020, pers. comms.). Therefore, the level of disturbance and/or unsuitable environmental conditions for sexual recruitment in the KNP, is/are possible reason(s) for the high prevalence of clonal reproduction within this population (Yang & Kim, 2016). Fruit abortion may also be due to self-pollination between clonal individuals, as hand self-pollinations occasionally resulted in early aborted fruit (Chapter 2) that were a similar size to the aborted fruit collected in 2020.

In Eston, fruit abortion of bagged fruit was also relatively high (74%), however natural fruit set in the orchard is still prolific. One explanation for the observed fruit abortion is here is the selective abortion of "low quality" fruit (e.g. with low seed set or produced through self-pollination) to mature "high quality" fruit (Stephenson, 1981). The trees in the Eston orchard were relatively taller (~ 8 m in height) than in KNP, therefore, some flowers on the lower portion of the tree were inaccessible and immature fruit were bagged on the branches that could

be reached. Some of the branches on which fruit were bagged lost their leaves, suggesting that resources (i.e. carbohydrates and nutrients) were not being directed to these branches (Stephenson, 1981). This was possibly due to less sunlight within the orchard canopy (Stephenson, 1981).

3.4.2 Pre-dispersal seed predation

The fruit fly, *Ceratitis cosyra*, was found to be the sole seed predator in the *W. salutaris* fruit from both KNP and Leshiba GNR. The fruit fly was also found in fruit from Eston, as well as a few additional flies (*Zaprionus sp.*) and unidentified Coleopteran larvae. The common name of *C. cosyra* is 'marula fly', as the marula tree (*Sclerocarya birrea)* is the principal host plant for the fruit fly. It is also a well-known commercial fruit pest in sub-Saharan Africa, mainly in mango fruit (Steck, 2012). Studies on the fly note that mature *C. cosyra* females oviposit into fruit at the start of ripening (CABI, 2020) and there are three larval stages which develop over a period of approximately 1 week (CABI, 2020). Analysis of fruit affected by *C. cosyra* confirmed that the fruit fly is ectophytic, meaning that oviposition occurs in the fruit pulp and not directly into the seeds (Ramirez & Traveset, 2010). Only once the *C. cosyra* larvae are fully developed do they begin eating through the seed coat into the stored food reserves (Ramirez & Traveset, 2010). Therefore, seeds can be successfully germinated from infested fruit if they are removed while the larvae are immature and have not yet penetrated the seed coat.

Previous studies note that *W. salutaris* fruit can be heavily infested by larvae – in emergence trials on fruit from the Skukuza Indigenous Plant Nursery, 400–3000 adult fruit flies were reared per kilogram of fruit (Hannweg *et al.*, 2015). However, in this study, the percentage of infested fruit in KNP (20%) was lower than expected. The percentage of fruit impacted by seed predators in KNP and Eston cannot be directly compared as potentially parasitised fruit were specifically selected in Eston for the insect emergence trials in October 2020. Pre-dispersal seed predation occurs in Eston; however because the trees have a high fruit set, the impact of seed predation on viable seed output is relatively low.

3.4.3 Seed dispersal

Warburgia salutaris seeds cannot be released from the fruit without the intervention of a frugivore. At the Random Harvest Nursery in Muldersdrift, *W. salutaris* fruit drop below the

tree and dry up, and no seedlings develop. However, in Eston, seedlings and saplings grow around the farm and this is attributed to dispersal by resident vervet monkeys (M. Stainbank, March. 2019, pers. comm). Reliance on frugivores as seed dispersal agents can be critical for the reproductive success of a species. For example, a study on the African palm (*Hyphaene petersiana* Klotzsch ex Mart.) in the KNP found that absence of elephants in an exclosure explained the observed lack of recruitment, despite the successful production of fruits (Midgley *et al.*, 2020). In addition, frugivores may alter the populations of seed-predators by removing the seeds before larvae have eaten into them (Peguero & Espelta, 2013). However, this would only be possible if frugivores eat the ripe fruit when the larvae are still immature.

In this study primates were the only frugivores seen eating *W. salutaris* fruits. Birds are reported to feed on the fruit of *W. salutaris* (CJM Growers, 2017; Senkoro *et al.,* 2020), however, this was not observed at any of the localities studied here. This may be because of the tough fruit exocarp that makes the seeds inaccessible to birds. However, frugivorous birds (e.g. Turaco species) may still be potential seed dispersers in other parts of *W. salutaris'* distribution. Elephants may also disperse the seeds in their dung, as suggested for *W. ugandensis* in Kenya (Muchugi *et al.*, 2008), but this was not observed in the KNP.

