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Seed germination behaviour of the narrow endemic *Daphne arbuscula* (Thymelaeaceae) compared to the more widespread *Daphne cneorum*

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Abstract Diminished reproduction success in species with narrow distribution ranges might be one of the factors responsible for their limited dispersal and colonization abilities. We investigated here various aspects of the seed biology of the West Carpathian endemic *Daphne arbuscula* (Thymelaeaceae) and compared it with its more widespread relative *D. cneorum*. In both species, we investigated (i) differences in seed viability and germination ability; (ii) differences between the two observed fruit morphotype groups, and (iii) the effect of cold stratification in breaking seed dormancy and enhance germination in stored seeds. To determine seed viability, a tetrazolium test and an imbibed cut test were performed. Several seed germination tests with gibberellic acid and with a sequence of cold and warm stratification, using different temperatures and durations, were carried out. We uncovered that (i) *D. arbuscula* seeds show significantly lower viability than *D. cneorum* seeds, but this difference is due to the smaller-fruit morphotype; (ii) seed quality and viability of the big-fruit morphotype are significantly greater than the smaller-fruit morphotype in both species, although the seed viability of the latter is not null and the dormancy level seems to differ between them; (iii) a warm stratification at 15°C for 13 weeks, followed by cold stratification at either 0 or 5°C for 28 weeks, followed by 4 weeks at 15°C, break physiological dormancy and allow the majority of seeds of *D. arbuscula* (63%) to germinate. We recommend including both fruit morphotypes when collecting seed of *Daphne* for *ex situ* conservation and reintroduction initiatives, to maintain the original genetic diversity of the species.

Keywords *Daphne arbuscula* · *Daphne cneorum* · Fruit polymorphism · Seed dormancy · Seed germination · Seed viability

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Introduction

Members of the genus *Daphne* L. (Thymelaeaceae Juss.) are widely known not only as valuable ornamental shrubs in horticulture but also as a textbook example of

a genus with extensive diversification of endemic entities (Tutin 1968; Halda 2001; Yinzheng et al. 2007). Indeed, a large number of *Daphne* species are endemics and restricted to only small areas of distribution. For instance, out of thirty European species, more than twenty are endemics of single mountain ranges or islands (Tutin 1968; Halda 2001; Pedrol 2011). The genus encompasses up to 95 deciduous or evergreen shrubs native to temperate and subtropical parts of Europe, Asia and North Africa (Tutin 1968; Halda 2001). Most of the species flower in late winter or early spring with colourful, scented flowers formed by petaloid sepals forming a flower cup (hypanthium). They are reported to be mostly self-incompatible and entomophilous. Fruits are dry or fleshy, one-seeded drupes; zoochory is the primary dispersal mechanism (Halda 2001).

In contrast to its horticultural and scientific attractiveness, the genus is, in general, understudied with regard to its reproduction strategies, seed biology and germination ability (but see e.g. Zhang and Smagula 2000; Alonso and Herrera 2001; White 2006; Filipovič 2011; Baskin and Baskin 2014; Fang et al. 2016). The viability and germination ability of seeds of the few *Daphne* species studied were shown to be considerably low, which points towards a problem with their generative reproduction (e.g. Erdelská and Turis 1995, 1996; Šedivá and Žlebčík 2010; Ari et al. 2014). One of the crucial factors considered responsible for the decreased germination ability in *Daphne* is physiological dormancy (hereafter abbreviated as PD; White 2006; Ari et al. 2014). However, PD might be overcome, for instance, by cold stratification treatment (Zhang and Smagula 2000; Fang et al. 2016).

