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Germination niche of the permanent wetland specialist, *Parnassia grandifolia* DC

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Abstract

Temperate wetland species often require light and warm temperatures for seed germination. However, recent studies indicate that species which specialize on permanently saturated wetlands that are maintained by groundwater discharge (fens, seeps and mountain springs), rather than wetlands with surface-water-driven hydrologic regimes, diverge from the typical wetland germination niche by germinating at cool temperatures and lacking photoblastic seeds. We conducted laboratory experiments that manipulated stratification conditions (non-stratified versus cold stratification in light and darkness), thermal regime (15/6, 25/15 and 35/20°C), and light (14 h photoperiod versus continuous darkness) to test whether seeds of the North American calcareous fen specialist *Parnassia grandifolia* diverged from the typical temperate wetland germination niche. After 30 d, fresh seeds were conditionally dormant and could only germinate to high percentages in light at 25/15°C. During 16 weeks of incubation, non-stratified seeds germinated to low percentages ($<40\%$) at all thermal regimes in darkness. In contrast, cold-stratified seeds germinated to high percentages in both light and darkness at all thermal regimes, although germination was incomplete (no cotyledon emergence) at 35/20°C. Further, seeds did not require light during cold stratification to germinate to high percentages when incubated in light or darkness. Thus, seeds diverged from the typical temperate wetland germination syndrome in lacking a light and warm temperature requirement for germination. Our results reinforce previous work from European fens and Mediterranean wetlands. This indicates that multiple germination strategies are found in fen wetlands that are maintained by the continuous or near-continuous discharge of cool groundwater.

Keywords: cold stratification, dark germination, dormancy, *Parnassia*, wetland

Introduction

In temperate wetland species, dormancy-break and germination timing are often linked to seasonal hydrologic regimes and the post-flooding drawdown environment (Grime *et al.*, 1981; Casanova and Brock, 2000). Seeds of most temperate wetland species are dispersed with physiological dormancy, require cold stratification for dormancy-break and need warm temperatures to cue germination (Baskin and Baskin, 2001). Indeed, most temperate wetland species cannot germinate at low temperatures ($<15°C$), which prevents seeds from germinating in early spring when seedlings have a lower probability of establishing due to flooding and anoxic conditions (Grime *et al.*, 1981; Baskin and Baskin, 2001). Light and increases in the amplitude of diurnal temperature fluctuations can also serve as important germination cues in temperate wetlands, allowing seeds to sense burial depth and the appropriate time to germinate in the drawdown environment (Thompson and Grime, 1983; Leck, 1996). For example, compared to sedges from dry habitats, wetland sedges (*Carex*) tend to produce dormant seeds that are more responsive to fluctuating temperatures, which allows them to detect vegetation gaps and burial depth (Schütz, 2000). Although dark germination can occur if seeds are near or at the soil surface where they can perceive high temperature fluctuations (Kettenring and Galatowitsch, 2007), most temperate wetland species maintain a persistent seed bank and have photoblastic seeds, or germinate to greater percentages in light than in continuous darkness (Baskin and Baskin, 2001; Kettenring *et al.*, 2006).

A majority of studies that report the classical wetland germination niche (warm temperatures and light) concern species that occupy surface-water-driven wetlands with distinct seasonal flooding

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regimes, including mudflats (Ignacio Galinato and Van Der Valk, 1986; Baskin *et al.*, 1993), marshlands (Grime *et al.*, 1981) and prairie wetlands (Seabloom *et al.*, 1998; Kettenring and Galatowitsch, 2007). Yet, much less is known about the germination syndromes of temperate wetland species with permanently or near-permanently saturated soils and less pronounced seasonal flooding, such as temperate fens, which are a type of wetland maintained by the continuous to near-continuous discharge of mineral-rich groundwater in the plant rooting zone and characterized by low nutrient availability (Bedford and Godwin, 2003; Hájek *et al.*, 2006). Unlike surface-water-driven wetlands, the soils of which are subjected to temperature and moisture variation due to seasonal flooding and drought, the near-continuous discharge of cool groundwater in fens maintains permanently or near-permanently saturated conditions and reduces diurnal and seasonal temperature fluctuations (Bedford and Godwin, 2003). Thus, environmental cues associated with the drawdown environment (i.e. warm temperatures and light) may be less important in shaping the germination niche of fen specialists. For example, in montane base-rich fens, seeds of some species germinated to greater percentages in cool than in warm temperatures, while germination in some species was inhibited at very warm temperatures (Fernández-Pascual *et al.*, 2012, 2013). In semi-natural fen grasslands of Europe, many fen specialists similarly germinated to high percentages at cool (<15°C) temperatures; however, most species also maintained wetland germination features, such as germinating to greater rates in light than darkness and forming persistent seed banks (Patzelt *et al.*, 2001; Jensen, 2004). In ephemeral wetlands and riparian systems of Mediterranean climates, germination in some species is cued by cool temperatures whereas other species require flooding (Carta *et al.*, 2013; Porceddu *et al.*, 2013).