A study on seed dispersal by baboon in South Africa found that seeds from woody species (e.g. of *Acacia* sp., *Diospyros* sp. i.e. jackalberry, and *Ziziphus mucronata* Willd, i.e. buffalo thorn) were still viable when removed from baboon faeces, and similarly, seeds of coastal dune forest trees ingested by vervet monkeys were not destroyed in the digestive tract (Foard *et al*., 1994). Seed ingestion can increase percentage germination and/or germination rate through scarification of a hard seed coat, separation of seeds from the pulp, and fertiliser effect of faecal matter (Levey *et al.,* 2002). Baboons' home ranges can vary from 4–40 km^2; therefore, if seeds remain viable after gut treatment, there is opportunity to disperse seeds a considerable distance from the parent tree (Slater & Du Toit, 2002). However, gut treatment may not be beneficial to the recalcitrant seeds of *W. salutaris* (Kiepiel & Johnson, 2019; Stringer *et al.,* 2020). Although vervet monkeys are suspected to ingest and disperse seeds around the Eston farm, seeds might be stored in cheek pouches (found in all primates in subfamily Cercopithecinae, including baboons and vervet monkeys; Chapman & Russo, 2011) and orally discarded at a later stage. Spitting out of seeds was not seen on the camera trap footage in Leshiba GNR, however, it may occur at a later stage once the primates have moved away from the trees. Orally-discarded seeds still benefit from fruit pulp removal which

enhances germination rates, as seen in samango monkey (*Cercopithecus albogularis schwarzi* Sykes) dispersal of the recalcitrant seeds of *Syzygium cordatum* Hochst. (Stringer *et al.*, 2020) and *Clivia miniata* (Lindl.) Bosse (Kiepiel & Johnson, 2019).

3.4.3 Seedling emergence trials

The seedling emergence trials confirmed that seeds removed from the few fruit produced in KNP are viable. Furthermore, KNP seeds had the highest germination success compared to those from Leshiba GNR and Eston. However, since fruit were only able to be collected from two trees, it is possible that this is not really representative for all trees in the KNP population. One possible explanation for the very low germination success of seeds from Leshiba is that the seeds held less moisture as the fruit in this population were comparatively smaller than either KNP or Eston, and in some cases only contained less than five seeds.

The mean number of days for seeds from all three populations to emerge was 48 days. It is important to note that a seed was considered germinated once the hypocotyl emerged above the soil, and at this point the root was approximately 5 cm in length (K. van den Bosch pers. obs.). Therefore, the mean number of days for the radicle to emerge from the seed (the typical indication of germination) would be less than 48 days. However this is still relatively long for a desiccation-sensitive seed, especially in a semi-arid environment like the KNP. Desiccation-sensitive seeds require a long enough window of soil moisture to match the time to germination in order to survive and for the radicle to extend below the evaporation zone (Stevens, 2020). For this reason, in *ex situ* situations, *W. salutaris* seeds need to be planted in moist soil immediately after being removed from the fruit (Nichols, 2005). A study on seedling establishment of *Acacia* species, which are not recalcitrant, reported that seeds required at least 3 mm water every second day for two weeks, thereby inferring that only in above-average rainfall years is seedling establishment likely to occur for these species in semi-arid environments (Wilson & Witkowski 1998).

In some plant species, ungerminated seeds are not necessarily nonviable, and may have an induced dormancy period (Murdoch & Ellis, 2000). For example, a study on *Kumara plicatilis* (L.) G.D. Rowley found that 100% of ungerminated 3-month old seeds were viable and germination continued for up to 6 months (Cousins *et al.*, 2014). However, dormancy is not a common trait associated with desiccation-sensitive seeds – all recovered *W. salutaris* seeds were soft, partially decomposed, and as a result were no longer viable. The rest of the

seeds were missing, and had most likely completely decomposed. It could not be confirmed whether the ungerminated seeds were initially nonviable, or whether they lost viability once in the soil, possibly as a result of pathogenic fungi (Berjak & Pammenter, 2001). Nevertheless, the results confirm that *W. salutaris* cannot rely on seed banks for population persistence when conditions are unsuitable for germination and for reestablishment after disturbance (Murdoch & Ellis, 2000).

3.4.4 Micro-habitat

Seedling establishment of *W. salutaris* in KNP is probably both seed-limited and microsite-limited. In the KNP, *W. salutaris* is distributed along the south-facing slopes, which suggests that the species cannot survive under heat stress on the north-facing slopes. The slope of the ridge may also negatively affect a seed's ability to establish, especially in the rainy season when rain could wash away the seeds and disrupt root security (Gross-Camp & Kaplin, 2011). While desiccation-tolerant seeds can persist in unpredictable environments, desiccation-sensitive seeds undergo rapid germination to escape seed predation and access soil water (Pammenter & Berjak 2000; Pritchard *et al.,* 2004). For this reason, desiccation-sensitive species have relatively larger seeds and comparatively smaller investments in seed protection, such as a thick seed coat or endocarp (Pritchard *et al.,* 2004). Lower investment in seed protection may be traded off with resources going towards rapid germination, such as high water content and being metabolically active when shed (Pritchard *et al.,* 2004). Evidence of self-seeded saplings in Eston suggests that conditions there provide suitable micro-habitats for establishment. This is probably due to several factors, such as higher rainfall, lower number of herbivores, lack of fire, and shade tolerance (as shown for *W. ugandensis*; Kinyamario *et al.,* 2008).