In this study, we focused on *Daphne arbuscula* Čelak., one of the well-known narrow endemics in the genus. It only occurs in the Muránska planina mountains in the Western Carpathians in Slovakia (Krippel and Goliašová 1988). It is an evergreen dwarf shrub, up to 25 cm tall (Krippel and Goliašová 1988; Turis 1994), with shiny revolute leaves and pink to purple-violet, sweetly scented flowers. It occupies sunny rocky slopes and rock terraces on limestone bedrock between 590 and 1,330 m a.s.l. (Erdelská and Turis 1996; Kochjarová et al. 1999). The second species of interest, *D. cneorum* L., is morphologically very similar to *D. arbuscula* but differs predominantly by its finer leaves and their position on the stem. It is widespread across Europe with an area of distribution ranging from Spain to Russia (Tutin 1968; Halda 2001). Both species are diploid with $2x = 2n = 18$ and are reported to be allogamous and entomophilous (Erdelská 1998; Marhold et al. 2007). Their fruits are one-seeded drupes. Interestingly, morphological fruit polymorphism has been observed in both species [*D. arbuscula* (Turis, unpublished data) and *D. cneorum* (Gajdošová 2018)]. Based on the colour and thickness of the mesocarp, fruits of both species can be assigned to two, essentially discrete, categories. The first fruit morphotype includes fruits that are bigger and have a large, fleshy mesocarp. They have a yellow to yellowish-brown colour (referred to below as the 'big-fruit morphotype'; Figs. 1a,b and 2a,b). The second fruit morphotype (referred to below as the 'small-fruit morphotype') consists of smaller and harder fruits, characterized by a reduced mesocarp, which is at first sight significantly thinner than the mesocarp of the 'big-fruit morphotype'. The fruit colour of the 'small-fruit morphotype' ranges between green reddish and reddish-brown in *D. arbuscula*, but is greenish in *D. cneorum* (Figs. 1d,e and 2d,e). In both species, the seeds of both fruit morphotypes do not show any noticeable morphological differences (Figs. 1c,f and 2c,f). Importantly, however, the fruits are dispersed at the same time (Gajdošová 2018; authors' own observations). It has been left unclear whether these two fruit morphotype groups represent actual fruit polymorphism or only a specific stage during the fruit ripening process. Furthermore, the seeds of both species are suspected to show PD, similar to other *Daphne* species (Erdelská and Turis 1995, 1996; White 2006). Several studies on various aspects of the biology, chorology and ecology including seed embryology of *D. arbuscula* have been published to date (Erdelská et al. 1989; Murín 1990; Erdelská and Turis 1995, 1996; Turis and Smetana 1997; Erdelská 1998, 1999; Kochjarová et al. 1999). Some of these studies even provided first insights into the reproductive strategies and mechanisms in *D. arbuscula*, highlighting decreased fruit formation, seed viability, germination ability, and a role of the fruit mesocarp as a factor inducing and prolonging the physiological dormancy of seeds (Erdelská and Turis 1995; Filipovič 2011). Previous experiments pointed towards a very low germination ability of *D. arbuscula* (Erdelská and Turis 1995, 1996) and *D. cneorum* (Šedivá and Žlebčík 2010). Furthermore, Filipovič (2011) examined the influence of various factors stimulating and controlling seed germination in *D. arbuscula* and showed that certain stimulators increase seed

Fig. 1 Fruits and seeds of *Daphne arbuscula*; big-fruit morphotype: **a** – fruit, **b** – fruit cross-section, **c** – seed; small-fruit morphotype: **d** – fruit, **e** – fruit cross-section, **f** – seed

germination. However, this study did not include crucial environmental factors such as the temperature which controls seed germination and physiological dormancy. Likewise, none of these studies addressed the morphological fruit polymorphism and any potential differences in seed viability between them.

For these reasons, based on previously obtained knowledge on seed production, viability and dormancy in *D. arbuscula* predominantly *in situ* (Erdelská and Turis 1995, 1996), we examined the *ex situ* seed germination of *D. arbuscula* after storage, also to give recommendations to seed conservation practitioners on the best protocol to germinate this threatened species, and therefore favouring its reinforcement in the wild. In particular, we focussed on the differences in viability and germination abilities between the observed fruit morphotypes, to inform the best seed collecting procedures for this species. We studied the influence of temperature regime and gibberellic acid to break potential physiological seed dormancy. We tested the following specific hypotheses:

1. The overall seed viability and germination ability of the endemic *D. arbuscula* is critically low compared to the closely related but much more widespread species *D. cneorum*.
2. Seeds of the two fruit morphotype groups of *D. arbuscula* differ in their levels of viability. Specifically, based on previous observations (Turis, personal communication), we expected a

Fig. 2 Fruits and seeds of *Daphne cneorum*; big-fruit morphotype: **a** – fruit, **b** – fruit cross-section, **c** – seed; small-fruit morphotype: **d** – fruit, **e** – fruit cross-section, **f** – seed

significantly lower or even no viability in the 'small-fruit' morphotype.

3. Long-term exposure of seeds to cold stratification breaks the dormancy and increases seed germination in *D. arbuscula*.

Material and methods

Ripe seeds of *D. arbuscula* (four populations) and *D. cneorum* (one population) were collected in June and July 2018 from natural populations in the Western Carpathians (Slovakia) at the time of their natural dispersal (see Table S1).

Because the populations of *D. arbuscula* were greatly restricted and the majority of individuals difficult to reach or not accessible at all (e.g. situated on vertical cliffs), and the collection was limited to the 20% of available seeds in order not to compromise the survival of the populations (Pedrini et al. 2020), the overall number of seeds collected and used in this study was limited. For this reason, it was not possible to perform replicates of the experiments. Instead, statistical analysis was performed considering each seed as an individual response, as previously done successfully by Newton

et al. (2013) and Davies et al. (2016). For all five populations, seeds from the two fruit morphotypes were collected and kept separately. Seeds were cleaned and removed from fruits shortly after collection to avoid any unintended influence of the mesocarp potentially blocking seed germination (Filipovič 2011). Afterwards, seeds were dried at 15% equilibrium relative humidity at 15°C, then shipped to the Millennium Seed Bank (MSB) for testing, which started in January 2019.

