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What are the consequences of water stress on the development of late postzygotic reproductive isolation in an orchid hybrid zone?

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[Correction added on 18 September 2025, after first online publication: The keywords 'ecophysiologyhybridization' and 'leaf anatomyOrchidaceae' have been

Abstract

Studies on reproductive isolation in angiosperms often focus on early postzygotic barriers, whereas research on established hybrid generations and their ecological interactions remains limited. This study aimed to compare the morphophysiological performance of *Epidendrum* × *purpureum* and its parental species, *Epidendrum orchidiflorum* and *Epidendrum denticulatum*, under different water-deficit conditions. To investigate the effects of water deficit on the parental species and the hybrids, we conducted an experiment comprising one control and two water-deficit treatments of different severity over time. We measured several ecophysiological and anatomical traits, including leaf water potential, leaf succulence, titratable acidity, and membrane resistance. We also compared growth and survival between parental species and hybrids to understand their performance under contrasting water conditions. The results showed hybrid plants had lower survival rates than parental species, with all plants dying by the end of the experiment. Membrane resistance was also lower in the hybrid plants, which led to increased electrolyte leakage and decreased the water storage potential. Cuticle width was significantly different between parental species and their hybrids. Significant changes in titratable acidity were observed, indicating alterations in the crassulacean acid metabolism. The greater vulnerability of the hybrids to water stress can be interpreted as a strong late postzygotic barrier that limits its distribution and survival. Such barriers may increase in climate change scenarios predicting the aridification in some regions, reducing the potential for heterospecific gene exchange between co-occurring species and influencing the occurrence and persistence of natural hybrid zones.

KEYWORDS

drought tolerance, ecophysiology, hybridization, leaf anatomy, Orchidaceae

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corrected to 'ecophysiology', 'hybridization', 'leaf anatomy', and 'Orchidaceae'.]

1 | INTRODUCTION

Hybridization is a key process in generating genetic diversity and facilitating gene flow between parental species (Goulet et al., 2017; Schley et al., 2022). Hybrids often exhibit heterosis, characterized by improved performance and unique phenotypic traits compared with their parent species (Hochholdinger & Baldauf, 2018). This enables hybrids to adapt and thrive successfully in different environments. Additionally, hybrid speciation can occur when hybrids form new populations that are reproductively isolated from their parent species, potentially leading to the emergence of new species (Vallejo-Marín & Hiscock, 2016). This process may be achieved particularly rapidly when hybrid speciation involves polyploidy, as hybrids with intermediate ploidy exhibit comparatively higher degree of postzygotic reproductive isolation (RI) from their progenitors (Ramsey & Schemske, 2002). While studies on RI in angiosperms often focus on early postzygotic barriers, research on established hybrid generations and their ecological interactions remains limited (Baack et al., 2015).

Once hybridization occurs, the fate of hybrids depends on their ability to overcome various barriers (Widmer et al., 2008). These barriers can be intrinsic, such as low viability and sterility, which are independent of environmental factors, or extrinsic, such as ecological selection (Baack et al., 2015). Hybrids may experience reduced fitness when they are unable to find a suitable ecological niche, even in the absence of inherent developmental issues (Coyne & Orr, 2004). For example, hybrids with intermediate phepnotypes may be selected against in the habitat of either parent, leading to ecological hybrid inviability (Toll & Willis, 2018). The specific factors driving this process remain unknown; however, the intermediate morphology of hybrids may make it challenging for them to successfully establish themselves in their parents' environments (Wang & Bradburd, 2014). Furthermore, the precise role of ecological barriers in impeding the establishment of recently formed hybrids has not been extensively investigated, especially in the Neotropical region (Schley et al., 2022). For instance, understanding how hybrids respond to extreme conditions, such as drought, is crucial for comparing their adaptation abilities with those of the parental species (Johnston et al., 2001). Given that arid environments are more vulnerable to global warming effects (Huang et al., 2017; Li et al., 2018), the impacts of water stress on hybrid viability may be especially important for understanding how late RI stages could be influenced by climate change scenarios (Vallejo-Marín & Hiscock, 2016).

Different environments impose biotic and abiotic stresses that act as selective pressures, shaping local adaptations and influencing species' evolutionary histories (Urban et al., 2020). Water restriction, in particular, is a significant abiotic stress factor that affects plant growth, development, and diversity (VanWallendael et al., 2019). Plant species exhibit various strategies to cope with water limitation, such as drought tolerance, drought avoidance, and drought escape, which are reflected in their functional traits (Fang & Xiong, 2015; Violle et al., 2007). In this context, different functional characteristics, including morphological, physiological, biochemical, and structural traits, provide insights into the performance and fitness of species under water stress (Fang & Xiong, 2015). For instance, variations in plant morphological traits, such as leaf succulence, stomatal density, and cuticle width, indicate potential plant responses to water stress in arid environments (Fradera-Soler et al., 2021). In addition, physiological traits, such as changes in water potential, electrolyte content, acidity, and leaf succulence, serve as indicators of plant responses to water deficit (Gilman et al., 2024). These responses vary among species, reflecting their ability to withstand drought events (Sun et al., 2014). Despite the importance of functional traits in understanding plant responses to environmental variation, information on these traits remains limited for several plant groups, including diverse families such as Orchidaceae (Cornwell et al., 2019).Orchids are an ecologically diverse group exhibiting numerous adaptations to cope with water limitation (Zhang et al., 2018). About 10% of the species in this family employ crassulacean acid metabolism (CAM), which enables them to inhabit more arid environments and improve water-use efficiency (Niechayev et al., 2019). Other adaptations, such as succulence, water storage organs, a thick cuticle, and superficial roots, are also present in this group (Rodrigues et al., 2013). Although hybridization is a common phenomenon in orchids (Schley et al., 2022), the performance of hybrids relative to their parents under environmental stress has been rarely investigated (Zhang et al., 2018). Such a comparison could provide important information about the ecology and evolution of hybrid individuals in natural populations, since the persistence of the hybrid in the habitat is a crucial step in the process of speciation by hybridization, as observed in other plant groups (Johnston et al., 2001; Oliveira et al., 2020).

