Evolutionary Ecology (2019) 33:811–824
https://doi.org/10.1007/s10682-019-10007-2

ORIGINAL PAPER

Artificial seed aging reveals the invisible fraction: Implications for evolution experiments using the resurrection approach

Steven J. Franks[1] · Michael R. Sekor[1] · Samuel Davey[1] · Arthur E. Weis[2,3]

Received: 25 April 2019 / Revised: 13 September 2019 / Accepted: 16 September 2019 /
Published online: 18 September 2019

Abstract
Non-random mortality is a key driver of evolution, but mortality that occurs early in life leaves adult traits of individuals that died unknown. This can lead to the invisible fraction problem, which causes difficulty in measuring selection and evolution in natural and experimental populations. Furthermore, seeds or other propagules that are stored intentionally or that persist in dormant states in nature can experience storage conditions that alter adult traits. Invisible fraction and storage condition effects can cause bias in evolutionary studies such as those using the resurrection approach of comparing ancestors and descendants in common environments. To investigate invisible fraction and storage condition effects, we subjected seeds of *Brassica rapa* Fast Plants to artificial aging under hot, humid conditions. We grew plants from artificially aged seeds alongside unaged control seeds for two generations and measured morphological and phenological traits on adult plants. We found that the plants from artificially aged seeds flowered later than those from unaged seeds in both the first and second generation, indicating storage condition and invisible fraction biases. However, the difference in flowering time was smaller in the second generation, indicating that the refresher generation decreased the storage condition effect. We also found that seeds that survived artificial aging were smaller than seeds that did not survive, indicating a potential physical basis for non-random mortality in storage. These results suggest that invisible fraction and storage condition effects can bias the results of resurrection experiments, and that the proper storage of seeds for use in resurrection experiments, as well as a refresher generation, are critical for valid results. The results also demonstrate that artificial aging can be used as a tool for studying mortality of propagules in nature, such as in soil seed banks, thus providing insight into evolutionary processes that would otherwise remain obscure.

Keywords Adaptation · *Brassica rapa* · Climate change · Contemporary evolution · Missing data problem · Phenology · Seed storage

✉ Steven J. Franks
franks@fordham.edu

Extended author information available on the last page of the article

Introduction

Given rapid changes in climatic and other environmental conditions (IPCC 2014), it has become especially important to understand rapid contemporary evolution in natural populations (Franks et al. 2014; Skelly 2010). While it was once commonly thought that evolution would be too slow to allow populations to adapt to contemporary environmental changes, there is now substantial evidence that such rapid evolution can occur, with examples drawn from a wide variety of taxa and habitats (Pelletier et al. 2009; Pulido and Berthold 2004; Salamin et al. 2010; Thompson 1998, 2013). Although many species might not be able to evolve fast enough to keep pace with current rates of climate change (Berteaux et al. 2004; Jezkova and Wiens 2016), evolutionary responses to changes in the environment are certainly happening, and offer us the opportunity to examine evolution as it occurs.

Detecting phenotypic evolution in response to environmental change is a formidable challenge. Traits in contemporary populations may shift relative to the values found among museum/herbarium specimens or other historical records, but if so, is this an evolutionary shift? Such differences could reflect plastic responses to a novel environment by a genetically unchanged population. One way to address this problem is through resurrection experiments, which are powerful tools for detecting phenotypic evolution (Franks et al. 2018). In this approach, ancestors are resurrected from a dormant condition (such as seeds, eggs or frozen cells) and raised alongside descendant (contemporary) populations under common conditions. Both generations experience the same developmental environment, and so their phenotypic differences can be attributed to evolutionary (genetic) change (Franks et al. 2008). While the total number of resurrection studies from natural populations remains relatively small, they have provided important insights into contemporary evolution (Franks et al. 2018). For example, one study using the resurrection approach found that the outcrossing weedy plant *Centaurea cyanus* evolved earlier flowering and a larger floral display between 1992 and 2010, a period of increased warming and pollinator declines (Thomann et al. 2015). Project Baseline, a recently established collection of seeds made explicitly to serve as ancestral generations in future resurrection experiments, will no doubt be an important resource in coming decades as biologists seek to determine evolutionary changes in response to global change in natural plant populations (Etterson et al. 2016).

Though very powerful, resurrection experiments are vulnerable to two biases (Weis 2018). The first of these arises if the storage environment affects propagule condition in ways that alter the phenotypes expressed after development resumes. In this case, phenotypic differences between generations reflect a plastic response by the ancestors to storage—the "storage condition effect" (Fig. 1). A second bias arises if survival of ancestral seed is genetically non-random. It is possible that genes that affect seed survival also influence adult phenotypes, causing a correlated response to selection imposed by storage (Fig. 1). For reasons made clear below, this is called the "invisible fraction problem" (Grafen 1988). Both of these effects could alter the frequency distribution of ancestral phenotypes, and thus distort the baseline for detecting evolutionary shifts in the descendant generation (Weis 2018). The following sections consider these hypothetical effects in the context of stored seed, but analogous processes could occur in other dormant/stored propagules, such as microbial spores, cladoceran resting eggs, or cryopreserved gametes and embryos. Although this paper deals with genetically non-random seed survival in the context of resurrection experiments, the Discussion section