3.4.5 Climate effects

The higher average annual rainfall in Eston (~ 850 mm) compared to northern KNP (~ 530 mm) may be an additional important reason why seedlings are able to establish more readily at Eston. Plants that produce recalcitrant seeds generally occur in tropical forest or aquatic environments, therefore, species that occur in drier climates or experience drought conditions are more vulnerable to desiccation (Murdoch & Ellis, 2000; Walters *et al.,* 2008). For this reason, species with desiccation-sensitive seeds are typically shed during months of relatively higher rainfall (e.g. > 60 mm) (Pritchard *et al.,* 2004). This was also seen for *W. salutaris* in the KNP, where fruit fall began in November which coincided with the onset of

the summer rainfall (78.7 mm in November, compared to 2.6 mm in October 2019; Appendix 3; Figure 2). Fruit fall would have most likely continued for approximately 3 months, into December and January which received 131.7 mm and 75.5 mm, respectively (Appendix 3; Figure 2). However, rainfall occurs in a few isolated events for example, in December 2019, there were 3 days of rainfall above 20 mm (i.e. 22 mm, 56 mm and 38 mm) followed by three dispersed rainfall events of less than 3 mm. The summer rainfall period also overlaps with higher temperatures, therefore, the relatively low volumes of irregular rainfall is combined with higher evaporation, exposing seeds and seedlings to desiccation. However, in order for seedlings to establish and survive they need to remain moist throughout their first growing season (Pritchard *et al.,* 2004). Therefore, recruitment in *W. salutaris* is most likely episodic as conditions for germination are only very rarely favourable (i.e. sufficient and frequent rainfall over many days).

3.4.6 Future work and recommendations

This study has revealed previously unstudied aspects of *W. salutaris*' seed ecology, and it has highlighted additional questions that can be clarified in future studies on the species. Fully developed fruit were only produced on two trees in sub-population 3 in the KNP. However, this may change in other fruiting seasons in response to climatic changes, and other trees in the KNP population may produce fruit in the future (Chapman *et al.,* 2005). Therefore, monitoring fruit production in relation to interannual rainfall and temperature changes may assist in understanding whether fruit abortion is a result of resource availability and unsuitable environmental conditions. Furthermore, germination trials on seeds from fruit produced on other trees may be useful to better assess overall seed viability within the KNP population. Tetrazolium staining would also be useful to assess viability of i) fresh seed, ii) seeds that are exposed to desiccation, iii) seed viability overtime to see if seeds decrease or increase in viability (i.e. undergo post dispersal maturation).

The seed fate of *W. salutaris* can be further studied to confirm whether the observed frugivores are in fact effective seed dispersers. This can be done by germinating and testing viability (using tetrazolium staining) of seeds found in fresh frugivore scats to assess whether the recalcitrant seeds remain viable after passing through the gut (Slater & Du Toit, 2002). Moreover, these germination data can be compared with the data collected in this study to establish whether gut treatment increases the germination rate and percentage of *W. salutaris.* Further frugivore observations should be carried out in KNP to confirm if seed dispersal is

occurring in this population. Data from baboon and vervet monkey populations in the area can also be used to assess whether their ranges overlap with the *W. salutaris* populations (Slater & Du Toit, 2002).

Although seed predation was relatively low in this study it is still a contributor to the low fruit set in the KNP *W. salutaris* population. Therefore, it would be beneficial to bag fruit at a very early stage in development in case the fruit develops successfully. Bags can be removed once fruit have matured to either be harvested to grow seedlings for the KNP pepperbark propagation programme and to expand the KNP gene pool found in nursery stocks, or be left on the trees to allow for the potential natural dispersal of the seeds. Having more mature fruit on the trees after removing the bags could also increase frugivore activity, making it worthwhile to carry out further observations in the KNP.

3.5 Conclusion

The results from this study have confirmed that the marula fly, *Ceratitis cosyra*, infests fruit from KNP, Eston and Leshiba GNR, reducing the number of seeds that could potentially contribute to seedling recruitment. However, 80% of fruit collected in KNP were healthy which suggests that seed-predation in the KNP population is not as high as other studies have expected. Instead, the early abortion of developing fruit, even when protected from seed predators, mainly contributes to the low fruit production in the KNP. Frugivore observations in Leshiba GNR revealed that baboons and vervet monkeys, rather than birds, eat *W. salutaris* fruit and ingest the seeds. In contrast, frugivore activity was not observed in KNP, but evidence that some fruit had been eaten (i.e. empty exocarps) was found. However, it is not known whether the seeds can survive passing through the digestive tract of the primates. In Eston, vervet monkeys are reported to eat *W. salutaris* fruit and are responsible for the seedlings seen around the farm. This suggests that the seeds survive gut treatment, or that the seeds are stored in cheek pouches and orally discarded. Nevertheless, *W. salutaris* relies on the action of a frugivore to remove the seeds from the pericarp before the fruit dries up. The few healthy fruit produced in KNP can successfully germinate under suitable nursery conditions. Furthermore, it was clarified that seeds are unable to remain viable in the soil (i.e. functioning as a seed bank), as all ungerminated seeds were partially or completely decomposed. Overall, successful germination and seedling establishment in KNP is most likely water-limited and only possible in seasons of above average rainfall. Comparing these results with the recent genetic analyses

on *W. salutaris* in KNP, suggests that a combination of low genetic diversity and ecological factors (e.g. lower rainfall and higher temperatures in contrast with Eston) are driving the high level of fruit abortion and low seedling establishment in this population.