Several hybrid zones have been studied in *Epidendrum* (Schley et al., 2022), an orchid genus often found in

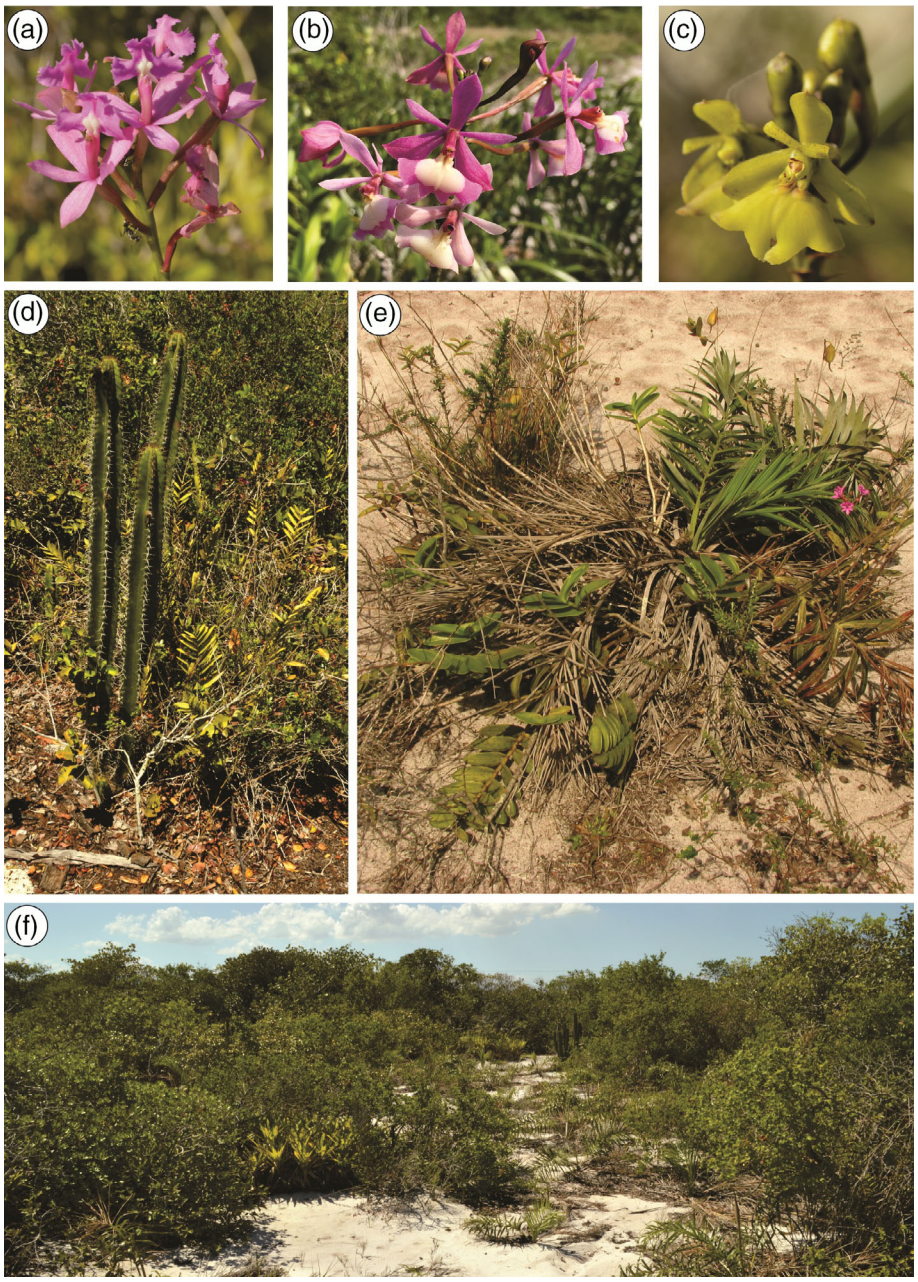


FIGURE 1 Flowers of *Epidendrum denticulatum* (a), *Epidendrum* × *purpureum* (b) and *Epidendrum orchidiflorum* (c) sampled at the hybrid zone located in the Restinga de Massambaba, Southest Brazil (d–f).

extreme environments such as rock outcrops and sand dunes (Pinheiro & Cozzolino, 2013). *Epidendrum* hybrid zones are found in these stressful environments, where hybrids often share the same habitat as parental species (Arida et al., 2021; Sujii et al., 2019). Abiotic stress tolerance has not yet been investigated in *Epidendrum* and its hybrids, preventing any conclusions about the role of late RI barriers driven by stressful conditions, such as water stress. Arida et al. (2021) investigated a hybrid zone between *Epidendrum denticulatum* Barb. Rodr. and *Epidendrum orchidiflorum* (Salzm.) Lindl., two species occurring in the dry sand dune vegetation along the Brazilian coast (Restinga de Massambaba, Rio de Janeiro State, Figure 1a,c). Although several RI barriers have been identified between the parental species and their hybrid, *E.* × *purpureum* Barb. Rodr. (Arida et al., 2021, Figure 1b), the effects of abiotic variables, such as water deficiency on hybrid viability and persistence remain unknown. This study aims to compare the morphophysiological performance of *E.* × *purpureum* with that of its parental species, *E. orchidiflorum* and *E. denticulatum*, under water-deficit conditions. We hypothesize that *E.* × *purpureum* will exhibit lower performance under water deficit than its parents, which could explain its distribution being limited to the Restinga de Massambaba (Figure 1e), the only known location where the hybrids currently occur.

2 | MATERIALS AND METHODS

2.1 | Area and species studied

The Massambaba Restinga is a coastal ecosystem located in the Massambaba Environmental Protection Area (APA), municipality of Arraial do Cabo, state of Rio de Janeiro (Supporting Information 1—Figure S1) (Araujo et al., 2009). The mean annual rainfall is c. 800 mm, with monthly precipitation usually less than 100 mm and minimum monthly precipitation of approximately 40 mm during the winter months, resulting in a soil water deficit throughout the year. The mean annual temperature is 25°C, and daily temperatures range from 12 to 36°C (Araujo et al., 2009).

