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Are seeds able to germinate before fruit color ripening? Evidence from six Neotropical bird-dispersed plant species

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Abstract. Many plants attract seed dispersers and indicate the presence of ripe, fleshy fruits with coloration changes. If seed maturation is simultaneous with visual signs for frugivorous, a portion of seeds can be removed before being ready for dispersal. In this study, we tested the delay visual signal hypothesis (DVSH), which implies high reproductive success when seed maturation precedes the visual signal of the fruit ripening, limiting the dispersal of unviable seeds. We evaluated this hypothesis with six tropical species that have fruits with discrete coloration for the immature, intermediate, and mature stages, testing the prediction that the development of mature and intermediate seeds is similar. First, we verified that the coloration of three categories was differentiable with color analysis. Next, we carried out germination and viability tests (i.e., electrical conductivity [EC] and tetrazolium) as an approach to evaluate the germination potential along development. We found that the EC of three species decreased constantly during fruit ripening, suggesting that the seed maturation is a gradual process, with a substantial germination of intermediate seeds. In the other species, the conductivity of seeds of fruits with intermediate coloration was lower than that of mature fruit, because mature seeds are more affected by predators and pathogens. The tetrazolium tests indicated that the viability of seeds of three species increased with maturity and smaller differences between intermediate and mature stages were observed, supporting the DVSH. Only two species achieved high percentages of germination, and for *Myrcia splendens*, the pattern was very consistent with the hypothesis. We concluded that the germination and viability rates of the seeds of each plant species depend on many factors (e.g., dormancy and time of exposure to predators); however, for all the species studied, there is some evidence that seeds can germinate before they reach maturity by color.

Key words: color analysis; delay hypothesis; development; electrical conductivity; germination potential; ripening stages; seed viability; visual signal of the fruit.

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Introduction

Many plant species depend on mutualistic processes, such as the seed dispersal by animals, to increase fitness. Fruit color in fleshy-fruited plants may be used as a signal to which seed dispersers can be attracted (Schaefer and Schmidt 2004). In fact, most plant species in tropical forests produce fleshy and colorful fruits to be consumed by animals (Ridley 1930, van der Pijl 1982, de Camargo et al. 2015). Traditionally, fruit coloration has been explained as an adaptation to facilitate the detection of fruits by dispersers (i.e., Snow 1970, Schaefer et al. 2008, Cazetta

et al. 2009). Fruit color could also act as a nutritional signal for dispersers which in turn play an important role in seed removal far from their parents, increasing plant survival (Janzen 1970, Howe and Smallwood 1982, Valenta et al. 2015). Therefore, the diversity of fruit color is commonly interpreted as the product of ecological and evolutionary processes involved in plant reproductive strategies (Schaefer and Schmidt 2004).

Many plants whose fruits are dispersed by diurnal animals, such as birds, change the color of their fruit as they reach maturity and become more conspicuous (Herrera 1982, Janson 1983). Changes in fruit color may be coupled with background coloration to increase the contrast with foliage, and these changes also affect frugivore preference (Burns and Dalen 2002). Therefore, once the fruit matures, it announces its condition to the dispersers through changes in color, but also in texture and smell (Herrera 1982, Schaefer et al. 2006, Schaefer et al. 2008). It is usually understood that the plant shows ripe fruit coloration when the seeds have the maximum possibility of germination (Sumner and Mollon 2000). However, in some cases, immature fruits are consumed, because there is no availability of ripe fruits, soft seeds are easier to digest, or fruits have few secondary compounds to protect seeds (Pizo et al. 1995, Francisco et al. 2002, Stevenson 2006, 2007, Levey et al. 2007, Schaefer 2011). Some frugivores (i.e., birds Psittacidae) are considered seed predators due to their mainly feeding on immature fruits regardless of the availability of mature fruits (Delgadillo and Páez 2011). Some plant species produce immature fruits with bright colors and ripe green fruits, to diversify seed dispersal vectors instead of restricting dispersal to birds (de Camargo et al. 2015). Foster (1977) found the long-tailed manakin (*Chiroxiphia linearis*, Pipridae) feeds on ripe fruits of *Ardisia revoluta* (Primulaceae), but changes its diet for green fruits in periods of food scarcity. In this sense, increased immature fruit consumption could decrease plant fitness because seeds would not be ready to germinate. However, immature seeds of *A. revoluta* germinated as often as mature seeds, indicating that birds that feed on unripe fruits of this species retain their role as dispersers because seed maturation is earlier than visual cues of maturity. Thus, if seed maturity is simultaneous with the visual signal that frugivores perceive, it is feasible that a portion of the seeds can be dispersed before being ready to germinate. In fact, dispersed seeds of fruits with an intermediate degree of development could have a chance to germinate, which has been called the delayed visual signal hypothesis (DVSH; Pardo and Stevenson 2008).

There are strategies that prevent seed germination when environmental conditions are not favorable for the development of seedlings (Baskin and Baskin 1998). Consequently, if the frugivores carry the seeds to sites unfavorable for germination, mature seeds of some species may enter dormancy and remain viable for some time. Thus, a species has dormant seeds when, after seed maturation, seeds do not germinate in a specified period of time under a combination of physical environmental factors (Baskin and Baskin 2004). The variation of this time depends on the type of dormancy, the physiological conditions of the seeds of each species, and the environmental characteristics that can break the latency (Baskin and Baskin 1998). Nevertheless, prolonging these periods can be detrimental to plants, as mature seeds and fruits are attractive not only to dispersers but also to many harmful agents such as bacteria, fungi, invertebrates, and vertebrate predators (Herrera 1982).

In this study, we tested the delay hypothesis in the visual signal, which implies that seed maturation of fleshy-fruited plants precedes the visual signal of fruit ripening to increase plant fitness. We tried to evaluate the ability of certain species of plants to produce seeds whose fruit or pulp is not yet mature visually but whose germination potential is similar to those of the seeds from ripe fruit. Initially, we determined whether fruit color during maturation can be established as a continuous (gradual) or discrete process for six plant species of the families Arecaceae, Araliaceae, Primulaceae, Phyllantaceae, and Myrtaceae. We also compared germination potential and seed viability from unripe, intermediate, and mature fruits. Therefore, we performed viability tests based on electrical conductivity (EC) of seed soaking solutions and tetrazolium as an approach to determine the germination potential of dormant seeds. Based on this, to support the DVSH, we did germination tests expecting that

the germination percentage of the seeds would increase with the ripening of the fruit and show no differences between the intermediate and mature stages. Thus, we predicted a decrease in EC with fruit maturation and eventually smaller differences in EC between intermediate and mature stages, because cellular membrane integrity has been related to high levels of conductivity and consequently to better germination performance (Bewley and Black 1994). At the same time, we predicted an increase in probability of seed viability measured by tetrazolium tests with fruit ripening and expected few differences between intermediate and mature fruit seeds. In cases where germination does not occur within the study period (e.g., dormancy), it is expected that there is support for the delay hypothesis if seeds from intermediate fruits show high viability and there are few differences between mature and intermediate stages. In contrast to the DVSH, it is possible to postulate an alternative hypothesis in which seeds exhibit a synchronous maturation with visual signal of fruits. Therefore, seeds can only germinate when fruits show mature coloration.