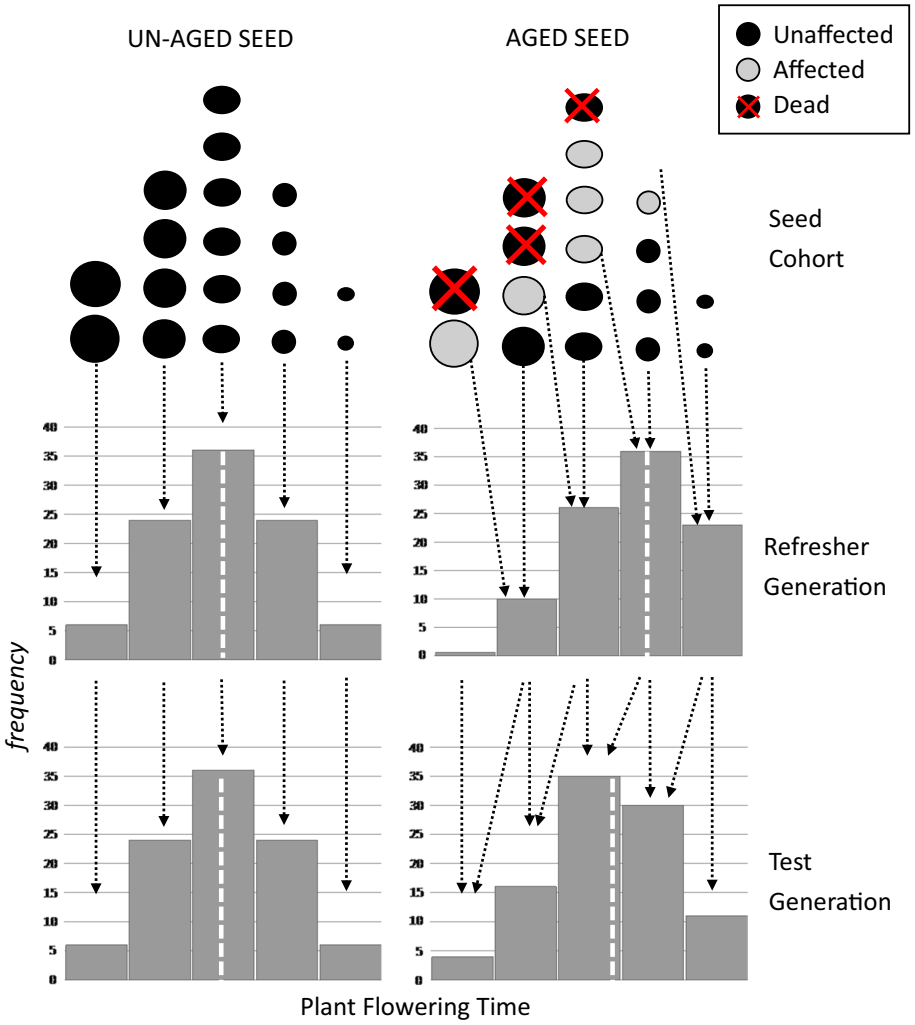

Fig. 1 A representation of invisible fraction and storage condition effect biases on resurrection experiments. Shown are seeds varying in some trait (here size), as well as the frequency distribution of a genetically corrected adult trait (here flowering time). Non-random mortality of aged seeds (marked X) biases the estimate of flowering time (dashed line) through invisible fraction effects, while changes in traits because of storage conditions (grey circles) causes bias in the estimate of flowering time though storage condition effects. Comparisons of aged (ancestral) and unaged (descendant) populations in a resurrection study would give spurious results because of these effects. A refresher generation reduces storage condition effects but not invisible fraction effects. See text for details

lays out its broader implications for ex situ plant conservation, and for evolution of natural plant populations through hidden trade-offs.

Impacts of seed age on viability and vigor

The environmental conditions experienced between seed maturation and germination can affect the quality of the seed, and thereby the vigor of the emerging plant (Vertucci and Roos 1990). Over the course of time, the proportion of viable seeds in a stored cohort declines sigmoidally (Walters 2008). Sequential samples from the cohort maintain a high germination rate for a period of time, but at some point germination declines, eventually to zero. Over the same time course, the vigor of the surviving seeds can deteriorate. Ex situ seed banks conserve seed quality by storage at low humidity and temperature (Li and Pritchard 2009). The optimal storage conditions varies with species, but general recommendations are for 10–27% relative humidity (Ellis et al. 2008; Vertucci and Roos 1990) and temperatures of − 20 to 4 °C, although some species are best stored cryogenically at temperatures of − 196 °C (Walters et al. 2013).

Even under the best conditions, however, seeds deteriorate and eventually die. The causes of this deterioration in seeds stored naturally or in ex situ seed banks include the buildup of reactive oxygen species (ROS) that degrade mRNA transcripts (Chaitanya and Naithani 1994; Fleming et al. 2018; Pammenter et al. 1974; Torres et al. 1997), as well as abscisic acid accumulation, sugar hydrolysis, and protein modifications (Murthy et al. 2003; Rajjou et al. 2008; Yin et al. 2015). Seeds also lose reserves and the ability to mobilize reserves, and become less efficient at detoxification (Rajjou et al. 2008). In artificial seed aging, seed biologists mimic this long-term process by subjecting seeds to high temperature and humidity (Delouche and Baskin 1973; Pammenter et al. 1974). This accelerates metabolism and causes buildup of harmful alterations (Chaitanya and Naithani 1994; Fleming et al. 2018; Murthy et al. 2003; Torres et al. 1997). Seeds quickly accumulate aging-related changes under hot, wet conditions that would otherwise take years, and prior research suggests that artificial seed aging effectively mimics natural seed aging because the same physical and chemical impacts occur through artificial and natural aging, and the same biochemical and physiological responses are involved (Delouche and Baskin 1973; Rajjou et al. 2008).