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3.7 Appendices

Appendix 1:

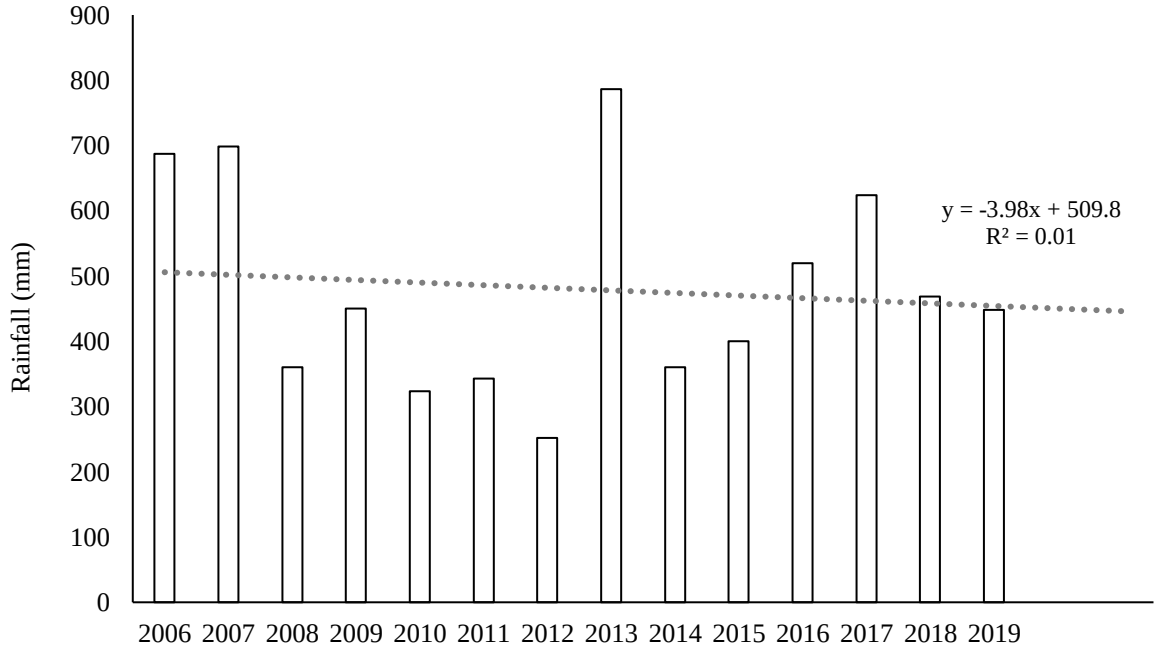

Figure 1: Total annual rainfall (mm) from 2006 to 2019 recorded at the Punda Maria weather station 0768011A8 in the Kruger National Park.

Appendix 2:

Table 1: First day of seedling emergence, last day of emergence (determined after no seedling emergence for > 2 weeks), time spread of emergence (days), mean ± SE emergence time (days), and final germination percentage (%) between *Warburgia salutaris* seeds in each seedling tray.

Tray	First day	Last day	Time spread of emergence (days)	Mean ± SE emergence time	Final germination (%)
Eston A	44	68	24	50 ± 1.77	47
Eston B	36	80	44	52 ± 3.46	53
Eston C	39	68	29	52 ± 2.73	40
KNP A	35	68	33	49 ± 2.57	53
KNP B	35	68	33	45 ± 1.40	67
KNP C	38	60	22	47 ± 2.45	64
Leshiba GNR A	42	56	14	48 ± 2.92	13
Leshiba GNR B	35	51	16	44 ± 1.93	69
Mean + SE	38 ± 1.23	65 ± 3.17	27 ± 3.51	48 ± 1.05	51 ± 6.46

Appendix 3:

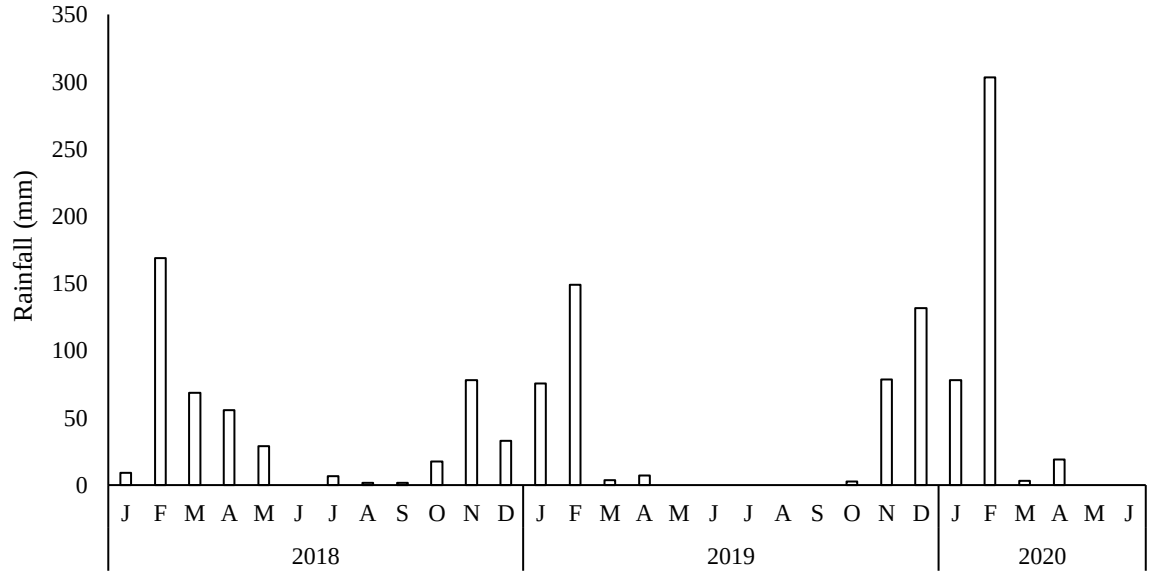

Figure 2: Seasonal variations in rainfall over 2018, 2019 and 2020 recorded at the Punda Maria weather station 0768011A8 in the Kruger National Park.