In addition, the interpretation of the results of the tests used in this study aims to determine the germination strategies of the seeds and, in this way, to establish the time and the manner to extract seeds of different native species to obtain high germination rates. These findings can be used for the development of restoration strategies in the places where the species are present.

Materials and Methods

Study site

We collected fruits in two main areas of Colombia: First, in a sub-Andean forest, more specifically, within Cueva de Los Guácharos National Park, located in the Department of Huila, Colombia (Fig. 1), we collected the fruits of *Chamaedorea linearis*, *Chmaedorea pinnatifrons*, and *Oreopanax microcephalus*. This 9000-ha park

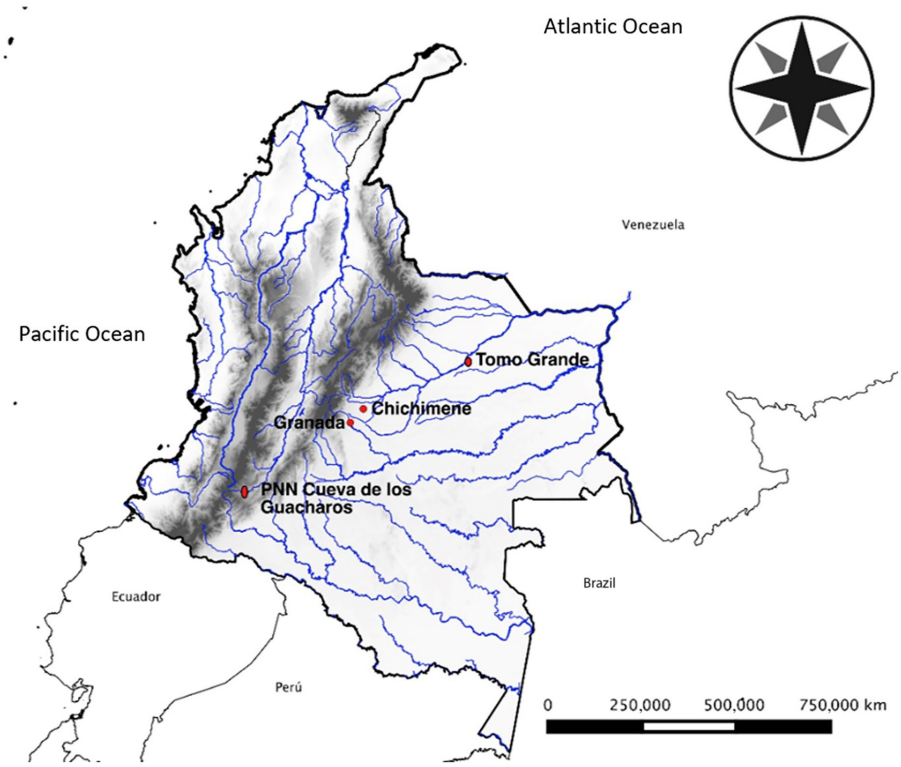


Fig. 1. Locations where the species samples of the study were collected. The red dots indicate the collection locations.

includes altitudes from 1630 masl at the confluence of La Cascajosa and Suaza rivers up to 2840 masl in Cerro Punta (the boundary with Department of Caquetá). The temperatures oscillate between 16° and 20°C, with humidity reaching up to 85%. The collection area is approximately 2000 masl and has an average annual rainfall of 3100 mm (Parques Nacionales Naturales 2005). There are several studies done in this park (Ramírez et al. 2014; Stevenson et al. *In press*); therefore, we have a prior knowledge about the plant species present in it and its dispersers.

In the same way, in the Orinoco basin, we collected the fruits of *Hieronyma alchorneoides* (Tomogrande Natural Reserve, Vichada), *Stylogyne longifolia* (Chichimene, Meta), and *Myrcia splendens* (Granada, Meta; Fig. 1). These sites have an average temperature of 25°–26°C and a unimodal pattern of rainfall with a mean ~2000–3000 mm. In these places, we have conducted various studies on plant composition and primate ecology, allowing us knowledge on plant phenology and fruit traits (e.g., Stevenson et al. 2000), which were basic criteria to choose adequate species for testing the DVSH.

Study species

We included six species in the study: *Cha. linearis*, *C. pinnatifrons*, *O. microcephalus*, *S. longifolia*, *H. alchorneoides*, and *M. splendens*. These species were chosen taking into account availability in the environment at the time of data acquisition and an obvious change in the coloration of the fruits throughout their development, so that at least three different colors could be observed while ripening (Fig. 2b). Despite the differences between the fruit type of these species (berries in *Oreopanax* and drupes in the rest of species), they are mainly dispersed by birds. Also, these species were selected because they are from different families (except *Chamaedorea*), so we could previously rule out an effect of phylogenetic bias. Main plant characteristics of all species are described in Appendix S1.

Seed collection

The number of individuals (4–11) and the number of fruits collected (124–539) varied depending on the species (Table 1). We tried to collect similar amounts of fruit for each individual, trying to collect the same number of fruits for each maturation stage, according to the visual perception of the collector. Before seed collection, we searched and marked individual trees providing fruits at different stages of development. Afterward, we selected the species with at least four fruiting individuals to collect the fruits, which were usually present in the same forest type, but not very close to each other. We collected fruits directly from the branches, and we did not take into account the fruits found in the soil. We processed fruits of only two individuals each day to have sufficient time to undertake color analysis (Fig. 2). Later, we removed the pulp manually from each fruit collected and washed the seeds. We assigned a unique identity for each extracted seed and separate them according to the fruit maturation state. The extracted seeds were stored in sealed bags in dark conditions until laboratory analyses, which occurred 1–10 d after collection.

For each test, we took the seed as a sampling unit, in the sense that each seed was analyzed individually in the tests described below (i.e., color, germination, and viability). For *Oreopanax*, that has a berry with several seeds, we randomly used a single seed of each fruit for further tests. However, for statistical tests of germination, we considered individual plants as replicates and three stages of fruit color as treatments.

Image capture and color analysis

The number of fruits collected from 4 to 11 individuals varied between species (range 124–539; Table 1), using similar number of fruits for the three ripening stages (immature, intermediate, and ripe). Color analysis was performed using the digital photograph method, which has advantages over other approaches (Stevens et al. 2007). Photographs of fruits belonging to one or two individuals were taken in a dark room to provide the same lighting conditions. A Canon Rebel T3i digital camera in RAW format was used to avoid loss of information (Stevens et al. 2007). Images were processed in TIF format (Tagged Image File Format) with 8-bit. We obtained a chromatic model based on primary colors, RGB (red, green, blue), which we simulated in the space of color using Image J