As seeds deteriorate in storage and some die, mortality may be non-random. Maternal vigor or genotype may influence seed quality at maturation, and thereby affect its chance of surviving. Gene expression within the seed may affect levels of the antioxidants, enzymes, and other molecules that break down harmful substances and repair damage, such that the loss of viability may be non-random with respect to genotype (Nguyen et al. 2012; Rajjou et al. 2008; Torres et al. 1997). One study, for instance, mapped multiple QTL associated with seed longevity in lettuce, indicating genetic variation in the probability of seed survival (Schwember and Bradford 2010). Non-random mortality with respect to trait values is a form of selection, and when the traits are heritable, evolution will occur (Falconer and Mackay 1996; Fisher 1958).

Non-random mortality early in the life cycle complicates the study of later-expressed traits. If a seed dies, it is impossible to know the phenotypes it would have expressed as a mature plant. When there is variation at loci that pleiotropically affect both seed mortality and subsequent plant development, the frequency distributions of later-expressed traits are distorted. If such trade-offs with early survival go undetected, inferences on natural selection and evolution of later-expressed traits are compromised because of this invisible

fraction problem. The severity of this problem depends on the strength of selection acting on the seed trait and the magnitude of its genetic correlation to the later-expressed trait (Weis 2018).

Disentangling storage condition and invisible fraction effects

Both the storage condition and the invisible fraction effects shift the frequency distribution of plant phenotypes from the one expected when all seeds in a cohort survive with undiminished vigor. A careful experimental design is needed to determine if one, the other or both act in a given storage environment (Fig. 1). One such design, which we used, first divides the freshly collected seed cohort into two treatment groups: un-aged and aged. The first group acts as a control, and is held under conditions that preserve viability and vigor. The second group is subjected to the relevant storage environment, or an experimental manipulation that mimics it. The two groups are then revived and propagated for two generations; first, the so-called refresher generation, followed by the test generation. As we show, both plasticity and selection imposed during storage can affect the plant phenotypic distribution in the refresher generation, but the test generation is affected by selection during storage (invisible fraction effects) alone (Fig. 1).

Suppose that a cohort of seed is variable for some heritable trait, such as size, and that this trait is genetically correlated with a trait in the mature plant, such as flowering time (larger seeds yield more vigorous plants that take fewer days to flower). Plants from the unaged seeds exhibit a distribution of flowering times that depends on their genotypes and the growth environment (Fig. 1). If the seeds are aged, some fail to germinate, and of those that do, some are affected by storage in ways that are generally harmful, such as a reduction in resources. When the aged seeds are grown, the mean time to flowering may increase because, for example, large seeds potentially deteriorate more quickly in storage. A fraction of the genes for early flowering that were present in the cohort before storage are now missing because these genes also led to a less viable, larger seed size. The greater deterioration of large seeds, and the resulting loss of vigor, also delays flowering in those that do germinate. The size of the missing fraction effect can be isolated from the storage condition effect by crossing the refresher generation plants within treatment groups, then rearing the resulting seeds, before they age, in a test generation (Fig. 1). The remaining flowering time differences between the two groups in this generation can be attributed to a change in gene frequencies that occurred during storage (invisible fraction effects).

In this hypothetical example, the seed trait affecting probability of survival in storage as well as adult viability and vigor is identified as seed size. In practice, however, seed traits that are influenced by storage conditions and that effect adult traits may be unknown. Nevertheless, running a seed cohort through one or more refresher generations can eliminate storage condition effects and reveal missing fraction effects even if the operative seed traits are unidentified. On another practical note, if seed survivorship is high (> 90%), missing fraction effects will be trivial even under moderately strong seed-parent genetic correlations (Weis 2018). In resurrection studies, the invisible fraction problem and storage condition effects are more likely to occur when seeds have not been intentionally stored under ideal conditions, such as seeds recovered from the environment or herbarium specimens, or when seeds have been kept for a long time at room temperature, which is suboptimal compared to more ideal cool (− 20 to 4 °C) and dry (10–20% RH) conditions (Walters et al. 2013). Both invisible fraction effects and storage condition effects also occur in natural populations such as seeds in soil seed banks. The invisible fraction problem has been

described and characterized through theoretical models (Weis 2018). However, we lack empirical data that would help to determine how much of a problem invisible fraction and storage condition effects would likely be for real seed collections. This is in part because the invisible fraction is generally invisible—it is usually not possible to determine traits of plants that failed to germinate.

In this study, we created invisible fraction and storage condition effects by artificial seed aging in replicate populations of the annual plant *Brassica rapa* (L.). We had two sets of populations: one containing artificially aged seeds and one containing control, unaged seeds. These sets of populations were drawn from the same seed pool and experienced identical conditions other than the aging treatment, thus any consistent differences in traits between them should be due to either invisible fraction or storage condition effects. In the context of resurrection studies, the aged seeds represent ancestors that have gone through storage, and the unaged seeds represent recently collected descendants (Fig. 1). Any difference between these groups would indicate a false positive result (it would appear as if evolution had occurred when it did not) caused by invisible fraction or storage condition effect bias. Furthermore, we can separate the storage condition and invisible fraction effects because we grew the plants for a second generation, which removed storage condition but not invisible fraction bias. We then compared, in several phenotypic traits, aged (representing ancestral) with control (representing descendant) populations. To investigate potential causes of non-random mortality in the artificially aged populations, we examined the relationships among seed size, plant traits and probability of mortality. We combined the results of these experiments to empirically investigate the effects of the invisible fraction and storage condition effect problems on drawing evolutionary inferences from resurrection studies with incomplete germination or a lack of a refresher generation.