Chapter 4 – Synthesis and conclusions

4.1 General discussion

The unsustainable harvesting of the pepperbark tree (*Warburgia salutaris* (Bertol.f.) Chiov.) for traditional medicine, combined with habitat loss, have resulted in the Endangered status of the species in South Africa (Cunningham, 1993). Various conservation efforts have been implemented to reduce demand on the few vulnerable natural populations that still exist. However, there is also concern over the long-term viability of the isolated population of *W. salutaris* in the Kruger National Park (KNP). Previous studies in KNP noted a lack of seedling establishment, low fruit set and seed viability (Botha *et al.*, 2004; Hannweg *et al.,* 2015). An assessment of the sexual reproduction of *W. salutaris* in the natural KNP population and, as comparison, a successfully reproducing orchard population, was therefore necessary to investigate factors that may be contributing to its limited sexual recruitment in KNP. This required an examination of the pollination biology (i.e. pollinator visitation, nectar characteristics, breeding system, pollen viability, and pollen tube growth), fruit and seed production, pre-dispersal seed predation, seed dispersal and seed germination of *W. salutaris.* In all instances (except for nectar measurements and seed dispersal) the findings from KNP were compared to those from the Eston orchard. In addition, due to very low fruit production in KNP, a nearby population situated outside of KNP in Leshiba Game and Nature Reserve (GNR), Soutpansberg, was selected to supplement data for analysis of seed predation, seed dispersal and seed germination. Figure 1 is a visual summary of the main findings in this study and recommendations for future work on the species, which are also discussed further below.

4.2 Summary of findings

4.2.1 Pollination biology of Warburgia salutaris

Three major findings from Chapter 2 are: 1) lepidopterans are the most likely effective pollinators of *W. salutaris*, 2) *W. salutaris* is a predominantly outcrossing species , and 3) there is a genetic and/or resource-based malfunction constraining fruit set. *Warburgia salutaris* has a range of floral visitors from the families Lepidoptera (moths and butterflies), Diptera, Coleoptera and Hymenoptera (bees and ants). The visitors are rewarded with a low volume of relatively concentrated nectar. Highest pollen load was seen on the proboscises of lepidopterans, which were identified as both diurnal (butterflies and day-flying moth) and nocturnal floral visitors (moths). The lepidopterans are the most likely effective pollinators of

W. salutaris as they are able to insert their proboscis between the enclosed petals to access nectar, and their comparatively higher vagility enables them to facilitate cross-pollination.