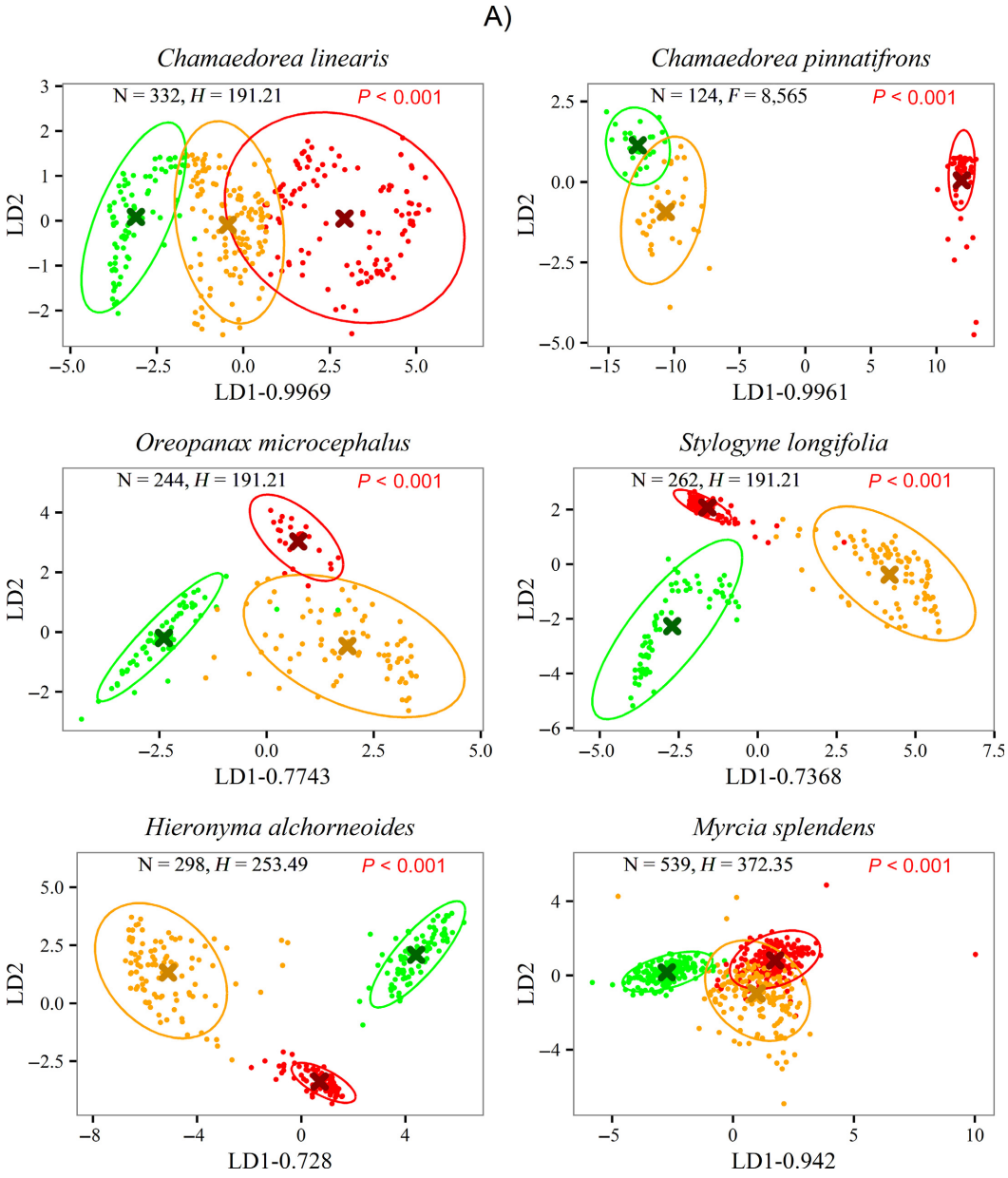


Fig. 2. Fruit color variation of each species. (a) Discriminant analysis of variation of the color of the fruit (from RGB values) with respect to its ripening stage. Green dots indicate immature fruits; the orange dots represent the intermediate fruits; the red dots show the ripe fruits; *H* and *F* show the statistic of an ANOVA and a Kruskal–Wallis test, respectively; the *P* represents the significance value of each test. (b) Fruits of the collected species. The colors of the immature, intermediate, and mature fruits are observed for each species.

1.47 software (Rasband 1997). Hence, we calculated the relative contribution of each channel to the color of the selected area in the image (with a standard area for all images). Thus, each value of R, G, B was expressed in relation to the total sum of these values (Eqs. 1, 2, 3; Endler 1990, Amézquita et al. 2009). Through this process, we obtained the variables that define the space of color in two dimensions (LM and S; Eqs. 4, 5).

b)

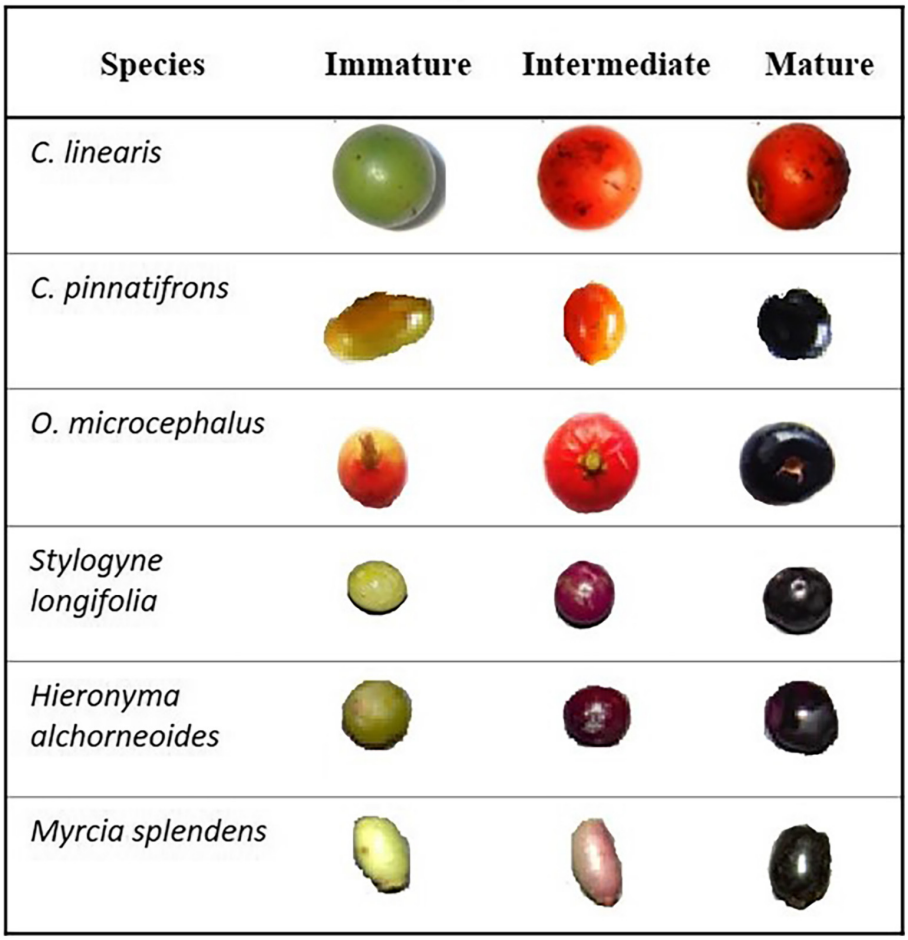


Fig. 2. (*Continued*)

$$R' = \frac{R}{R + G + B} \quad (1)$$

$$G' = \frac{G}{R + G + B} \quad (2)$$

$$B' = \frac{B}{R + G + B} \quad (3)$$

$$LM = R' - G' \quad (4)$$

$$S = B' \quad (5)$$

Viability analysis

Electrical conductivity test.—To recognize seed viability, we first used the International Seed Testing Association—ISTA method of EC of the water leached by the seeds, which gives an indirect measure of deterioration degree of cell membranes and seed cover. Thus, high conductivity values indicate seed deterioration and hence low germination (Matthews and Powell 2006).