In this study, we ask whether the germination niche of *Parnassia grandifolia*, an obligate wetland species that is endemic to the south-eastern United States (SE USA) (Lichvar, 2013; NatureServe, 2013), is similar to or divergent from the classical wetland germination niche. Although little is known about germination syndromes of fen specialists in the SE USA, two previous studies suggest that germination characteristics differ among species. In *Xyris tennesseensis*, which is restricted to calcareous fens and co-occurs with *P. grandifolia*, germination characteristics were consistent with the wetland germination niche: a light requirement for germination, inability to germinate at cool thermal regimes (15/6°C), and formation of a short-term persistent seed bank (Baskin and Baskin, 2003). In contrast, *Parnassia asarifolia*, which is also restricted to partially shaded to open fens in the SE USA but grows in more acidic soils and at higher elevations than *P. grandifolia*, disperses conditionally dormant seeds that, after cold stratification, germinate to high percentages at both cool and warm temperatures in either light or darkness (Farmer, 1980). Here, we conducted a factorial laboratory experiment in which temperature and light availability were manipulated in order to distinguish the more typical wetland germination pattern from the cool germination syndrome recently reported in European fen habitats (Fernández-Pascual *et al.*, 2012, 2013). Specifically, we test the hypothesis that the germination niche of *P. grandifolia* diverges from the 'classical' wetland germination niche in that seeds germinate to similar or greater proportions in cool than warm thermal regimes, and germination rates are similar in light and darkness. Due to phylogenetic relatedness, we expect *P. grandifolia*'s germination niche to be more similar to that of *P. asarifolia* than that of *X. tennesseensis*. However, because dormancy and germination characteristics can differ among closely related species from similar climates and habitats (Van Assche *et al.*, 2002; Karlsson and Milberg, 2007), we also expect subtle differences in germination features between *P. grandifolia* and *P. asarifolia.*

Materials and methods

P. grandifolia (Celastraceae, formerly Saxifragaceae), or 'largeleaf grass of Parnassus', is an herbaceous perennial endemic to the south-eastern United States. *P. grandifolia* is considered to be at a moderate risk of range-wide extinction, with a high or very high risk of extinction in 11 of the 15 states in which it occurs (NatureServe, 2013). Habitat for *P. grandifolia* is restricted primarily to open areas in calcareous fens and fen-like seeps with thin soils, and on rocky ledges and openings along springs and headwater streams (NatureServe, 2013). Flowering occurs from August through October, followed by the formation of small capsules producing copious quantities of small (0.16 mg), light brown seeds. To our knowledge, no prior studies have reported the germination phenology of the species.

Seeds for all experiments were bulk collected on 11 November 2011 from ~50 *P. grandifolia* plants whose fruits had ripened and were beginning to dehisce. Plants were growing in a calcareous seepage fen located on wooded slopes adjacent to a headwater stream in the Western Highland Rim of Lewis County, Tennessee. In addition to *X. tennesseensis*, other common associates include *Amphicarpaea bracteata*, *Rudbeckia fulgida* var. *umbrosa* and *Carex* spp. After collection, bulk material was cleaned by hand to remove chaff; seeds were maintained at ambient room conditions of approximately 20°C and 40% relative humidity until germination experiments commenced on 1 December 2011.