Epidendrum orchidiflorum, *E. denticulatum*, and their natural hybrid, *E.* × *purpureum* are terrestrial herbs with succulent leaves, growing directly on sandy soil (Figure 1d). Reinert et al. (1997) identified an obligatory CAM for both parental species. Pre- and postzygotic barriers were studied by Arida et al. (2021), suggesting introgression may be rare between parental species. Despite the variable fertility of hybrid plants, which can attract pollinators during the day and at night (Arida et al., 2021), very few individuals were found in the Massambaba Restinga, suggesting that the presence of strong late postzygotic barriers likely decreases the viability of *E.* × *purpureum*.

2.2 | Sampling and water-deficit experiment setup

We sampled 15 individuals of each parental species and the hybrid species in the Massambaba Restinga. From each individual (genet), we collected three clones (ramets) with a height of 19–21 cm. We weighed all plants and planted the clones in plastic pots using an orchid substrate medium composed of equal parts of pine bark and charcoal (Mogifértil, São Paulo, SP, Brazil). The plants were allowed to acclimate for 2 months in a greenhouse, where the experiment took place. Before the experiment began, we excluded any plant that had not developed new roots and leaves during acclimation. We ended up with 135 plants arranged in a three-block design (Supporting Information 1—Figure S2). Each block contained 15 genets of each parental species and the hybrid, and all blocks contained ramets from the same genets. This design allowed us to conduct different water-deficit experiments using the same clones as replicates, thereby avoiding biases in the responses of functional traits across environmental gradients (Sun et al., 2014). In each block and for each species, 5 plants were used for nondestructive analysis and 10 plants for destructive analysis (Supporting Information 1—Figure S2). The experiment was conducted from June to December 2019 in the greenhouse of the Instituto de Biologia, Universidade Estadual de Campinas, in southeastern Brazil.

The experiment followed a completely randomized design, with one control and two water-deficit treatments per species: (a) Control—plants watered three times per week with 50 mL of water; (b) Moderate stress (MS)—plants watered approximately every 25 days with 50 mL of water (after 26, 54, 82 days of stress), and (c) Severe stress (SS)—plants not watered during the entire experimental period. To investigate the effects of water deficiency on the parental and hybrid species, the following traits were measured during the experiment: growth, leaf water potential (Ψ_l), leaf succulence, leaf titratable acidity, leaf membrane damage, and survival. At the end of the experiment, undamaged plants were used to estimate growth based on dry matter production. Detailed experimental procedures are provided in the Supporting Information 2.

2.3 | Anatomical analyses

Six different anatomical traits, expected to have functional relevance for the plant performance under water-deficit conditions, were examined: stomatal density, mesophyll thickness, adaxial and abaxial cuticle thickness, and fiber bundles in the adaxial and abaxial zones. We collected fully expanded leaves from five individuals of each species and immediately fixed them in 10% neutral buffered formalin (NBF, Boon & Drijver, 1986). A vacuum pump was used to remove air from tissues, and after 48 h, the samples were dehydrated in an ethanolic series of increasing concentrations and stored in 70% ethanol.

The middle region of the leaf blades (equidistant from the base and the apex) was selected to obtain transverse sections, excluding the midrib. This material (previously stored in 70% ethanol) was further dehydrated in an ethanolic series (80–100%) and embedded in glycol methacrylate resin (Leica Historesin®, Leica Microsystems®, Wetzlar, Germany) according to the manufacturer's instructions. Cross-sections (8 μm thick) were obtained using a manual rotary microtome (Leica Microsystems®) with a C-type knife. The sections were stained with toluidine blue O (TBO) 0.05% (Sakai, 1973) in phosphate buffer (pH 4.5) and mounted in Entellan® (Merck KGaA®, Darmstadt, Germany) synthetic resin. Different histochemical tests were conducted on free-hand transverse sections using fresh leaves: 0.3% Sudan III in glycerin for lipids (modified from Pearse, 1960), 0.01% Calcofluor White to detect β-glucans (Hughes &

McCully, 1975), 0.002% ruthenium red for pectins (Johansen, 1940), and 2% phloroglucinol in 25% hydrochloric acid for lignin (Johansen, 1940). Sections incubated in Calcofluor White were analyzed under an Olympus BX51 microscope (Olympus Corporation®, Tokyo, Japan), equipped for Olympus® epi-illumination filter, providing excitation (bandpass filter 440 nm) and emission suppression (long-pass filter 500–520 nm), and connected to an Olympus DP71 video camera (Olympus Corporation®).

Paradermal sections were obtained from the abaxial side of fresh leaves, in the middle region of the blade, and mounted in water. Images from 20 fields per leaf (10 from each side of the blade, excluding the midrib) were captured in an Olympus BX51 microscope (Olympus Corporation®) connected to an Olympus DP71 video camera (Olympus Corporation®). Stomata were counted using the cellSens® Imaging Software (Olympus Corporation®). The total number of stomata was then divided by the field area, yielding the stomatal density in the unit of stomata mm^{-2}.

Scanning electron microscopy (SEM) of the blade surface was conducted on the samples fixed in NBF, which were then further dehydrated in an ethanol series (80%–100%), subjected to critical point drying with CO_2 using a Balzers model CPD 030 apparatus (BalTec AG®, Pfäffikon, Switzerland), mounted on metal supports, and coated with colloidal gold for 180 s using a Bal-Tec SCD model 050 system (BalTec AG®). The samples were analyzed and micrographs were recorded using a scanning electron microscope model JSM 5800LV (Jeol®, Peabody, MA) operated at 10 kV at the Laboratory of Electron Microscopy (LME), Instituto de Biologia, Universidade Estadual de Campinas, Brazil.