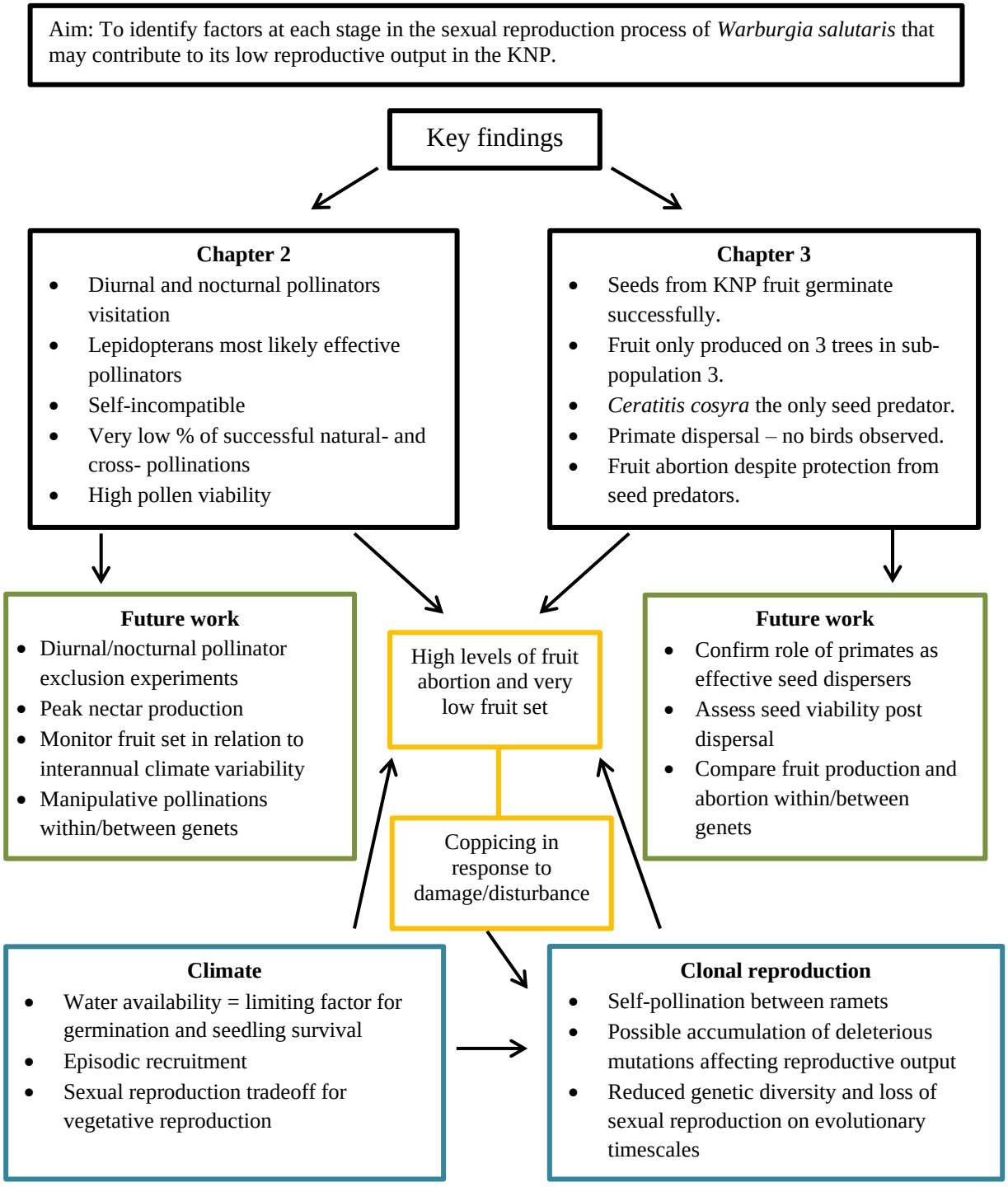

Figure 1: Summary flow diagram of main results in Chapters 2 and 3 (black boxes), major findings in the Kruger National Park (KNP) (orange boxes), possible explanations for impaired fruit production in KNP (blue boxes) and recommendations for future work on *Warburgia salutaris* (green boxes).

The pollination trials revealed that *W. salutaris* cannot autonomously self-pollinate, and self-pollination resulted occasionally in small aborted fruit, but no fully developed fruit. In KNP, no cross-pollinations formed fully developed fruit either, but a very small proportion (2%) resulted in small aborted fruit like those produced from self-pollination. Early abortion of fruit was also seen in the natural-bagged treatment where 34/36 fertilised flowers were aborted. On the other hand, in Eston, all successful cross-pollinations produced fully developed fruit and natural-bagged fruit also developed fully. The results from this study show that *W. salutaris* is primarily out-crossing, as there was no evidence of a fully developed self-pollinated fruit.

Pollen viability is not a contributing factor to the low reproductive output in the KNP population. However, it was noted that one sub-population had non-dehiscent anthers – one of the ways in which male sterility can manifest (Vinod, 2005). Pollen tube growth was assessed to identify if and/or at what point fertilisation is inhibited. In KNP, the higher number of flowers that produced pollen tubes relative to the fruit set from the pollination trials indicates that a genetic and/or resource based postzygotic malfunction may be the cause of the observed early fruit abortion (Krebs & Hancock,1990). For the hand self-pollinations this may be late-acting self-incompatibility, however, the even lower success of hand cross-pollinations suggests that it may be the result of expressed deleterious mutations after the ovule is fertilised, ceasing the development of the fruit (Krebs & Hancock,1990; Hao *et al.*, 2012).

4.2.2 Seed ecology of Warburgia salutaris

Three major findings from Chapter 3 are: 1) primates were the only frugivores observed eating the *W. salutaris* fruit, 2) *W. salutaris* seeds harvested from KNP fruit germinate successfully, and 3) fruit production is extremely low and levels of fruit abortion are high. *Ceratitis cosyra* Walker, the marula fly, infests fruit from KNP, Eston and Leshiba GNR, which reduces the number of seeds that could contribute to sexual recruitment. This is critical in KNP when fruit production is already extremely low. However, 80% of fruit collected in KNP in 2019 were healthy, which suggests that seed-predation in the KNP population is not as high as originally expected. Instead, the early abortion of developing fruit, even when protected from seed predators, appears to be the main contributor to the low fruit production in the KNP. Baboons and vervet monkeys (in Leshiba GNR, Soutpansberg), rather than birds, were captured on camera trap footage eating *W. salutaris* fruit and swallowing the seeds. In contrast, frugivore activity was not observed in KNP, however, empty exocarps were found on the

ground by the trees, which indicates that a frugivore had eaten them. However, it is not certain whether the seeds remain viable after passing through the digestive tract of the primates. In Eston, vervet monkeys are reported to eat *W. salutaris* fruit and the dispersal of seed is responsible for the seedlings seen around the farm. This either means that the seeds survive gut treatment, or can be stored in cheek pouches and orally discarded in the sites where seedlings are found. Nonetheless, *W. salutaris* seeds cannot be released from the fruit unless handled by a suitable frugivore. Seedling emergence trials confirmed that seeds from healthy fruit in KNP can successfully germinate under nursery conditions and all ungerminated seeds partially or completely decomposed. Therefore, seeds must germinate soon after release from the pericarp to avoid desiccation.

4.3 Potential explanations for the impaired sexual reproduction of **Warburgia salutaris** *in the Kruger National Park*

4.3.3 Fruit abortion

This study has confirmed that *W. salutaris* fruit production in KNP is extremely constrained and it was established that immature fruit are aborted very early in development. Fruit abortion was noted in the cross-pollination and natural-bagged treatments, and during observations of natural fruit found on the trees late in the 2020 flowerings season. Three possible explanations for these findings are the predominance of resprouting, widespread clonal reproduction, and unsuitable environmental conditions inhibiting investment in sexual recruitment.