Materials and methods

Study species

Rapid-cycling *B. rapa* L. Fast Plants were used in this study. These plants have been artificially selected for rapid growth and development (Williams and Hill 1986). More information about Fast Plants can be found at https://fastplants.org/. Fast Plant seeds were obtained from Carolina Biological Supply Company (Burlington, NC), grown under laboratory conditions, and the seeds collected and stored at 4 °C for approximately two weeks for use in this study. *Brassica rapa* is an outcrossing plant (Kitashiba and Nasrallah 2014), and Carolina fast plants are genetically variable, with prior studies showing substantial responses to selection (Agren and Schemske 1992; Zu et al. 2016).

Germination trials

We conducted a germination trial to determine the length of seed aging required to induce ~50% seed mortality, which should be sufficient to potentially induce a substantial invisible fraction. Artificial aging was conducted in a drying oven with bowls of water to raise humidity, and temperature and relative humidity were measured using a thermometer-hygrometer. We aimed for approximately 40 °C and 90% RH, which has been used in other studies utilizing an artificially aging seed treatment (Rajjou et al. 2008; Yin et al. 2015). During the study, temperature varied between 40–50 °C and relative humidity varied from

79 to 99%. Seeds were placed in petri dishes with 20 seeds per dish and 30 total dishes. The petri dishes were placed in the hot and humid conditions for 0, 1, 2, 3, 4, or 5 days (5 dishes per each of the temporal treatments), transferred to moist pieces of filter paper, and sealed in new petri dishes to germinate. Germination was assessed in the lab, where the temperature was ~22 °C. The samples were checked daily and germination, defined as the emergence of the radicle, was recorded. We continued to monitor for germination for two weeks, but no seeds germinated after 4 days.

Comparison of artificially aged and control samples

Artificially aged seeds (n = 90), as well as control seeds that had not been artificially aged (n = 45), were grown under common conditions. The seeds were randomly assigned to one of the treatments. Artificial aging was conducted for 2 days at 80–99% relative humidity. As the seeds germinated, they were planted in potting soil in individual pots and randomly distributed under grow lights. The plants were grown under constant light, fertilized weekly with Miracle-Gro 15–30–15 fertilizer, and bulk-pollinated within each treatment. Seeds from this first generation were used to grow a second generation under the same growing conditions. For the first- and second-generation plants, we recorded flowering time, leaf count, length of longest leaf, and width of widest leaf for all plants. Flowering time was recorded as the number of days after germination for the first flower to open on each plant. All morphological characteristics were measured 30 days following the first day of average seed germination (all seeds germinated within two days). The length of the longest leaf was measured from the stem to its tip and the width of the widest leaf was measured at its widest point. In order to determine if there was a significant difference between the artificially aged and control samples, two-tailed two sample *t* tests assuming unequal variances were performed in R (R Core Team 2018).

Effect of seed weight on germination after artificial aging

In order to examine the effect of seed size on its ability to remain viable through artificial aging, the individual mass of 96 seeds was measured to the nearest hundredth of a milligram. Seeds were artificially aged for two days as described above, placed on damp filter paper in a sealed petri dish, and observed daily for seed germination. To determine if the seeds that germinated differed in mass from the seeds that did not germinate, we performed Mann–Whitney–Wilcoxon test in R (R Core Team 2018) due to the non-normality of the seed weight data.

Results

Germination trials

The different artificial aging treatments resulted in varying germination rates from 0% in the seeds aged for 5 days to 95% in the control seeds that were not aged (Fig. 2). The sample with the mortality rate closest to 50% was the sample aged for 2 days, which had 60% germination after 4 days in the germination trial. Therefore, we used 2 days as our length of artificial aging in the remainder of our experiments.

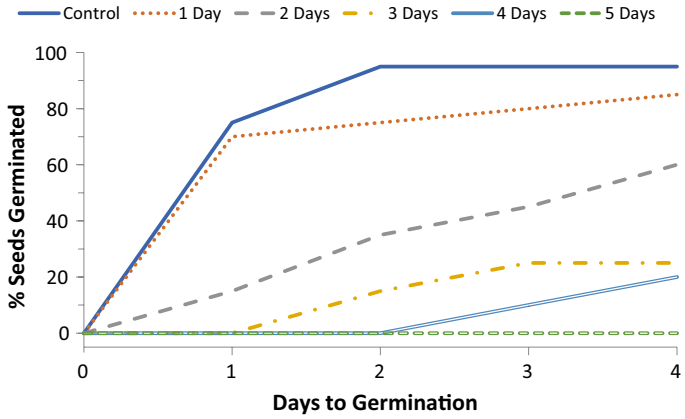

Fig. 2 The effect of artificial aging at 40 °C and 90% RH on the germination rate of *Brassica rapa* fast plant seeds. Samples of 20 seeds were artificially aged for 0, 1, 2, 3, 4, or 5 days and the number of seeds that had germinated was recorded daily

Comparison of artificially aged and control samples

The seeds that were artificially aged for two days had a 31% germination rate and only 14% (13 plants) survived to seed set, while the control population had a 93% germination rate and 87% (39 plants) survival to seed set. Since there were three times as many seed-producing plants in the control sample as in the artificially aged sample, we grew one seed from each control plant and three seeds from each aged plant to create a second-generation sample that equally represented the surviving plants from each sample. Of the 39 seeds planted for each sample, 31 aged plants and 35 control plants grew to be measured, although one of the control plants did not flower.