2.4 | Statistical analysis

Growth, water potential, succulence, acidity, electrolyte leakage, and survival data were first tested for normality using the Shapiro–Wilk test. For normal measurements over time, we applied repeated measures analysis of variance (ANOVA) followed by Tukey's test (5% significance) in order to identify possible differences between treatments. For the survival data, we used generalized linear models (GLM) with a binomial link function. We built all possible additive models, considering time and species as explanatory variables, and compared them using the Akaike information criterion (AIC). To explore the potential threshold-like behavior of the survival curve, we fitted segmented linear models (Muggeo, 2008). We were particularly interested in assessing potential differences in the timing of the survival threshold (i.e., the breaking point [BP] after which $P(\text{death}) \neq 0$) and the mortality rate after the threshold (α_2) (see Supporting Information 2). We only discussed segmented models that explained the data better than their linear counterparts ($\Delta AIC > 10$) (Burnham & Anderson, 2004). We fitted the GLM and segmented models separately for the moderate and severe drought treatments. All analyses were performed using the "ggplot," "cowplot," "tidyverse," and "rstatix" packages in R (R Core Team, 2020). The segmented models were fitted using the "segmented" R package (Muggeo, 2008).

Anatomical data were also assessed for normality using Shapiro–Wilk test. To test for differences among species, we used a one-way ANOVA followed by Tukey's post hoc test (for homoscedastic data) or Games-Howell post hoc test (for heteroscedastic data) using IBM® SPSS® Statistics (version 28.0.1.1; IBM®, Armonk, NY). Data that did not follow a normal distribution were resampled using bootstrapping (1000 resamples, 95% CI BCa).

3 | RESULTS

3.1 | Growth

Most comparisons of growth parameters showed nonsignificant results. Dry mass, height, and number of leaves did not differ significantly between the beginning and the end of the experiment, or between species within each treatment (Supporting Information 3—Figures S3–S5). The only exceptions were the significant increase in dry mass over time in the control treatment in all species and the higher dry mass of *E. denticulatum* compared with the other species at the start and end of the experiment in the control treatment (Supporting Information 3—Figure S3).

3.2 | Water relations

Considering each treatment separately, water potential did not differ significantly among the species (Supporting Information 3—Figure S6). Moreover, leaf succulence did not differ among species in each treatment. Overall, average leaf succulence values showed a decreasing tendency during the experiment (Supporting Information 3—Figure S7). A significant difference in this trait ($p = 0.02$) was observed between control and treated plants on the 57th day for *E. orchidiflorum*, and on the 85th day for *E. denticulatum*. On the other hand, no difference in average leaf succulence was detected between treated and control plants in *E.* × *purpureum*.

3.3 | Titratable acidity and membrane damage

Leaf acidity was the physiological parameter most impacted by drought. By the end of the experiment, leaf acidity showed a tendency to decrease only in treated plants, regardless of the species (Figure 2). No significant difference was found among species within the same treatment, indicating that all species ultimately had the same response to drought in terms of leaf acidity. However, the reduction timing was different. *Epidendrum denticulatum*-treated plants showed reduced acidity from the 55th day onward, while in *E. orchidiflorum* and *E.* × *purpureum*-treated plants, this difference was only detected from the 83rd day.

We detected drought-induced membrane damage, inferred from the average electrolyte leakage difference ($p = 0.04$) between control and treatment plants only in *E.* × *purpureum* from the 57th day onward (Figure 3). No difference was detected among species or hybrids within the same experimental group.

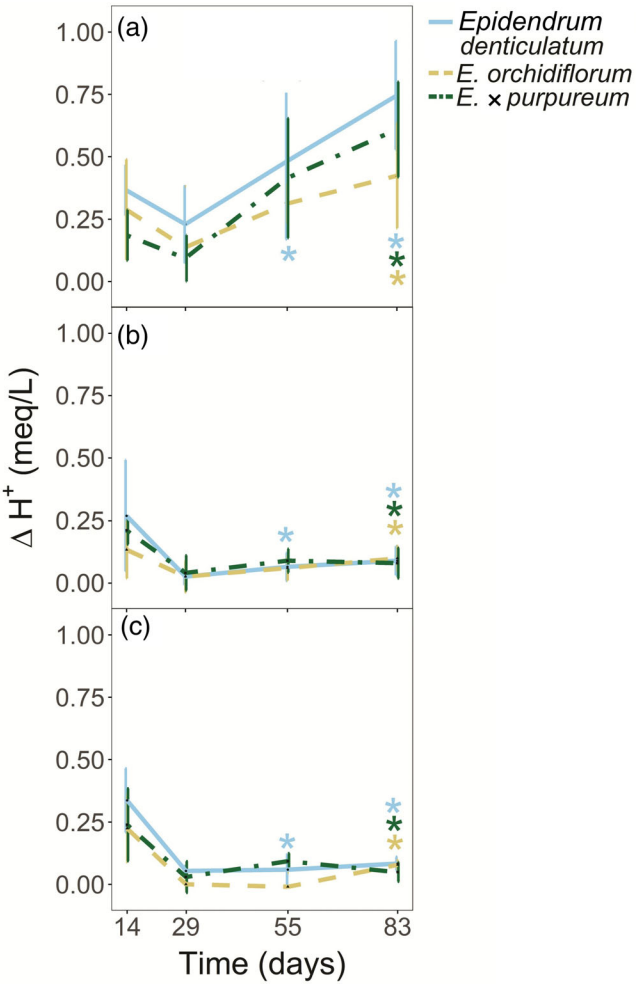


FIGURE 2 Titratable acidity (ΔH+) mean values in *Epidendrum denticulatum*, *Epidendrum orchidiflorum* and *Epidendrum* × *purpureum* subjected to different watering regimes over time, including control (a), moderate stress (b) and severe stress (c). Significant differences ($p < 0.05$) according to Tukey's post hoc pairwise comparisons are indicated by an asterisk (*), and its color indicate the species being compared across different treatments. Bars indicate standard deviation.