Viability tests

Prior to germination tests, we studied the overall viability of *D. arbuscula* seeds from both fruit morphotypes. To verify the potential difference in seed viability between seeds from the small-fruit morphotype vs the big-fruit morphotype, two different viability tests (tetrazolium test and imbibed cut test) were performed (Davies et al. 2015a). To avoid possible population effects, seeds of *D. arbuscula* from both morphotypes collected in the four populations as shown in Table S1 were mixed prior to the tests. Because of variable seed availability, the number of seeds per collection was not equal, but within each population we used the same number of seeds for each morphotype so as to ensure the same contribution of each population to the morphotype groups. Both tests were also performed on seeds of the closely related species *D. cneorum* from the locality Baba (see Table S1).

Fifty seeds of each morphotype group of each species were used for the tetrazolium (TZ) test, following the MSB standard procedure (24 h rehydration over water at 20°C, 48 h on 1% agar at 20°C, seed excision at cotyledons' end, followed by 48 h in 1% TZ solution at 30°C in the darkness). The seeds were scored under a stereomicroscope and different colour categories were noted. As no literature is available to guide the interpretation of the TZ results for these species, two possible viability interpretations were recorded, one where only the fully red seeds were counted as viable (referred to below as 'Low TZ'), and another one where both, the fully red and the red with small patches of pink colour were considered viable (referred to below as 'High TZ'). White, pink or mouldy seeds were always considered not viable. The number of empty seeds was recorded, too.

At the same time, fifty seeds of each morphotype group of each species were used for the imbibed cut test, which was performed by dissecting the seeds under a stereomicroscope after 4 weeks of imbibition on 1% agar at 15°C. The following categories were identified: viable (fresh, healthy tissues), not viable (mouldy, necrotic, soft tissues) and empty seeds. This test was used to validate the TZ interpretations described above, so only the most reliable interpretation was used in the study.

Daphne arbuscula germination tests

Because of the limited number of available seeds in this study, we were able to test only few germination and stratification temperatures. Only seeds from the big-fruit morphotype of *D. arbuscula* of the population in Šiance were used for the germination tests. To choose the most likely successful ones, we used available actual climatic data at the sites of natural occurrence as a proxy. Actual climatic data of Šiance for long-term average monthly temperatures measured between 1994 and 2019 were extracted from SolarGIS (Bratislava, Slovakia) and used, with reasonable approximation, as germination and stratification temperatures (Table S2).

Four germination tests with fifty seeds each were set up (Table 1). Seeds were exposed to initial warm stratification (4 weeks), followed by a period of cold stratification at 0 or 5°C (8 weeks), followed by the spring temperature regime (10 weeks), when one would expect the seeds to germinate. For both, warm stratification and spring temperatures, constant (15°C) and alternating temperature (25/5°C) regimes were tested. At each stage, we used a photoperiod of 12 h. The lengths of the different steps were shorter than the natural seasons to make the tests doable in the laboratory. However, as suggested for move-along experiments (Kildisheva et al. 2020), an additional two germination tests were set up using the full length of seasons (13 weeks warm stratification and 28 weeks for cold stratification). For these tests, only a constant temperature (15°C) for warm stratification and the spring temperature regime was applied whereas both 0°C and 5°C were tested for cold stratification.

Germination tests were performed using Petri dishes with a 1% agar substrate. Germinated seeds were counted and discarded weekly, and tests were abandoned after a minimum of 4 weeks with no further germination event. Final cut tests were done by dissecting the non-germinated seeds under a stereomicroscope and these seeds categorized as either fresh and healthy (f), mouldy and dead, empty (e), or infested (i). Following standard protocols (Davies et al. 2015a), germination (G) and estimated viability (V) percentages were calculated as: $G = g \times 100/(\text{s-e-i})$ and $V = (g + f) \times 100/(\text{s-e-i})$, where g

Table 1 Constant and alternating temperatures used for warm stratification, cold stratification and spring temperature treatments (with lengths of exposure) in the six seed germination tests of *D. arbuscula* and resulting final germination, viability percentage, mean germination time (MGT) and germination synchrony (SYN).

Warm stratification	Cold stratification	Spring temperatures	Germination [%]	Viability [%]	MGT [days]	SYN
15°C (4 weeks)	0°C (8 weeks)	15°C (10 weeks)	51	91	99.9	0.6
15°C (4 weeks)	5°C (8 weeks)	15°C (10 weeks)	42	78	100	0.34
25/5°C (4 weeks)	0°C (8 weeks)	25/5°C (10 weeks)	11	15	92.6	0.3
25/5°C (4 weeks)	5°C (8 weeks)	25/5°C (10 weeks)	16	24	84	0.29
15°C (13 weeks)	0°C (28 weeks)	15°C (4 weeks)	63	63	297.7	0.44
15°C (13 weeks)	5°C (28 weeks)	15°C (4 weeks)	57	57	271.3	0.5

= number of germinated seeds and s = number of sown seeds. The viability calculated here does not indicate the original viability of the seed collections, as the effect of long germination tests might affect seed viability. For this reason, the intention in this case was to show the effect of certain germination conditions on seed viability.