Experimental design

The germination experiment was conducted in four temperature- and light-controlled germination incubators (Model I-36VL, Percival Scientific, Inc., Perry, Iowa, USA). All treatment combinations consisted of five Petri dishes each containing 20 seeds placed on top of Quickrete® white quartz sand. Seeds were kept constantly imbibed during the duration of the experiment with distilled water. For dark treatments, all Petri dishes were wrapped in two layers of aluminium foil and maintained in continuous darkness for the duration of each experimental treatment, at which point they were checked for germination. In the light treatments ($\sim 35\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ at seed level), dishes were checked weekly and all germinates were removed; radicle emergence was the criterion for germination. Seeds were placed in germination incubators set at a 14/10 h light/dark daily cycle with alternating thermal regimes of 15/6°C, 25/15°C or 35/20°C. Thermal and light regimes were chosen because they: (1) allow for direct comparison with the results of germination experiments conducted with the co-occurring *X. tennesseensis* from the same habitat and with the closely related *P. asarifolia*; and (2) simulate the average maximum and minimum temperatures in spring, autumn and summer in Lewis County, Tennessee (Baskin and Baskin, 2003).

To test the effects of cold stratification on germination, we used a 3 × 3 × 2 factorial design consisting of three stratification treatments (non-stratified, cold stratified in light and cold stratified in darkness), three incubation thermal regimes (15/6°C, 25/15°C and 35/20°C), and two incubation light regimes (14/10 h light/dark photoperiod and continuous darkness). Seeds were cold stratified (5/5°C) in light (14/10 h light/dark photoperiod, $n = 30$ Petri dishes) or continuous darkness ($n = 30$ Petri dishes). After 12 weeks, five dishes each were then transferred to each of the six treatment combinations (3 thermal × 2 light regimes), where they were incubated for 4 weeks. Non-stratified seeds were held in light or continuous darkness for 16 weeks at each of the three thermal regimes. At the end of the experiment, non-germinated seeds were scored as non-viable if they collapsed when pinched with forceps (Baskin and Baskin, 2001). For each treatment combination, we calculated the cumulative germination percentage as the ratio of the total number of seeds germinated by the number of seeds that were started in each dish ($n = 20$) and the cumulative non-viable percentage as the total number of seeds that rotted during incubation.

Statistical analyses

Generalized Linear Models (GLM) with a binomial distribution, or a quasibinomial distribution to control for overdispersion when necessary, were used to examine differences in final germination percentages among treatments. In the first GLM, the effects of stratification light regime, incubation thermal and light regime were tested using a three-way GLM. In the second GLM, we examined the effect of pretreatment (non-stratified versus cold stratified in the dark) incubation thermal and light regime on final germination percentages. In this analysis, we only considered seeds that were cold stratified in the dark since there were no significant differences in germination percentages among stratification light regimes (see Results section). We used the model simplification procedures outlined in Crawley (2007) by starting with the maximal model (all main effects and interactions) and only retaining variables that caused a significant increase in deviance when removed from the model. For the final model, differences among treatments were compared using pairwise least-squares means with Tukey adjusted P values.

We quantified relative differences in germination rates of non-stratified seeds over time by fitting germination progress curves using a three-parameter log-logistic event-time model (Ritz *et al.*, 2013). Semi-parametric event-time models are preferred over linear regression and non-linear regression models because germination data exhibit strong temporal autocorrelation and are highly variable over time, which violates the homogeneity of variance and data independence assumptions implicit in the latter approach (Ritz *et al.*, 2013). We tested whether the slopes of germination progress curves and t_{50} (number of weeks to reach 50% of cumulative germination) differed among thermal regimes for non-stratified seeds (Ritz *et al.*, 2013). This analysis only included seeds that were incubated in light; curves could not be fitted for seeds incubated in dark because they were not checked for germinates until the end of the incubation period. Similarly, progress curves fitted to stratified seeds using time to event analysis did not converge, because most cold-stratified seeds germinated in the first or second week of incubation, with little or no germination thereafter. All analyses were conducted in R version 3.0.0 (R Development Core Team, 2013), using the drc (Ritz and Streibig, 2005) and lsmeans (Lenth, 2013) packages.

Results

Stratification light regime did not significantly affect final germination percentages of *P. grandifolia* (main effect: $P = 0.27$; two- and three-way interactions with incubation thermal and light regimes: all P values > 0.07; Table 1). Further, seeds did not require light during cold stratification to attain high germination percentages at any combination of incubation thermal regime and photoperiod. For seeds that were cold stratified in darkness and then incubated in darkness, germination percentages were $> 90\%$ at all thermal regimes (Table 1). No seeds germinated in light or darkness during cold stratification.