We placed six individuals of each species, each with approximately 30 seeds, in leach racks submerged in 8 mL (*Chamaedorea linearis* and *C. pinnatifrons*) and 5 mL (for *O. microcephalus, S. longifolia, H. alchorneoides,* and *Myrcia splendens*) of deionized water for 24 h. Subsequently, we measured the permeability and EC of the

Table 1. Number of fruits collected per species in each place.

Species	Number of fruits	Number of individuals	Site collection
Chamaedorea linearis	332	11	Cueva de Los Guácharos National Park, Huila
Chamaedorea pinnatifrons	124	5	Cueva de Los Guácharos National Park, Huila
Oreopanax microcephalus	263	6	Cueva de Los Guácharos National Park, Huila
Stylogyne longifolia	262	4	Chichimene, Meta
Hieronyma alchorneoides	298	7	Tomogrande Natural Reserve, Vichada
Myrcia splendens	539	6	Granada, Meta

leached water with an Automatic Seed Analyzer SAD 9000. From the tetrazolium tests described below, we obtained a cutoff value that allowed us to establish the viability limit of seeds of each species.

Tetrazolium tests.—These tests may be useful to estimate the viability of those seeds that present dormancy or slow germination (Association of Official Seed Analysts 2000). In this study, in addition to examining dormancy in seeds that did not germinate, we used a separate tetrazolium test to determine seed viability in each stage of development and establish their relationship with the EC. For this, we used 10 seeds per stage from six individuals to determine viability (independently of germination test), through staining with 2,3,5-triphenyltetrazolium chloride (TTC; Delouche 1962). TTC solution was prepared with 0.5 g of the chemical in 1 L of distilled water (0.5% solution). We stored this solution at 20°C in the dark (Waes and Debergh 1986). After seeds were submerged in deionized water for the amount of time established by the EC test, we applied the tetrazolium salt for 24 h at 30°C. Criteria for determining viability as established by Association of Official Seed Analysts (2000) were taken into account (Appendix S2: Fig. S1).

Germination test.—To evaluate the germination potential of these seeds as an indicator of their maturity, we performed germination tests for all species in different substrates (Appendix S3: Table S1). Before placing them on the germination substrate, we subjected the seeds of some species (*Cha. linearis*, *C. pinnatifrons*, and *O. microcephalus*), according to preliminary germination tests and the environmental conditions that we could establish for this test, to a cleaning process with 95% ethanol (10 s), 0.7% sodium hypochlorite (2 min), and 70% ethanol (2 min), to eliminate surface pathogens (Gallery et al. 2007).

Due to the fact that the end of the germination process occurs with the emergence of the epicotyl (Bewley 1997), on a weekly basis we recorded whether or not this structure was visible. The duration of the germination test for each species was different (Appendix S3: Table S1). During these tests, we took into account the percentage of germination and the average germination time that corresponds to the average time that seeds of a single replica took to germinate. Seeds that did not reach germination were also analyzed with tetrazolium tests to determine their state of dormancy (Zalamea et al. 2015).

Statistical analysis

We performed a discriminant analysis to distinguish color among stages of fruit maturation. LD1 was obtained as a new variable that explained changes between groups. With this new variable, we ran ANOVA or Kruskal–Wallis, Tukey or pairwise Wilcoxon, depending on data distribution for each species, to show the differences between three ripening stages. As we found significant differences between the three stages, variable denominated "ripeness" was treated as a discrete factor for all analyses.

To explain the germination potential of the seeds in relation to their ripening stage, we first established the relationship between the ripening stage and the EC of the water leached by the seeds. We performed parametric (ANOVA) and nonparametric (Kruskal-Wallis), and post hoc (Tukey and parwise Wilcoxon) variance analyses to compare the EC of each of the ripening stages. In addition, to determine whether the EC could be a good indicator of the viability of the seeds and hence their germination potential, we determined the effect of the EC on the probability of germination of the seeds through a logistic model. We also used a logistic model to determine whether EC affected seed viability measured by tetrazolium tests.

We calculated germination percentages as measures of comparison between the three stages of development. These percentages were obtained from the number of germinated seeds in the observation period (Appendix S3: Table S1) and the number of seeds placed (Bewley and Black 1986).

We also used parametric and nonparametric tests to compare different stages of seed maturation. Those seeds that did not germinate were analyzed with logistic models in which the predictor was the EC and the response variable was the probability of dormancy. The significance of all analyses was established from $\alpha = 0.05$. Each statistical analysis was performed using R version 3.3.1 (R Development Core Team 2007).

Results

Image capture and color analysis

For all species, we observed changes in fruit color associated with the three ripening stages (i.e., immature, intermediate, and mature; Table 2 and Fig. 2a).

Viability analysis

Electrical conductivity.—The lowest EC values were observed in fruits with mature ripening coloration in *Chamaedorea linearis* ($H_{2df} = 56.29$, $P < 0.001$), *Chamaedorea pinnatifrons* (ANOVA, $F_{2df} = 39.91$, $P < 0.001$) and *M. splendens* ($H_{2df} = 128.40$, $P < 0.001$), suggesting that higher seed viability is present in ripe fruits. This means that the conductivity decreases as the ripening stage progresses (Fig. 3a, b, f). Also, with the post hoc tests, we obtained significant differences in EC of the seeds of the three stages of maturation, suggesting a gradual development for these three species. We also found differences in EC between the three ripening stages for the remaining species, where *Oreopanax microcephalus* ($H_{2df} = 15.03$, $P < 0.001$) and *Stylogyne longifolia* ($H_{2df} = 63.48$, $P < 0.001$) showed the lowest EC for intermediate seeds (Fig. 3c, d), being the stage with highest germination potential. Finally, EC for *H. alchorneoides* seeds was 0 for almost all seeds (Fig. 3e).

Table 2. Statistic tests for fruit classification in the three ripening stages.