4.3.3.1 Resprouting in response to damage

Multi-stem variants of *W. salutaris* are common in KNP as the species readily resprouts when damaged by elephants and fire (Botha *et al.*, 2004). The ability to resprout is considered a resource trade-off promoting vegetative growth over sexual reproduction (Kruger *et al.*, 1997). The three KNP trees that had matured fruit were relatively undamaged and this may contribute to their successful sexual reproduction. While resprouting allows the KNP population to buffer the impact of disturbances (e.g. by elephants), the species has not managed to withstand the unsustainable harvesting of bark in unprotected areas.

4.3.3.2 Clonal reproduction

It was confirmed that *W. salutaris* is self-incompatible and that some self-pollinated flowers were fertilised and then aborted early in development, which is likely a late-acting self-

incompatibility response. It has also been confirmed that there are pockets of clonal reproduction within the KNP population, therefore, pollination between ramets within a single clonal genet would not result in fruit as this is essentially self-pollination. However, the equally low success of cross-pollinations and abortion of naturally pollinated fruit suggests that fruit set may be constrained as a result of underlying genetic abnormalities. This is possible in plants that have undergone long periods of clonal reproduction and have accumulated mutations in the reproductive structures. An explanation for the "loss of sex in clonal plants" is that ecological factors inhibit sexual recruitment, which allows a period where sterility mutations can accumulate (Eckert, 2002). There are a range of ecological factors that may impact a plant's dependence on sexual or asexual reproduction. However, the water-limiting climate in KNP is the most plausible explanation for *W. salutaris'* reliance on clonal reproduction.

4.3.3.2 Climate

In semi-arid areas like northern KNP, water availability is a major limiting factor for plant growth and reproduction (Wilson & Witkowski, 1998). In contrast, the climate in the Eston orchard, KwaZulu-Natal, is cooler and receives a higher mean annual rainfall (~ 850 mm) than northern KNP (~ 530 mm). Interannual rainfall is highly variable as seen from the Punda Maria records over the last 15 years and the mean annual rainfall over this period (480 mm) is lower than the long-term annual average (Chapter 3, Appendix 1; Figure 1). Water availability plays a critical role in several stages of the reproductive process of species with desiccation-sensitive seeds, and is especially critical for desiccation-sensitive seeds in semi-arid environments (Pritchard *et al.,* 2004). Fruit fall for such species should coincide with onset of the rainy season to ensure that seeds are deposited into a moist environment in which they can rapidly germinate. In KNP, fruiting coincides with the rainy season, however, rainfall in this area of KNP is erratic, consisting of a few high volume rainfall events interspersed with a few low volume showers (i.e. < 10 mm). The rainy season also occurs over summer when temperatures are very high. Therefore, the relatively low volumes of irregular rainfall are combined with higher evaporation (Kusangaya *et al.,* 2014), exposing seeds and seedlings to desiccation. However, in order for seedlings to establish and survive they need to remain moist throughout their first growing season (Pritchard *et al.,* 2004), which is likely essential for *W. salutaris* as well.

4.4. Recommendations and future work

This study has revealed aspects of *W. salutaris*' reproductive ecology that were previously undocumented. However, examining the intricacies of the reproductive process has also generated more questions that can be answered in future studies on the species.

4.4.1 Future work to further our understanding of the reproductive biology of W. salutaris.

4.4.1.1 Pollination biology

Our understanding of the pollination biology of *W. salutaris* can be refined with further exploration. Diurnal and nocturnal pollinator exclusion experiments could be set up to quantify the relative contribution of nocturnal/diurnal pollinators to *W. salutaris* fruit set (Balmford *et al*., 2006), i.e. the relative importance of butterflies (day) and moths (night). The importance of nocturnal pollinators can also be further established by measuring nectar production at different times of the day to see when nectar volume is at its peak (Young, 2002). In addition, floral scent analysis and floral spectral reflectance analysis would also prove valuable to isolate particularly important pollinators of *W. salutaris* (Walsh *et al.*, 2019). These methods may also help confirm the potential role of bees as pollinators, which some studies on *W. salutaris* (Senkoro *et al.,* 2020) and *W. ugandensis* Sprague have suggested (Muchugi *et al.,* 2008).

4.4.1.2 Confirming the role of frugivores as seed dispersers

While it was confirmed in this study that primates eat the *W. salutaris* fruit and ingest the seeds, the role of these frugivores as effective dispersal agents is not certain. Therefore, further germination trials should be carried out on seeds from fresh frugivore scats to assess whether the seeds remain viable after passing through the gut (Slater & Du Toit, 2002). Moreover, this germination data can be compared with germination of seeds which have not passed through the gut of a baboon and/or vervet monkey to establish whether gut treatment increases or decreases the germination rate and/or percentage of *W. salutaris.* Further frugivore observations should be carried out in KNP to confirm if seed dispersal is occurring in this population, as it is possible that the low level of fruit production hinders frugivore activity. Data from baboon and vervet monkey populations in the area can also be used to assess whether their ranges overlap with the *W. salutaris* populations.