Mean germination time (MGT), expressed in days, was calculated for each test as:

MGT= $\sum(n,t)/\sum n$, where t is time from the beginning of the germination test in terms of days, and n is the number of newly germinated seeds at time t (Ranal et al. 2009; Lozano-Isla et al. 2019). Germination synchrony (SYN), expressed as a value between 0 and 1, was calculated as: SYN = $(\sum Cn_{1,2}) / N$, where $Cn_{1,2} = [n_i (n_i - 1)] / 2$ and $N = [\sum n_i (\sum n_i - 1)] / 2$, and n_i is the number of seeds germinated in the i^{th} time. When synchrony is equal to 1, seed germination occurs at the same time whereas synchrony near 0 denotes that at least two seeds complete the germination process at different times (Ranal et al. 2009; Lozano-Isla et al. 2019).

GA3 tests

To assess the difference in dormancy level between the two morphotypes of both species *D. arbuscula* and *D. cneorum*, as well as between the species, a further germination test with gibberellic acid (GA3) was performed. Prior to the test, seeds of *D. arbuscula* from four populations were mixed as described above. Fifty seeds of each fruit morphotype of each species were sown on 1% agar with dissolved GA3 at a concentration of 250 mg/l, as described in Davies et al. (2015a). Plates were incubated at 15°C and under a 12-h photoperiod. Germinated seeds were counted and discarded weekly, and tests were run for 13 weeks. Final cut tests were done by dissecting the non-germinated seeds under a stereomicroscope. Germination percentages were calculated as described above.

Statistical analysis

To assess the best TZ interpretation, the two results Low TZ and High TZ were compared with the imbibed cut test using the Pearson's chi-square test, or the Fisher's exact test where any of the expected values were < 5. The same statistical tests were used to compare the viability of the two fruit morphotypes for each species and to compare the viability between the two species.

To assess the effect of each variable tested with the germination tests (germination temperature, stratification temperature and stratification length), a logistic regression with categorical variables was performed. Both, germination and viability estimated through final cut tests were analysed.

To calculate MGT and SYN, the GerminaR package was used (Lozano-Isla et al. 2019).

Differences in the levels of dormancy between the fruit morphotypes and between the species were assessed through a Pearson's chi-square test, using the number of seeds germinated and non-germinated, excluding empty and infested seeds in order to assess dormancy in full seeds only, as the only ones potentially viable (Davies et al. 2015a).

All statistical analyses were run with RStudio (RStudio Team 2019) and R (R Core Team 2020). Graphs were produced with Microsoft Office Excel.

Results

Viability tests

For both fruit morphotypes of both the species *D. arbuscula* and *D. cneorum*, the results of the High TZ test and the imbibed cut test were not significantly different (in *D. cneorum* $P = 0.229$ and $P = 0.117$ for the small- and big-fruit morphotype, respectively; in *D. arbuscula* $P = 0.08$ and $P = 1$ for the small- and big-fruit morphotype respectively, Fisher's exact test; Table 2). On the other hand, for the big-fruit morphotype of both species, the results of the Low TZ test were significantly different ($P = 0.001$ and $P = 0.031$ for *D. arbuscula* and *D. cneorum*, respectively) from the results of the imbibed cut test. Therefore, the results of the High TZ test were the most accurate for the two species, and it was used in this study to analyse the difference between the seeds of the two fruit morphotypes.

The viability of seeds contained in the big-fruit morphotype was very high for both species (94% in *D. arbuscula*, 100% in *D. cneorum*), and the smaller-fruit morphotypes contained fewer viable seeds (58% in *D. arbuscula*, 86% in *D. cneorum*).

As is apparent already from the percentages of seed viability, there was a statistically highly significant difference between the two fruit morphotypes in terms of the quality (expressed as the proportion of full vs empty seeds) and viability of the seeds of both species, *D. arbuscula* ($P < 0.001$, Fisher's exact test) and *D. cneorum* ($P = 0.006$, Fisher's exact test).

Seed viability differed between the two species, being greater in *D. cneorum* (Table 2). This difference was significant if all seeds were taken into account ($P = 0.007$, Fisher's exact test), but that was due to the lower viability of the seeds of the small-fruit morphotype in *D. arbuscula* ($P = 0.011$, Fisher's exact test), as no significant difference ($P = 0.242$, Fisher's exact test) was found between the seeds of the big-fruit morphotype.

Daphne arbuscula germination tests

The highest seed germination (63%) of *D. arbuscula*, of seeds collected from the big-fruit morphotype, was achieved after 13 weeks of warm stratification at 15°C incubation, followed by 28 weeks of cold stratification at 0°C, followed by 15°C incubation (Table 1).