The effects of thermal regime and light on germination percentages depended upon whether seeds were cold stratified or not (three-way interaction, $P < 0.0001$). Overall, cold stratification significantly enhanced germination relative to non-stratified seeds in four of the six incubation treatments (Fig. 1). In pairwise comparisons, cold-stratified seeds germinated to significantly greater percentages than non-stratified seeds in light at the warm and hot thermal regimes (Fig. 1), while in darkness cold-stratified seeds germinated to significantly greater percentages than non-stratified seeds at all thermal regimes (Fig. 1).

Germination of fresh (non-stratified) seeds exposed to light began after 2 weeks at 25/15°C, but was substantially more delayed at the cool (15/6°C) and the hot (35/20°C) thermal regimes (Fig. 2). While the cool thermal regime ultimately yielded the highest cumulative germination, t_{50} was significantly delayed relative to the warm and hot thermal regimes (15/6°C < 25/15°C: $P < 0.001$; 15/6°C < 35/20°C: $P = 0.01$; Fig. 2). In contrast, there was no difference in t_{50} among 25/15°C and 35/20°C thermal regimes ($P = 0.34$), although final germination percentages were markedly greater in the 25/15°C thermal regime (Fig. 1). Overall, the lowest germination percentages occurred at the hot thermal regime, due to loss of seed viability; after several weeks of incubation, seeds turned watery and dark brown, and collapsed when pinched with forceps. The percentage of non-stratified seeds that lost viability when incubated in light was 92 ± 4% (mean ± SE) at 35/20°C, 9 ± 3% at 25/15°C and 3 ± 2% at 15/6°C. For non-stratified seeds incubated in darkness, the percentage of non-viable seeds after 16 weeks was $< 5\%$ in all thermal regimes. All non-germinated seeds in the cold stratification treatment were viable at the end of the experiment. Although germination percentages in light and darkness were much greater at the hot thermal regime after cold stratification (Fig.1, Table 1), only the radicles on seeds emerged at 35/20°C in darkness; conversely, radicles and shoots emerged simultaneously across all other treatment by incubation conditions.

Table 1. The effects of light regimes during stratification and incubation on germination percentages (mean ± SE) of cold-stratified *Parnassia grandifolia* seeds in three alternating temperatures. Stratification light regime did not significantly ($P = 0.27$) affect final germination percentages

Light regime		Incubation temperature (°C)		
Stratification	Incubation	15/6	25/15	35/20
Light	Light	97 ± 3	95 ± 2	63 ± 15
Light	Dark	92 ± 3	95 ± 2	86 ± 3
Dark	Light	71 ± 4	96 ± 2	55 ± 16
Dark	Dark	94 ± 3	96 ± 2	94 ± 5

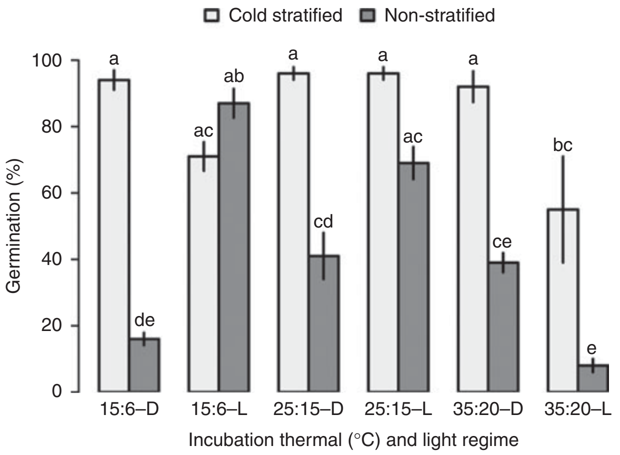

Figure 1. Effects of cold stratification in darkness on germination percentages (mean ± SE) of *Parnassia grandifolia* seeds in six different combinations of thermal and light regimes. L, 14/10 h light/dark photoperiod; D, continuous darkness. Different letters indicate significant ($P < 0.05$) differences among treatments based on pairwise least-squares means with Tukey adjusted P values.