Species	Statistic	*P*-value	Post hoc HSD Tukey	Pairwise Wilcoxon test
Chamaedorea linearis	$H = 191.21$	<0.001		***
Chamaedorea pinnatifrons	$F = 8565$	<0.001	***	
Oreopanax microcephalus	$H = 191.21$	<0.001		***
Stylogyne longifolia	$H = 191.21$	<0.001		***
Hieronyma alchorneoides	$H = 253.49$	<0.001		***
Myrcia splendens	$H = 372.35$	<0.001		***

*** Probability values of differences between groups <0.001.

Tetrazolium tests.—Seed viability was independent of ripening stage in *Chamaedorea linearis* ($\chi^2_{2df} = 5.26$, $P = 0.07$) and *O. microcephalus* ($\chi^2_{2df} = 1.19$, $P = 0.55$; Fig. 4a, c), for seeds that were not used in germination tests. Therefore, there were no significant differences in number of viable seeds between the three ripening stages. However, we found that viability depends on the ripening stage in *C. pinnatifrons* ($\chi^2_{2df} = 7.77$, $P = 0.02$), *S. longifolia* ($\chi^2_{2df} = 46.15$, $P < 0.001$) and *Myrcia splendens* ($\chi^2_{2df} = 19.39$, $P < 0.001$). The subsequent contingency analysis that included only the intermediate and mature stages indicated that there were no significant differences in the viability of the seeds of these two stages for *C. pinnatifrons* ($\chi^2_{1df} = 0.86$, $P = 0.35$) and *M. splendens* ($\chi^2_{1df} = 1.77$, $P = 0.18$; Fig. 4b, e). In the case of *S. longifolia*, this relationship was a gradual process, and therefore, the quantity of viable seeds of intermediate fruits was significantly lower than the number of viable seeds of mature fruits ($\chi^2_{1df} = 20.13$, $P < 0.001$; Fig. 4d). For five species, we observed that EC could be a reliable indicator of seed viability, where high EC values showed low viability (Fig. 5) and particular models were established for each species (Appendix S4: Table S1).

Germination tests

Chamaedorea linearis.—After 10 months in the greenhouse, no seed germination was observed for this species. Nevertheless, we applied the tetrazolium test to all samples collected and we found 36% of ungerminated seeds were viable. Therefore, these seeds were considered dormant. The seeds that were no viable were died. Additionally, we found that ripening stage influenced

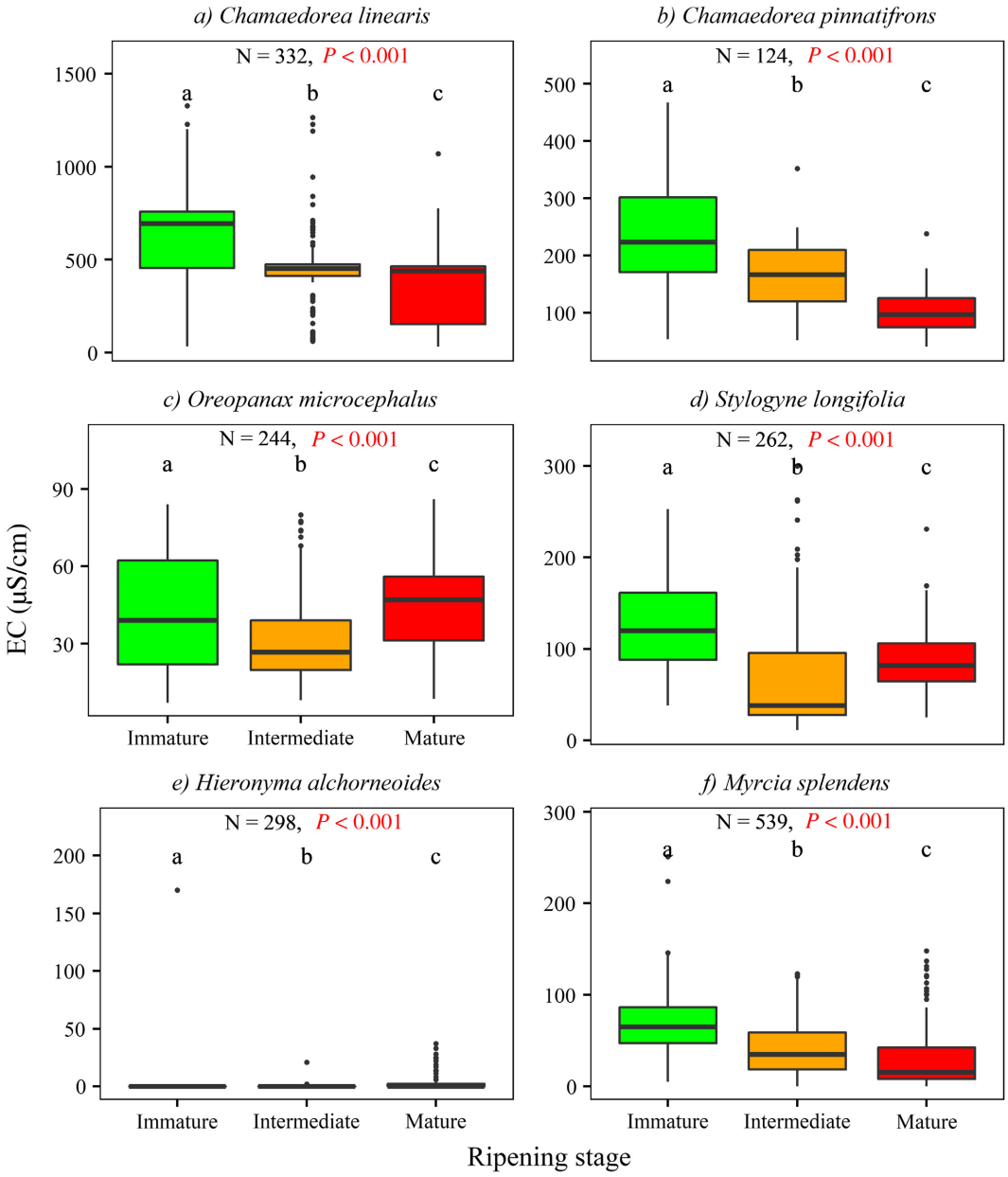


Fig. 3. Differences in the electrical conductivity of the water leached by the seeds with respect to their state of maturation. The letters above the boxes represent the estimated groups with a post hoc HSD (b) or Wilcoxon (a, c, d, e, f) test.

dormancy (χ^2_{2df} = 19.43, P < 0.001). With a later contingency analysis, we found that there were no differences in the number of dormant seeds between intermediate and mature stages (χ^2_{1df} = 0.18, P = 0.66; Fig. 6a).

Chamaedorea pinnatifrons.—We did not register germination of these seeds but tetrazolium tests showed that 39% of the seeds were viable, and thus, these seeds were considered as dormant. We also found that dormancy depends on the ripening stage (χ^2_{2df} = 9.33, P = 0.009). However, the subsequent contingency analysis showed that the number of dormant seeds of intermediate fruits was lower than the number of