Germination at a constant temperature of 15°C (42 to 63%) was notably greater than at alternating temperatures of 25/5°C (11 to 16%), confirmed by the logistic regression ($P < 0.001$). Similarly, seed viability estimated by a cut test after seeds were exposed to constant temperature (57 to 91%) was significantly greater than when seeds were exposed to alternating temperatures (15 to 24%), also confirmed by logistic regression ($P < 0.001$).

In the germination tests at constant temperatures, the lower temperature used for cold stratification, 0°C, achieved greater germination success but the difference was not significant ($P = 0.586$). This result was confirmed even when the logistic regression was applied to the tests at constant temperatures only.

Finally, the duration of the stratification treatments had a slight impact on the final germination results, with the longer time (13 weeks for warm and 28 weeks for cold stratification) resulting in higher germination percentages; however, this difference was not confirmed by logistic regression ($P = 0.075$).

Table 2 Numbers of viable, not viable and empty seeds in TZ viability and imbibed cut viability (ICT) tests for each fruit morphotype in both species. BFM – the big fruit morphotype, SFM - the small fruit morphotype

	D. arbuscula SFM			*D. arbuscula* BFM			*D. cneorum* SFM			*D. cneorum* BFM		
	High TZ	Low TZ	ICT	High TZ	Low TZ	ICT	High TZ	Low TZ	ICT	High TZ	Low TZ	ICT
Viable	29	24	18	47	32	46	43	33	37	50	37	46
Not viable	15	20	21	3	18	4	5	15	11	0	13	4
Empty	6	6	11	0	0	0	3	3	2	0	0	0

MGT varied from 84 to 100 days in the short tests and from 271 to 298 days in the long tests (Table 1). Germination was concentrated immediately after the cold stratification, with just a few seeds germinating during the warm stratification. Exceptionally, when the cold stratification at 5°C lasted for the full length of the winter, seeds started to germinate after 24 weeks under the winter temperature regime (Fig. 3).

Germination synchrony (SYN) varied from 0.29 to 0.6 (Table 1). These values, distant from 0, were due to the germination being spread over two periods (warm stratification and the spring temperature regime), and in one case to the cold stratification period.

GA3 tests

When comparing seed germination with GA3 between the fruit morphotypes, the two species behaved in opposite ways (Table 3). In *D. cneorum*, seeds of the small-fruit morphotype (39%) achieved significantly greater germination than seeds from the big-fruit morphotype (8%; $P < 0.001$). On the other hand, in *D. arbuscula* more seeds of the big-fruit morphotype (46%) germinated compared to the small-fruit morphotype (30%), but the difference was not significant ($P = 0.12$).

Table 3 Germination percentages after 13 weeks of incubation at 15°C under a 12-h photoperiod on 1% agar with dissolved GA3 at a concentration of 250 mg/l. Statistically significant differences between fruit morphotypes and species are shown (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

	Daphne arbuscula	*D. cneorum*	χ^2 between species
Big-fruit morphotype	46%	8%	***
Small-fruit morphotype	30%	39%	–
χ^2 between fruit morphotypes	–	***	–

Overall, comparison of the two species, revealed that seeds of *D. arbuscula* germinated significantly better than those of *D. cneorum* ($P = 0.019$), but only due to the highly significant difference between the big-fruit morphotypes of the two species ($P < 0.001$).

Discussion

Relationship between fruit polymorphism and seed viability in *Daphne arbuscula* and *D. cneorum*