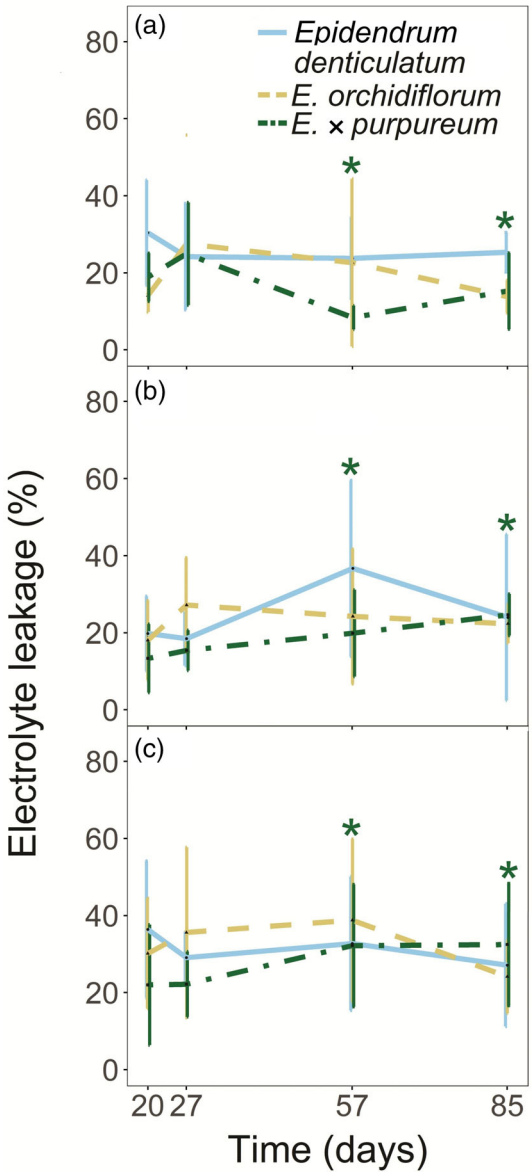


FIGURE 3 Percentage of electrolyte leakage in *Epidendrum denticulatum*, *Epidendrum orchidiflorum* and *Epidendrum* × *purpureum* subjected to different watering regimes over time, including control (a), moderate stress (b) and severe stress (c). Significant differences between control and treatment groups of *E. purpureum* ($p < 0.05$) according to Tukey's post hoc pairwise comparisons were indicated by an asterisk (*). Bars indicate standard deviation.

3.4 | Survival

The full GLM, which included both treatment and species, performed better than all other models (Table 1). Therefore, both species identity and treatment (MS vs. SS) were factors that influenced the plants' survival during the water-deficit experiment (Figure 4a,b). No significant difference in survival among the three species was found in the MS treatment, as the segmented models had broad confidence intervals for the two estimated parameters—BP and mortality rates after the BP (α_2) (Figure 4c,d, Table 2). However, the segmented model for *E. orchidiflorum* should be disregarded, as it did not perform better than its GLM counterpart ($\Delta AIC < 10$). Therefore, the conclusion drawn from the GLM is the same, as model comparison showed that the model considering only time (survival ~ time) was as good as the one considering time and species (Table 1, survival ~ time + species, $\Delta AIC < 2$).

TABLE 1 Generalized linear models (GLM) with binomial link function.

Models	df	ΔAIC
Survival = day + treatment + species	6	0.00
Survival = day + treatment	4	12.20
Survival = day + species	4	72.10
Survival = intercept	1	217.50

Note: These models estimate the effect of drought treatment (severe or moderate) and species (*Epidendrum orchidiflorum*, *Epidendrum denticulatum*, or *Epidendrum* × *purpureum*) on survival over time.
Abbreviation: AIC, Akaike information criterion.

In the SS treatment, all models had a $\Delta AIC > 10$ when compared with their GLM counterparts (Figure 4a,b, Table 3). The BP values for the three species were not significantly different, as all confidence intervals overlapped. On the other hand, the absolute value of α_2 of *E.* × *purpureum* was significantly larger than that of *E. denticulatum* (Table 3). *Epidendrum orchidiflorum* had an intermediate α_2 mean value compared with *E. denticulatum* and *E.* × *purpureum* and did not differ significantly from either. At the end of the experiment, all individuals of *E.* × *purpureum* perished in both MS and SS treatments.

3.5 | Anatomical characteristics

According to the ANOVA (Supporting Information 4—Table S1), differences between the species were observed only in the adaxial cuticle thickness ($F(1, 12) = 13.06$, $p = 0.001$) and the number of fiber bundles in both the adaxial ($F(1, 12) = 19.12$, $p < 0.001$) and abaxial ($F(1, 11) = 4.06$, $p < 0.05$) zones of the mesophyll. For these variables, the effect size was large (evidenced by an eta-squared value ≥0.14, Supporting Information 4—Table S1). No significant differences in the other variables were found between the species analyzed. The Tukey's post hoc test showed significant differences in adaxial cuticle thickness between *E. denticulatum* and *E. orchidiflorum* ($\Delta M = -10.01$, $p = 0.001$) and between *E. orchidiflorum* and *E.* × *purpureum* ($\Delta M = 5.58$, $p < 0.05$) (Supporting Information 4—Table S2). Only *E. denticulatum* and *E. orchidiflorum* showed a statistically significant difference in the number of fiber bundles in the abaxial