4.4.2 Future work to monitor reproduction of W. salutaris *in KNP and understand the underlying causes for low fruit production*

4.4.2.1 Fruit production

This is the first study to quantify fruit set within the KNP population and it was confirmed that fruit were only produced on two trees in sub-population 3 in 2019, and three trees in sub-population 3 in 2020. Fruit set should be monitored in each sub-population to better understand the patterns of reproduction in the KNP and establish whether fruit set is getting progressively less or remaining stable, and whether fruit maturation is restricted to only one or few genets. Fruit production may vary between seasons, possibly in response to interannual changes in rainfall and temperature (Chapman *et al.,* 2005). Therefore, climate-related links may give more insight into the low fruit set in this population.

4.4.2.2 Pre-dispersal seed predation

While bagged fruit were mostly aborted early in development (see Chapters 2 and Chapter 3), a few successfully matured on two (2019) and three (2020) three trees in sub-population 3, suggesting that there is some benefit to protecting the fruit from pre-dispersal seed predation. Fruit should be bagged when immature and bags only removed once fruit are fully developed. These fruit could be used to provide seed for the KNP pepperbark propagation programme, and expand the KNP gene pool in nursery stocks. Alternatively, fruit could be left on the trees *in situ* to allow for natural dispersal of the seeds.

4.4.2.3 Population structure and genetic diversity

The population size and structure of the KNP *W. salutaris* population are monitored by SAEON (South African Environmental Observation Network). In addition, it would be useful to get an idea of the age of *W. salutaris* trees as this may be another important difference between the trees in KNP and the relatively young Eston orchard which was only established in 1992. Evidence of root suckering has been observed in KNP and genets of clonal individuals have been identified through recent genetic analyses of approximately 25% of the total population (D. Thompson, Nov. 2020, pers. comms. 2020). Confirming clonality more broadly is important as it would mean the effective population size (in terms of genets) is smaller than what is currently recognised, which is critical to confirm for an Endangered species. Furthermore, flowering and fruit production can be assessed in ramets of clonal pockets and compared with genetically distinct individuals to confirm the trade-off between asexual and

sexual reproduction. Investigating the relationship between genotypes of the three successful reproducing trees would also give further insight into reasons for their successful reproduction. Cross-pollinations should be conducted between different genets in sub-population 3 (identified using the new genetics findings) as fruit may be successfully produced between genets that are in close proximity. The genetic structure of the population has important implications for its survival – low genetic diversity comes with the risk of a lowered ability to survive disturbances such as environmental changes and a lower availability of compatible mates (Aigner, 2004). Therefore, conserving the genetic diversity is especially critical for threatened species in confined distributions (Senkoro *et al*., 2020).

4.4.2.4. Socio-economic value of the Pepperbark tree

The Covid-19 pandemic reiterated the importance and widespread use of medicinal plants. In Madagascar, the herb, *Artemisia annua* L., was reported as a herbal cure for the Covid-19 virus (BBC, 2020). This information can influence the current demand on medicinal plant species; therefore, extra precautions need to be taken to ensure their sustainable trade. It also emphasises the need for continual monitoring and protection of endangered medicinal plants like *W. salutaris*. Furthermore, conservation programmes such as the Pepperbark propagation programme need to continue so that the communities who rely on the species can access the plant material in a sustainable manner in order to preserve few natural populations.

4.5. Conclusion

This study has revealed that *W. salutaris* is visited by a variety of insects, and Lepidoptera are the most likely effective pollinators. The flowers are protogynous and produce low volumes of relatively concentrated nectar. The species is self-incompatible as no fully developed fruit from self-pollination were observed, despite pollen tubes being able to reach the ovules in some cases. Male function was not impaired as pollen viability was high overall, however, very little pollen was found to be carried by insects visiting the flowers, and very little pollen was available for hand pollinations. In KNP, only two (2019) and three (2020) trees in one sub-population fully matured fruit. Even when hand pollinated, the fruit that were initially fertilised were aborted early in development. Pollen tube occurrence was higher than expected (based on low fruit set in the initial pollination trials), suggesting that a postzygotic mechanism is negatively influencing reproductive output. On the rare occasion that a fruit is successfully produced through natural pollination in KNP, seeds are viable and successfully germinate under nursery conditions. The marula fly, *Ceratitis cosyra*, was found in fruit from

KNP, Leshiba GNR and Eston. Therefore, low fruit/seed production in the KNP population is reduced further, limiting the number of seeds that could potentially contribute to seedling recruitment. However, the early abortion of developing fruit, even when protected from seed predation, appears to be the main contributor to the low fruit production in KNP. Primates, rather than birds, eat *W. salutaris* fruit and possibly ingest the seeds. Nevertheless, *W. salutaris* is reliant on frugivores to remove the seeds from the fruit. Self-seeded saplings in Eston suggest that conditions there are, or have recently been, suitable for establishment. This is probably due to higher rainfall, lower number of herbivores, and lack of fire. Alternatively, sexual recruitment in KNP is likely seed- and microsite-limited, and possibly episodic, where recruitment occurs in periods of high rainfall. Therefore, in KNP, *W. salutaris* relies on clonal reproduction and coppicing to persist. However, long-term reliance on clonal reproduction may impair the ability to reproduce sexually, and it will ultimately be necessary to establish genetically diverse genets that have the potential to adapt to future environmental changes.

4.6 References

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