Artificial seed aging significantly influenced estimates of the population average flowering time in both the first ($t_{1,14}=2.65$, $p=0.019$) and the second ($t_{1,59}=2.21$, $p=0.031$) generation. The plants in the artificially aged sample flowered 11% (2.9 days) later than the plants in the control sample in the first generation and 4% (0.6 days) later in the second generation (Fig. 3a).

Artificial seed aging also influenced estimates of leaf width, but only in the first generation. The plants in the artificially aged sample had a leaf width that was 21% (1 cm) less than the control plants in generation 1 ($t_{1,22}=2.55$, $p=0.018$), but the difference was not significant in generation 2 ($t_{1,58}=0.57$, $p=0.57$) (Fig. 3b). There was no significant difference between the plants in the aged and control samples in leaf count (gen 1: $t_{1,20}=1.00$, $p=0.33$; gen 2: $t_{1,63}=0.20$, $p=0.84$) or length of longest leaf (gen 1: $t_{1,19}=1.16$, $p=0.26$; gen 2: $t_{1,62}=0.96$, $p=0.34$) in either generation.

Effect of seed weight on germination

Of the 96 seeds weighed and artificially aged, 38 germinated. The seeds that germinated had significantly less mass than those that did not germinate ($W=1427.5$, $p=0.015$). The average mass of the seeds that germinated following aging was 1.71 mg and the average mass of the seeds that did not germinate was 1.89 mg.

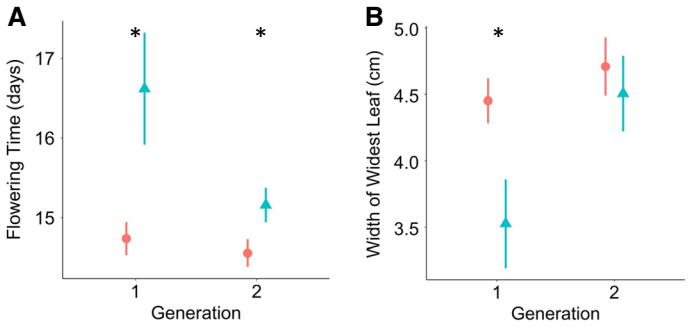

Fig. 3 Flowering time (**a**) and width of widest leaf (**b**) of *Brassica rapa* for the unaged (red, left line) and artificially aged (blue, right line) samples in the first and second generation. Flowering time was measured as the number of days between germination (emergence of the radicle) and flowering (opening of the first flower) of an individual plant. The width at the widest point of the widest leaf of each individual was measured 30 days after average germination. Significant differences in means according to two-tailed two sample *t* tests assuming unequal variances are indicated as * ($p < 0.05$)

Discussion

Mortality experienced by seeds or other propagules as they age, as well as the effects of conditions that dormant propagules experience, help to shape adult populations in natural environments, such as soil seed banks, and in ex situ collections, in important ways. However, these are vexing processes to study because what the adult traits would have been in the fraction of propagules that died had they lived, and in the propagules that aged had they not experienced the effects of aging, is usually unknown. In this study, we subjected seeds to artificial aging to create invisible fraction and storage condition effects and to quantify their influences on inferences drawn from experiments such as resurrection studies.

Our experiments confirm that the invisible fraction problem can arise when poor storage conditions substantially reduce seed viability. We found that plants from the artificial aging treatment differed in some traits from control plants, indicating that the artificial aging treatment created an invisible fraction. Because of this invisible fraction, had these seeds been used in a resurrection study, evolutionary inferences could have been biased and incorrect. The effect of aging was reduced in the second generation, showing the presence of storage condition effects and indicating that including a refresher generation is important in resurrection studies. In this case, smaller seeds were more likely than larger seeds to survive artificial aging. These results, which we further discuss below, have implications for our understanding of seed survival and storage in natural populations and in collections, and of the relationship between seed and post-emergence traits. The results also help us to optimally design and draw appropriate conclusions from resurrection studies such as those using stored seed to investigate evolution.

For the *B. rapa* fast plants we used in our study, placing seeds in conditions of 40–50 °C and relative humidity of 79 to 99% for 2 days induced a mortality of 40% in the trial and 69% in the main experiment. The difference between the trial and main experiment could have been due to slight differences in conditions or to variation between the sets of seeds used in the two experiments. In any case, hot, humid conditions induced substantial mortality. This result is in keeping with other studies that have used artificial seed aging (Rajjou et al. 2008; Yin et al. 2015). This also supports the

practice of keeping seeds under cold, low humidity and constant conditions for optimal storage (Walters et al. 2013). Artificial aging has previously been used for studies examining, for example, the mechanisms of seed deterioration (Yin et al. 2015). Here we show that this technique can also be used to study the invisible fraction, which is a portion of a population that is unmeasured due to mortality (Grafen 1988).