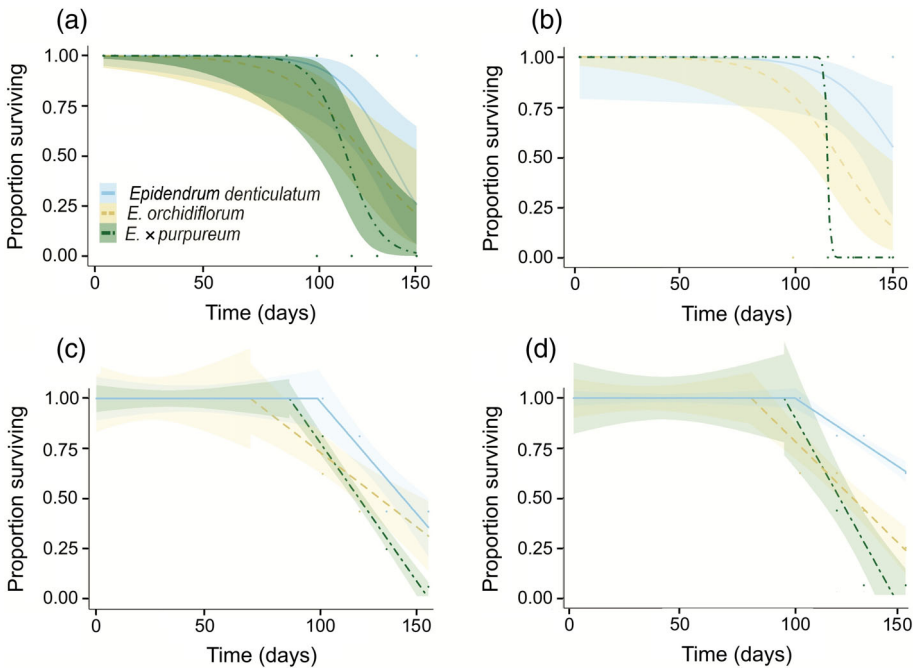


FIGURE 4 Generalized linear models (GLM) with binomial link function (a, b) and segmented models (c, d) of the survival experiment for the three studied species (*Epidendrum denticulatum*, *Epidendrum orchidiflorum* and *Epidendrum* × *purpureum*) under moderate (a, c) and severe (b, d) stress treatments. Shadows around models represent 1 standard error. Note that in the severe drought treatment (b) the *E.* × *purpureum* model does not have its standard error represented, as it was not possible to calculate it.

TABLE 2 Segmented models for the moderate drought treatment.

Moderate models	α_2 estimate ± CI	Threshold estimate ± CI	df	ΔAIC
Epidendrum orchidiflorum	−1.01 ± 0.73	69.60 ± 32.40	7	6.74
Epidendrum denticulatum	−1.52 ± 0.61	96.99 ± 14.61	7	33.50
Epidendrum × *purpureum*	−1.87 ± 0.13	85.50 ± 7.08	7	15.84

Note: The model estimates the threshold and the rate of decline in survival after that point (α_2) for *Epidendrum orchidiflorum*, *E. denticulatum* and *E.* × *purpureum*.
Abbreviations: AIC, Akaike information criterion; CI, confidence interval.

TABLE 3 Segmented models for the severe drought treatment.

Severe models	α_2 estimate ± CI	Threshold estimate ± CI	df	ΔAIC
Epidendrum orchidiflorum	−1.29 ± 0.44	79.24 ± 14.62	7	18.06
Epidendrum denticulatum	−0.87 ± 0.21	97.07 ± 8.79	7	25.75
Epidendrum × *purpureum*	−2.37 ± 1.04	92.61 ± 16.22	7	15.57

Note: The model estimates the threshold and the rate of decline in survival after that point (α_2) for *Epidendrum orchidiflorum*, *E. denticulatum* or *E.* × *purpureum*.
Abbreviations: AIC, Akaike information criterion; CI, confidence interval.

zone ($\Delta M = -5.15$, $p < 0.05$) (Supporting Information 4—Table S2). Significant differences in the number of fiber bundles in the adaxial zonewere found between *E. denticulatum* and *E. orchidiflorum* ($\Delta M = -8.0$, 95% CI BCa [−9.80 to 6.12]) and between *E. denticulatum* and *E.* × *purpureum* ($\Delta M = -6.0$, 95% CI BCa [−8.12 to 3.75]) (Supporting Information 4—Table S3) as determined using the bootstrap resampling method. *Epidendrum orchidiflorum* had a thicker adaxial cuticle (Figure 5g) compared with the other two species (Figure 5b,l) and more fiber bundles (arrowheads, Figure 5f) than *E. denticulatum* (arrowheads, Figure 5a) in both the adaxial and abaxial zone. *Epidendrum* × *purpureum* had significantly more fiber bundles in the adaxial zone (arrowheads, Figure 5k) compared with *E. denticulatum* (arrowheads, Figure 5a). For a detailed description and images of the anatomical traits mentioned, see Supporting Information 4 (Figures S8 and S9).

4 | DISCUSSION

The results from the survival analysis revealed that when exposed to severe drought, the hybrid species *E.* × *purpureum* performed worse than *E. denticulatum*, one of its parents (Figure 4). The results for *E. orchidiflorum* were less clear, as no differences were found compared with either of the other two species in terms of mortality rate after the BP (Table 3). However, this could be interpreted as *E. orchidiflorum* having an intermediate performance, suggesting that *E.* × *purpureum* performs worse than both parents in terms of survival to drought, which characterizes a transgressive pattern of expression (e.g., Schwarzbach et al., 2001). This seems to be a reasonable conclusion, as some individuals of *E. orchidiflorum* survived the moderate and severe water-deficit treatments, while all *E.* × *purpureum* individuals perished before the end of the experiment. The conclusion that *E.* × *purpureum* performed worse than its parental species is also supported by the detection of drought-induced membrane damage, inferred from the average electrolyte leaking difference between control and treatment plants of this species (Figure 3). Electrolyte leakage is a general indicator of tissue injury and can be induced by various stressful conditions (Demidchik et al., 2014). Therefore, higher electrolyte leakage may have contributed to the increased mortality in hybrid plants under drought stress.