Discussion

Seed germination characteristics in *P. grandifolia* diverged from those reported for other herbaceous perennials of temperate zone wetlands in several ways. First, the minimum and maximum temperature ranges at which *P. grandifolia* seeds can germinate differed from other co-occurring fen specialists. Like *P. asarifolia*, non-dormant seeds of *P. grandifolia* can germinate in cooler (15/6°C) thermal regimes than *X. tennessensis*, and well below the reported average temperature optimum (24°C) for seed germination in wetland species (Baskin and Baskin, 2001). However, the upper temperature threshold for germination of *P. grandifolia* seeds was much lower than expected for a species of wet, open habitats. At the warmest thermal regime (35/20°C), *P. grandifolia* seeds lost viability in light and those maintained in continuous darkness survived but germination was incomplete (i.e. no hypocotyls emerged). This contrasts with the rapid

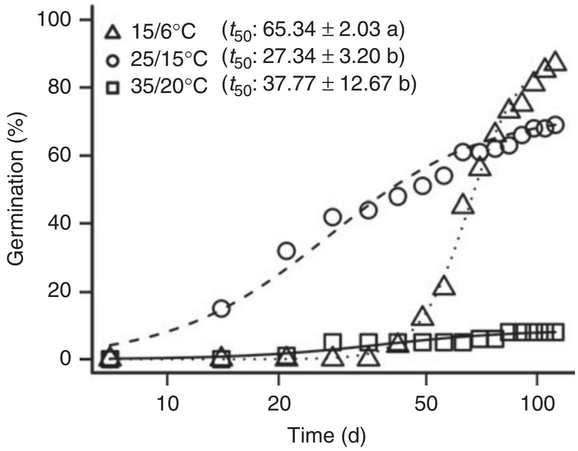

Figure 2. Germination progress curves of non-stratified *Parnassia grandifolia* seeds in different thermal regimes (14 h daily photoperiod). Curves were fitted using a three-parameter log–logistic function. Data are means for five replicates in each thermal regime and t_{50} values (mean ± SE, days) for each thermal regime are in parentheses. Values followed by different letters are significantly different based on *t*-tests.

and high germination observed with *P. asarifolia* seeds in light and darkness at 29/24°C (Farmer, 1980), but aligns with previous reports that seed temperature responses can differ among closely related species in wetland habitats (Brändel, 2006; Kettenring and Galatowitsch, 2007).

Another divergence from the wetland germination niche was the germination patterns of *P. grandifolia* seeds in light and darkness. Although non-stratified *P. grandifolia* seeds germinated to greater percentages in light than in darkness at two of the three thermal regimes, cold-stratified seeds germinated to equal or greater percentages in darkness than in light. In contrast, seeds of the co-occurring *X. tenneseensis* and most other temperate wetland species, including those with germination optimums at cool temperatures (Fernández-Pascual *et al.*, 2012), are either photoblastic or germinate to greater percentages in light than darkness. Further, *P. grandifolia* seeds do not need to first experience light during cold stratification to germinate in darkness, which is unexpected given that in fens dark germination is usually confined to larger-seeded species with transient soil seed banks, and small-seeded species usually require light before, during or after dormancy release to germinate (Jensen, 2004). Alternating temperatures can override a light requirement for germination in temperate wetland species (Thompson and Grime, 1983), although we did not examine *P. grandifolia* germination in light and darkness at constant temperatures to test this hypothesis. Nevertheless, our results suggest that *P. grandifolia* seeds that become shallowly buried following dispersal can probably germinate when alternating diurnal temperatures become favourable in early spring, and are unlikely to carry-over in the soil seed bank.

While *P. grandifolia* seeds do not appear to perceive signals associated with drawdown environments and vegetation gaps like other temperate wetland species, they do require cold stratification to break conditional dormancy. After 16 weeks, germination percentages of non-stratified *P. grandifolia* seeds remained low (<50%) at all incubation temperatures in darkness, whereas germination percentages ranged from 10–80% in light. In contrast, seeds that were cold stratified germinated to high percentages (>75%) in light and darkness in all three incubation temperatures. Some fresh seeds can germinate at intermediate temperatures and cold stratification broadens the low and high temperature range at which seeds can germinate, which indicates that seeds of *P. grandifolia* have type 3 non-deep physiological dormancy (Baskin and Baskin, 2004).