We found that artificial aging induced non-random mortality that significantly affected the mean of some post-emergence phenotypic traits. For example, flowering time was significantly earlier in the control compared to the aged seeds in both the first and second generation. In the first generation, differences in flowering time could have been caused by invisible fraction effects, storage effects, or both (Fig. 1). If these seeds had been used in a resurrection study without a refresher generation, with ancestors represented by the aged seeds and descendants represented by the controls, the results would have been biased, and evolutionary inferences would have been incorrect. Specifically, flowering time would have been estimated to have evolved to be about 3 days earlier, a false positive result. The plants from the second generation represent a resurrection study with a refresher generation. Here, storage effects are minimized because the seeds from the first generation were only stored for a very short time under optimal conditions, resulting in high germination rates, but any invisible fraction effects remain. Following the refresher generation, flowering time was about a half a day earlier, indicating a false positive caused by invisible fraction bias. Taken together, these results indicate that for flowering time, the total bias caused by invisible fraction and storage effects was 3 days, with 83% caused by storage effects and 17% caused by the invisible fraction. The invisible fraction and storage condition effects detected indicate that mortality during storage was non-random with respect to flowering time, and that flowering time may be genetically correlated with traits related to seed survival in storage.

One can ask, how strong was selection in storage on underlying seed traits to produce the 0.6 day shift in flowering time seen in test generation? For that we would need to know the genetic correlation between survival and flowering time, which could not be determined in this study. Nevertheless, we can lay out some possibilities based on prior theoretical work (Weis 2018). The observed flowering time difference between the un-aged and aged groups was ~0.25 standard deviations. If storage-imposed selection was acting on a single trait with a modest genetic correlation to flowering time of 0.25, the selection gradient would be 1. In other words, the seed trait mean among the survivors would be a full standard deviation greater than the mean of the cohort as a whole. A more robust genetic correlation of 0.5 would indicate a more modest selection gradient of 0.25. Based on these assessments, it thus appears that flowering time could be strongly influenced by invisible fraction bias in resurrection experiments with low rates of germination, and that flowering time of natural populations of adults could also be highly influenced by mortality of propagules such as seeds in soil seed banks.

Flowering time in particular has been the subject of investigation of many studies of responses to climate change, with multiple previous resurrection studies finding evidence for evolution of earlier flowering time in natural populations e.g. Franks et al. (2007), Nevo et al. (2012) and Sekor and Franks (2018). Fortunately, these studies had high rates of germination and used a refresher generation, making the invisible fraction and storage effects unlikely to have caused bias in these cases. However, this problem remains a concern particularly for any studies in which germination rates are more moderate, such as when seeds are recovered from suboptimal storage conditions. Previous work has shown that germination timing influences flowering time and selection on flowering time, indicating that there may be direct linkages, possibly due to pleiotropy, between phenology at these two life

stages (Donohue 2002). Thus if mortality during storage influences germination, it can cascade onward to effects at later life-history stages.

The significant difference between the aged and control samples in width of widest leaf found only in the first and not the second generation suggests that this difference may be due to storage conditions effects. This further highlights the importance of a refresher generation, which alleviates the effects of storage conditions, in resurrection experiments (Franks et al. 2018). It also indicates that there may be substantial differences among traits in the extent to which invisible fraction and storage condition effect biases occur, likely because of differences among adult traits in the degree of association with seed traits linked to mortality in storage or to the effects of storage conditions.

This study also found that smaller seeds were more likely to survive the artificial aging treatment than larger seeds. A number of prior studies have examined the relationship between seed size and longevity or viability across species e.g. Nagel and Börner (2009), but little prior research has looked at this relationship within species (Elwell et al. 2007), and especially not with regard to artificial seed aging. The exact mechanism that would provide small seeds with increased seed longevity in an artificial seed bank is unknown and would make an interesting topic of future research.

The idea that small seeds are more likely to survive aging also helps explain why the average flowering time would be significantly later in the sample of seeds that survive aging compared to the average flowering time of the population at large. Seeds that have reduced mass have fewer resources stored within them, which may hinder initial growth and delay flowering time (Elwell et al. 2011; Wulff 1986). It thus appears likely that seeds that do not survive artificial aging or long-term storage would be a non-random subset of the original population, and would differ in characteristics related to seed longevity such as seed size. These seed characteristics would likely relate to adult traits such as flowering time and other characteristics, resulting in an invisible fraction.

Substantial aging-related mortality of seeds in storage makes it is impossible for a resurrection experiment to distinguish between change due to evolution and change due to seed aging. It is thus crucial that resurrection studies should only be conducted using seeds stored properly to prevent the seed aging process from occurring (Franks et al. 2018). Artificial seed banks dedicated to the careful storage and preservation of seeds of a variety of plants are necessary to ensure that we are able to monitor evolutionary trends in populations as the climate changes (Etterson et al. 2016).

Besides the invisible fraction and storage effects, maternal effects can also be a source of bias in resurrection studies (Franks et al. 2018). Maternal effects occur when the maternal environment influences offspring traits (Roach and Wulff 1987). In resurrection studies, maternal effects are more likely to be a problem if ancestors and descendants have experienced different conditions in the field. For example, a prior study collected seeds of plants before and after a multi-year drought (Franks et al. 2007). In this case, the pre-drought adult plants had more resources to provision offspring than the post-drought plants, which could result in bias because of maternal effects. The previous study minimized maternal effects by using a refresher generation (Franks et al. 2007). In the current study, maternal effects could be present, but because all adult plants experienced identical growing conditions, maternal effects are unlikely to be a source of systematic bias. Maternal effects should, however, be considered in the design and interpretation of resurrection studies and in many studies of evolution in natural populations.