Although the main goal of this work was to determine the importance of drought as a factor in hybrid inviability, we were also interested in understanding the underlying physiological differences that could explain the differential performance. In this context, leaf succulence provides an initial insight into understanding the physiological traits that contribute to the observed difference in performance. In our experiment, *E. orchidiflorum* individuals subjected to severe drought stress exhibited reduced leaf succulence compared with the control group on the 57th day, while severe and moderate stress led to decreased leaf succulence in *E. denticulatum* on the 85th day of the experiment (Supporting Information 3—Figure S7). In both species, plants under severe drought showed a consistent reduction in Ψ_1 (Supporting Information 3—Figure S6). Hybrids experienced a sudden reduction in Ψ_1 on Day 56 under the severe drought treatment, and no reduction in leaf succulence over time (i.e., no

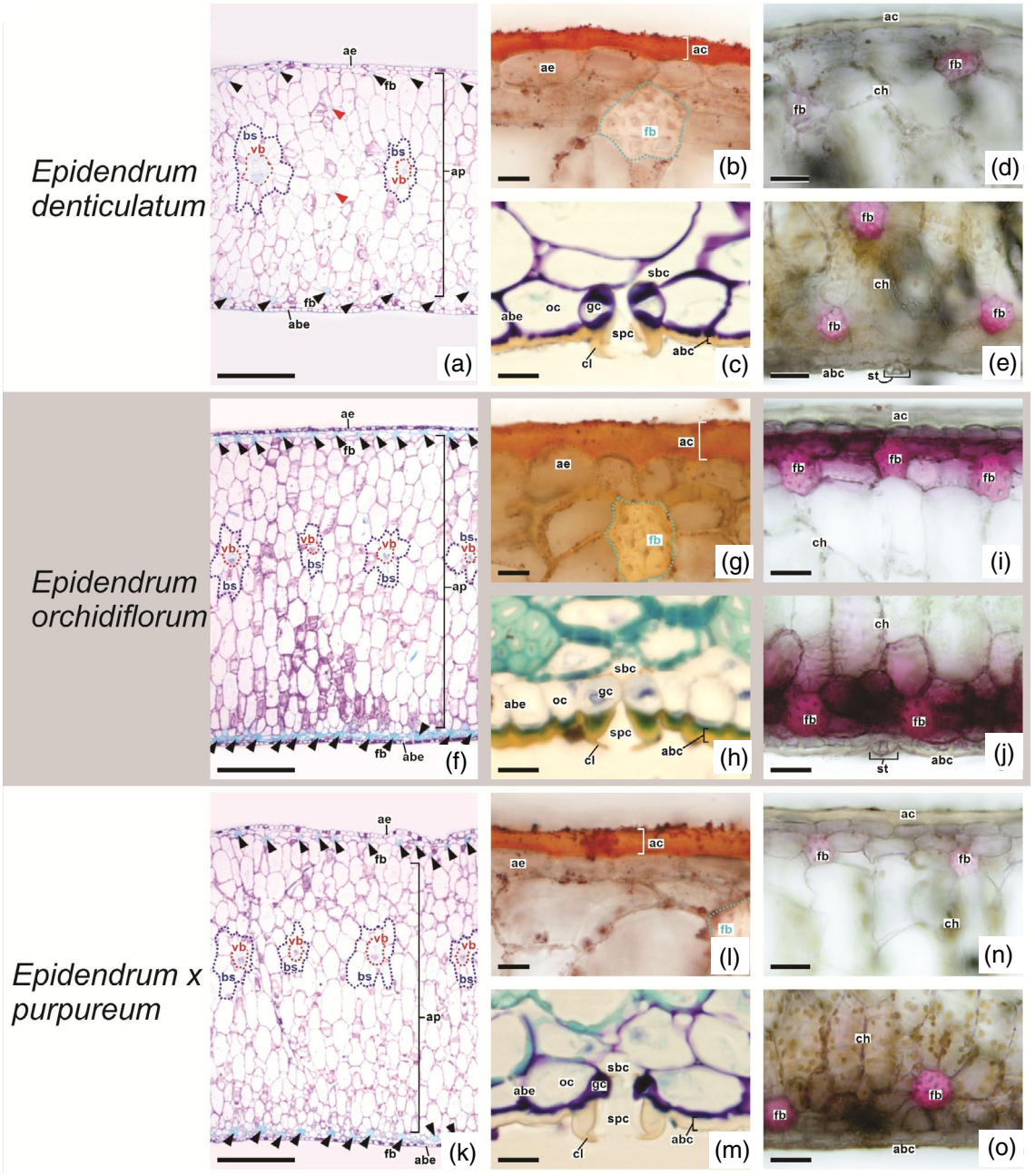


FIGURE 5 Transverse sections of *Epidendrum denticulatum*, *Epidendrum orchidiflorum*, and *Epidendrum* × *purpureum* leaves stained with toluidine blue O (a, f, k, c, h, m) and tested for lipids (Sudan III; b, c, g, h, l, m) and lignin (phloroglucinol; d, e, i, j, n, o). Arrowheads (black and red): Fiber bundles. Abc, abaxial cuticle; abe, abaxial epidermis; ac, adaxial cuticle; ae, adaxial epidermis; ap, aquifer parenchyma; bs, bundle sheath; ch, chlorenchyma (composed of photosynthetic cells); cl, cuticular ledge; fb, fiber bundle; gc, guard cell; oc, ordinary epidermal cell; sbc, substomatal chamber; spc, suprastomatal chamber; st, stoma; vb, vascular bundle. Scale bars: a, f, k = 500 μm; b, c, g, h, l, m = 20 μm; d, e, i, j, n, o = 50 μm.

use of stored water). Under drought conditions, succulent plants often use tissue-stored water as a buffer to maintain photosynthesis (e.g., Nobel, 2006) and to prevent a decline in Ψ_l, which rarely reach −1.2 MPa (Griffiths & Males, 2017). Our results are consistent with previous findings, as the mean Ψ_l did not fall below −1 MPa for any species under any treatment. Given these results, it can be assumed that drought-induced changes in photosynthesis in parental plants were buffered by the use of the stored water. Although we lack direct data on photosynthesis, leaf titratable acidity can offer a relative measure of CO_2 assimilation in CAM plants, which is usually reduced under drought conditions (e.g., Wang et al., 2019; Zotz & Tyree, 1996). Here, leaf titratable

acidity was largely reduced in all species under both moderate and severe stress (Figure 2), indicating that drought induced a notable reduction of photosynthetic activity in all species. Therefore, the use of stored water does not seem to have prevented this decrease in photosynthetic activity.