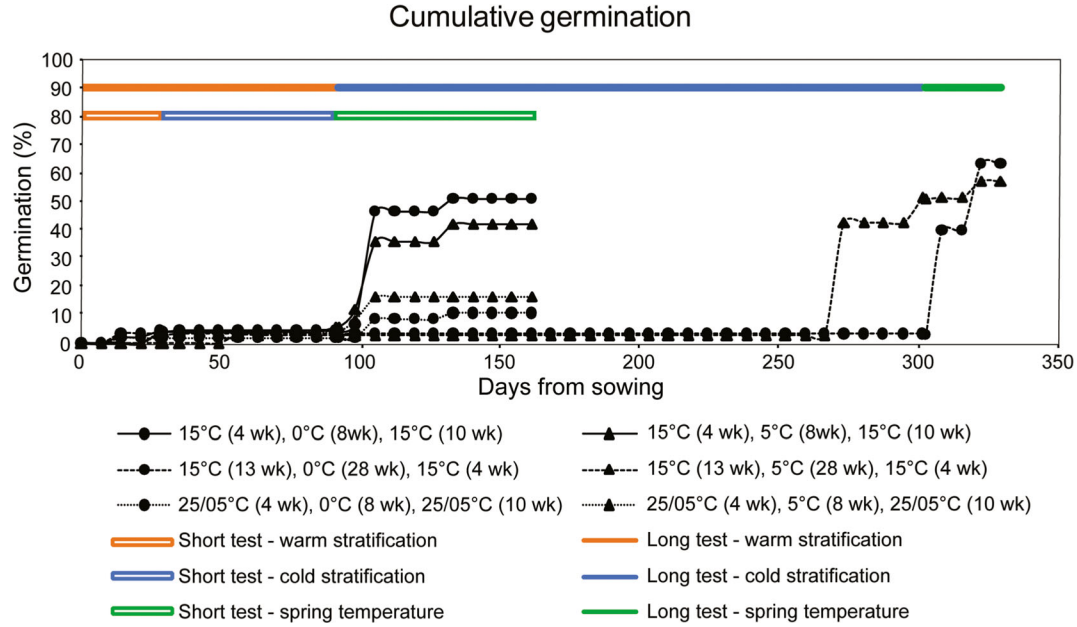

Fig. 3 Cumulative germination percentages for the six seed germination tests of *Daphne arbuscula* for each day; stratification lengths are shown, too

Our study shows that *D. arbuscula* has statistically lower seed viability compared to the more widespread *D. cneorum*. This finding supports the hypothesis about decreased seed viability in this steno-endemic species which, in consequence, might negatively affect its overall generative reproduction. Indeed, the proved decrease in seed viability in *D. arbuscula* might represent one of the factors previously hypothesized to be responsible for the restricted distribution and rarity of the species (see Erdelská and Turis 1995, 1996).

Significant differences between the viability of seeds of big- and small-fruit morphotypes in both species were, however, apparent at first sight (see the subsection Results – Viability tests). The seeds extracted from the big-fruit morphotype reached high levels of viability, indicating no substantial problems with embryo development and the formation of viable seeds. By contrast, seeds from the-small-fruit morphotype showed significantly lower viability. Although the viability of seeds from the small-fruit morphotype exceeded in both species a 50% threshold, their formation and dispersal are likely to be suboptimal for the generative reproduction of these species.

The precise reason for the decreased seed viability of the small-fruit morphotype is left unresolved and calls for further investigation. Indeed, the significantly lower viability of seeds from the small-fruit morphotype could point towards an earlier ontogenetic stage of the fruits. However, even if both fruit morphotypes represent only different ontogenetic stages of the fruit ripening process, we can only speculate which specific intrinsic or extrinsic factors or their combination are responsible for the early dispersal of at least partially unripe fruits. By contrast, the fact that both morphotypes are dispersed at the same time could rather support the existence of actual fruit polymorphism. If this is the case, one would have to ask about its origin and functionality. The thickness and structure of the mesocarp have an impact on water absorption, gas exchange or protection against pathogens and on the germination ability of seeds (Mohamed-Yasseen et al. 1994; Imbert 2002). In both species, the formation of fruits with large and thick mesocarps might be a strategy to protect seeds against desiccation at their localities during the relatively dry and hot summer period. The formation of a fleshy mesocarp, restricted to only some fruits, might also be associated with a somehow restricted availability of certain resources, specifically water. Indeed, a similar type of fruit dimorphism was detected in *Thymelaea velutina* (Pourr. ex Cambess.) Endl. (Thymelaeaceae). This species, which is endemic to the Balearic Islands, is known for its fruit heterocarpy. It forms two types of drupes: a 'dry' form with a thin mesocarp and a yellow form with thick, fleshy fruits (Tebar and Llorens 1993; de la Bandera and Travaset 2006). The morpho-anatomical difference of the mesocarp might be a response to different ecological conditions, especially water availability and/or temperature. A third possible explanation is that fleshy mesocarps might serve to attract insects or small vertebrates and support seed dispersal via endozoochory (e.g. Halda 2001; de la Bandera and Travaset 2006). The presence of fleshy tissues in fruits might not only be a mechanism protecting seeds or attracting dispersal vectors, but it can also have a direct function in controlling the seed germination process. Indeed, fleshy tissues that contain inhibitors which hamper seed germination have repeatedly been evidenced in the genus *Daphne* (Mathew 1989; Zhang and Smagula 2000; Filipovič 2011). It was found that the germination ability of uncleaned seeds of *D. arbuscula* including also mesocarp tissue decreased sharply to 24% whereas the germination of cleaned seeds without mesocarp tissue reached 66% (Filipovič 2011). Furthermore, Zhang and Smagula (2000) reported for *D. mezereum* L. that a colour variation of its fleshy fruits (green, red and brown) was associated with the maturation stage and germination ability. All colour-associated fruit morphotypes encompassed mature and viable seeds, but the germination of seeds from red drupes was significantly greater than that of seeds from brown ones. This mechanism might facilitate the prolongation of seed germination and its spread along a larger period of time, thus assuring the successful germination and establishment of seedlings at least for a certain proportion of its offspring.