We suggest that the experimental procedures that we describe here can be used to gain insight into ecological and evolutionary processes in natural systems not only in the context of resurrection studies. Non-random mortality of propagules, as well as plastic changes

in adult traits caused by conditions of natural propagule storage, are important ecological processes that influence population and evolutionary dynamics, and that can be studied using artificial aging of propagules. For example, artificial aging can be a useful tool to study evolution in species that regenerate from soil seed banks. Recruitment from the soil can slow genetic drift just as does migration (Falahati-Anbaran et al. 2014; Nunney 2002)—effectively it is gene flow through time. Are there hidden trade-offs between long-term survival of buried seed and later-expressed phenotypes? If so, selection within the seedbank could impact the evolution of adult plants. This possibility could be addressed by collecting a sample of seeds from a wild population and storing groups of seeds under various conditions for many years. By storing one sample in a cool, dry environment typically ideal for preserving seeds, and storing another sample in an environment that would allow seeds to age naturally, researchers could compare traits in the plants grown from these seeds to determine if the natural aging process causes non-random mortality. Further studies of seed aging in soil seed banks that included experimental manipulations of temperature and moisture would also be useful for determining how seeds will respond to changes in climate. Increases in temperature occurring widely and increases in precipitation that are occurring in some areas (IPCC 2014) would be particularly likely to increase seed mortality, with subsequent effects on the structure of populations. These effects may be particularly important for the conservation of plant populations as climatic conditions change.

Acknowledgements We thank the Fordham University Honors Program for providing support to S.D. for his research.

Funding This work was supported by grants from the National Science Foundation (DEB-1142784 and IOS-1546218) to S.J.F., and an NSERC Discovery Grant to A.E.W.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

References

Agren J, Schemske DW (1992) Artificial selection on trichome number in *Brassica rapa*. Theor Appl Genet 83:673–678

Berteaux D, Réale D, McAdam AG, Boutin S (2004) Keeping pace with fast climate change: can arctic life count on evolution? Integr Comp Biol 44:140–151. https://doi.org/10.1093/icb/44.2.140

Chaitanya KSK, Naithani SC (1994) Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn.f. New Phytol 126:623–627. https://doi.org/10.1111/j.1469-8137.1994.tb02957.x

Delouche JC, Baskin CC (1973) Accelerated aging techniques for predicting the relative storability of seed lots. Seed Sci Technol 1:427–452

Donohue K (2002) Germination timing influences natural selection on life-history characters in *Arabidopsis thaliana*. Ecology 83:1006–1016

Ellis RH, Hong TD, Roberts EH (2008) Seed moisture content, storage, viability and vigour. Seed Sci Res 1:275–279. https://doi.org/10.1017/S0960258500001008

Elwell A, Durham TL, Miller ND, Spalding E (2007) Environmental effects on seed size and effects of seed size on seedling development in Arabidopsis. Plant Biol Rockv 2007:16

Elwell AL, Gronwall DS, Miller ND, Spalding EP, Brooks TL (2011) Separating parental environment from seed size effects on next generation growth and development in Arabidopsis. Plant Cell Environ 34:291–301. https://doi.org/10.1111/j.1365-3040.2010.02243.x

Etterson JR et al (2016) Project baseline: an unprecedented resource to study plant evolution across space and time. Am J Bot 103:164–173. https://doi.org/10.3732/ajb.1500313
Falahati-Anbaran M, Lundemo S, Stenøien HK (2014) Seed dispersal in time can counteract the effect of gene flow between natural populations of *Arabidopsis thaliana*. New Phytol 202:1043–1054. https://doi.org/10.1111/nph.12702
Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics. Longman, Harlow
Fisher RA (1958) The genetical theory of natural selection. Claredon, New York
Fleming MB, Patterson EL, Reeves PA, Richards CM, Gaines TA, Walters C (2018) Exploring the fate of mRNA in aging seeds: protection, destruction, or slow decay? J Exp Bot 69:4309–4321. https://doi.org/10.1093/jxb/ery215
Franks SJ, Sim S, Weis AE (2007) Rapid evolution of flowering time by an annual plant in response to a climate fluctuation. Proc Natl Acad Sci USA 104:1278–1282. https://doi.org/10.1073/pnas.0608379104
Franks SJ et al (2008) The resurrection initiative: storing ancestral genotypes to capture evolution in action. Bioscience 58:870–873. https://doi.org/10.1641/B580913
Franks SJ, Weber JJ, Aitken SN (2014) Evolutionary and plastic responses to climate change in terrestrial plant populations. Evol Appl 7:123–139. https://doi.org/10.1111/eva.12112
Franks SJ, Hamann E, Weis AE (2018) Using the resurrection approach to understand contemporary evolution in changing environments. Evol Appl 11:17–28. https://doi.org/10.1111/eva.12528
Grafen A (1988) On the uses of data on lifetime reproductive success. In: Clutton-Brock TH (ed) Reproductive success. University of Chicago Press, Chicago, pp 454–471
IPCC (2014) Climate change 2014: impacts, adaptation, and vulnerability. Part A: global and sectoral aspects. Contribution of working group II to the fifth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge
Jezkova T, Wiens JJ (2016) Rates of change in climatic niches in plant and animal populations are much slower than projected climate change. Proc R Soc B Biol Sci. https://doi.org/10.1098/rspb.2016.2104
Kitashiba H, Nasrallah JB (2014) Self-incompatibility in Brassicaceae crops: lessons for interspecific incompatibility. Breed Sci 64:23–37. https://doi.org/10.1270/jsbbs.64.23
Li DZ, Pritchard HW (2009) The science and economics of ex situ plant conservation. Trends Plant Sci 14:614–621. https://doi.org/10.1016/j.tplants.2009.09.005
Murthy UM, Kumar PP, Sun WQ (2003) Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. J Exp Bot 54:1057–1067
Nagel M, Börner A (2009) The longevity of crop seeds stored under ambient conditions. Seed Sci Res 20:1–12. https://doi.org/10.1017/S0960258509990213
Nevo E, Fu Y-B, Pavlicek T, Khalifa S, Tavasi M, Beiles A (2012) Evolution of wild cereals during 28 years of global warming in Israel. Proc Natl Acad Sci 109:3412–3415. https://doi.org/10.1073/pnas.1121411109
Nguyen TP, Keizer P, van Eeuwijk F, Smeekens S, Bentsink L (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in Arabidopsis. Plant Physiol 160:2083–2092. https://doi.org/10.1104/pp.112.206649
Nunney L (2002) The effective size of annual plant populations: the interaction of a seed bank with fluctuating population size in maintaining genetic variation. Am Nat 160:195–204. https://doi.org/10.1086/341017
Pammenter NW, Adamson JH, Berjak P (1974) Viability of stored seed: extension by cathodic protection. Science 186:1123–1124. https://doi.org/10.1126/science.186.4169.1123
Pelletier F, Garant D, Hendry AP (2009) Eco-evolutionary dynamics. Philos Trans R Soc B Biol Sci 364:1483–1489. https://doi.org/10.1098/rstb.2009.0027
Pulido F, Berthold P (2004) Microevolutionary response to climatic change. Birds Clim Change 35:151–183. https://doi.org/10.1016/s0065-2504(04)35008-7
Rajjou L, Lovigny Y, Groot SPC, Belghazi M, Job C, Job D (2008) Proteome-wide characterization of seed aging in Arabidopsis: a comparison between artificial and natural aging protocols. Plant Physiol 148:620–641. https://doi.org/10.1104/pp.108.123141
R Core Team (2018) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
Roach DA, Wulff RD (1987) Maternal effects in plants. Annu Rev Ecol Syst 18:209–235. https://doi.org/10.1146/annurev.es.18.110187.001233
Salamin N, Wuest RO, Lavergne S, Thuiller W, Pearman PB (2010) Assessing rapid evolution in a changing environment. Trends Ecol Evol 25:692–698. https://doi.org/10.1016/j.tree.2010.09.000