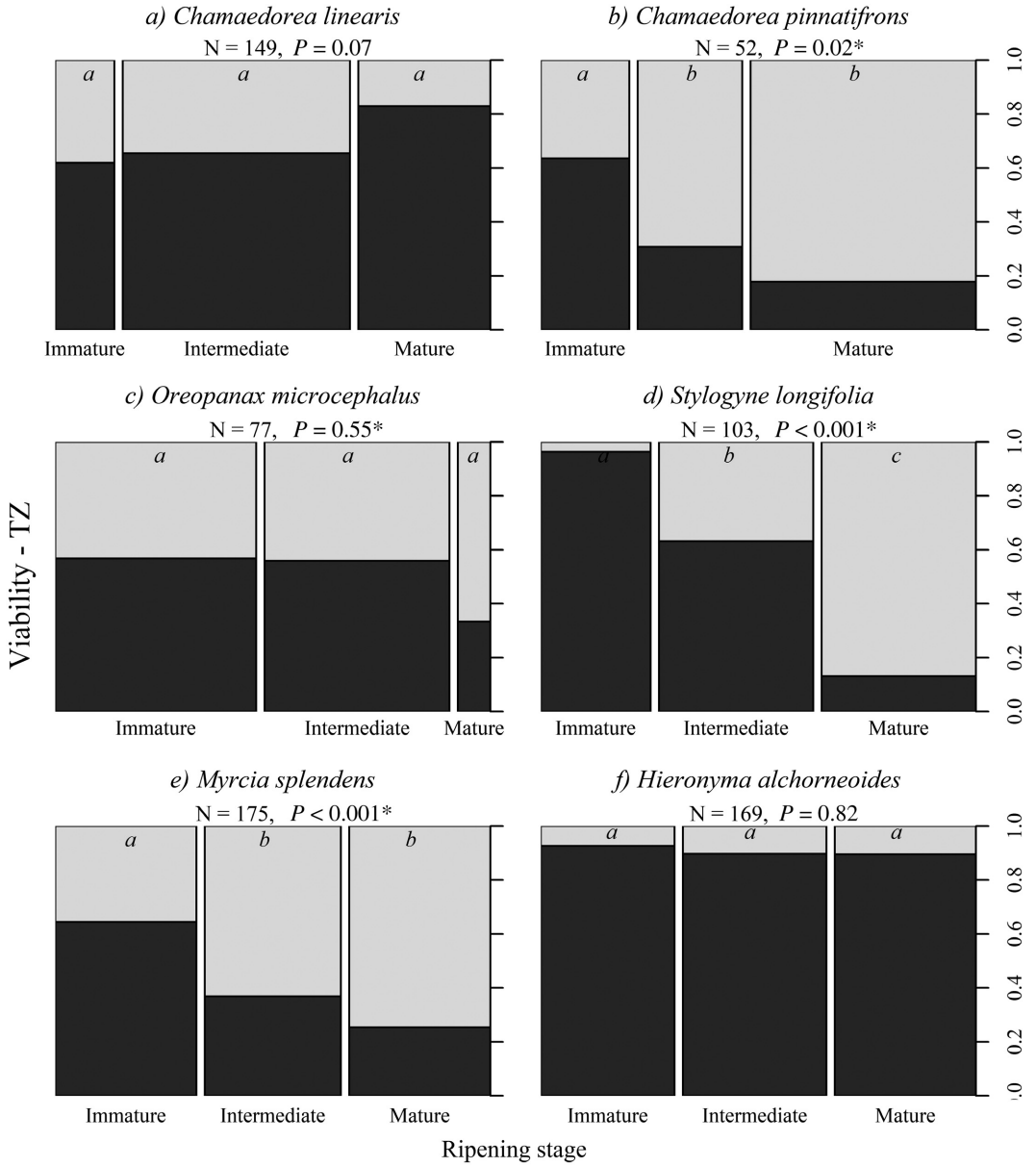


Fig. 4. Relationship between viability from the tetrazolium tests and the ripening stage of the fruit. In *y*-axis, the light color represents viable seeds; the dark color represents inviable seeds. In the right side, are showing the percentages of viable and inviable seeds for each of the stages in each species. The *P* represents the level of significance of the contingency analysis performed from the frequencies of each group; * indicates a significant *P*-value. The letters represent the estimated groups with a later contingency analysis with a square matrix. (a–e) Percentages of seeds that were not used in germination tests. (f) Percentages of non-germinated seeds of *Hieronyma alchorneoides*. The percentage of viable seeds for immature fruits is 7.4%, and intermediate is 10.34% and mature is 10.52%.

dormant seeds of mature fruits ($\chi^2_{1df} = 5.73$, $P = 0.016$; Fig. 6b).

Oreopanax microcephalus.—We did not observe germination in this species. Based on results of tetrazolium tests after the seeds passed through the germination test, we determined that these seeds might be non-dormant since only 2.3% of the non-germinated seeds

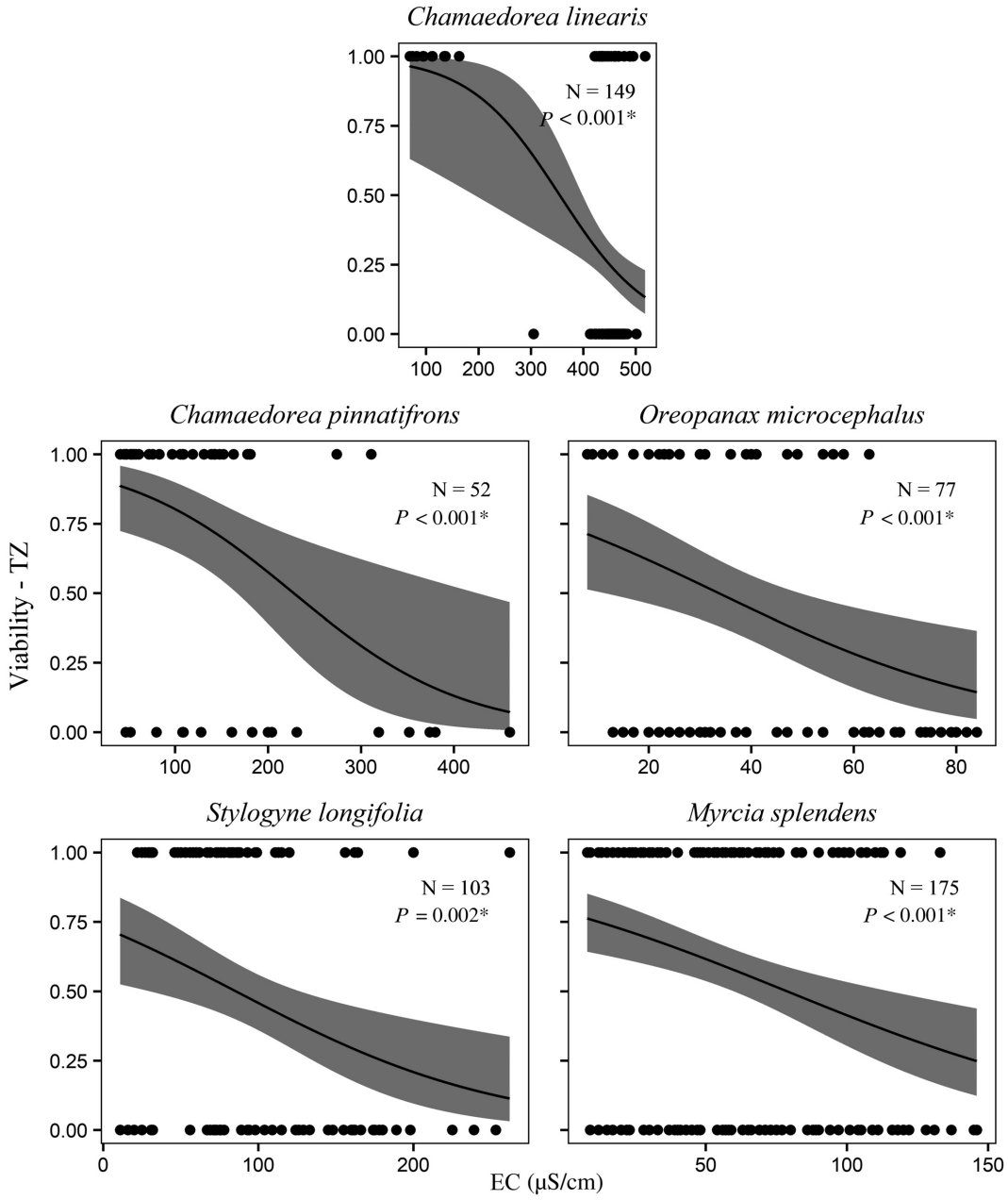


Fig. 5. Seed viability from tetrazolium test and explained by electrical conductivity (EC). The *P*-value represents the significance level obtained with the evaluation of a logistic model; * indicates a significant *P*-value.

were found to be viable ($\chi^2_{2df} = 0.31$, $P = 0.85$; Fig. 6c).