The critical combination of intensity and duration of drought leads to irreversible damage and plants mortality. The inflection point was reached under both water-deficit treatments in all three species, although severity differed (Figure 4). However, the sequence of events leading to mortality and the reasons it differed among species, remains unclear. For that reason, we can only comment on the potential processes involved in the higher mortality of hybrid plants. Plant death is a complex phenomenon with multiple causes (Anderegg et al., 2012; McDowell et al., 2022), but it is thought to result from two main processes: carbon starvation and dehydration due to hydraulic failure (McDowell et al., 2008, 2022). Our results do not support the dehydration hypothesis, since *E.* × *purpureum*, as previously mentioned, showed no reduction in leaf succulence and suffered marginal reduction in Ψ_1. On the other hand, our results regarding photosynthesis and carbon storage are indirect and do not allow testing the hypothesis of carbon starvation. Nevertheless, the difference in mortality between species is notable, especially between *E. orchidiflorum* and *E.* × *purpureum*.

The morphoanatomical leaf traits of the species examined here are adaptations to the prevailing xeric conditions, similar to those observed in other plant species from dry environments (Fradera-Soler et al., 2021; Pérez et al., 2021; Smith et al., 1998). The characteristic presence of water-storing tissue and a thick cuticular layer in *Epidendrum* leaves, for instance, implies drought tolerance and water-conserving strategies. In fact, these traits are a common feature of all *Epidendrum* species within subgenus *Amphyglottium*, a group characterized by having a prevalent CAM metabolism (Torres-Morales et al., 2020). This group is also well known for thriving in harsh natural environments such as sand dune vegetation, rocky outcrops, and nutrient-poor soils (Pinheiro & Cozzolino, 2013). Several studies (reviewed by Benzing, 1990) have highlighted the prevalence of succulent tissues, particularly in leaves and pseudobulbs, as a key structural adaptation that helps orchids to endure harsh drought conditions. This succulence serves a dual purpose: it acts as a reservoir for water and nutrients during dry periods (Benzing, 1990) and it is also believed to be a prerequisite for CAM photosynthesis (Gilman et al., 2024; Silvera et al., 2010). The increased thickness of the leaves in CAM plants is attributed to their enhanced capacity for nocturnal storage of organic acids within vacuoles during the CAM cycle (Silvera et al., 2010). Consistent with the observations of tightly packed mesophyll cells and minimal intercellular air spaces in both parental species and hybrids (Figure 5, Supporting Information 4—Figure S9), these anatomical features may potentially limit CO_2 conductance. This could enhance the capacity of the CAM pathway by restricting internal CO_2 efflux, thereby promoting high intercellular CO_2 concentrations during the daytime refixation process (Gilman et al., 2024).

The adaxial leaf cuticle thickness and the number of fiber bundles were the only traits exhibiting significant differences among the studied species. Both traits showed higher values in *E. orchidiflorum* and lower values in E. *denticulatum* (Figure 5). Hybrid plants showed intermediate values for both traits, which might be determined by additive effects in the inheritance of these traits. The leaf cuticle helps plants to maintain a favorable water status, especially under drought conditions (Domínguez et al., 2017). In fact, during periods of maximal stomatal closure, the rate of water loss through the cuticle becomes the primary determinant of minimal water loss (Jeffree, 2006). The differences in the cuticle observed here may be associated with the divergent ecological preferences of the parental species. While *E. denticulatum*, the species with thinner cuticle, occurs in seasonally flooded habitats, *E. orchidiflorum*, with thicker cuticle, occurs in nonflooded, closed shrubby vegetation, where water limitation is more severe (Arida et al., 2021; São Leão et al., 2019). However, we cannot draw any conclusions about the role of the cuticle in our drought experiment because most of the comparisons using the physiological data returned nonsignificant results. For instance, the species with the thinner cuticle (*E. denticulatum*) showed the highest survival in our experiment, suggesting additional roles for the cuticle layer, for example, protection from abiotic and biotic stresses such as ultraviolet radiation, insects, fungi, and other organisms (Domínguez et al., 2017).

5 | CONCLUSIONS

The higher mortality levels of *E.* × *purpureum* under drought stress suggest that hybrid plants are less fit than the parental species in the dry environment where they occur. According to Arida et al. (2021), several pre- and postpollination barriers act in concert to prevent interspecific gene exchange between *E. denticulatum* and *E. orchidiflorum*. However, even under scenarios of strong hybrid sterility, the persistence of hybrid plants in the habitat may increase the likelihood for interspecific gene exchange or hybrid speciation (Vallejo-Marín & Hiscock, 2016). In this study, we showed that drought can severely affect the persistence of the hybrid under the dry conditions of the restinga. In such a scenario, only recurrent formation may enable hybrid plants

to persist in such extreme conditions. Hybrid plants may respond to abiotic stresses in different ways, which may influence the intensity of gene exchange between parental taxa (Johnston et al., 2001). For instance, Favre and Karrenberg (2011) found that both shade and drought stress reduced the hybrid fitness in a *Silene* hybrid zone, precluding interspecific gene exchange. On the other hand, Campbell and Wendlandt (2013) found increased reproduction in hybrid plants under drought stress, suggesting that late post-mating barriers may break down under dry conditions. Thus, given the variable responses of hybrid plants to abiotic stresses such as drought, further studies are needed in hybrid zones of nonmodel organisms. Considering the predicted climate change scenarios and their effects on plant geographic ranges (Parmesan, 2006), the extent to which hybrid plants respond to abiotic stresses will shape the occurrence and persistence of natural hybrid zones. In fact, since heterospecific gene exchange is considered an important source of genetic variation in plants (Schley et al., 2022; Vallejo-Marín & Hiscock, 2016), the vulnerability of hybrid plants may also have long-term effects also on parental species.

AUTHOR CONTRIBUTIONS

Karolyne W. de Jesus and Fabio Pinheiro designed the plan of work, carried out the fieldwork, experimental work and collected data. Luliana L. S. Mayer and Matheus Pena-Passos carried out the anatomical analysis. Karolyne W. de Jesus and Thales M. de Lima carried out the statistical analysis and first draft of the manuscript. Thales M. de Lima, Bárbara S. S. Leal, Rafael S. Oliveira and Fabio Pinheiro edited the revised drafts and finalized the manuscript. Subsequently, all authors approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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