Schwember AR, Bradford KJ (2010) Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. J Exp Bot 61:4423–4436. https://doi.org/10.1093/jxb/erq248

Sekor MR, Franks SJ (2018) An experimentally introduced population of *Brassica rapa* (Brassicaceae). 2. Rapid evolution of phenotypic traits. Plant Ecol Evol 151:293–302. https://doi.org/10.5091/plecevo.2018.1401

Skelly D (2010) A climate for contemporary evolution. BMC Biol 8:136–136. https://doi.org/10.1186/1741-7007-8-136

Thomann M, Imbert E, Engstrand RC, Cheptou PO (2015) Contemporary evolution of plant reproductive strategies under global change is revealed by stored seeds. J Evol Biol 28:766–778. https://doi.org/10.1111/jeb.12603

Thompson JN (1998) Rapid evolution as an ecological process. Trends Ecol Evol 13:329–332. https://doi.org/10.1016/s0169-5347(98)01378-0

Thompson JN (2013) Relentless evolution. University of Chicago Press, Chicago

Torres M, De Paula M, Pérez-Otaola M, Darder M, Frutos G, Martínez-Honduvilla CJ (1997) Ageing-induced changes in glutathione system of sunflower seeds. Physiol Plant 101:807–814. https://doi.org/10.1111/j.1399-3054.1997.tb01067.x

Vertucci CW, Roos EE (1990) Theoretical basis of protocols for seed storage. Plant Physiol 94:1019–1023. https://doi.org/10.1104/pp.94.3.1019

Walters C (2008) Understanding the mechanisms and kinetics of seed aging. Seed Sci Res 8:223–244. https://doi.org/10.1017/S096025850000413X

Walters C, Berjak P, Pammenter N, Kennedy K, Raven P (2013) Preservation of recalcitrant seeds. Science 339:915–916. https://doi.org/10.1126/science.1230935

Weis AE (2018) Detecting the "invisible fraction" bias in resurrection experiments. Evol Appl 11:88–95. https://doi.org/10.1111/eva.12533

Williams PH, Hill CB (1986) Rapid-cycling populations of Brassica. Science 232:1385–1389

Wulff RD (1986) Seed size variation in *Desmodium paniculatum*: II. Effects on seedling growth and physiological performance. J Ecol 74:99–114. https://doi.org/10.2307/2260351

Yin X, He D, Gupta R, Yang P (2015) Physiological and proteomic analyses on artificially aged *Brassica napus* seed. Front Plant Sci 6:112. https://doi.org/10.3389/fpls.2015.00112

Zu P, Blanckenhorn WU, Schiestl FP (2016) Heritability of floral volatiles and pleiotropic responses to artificial selection in *Brassica rapa*. New Phytol 209:1208–1219. https://doi.org/10.1111/nph.13652

Affiliations

Steven J. Franks[1] · Michael R. Sekor[1] · Samuel Davey[1] · Arthur E. Weis[2,3]

[1] Department of Biological Sciences, Fordham University, Bronx, NY 10458, USA

[2] Department of Ecology and Evolutionary Biology, University of Toronto, Toronto M5S 3B2, Canada

[3] Koffler Scientific Reserve at Jokers Hill, University of Toronto, Toronto M5S 3B2, Canada