Stylogyne longifolia.—No germination percentages were obtained for the seeds of this species. We found that 36% of the seeds were viable after they passed through the germination test; therefore, they were considered dormant. Similarly, we observed that dormancy depends on ripening stage ($\chi^2_{2df} = 6.82$, $P = 0.03$). From a subsequent contingency analysis, we determined that there were no differences between intermediate and mature stages ($\chi^2_{1df} = 0.25$, $P = 0.62$; Fig. 6d).

Hieronyma alchorneoides.—We found there were no significant differences in the percentages

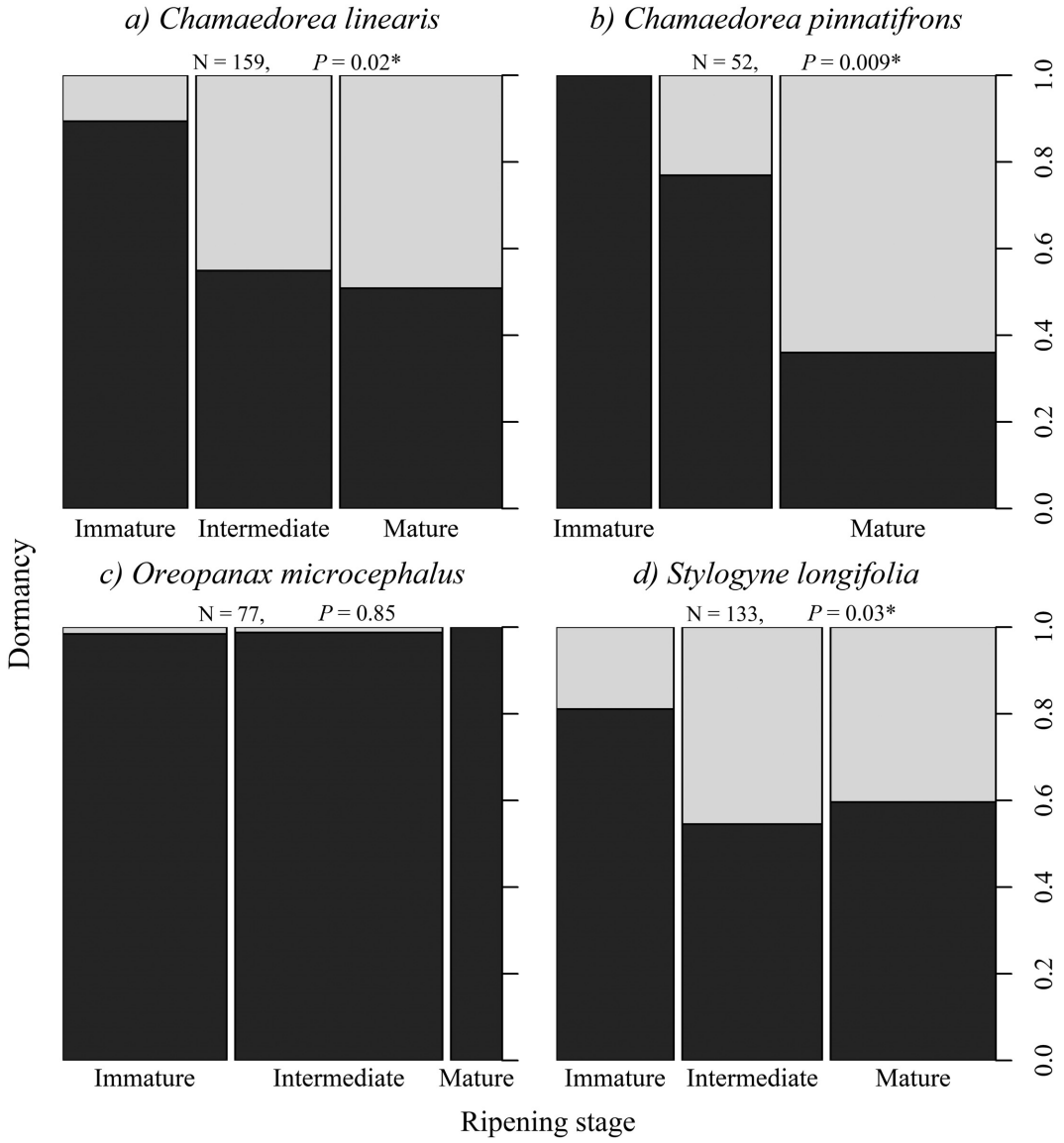


Fig. 6. Percentages of seeds that entered dormancy after a germination process. In *y*-axis, the light color represents dormant seeds; the dark color represents inviable seeds. In the right side, are showing the percentages of dormant and inviable seeds. The *P* represents the significance level of the contingency analysis performed from the frequencies of each group; * indicates a significant *P*-value. The letters represent the estimated groups with a later contingency analysis with a square matrix. The percentages of dormant seeds for each species are (a) 36%, (b) 39%, (c) 2.3%, and (d) 36%.

of germination among three stages of development (ANOVA, $F_{2df} = 0.025$, $P = 0.97$; Appendix S6: Fig. S1). Also, we observed that EC is not a faithful indicator of germination probability ($P = 0.12$). Only 9.67% of ungerminated seeds were viable, and no differences were observed in seed viability between ripening stages ($\chi^2_{2df} = 0.39$, $P = 0.82$; Fig. 4f).

Myrcia splendens.—First, we found that although there is a high percentage of germination of intermediate and mature seeds, there are no significant differences in the percentages of

Table 3. Summary of statistical results obtained with each of the species of the study.