Based on our results, we think that further comprehensive experiments, including a detailed observation of all ontogenetic stages of fruit development and morpho-anatomical analyses of the mesocarp, would help decipher whether the observed fruit morphotypes represent real fruit polymorphism or simply various stages of the ripening process. Ultimately, however, it is necessary to emphasize that, irrespective of which of the discussed hypotheses about the origin of fruit polymorphism is correct, fruits of both morphotypes are dispersed and are at least partially viable and thus play an essential role in the sexual reproduction of the species. Knowledge about their viability and germination ability is therefore crucial

for the long-term survival of the species and future conservation activities. In consequence, because the seeds of small-fruit morphotypes get dispersed and have at least some level of viability, they should not be excluded from conservation actions such as seed collecting, storage or re-introduction programmes.

Seed germination in *Daphne arbuscula* and *D. cneorum*: the cold treatment breaks down physiological dormancy most effectively

The overall germination ability of the endemic *D. arbuscula* was generally low, irrespective of the temperature regime or the use of GA3. The greatest seed germination percentage (63%) was achieved only after 13 weeks of warm stratification at 15°C, followed by 28 weeks of cold stratification at 0°C, followed by incubation at 15°C, indicating the presence of deep physiological dormancy. In our study, even if a control test at 15°C without cold stratification was not performed, in the initial step of this test, 13 weeks of warm stratification at 15°C, only 3% of the seeds germinated. This confirms that the seeds of *D. arbuscula* are physiologically dormant and require stratification to germinate. Indeed, this finding is also in good accordance with previous *in situ* experiments performed in an alpine garden or directly in natural sites of *D. arbuscula*, which revealed meagre (3–10%) or no germination of the seeds used, respectively (Erdelská and Turis 1995, 1996).

Seeds of the genus *Daphne* are known to possess PD and to be challenging to germinate (Baskin and Baskin 2014; Zhang and Smagula 2000). PD is often overcome in the laboratory by a period of warm and/or cold stratification, or by the addition of chemical stimulants such as gibberellic acid (Baskin and Baskin 2014; Davies et al. 2015b; Kildisheva et al. 2020). Cold stratification was shown to be efficient at increasing germination in *D. mezereum* up to 82% (Zhang and Smagula 2000) or even up to 86.5% in the Chinese endemic *D. giraldii* Nitsche (Fang et al. 2016). The germination tests with various *Daphne* species performed *in vivo* revealed a distinctly low ability of seeds to germinate. Šedivá and Žlebčík (2010) analysed the seed germination of *D. cneorum* during a nine-year study in which the annual seed germination oscillated between 18 and 63%. Germination experiments on other species, conducted in a greenhouse, revealed 33% germination in *D. gnidioides* Jaub. & Spach but, unexpectedly, no germination at all in the cases of *D. oleoides* Schreb. and *D. sericea* Vahl (Ari et al. 2014), indicating strong PD in those species. Likewise, various members of closely related genera of the family Thymelaeaceae were also found to have significantly low seed germination ability. Seed germination of *Thymelaea velutina*, *T. hirsuta* (L.) Endl. and *Pimelea arenaria* A. Cunn. in *in situ* experiments were meagre, reaching in most cases only low percentages (Minuto et al. 2004; Dawson et al. 2005; de La Bandera and Travaset 2006).

The germination ability of seeds belonging to the big-fruit morphotype of *D. arbuscula* differed between the constant vs alternating temperature regimes, with a strong preference for the former. Indeed, seed viability estimated at the time of the cut test after the seeds were exposed to alternating temperatures was significantly lower than when seeds were exposed to constant temperatures, showing a negative impact of either high temperature such as 25°C or diurnal differences at a temperature of 20°C. Our data are not exhaustive enough to clarify whether the low success of the tested alternating temperature was caused by a negative impact of the higher temperature of 25°C or by an excessive daily difference of 20°C. Such temperatures might occur at the collecting site (Šiance) during the vegetation period, but the microclimate of the precise areas where the seeds lay between seed dispersal and germination might differ notably. It is known that soil temperatures do not reach the same temperature extremes registered in the air, therefore having a narrower amplitude between day and night and between seasons. According to Fernández-Pascual et al. (2015), who studied soil temperatures at three sites in Slovakia, during the day the soil temperature is not always significantly lower than the air temperature whereas during the night soil temperatures remain between 4 and 8°C higher than the air temperatures, especially when air temperatures drop below 0°C. The seeds of *D. arbuscula* are dispersed to niches surrounding mother plants that are mostly formed by limestone crevices or cushions and tussocks of saxicolous vegetation. Such specific microhabitats might also buffer temperature conditions, but it is not clear whether this would be comparable to the 'normal' soil studied by Fernández-Pascual et al. (2015). Nevertheless, taken these facts into account, we might suspect that, in this case, the factor which affected seed germination and viability the most are greatly alternating temperatures, rather than maximum diurnal temperatures.