At the time of dispersal in late autumn, *P. grandifolia* produces conditionally dormant seeds that can only germinate immediately at thermal regimes (25/15°C) that exceed those in the natural habitat (Baskin and Baskin, 2003). Fresh *P. grandifolia* seeds can also germinate to high percentages at 15/6°C, indicating the progressive loss of dormancy and lowering of the base temperature for germination in the cool thermal regime. However, slow dormancy loss and delayed germination at cool temperatures probably reduces the risk of germinating shortly after dispersal. In nature, temperatures are too cold (≤10°C) for seeds of *P. grandifolia* to germinate if dormancy was broken. Thus, our laboratory experiments predict that after dispersal, *P. grandifolia* seeds would quickly imbibe in the near-permanently saturated fen soils, lose dormancy over winter, and germinate from late winter to early spring.

Whether the dormancy and germination traits observed in *P. grandifolia* are ancestral to the genus or have evolved for specializing on calcareous fens and seeps remains unclear. According to Gu and Hultgård (2001), approximately 70 species of *Parnassia* are recognized and most are restricted to moist, open habitats, including calcareous shores, wet meadows and bogs. In addition to *P. asarifolia*, the only other information on seed dormancy and germination in *Parnassia* is for the most geographically widespread member of the genus, *P. palustris*, which is circumpolar in distribution. Cold-stratified seeds of *P. palustris* from populations in montane, base-rich fens (Fernández-Pascual *et al.*, 2013) and dune slacks of north-western Europe (Schat, 1983) also exhibited conditional seed dormancy and germinated over a broader range of temperatures than non-stratified seed. However, the germination niche of *P. grandifolia* differed from those of *P. palustris* and *P. asarifolia* in that seeds of the latter two species did not germinate incompletely or lose viability at very warm temperatures. Further, the response of *P. palustris* seeds to light and darkness varies across geographical regions and experimental conditions (Bliss, 1958; Maas, 1989; Jensen, 2004), making it difficult to determine whether high germination in the dark is unique to *Parnassia* in the SE USA or prevalent in other habitats and geographical regions. To elucidate the phylogenetic origin of seed dormancy and germination traits in *Parnassia*, future germination studies are needed with other species in the SE USA, China and the Himalayas, which are the primary centres of diversification and endemism in the genus (Gu and Hultgård, 2001).

In conclusion, our study of a fen specialist in the SE USA supports recent studies suggesting that a wide range of germination traits can be found in specialists of temperate fen wetlands. Compared to most other previously studied fens, which occur primarily in an open, grassland matrix, calcareous fens and seeps in the SE US usually occur as small openings on steep forested hillsides and alongside small streams. Interestingly, the germination characteristics of *P. grandifolia* (and *P. asarifolia*) are more similar to those of herbaceous perennials in the surrounding woodlands (i.e. cold stratification requirement, germination at low temperatures and in darkness) (Baskin and Baskin, 2001; Albrecht and McCarthy, 2011), rather than the more typical wetland germination niche described for species of prairie wetlands, fen grasslands and more open aquatic habitats. Schütz (1997) similarly found that woodland sedges (*Carex*) had cooler germination temperature thresholds than sedges from open wetlands, suggesting that germination characteristics in wetland plants are contingent on habitat-specific differences. Given that flooding and drawdown environments may be less important in shaping germination niches in SE USA fens, we hypothesize that fen and fen-like habitats in a forested matrix contain a combination of species that utilize different germination windows in time; seeds of some species germinate in the brief period between late winter and early spring, just prior to canopy leaf-out, whereas others (e.g. *X. tennesseensis*) germinate in gaps following disturbance (e.g. litter removal and reduction in competition) in late spring and early summer. In ephemeral wetlands of Mediterranean climates (Carta *et al.*, 2013), and in European fens and mountain springs (Fernández-Pascual *et al.*, 2013), divergent germination strategies and seed temperature responses were similarly reported. Future germination studies with a greater number of fen specialists are needed to better understand the range of dormancy and germination traits that occur in fens with different ecological and climatic contexts.

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Conflicts of interest

None.

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