Between the tests with constant temperatures (15°C), the use of 0°C for cold stratification led to greater germination than the use of 5°C. This difference in

germination success was, however, not significant statistically. In the majority of germination tests carried out in the laboratory, cold stratification using the temperature of 5°C is applied (Baskin and Baskin 2014; Ellis et al. 1985). In common practice, temperatures below 10°C, similar to the climate of origin (Kildisheva et al. 2020), are used in germination experiments of this type. Both 0°C and 5°C are likely to be the actual soil temperatures at the collection site. In fact, despite the climate data showing minimum winter temperatures far below 0°C, it is unlikely that soil temperatures reach such extreme lows (Fernández-Pascual et al. 2015). Zhang and Smagula (2000) reported the use of 4.44°C for *D. mezereum*, while 0°C is suggested for *D. papyracea* Wall. ex Steud. (Baskin and Baskin 2014). An important practical consequence of our experiments is the choice of the most relevant cold stratification temperature. Specifically, we showed that after the 24 weeks cold stratification, seeds germinated well if the temperature of 5°C was used, but no germination occurred in cases where 0°C was used.

With this experiment it is not possible to verify the need for warm stratification prior to cold stratification. However, the high percentage of mouldy/dead seeds found at the time of the final cut test in the long germination tests (57 to 63% viability), compared with the short germination tests using constant temperatures (78 to 91% viability), indicates that the conditions in the long test had led to the death of many seeds. We suspect that the longer stratification could be responsible for the ageing of the seeds, similarly to what Zhang and Smagula (2000) found for *D. mezereum*, for which 1 month of cold stratification was more successful than longer periods.

Interestingly, seeds of the two species studied differed in their response to GA3 treatment and its influence on dormancy. Results in *D. arbuscula* were in line with standard germination tests; that is, the germination success of the big-fruit morphotype after GA3 treatment was greater than of the small-fruit morphotype. On the other hand, the germination level of *D. arbuscula* seeds was not significantly increased after GA3 treatment, as previously indicated by Filipovič (2011). Completely opposite results were obtained in *D. cneorum*, where the small-fruit morphotype seeds germinated significantly better than those of big-fruit morphotype after exposure to GA3.

One plausible explanation for this unexpected finding could be that physiological dormancy increased during the seed ripening process, which is a common phenomenon in seed development (Baskin and Baskin 2014). A decrease in germination in correlation with the level of maturation was evidenced in *D. mezereum* by Zhang and Smagula (2000). Thus, it is tempting to speculate about potential advantages of the strategy of *D. cneorum* and *D. mezereum* to leave parts of the fruit not fully ripe at dispersal in order to differentiate even more the levels of dormancy of their offspring. In that way, germination would be spread from the immediate summer after dispersal to up to two or more following springs, consequently minimizing the likelihood of the death of all seedlings and helping establish at least a proportion of them. On the other hand, the absence of this mechanism in *D. arbuscula* could be related to its limited area of distribution and its rarity. It is to be noted that the difference in the level of dormancy between *D. arbuscula* and *D. cneorum*, shown by the germination tests with the use of GA3, was not due to the small-fruit morphotype, but it was entirely attributed to the big-fruit morphotype, which in *D. arbuscula* showed a significantly lower level of dormancy.

Practical implications for seed conservation and re-introduction

None of the germination tests achieved the germination percentage of 85% required by international seed banking standards (FAO 2013) and further investigation is needed. In particular, multiple cycles of exposure to cold-warm temperatures should be tested to increase germination percentages, as suggested by Zhang and Smagula (2000) for *D. mezereum* and by Alonso and Herrera (2001) for *D. laureola* L. In fact, spreading the seed germination across several consecutive years is a known survival strategy for plant species occurring in areas where winters are extremely cold and chances of losing their offspring in the first year are high (Kildisheva et al. 2020).

We recommend including both fruit morphotypes when collecting seed of *Daphne* for *ex situ* conservation and re-introduction initiatives. This enables to maintain the original genetic diversity of the species and secure their future survival. Knowing about the variability in fruit morphology and associated viability of the seeds of the small-fruit morphotype informs seed bank managers and seed conservation practitioners and determines best practice for seed collection and subsequent seed processing of *D. arbuscula* and *D. cneorum*.

Concerns arise in relation to seed banking potentially immature seeds, as these could not have developed full desiccation tolerance or full longevity (Way and Gold 2014). Reassuringly, we found seeds of small green/brown fruits to be tolerant of desiccation, as the seeds used here were dried at 15% equilibrium relative humidity at 15°C and kept at these conditions for several months prior to testing. However, as not all seeds were viable after storage, testing the viability of freshly collected seeds would be needed to exclude potential negative effects of drying on part of the seeds. Furthermore, the effect of freezing at −20°C following international standards for conventional seed banking (Breman and Way 2018) was not tested, and further investigation is needed to assess the orthodox behaviour of these seeds and their longevity *ex situ*.

When performing tetrazolium tests on seeds of *Daphne,* we recommend including seeds coloured mostly in red but with slight patches of pink as viable.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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