Species	Germination	EC†	TZ‡	HS
Chamaedorea linearis	No germination (MPD)	RN ($P < 0.001$)	IV ($P = 0.07$)	
	DV:Imm≪Int≈Mat	GP: Imm≫Int≫Mat	Imm≈Int≈Mat	±
	+	±	−	
Chamaedorea pinnatifrons	No germination (MPD)	NR ($P < 0.001$)	PR ($P = 0.02$)	
	DV: Imm≈Int≪Mat	Imm≫Int≫Mat	Imm≪Int≈Mat	±
	−	±	+	
Oreopanax microcephalus	No germination	SDi ($P < 0.001$)	IV ($P = 0.55$)	
	Seed death	Imm≈Mat≫Int	Imm≈Int≈Mat	±
	NC	±	±	
Stylogyne longifolia	No gemination (PD)	SDi ($P < 0.001$)	PR ($P < 0.001$)	
	DV: Imm≪Int≈Mat	Imm≫Mat≫Int	GP: Imm≪Int≪Mat	±
	+	±	±	
Hieronyma alchorneoides	%Germ	SDi ($P < 0.001$)	No tetrazolium tests	
	Imm≈Int≈Mat	Imm≈Int≪Mat		±
	VNG: Imm≈Int≈Mat			
	±	−	NC	
Myrcia splendens	%Germ	NR ($P < 0.001$)	PR ($P < 0.001$)	
	Imm≈Int≈Mat	GP: Imm≪Int≪Mat	Imm≪Int≈Mat	+
	+	±	+	

Notes: HS, hypothesis support; Imm, seeds of immature fruits; Int, seeds of intermediate fruits; Mat, seeds of mature fruits. Symbols are ≪, significantly lower; ≫, significantly higher; ≈, no significant differences; +, supports the delay hypothesis; ±, partially supports the delay hypothesis; −, does not support the delay hypothesis; NC, not possible to draw conclusions; EC, electrical conductivity; MPD, morphophisiological dormancy; PD, physiological dormancy; DV, dormant seed viability; VNG, viability of ungerminated seeds; %Germ, germination percentages.

† EC shows the relationship between the seeds' EC and the ripening stage of the fruit; NR, negative relationship; SDi, significant differences; GP, gradual process.

‡ TZ shows the relationship between the seeds viability from the tetrazolium tests and the ripening stage of the fruit; IV, independent variables; PR, positive relationship.

germination among the three stages of development (ANOVA, $F_{2df} = 3{,}324$, $P = 0.06$; Appendix S6: Fig. S1).

Also, we observed EC could be an indicator of the probability of germination of these seeds ($P < 0.001$). Thus, germination probability of *M. splendens* seeds decreased while EC increased (Appendix S6: Fig. S1). Since germination of the first seeds occurred immediately after the EC test or even during the imbibition process, these seeds were not considered dormant. In addition, Baskin and Baskin (1998) have reported that several genera of Myrtaceae do not present dormancy. Thus, in this species, we do not contemplate necessary to perform the tetrazolium tests for ungerminated seeds, and we considered them to be inviable seeds.

Discussion

Fruits of all six species were classified in three stages of color (immature, intermediate, and mature); however, the results of the study showed that in general, seed maturation is a gradual process. This partially supports the delayed signal hypothesis, since we show evidence that some seeds in intermediate maturity stages (or even when unripe) are viable and have high germination capacity. In other words, some seeds do not require fruit color maturity to germinate. For the species with highest germination rates (*Myrcia splendens*), immature seeds germinate at higher rates supporting the DVSH, as it was found for *Miconia nervosa* in the only study that we are aware of, which directly has tested this hypothesis (Pardo and Stevenson 2008). These results also agree with those found for *Ardisia revoluta* by Foster (1977). Taking into account all variable evaluated, it should be noted that *M. splendens* showed support for all the traits measured and five other species showed partial support (Table 3). In this sense, we evaluated each species with the three tests explained before (germination, EC, and tetrazolium) and we obtained a final conclusion according to the support of the hypothesis with each one of these

tests. Thus, the partial support means that one of the three tests evaluated for each species cannot support the hypothesis.

Overall, the most consistent pattern we found from the EC analyses revealed gradual ripening processes for seeds. This is not a new finding because in general, fruit and seed maturations have been described as a gradual process (i.e., Zaidan 1985, Grzesiuk and Górecki 1989, Tekrony and Egli 1997). For example, chemical changes during development have been reported to determine several elements such as pigmentation (Gil et al. 1995). Additionally, the state of the cell membranes depends on the degree of maturity of the seeds (Zaidan 1985). However, the graduality observed in three species indicates that seeds of intermediate fruits present some germination potential. In the other cases, high conductivity values (indicating less viability) in mature seeds suggest that these seeds could suffer more from predators than seeds from unripe fruits, because it has probably been a long time before the collection of these seeds (Lazar et al. 2014). Although this pattern was not expected to support the delayed signal hypothesis, these results show that seed viability of intermediate-color fruit is highest for two species, which is congruent with the hypothesis. Finally, EC measure was an indicator of seed viability for most of the species, except for *Hieronyma alchorneoides.* It means that an increase in EC in seeds of five species reflected a decrease in seed viability (Lazar et al. 2014).

Two palm species of *Chamaedorea* and *Myrcia* seeds showed a progressive increase in germination capability during their development when using the EC test. However, it is worth noting that seeds of intermediate and mature fruits of *Chamaedorea linearis* showed marginal statistical differences in the EC. In consequence, seeds of incompletely ripe fruits had similar viability than those of ripe fruits. However, this is not evident in the seeds of *Chmaedorea pinnatifrons* and *M. splendens,* which exhibited a more gradual process. *Oreopanax*, *Stylogyne*, and *Hieronyma* showed higher values of conductivity in mature seeds. This could be explained by longer exposure to predators of mature seeds (i.e., Lazar et al. 2014) which may have caused seed damage and this was reflected in the increase in conductivity values. This was not the expected pattern, but the results do not invalidate the delay hypothesis. On the contrary, it suggests that seed maturation could be modified by plant enemies when fruit reaches ripe coloration. In addition, it is not clear that we can assume that the same relationship between EC and seed age or fruit maturity occurs at all stages of seed development (Mohamed-Yasseen et al. 1994), for instance, because seed size varies through development and may alter the EC values. Similarly, other factors that affect conductivity were not taken into account, such as moisture content of seeds, imbibition time of seeds between species, genotype within species, and ontogeny of particular seeds (Panobianco and Vieira 1996). In fact, significant differences were observed between the EC of some seeds from the same *Oreopanax* tree, which would imply that the ontogeny of particular seeds may be influencing the results obtained for the conductivity of at least this species.

On the other hand, tetrazolium viability tests showed that there are no differences between intermediate and mature stages for seeds of *C. pinnatifrons* and *M. splendens*. It also represents evidence that some seeds were viable before their fruits were completely mature. In *Oreopanax*, seed viability was independent of their ripening stage, and therefore, there is a similar quantity of both viable and inviable seeds for the three stages of maturation, which provides support for the delay hypothesis. Additionally, it is important to note that most of these seeds showed viability (45%). On the contrary, the same analysis indicated a gradual viability increase for *Stylogyne* seeds, suggesting again that there were viable seeds whose fruits were not completely ripe and partially supporting the delay hypothesis.

The absence of germination of most species is explained by the physiology of their seeds. We cannot remove the fact that palm seeds present dormancy (the most common is the morphophysiological, i.e., Pérez 2009, Baskin and Baskin 2014), and therefore, they must break it to germinate. Carpenter (1988) reports that palm seeds require exposure to high temperatures (25–30°C) in order to promote seed germination. In our study, although greenhouse temperature range oscillated between 10° and 35°C during the day, germination was not observed. It is possible that germination conditions were not adequate and seeds did not break dormancy, perhaps waiting

for conditions suitable for seedling establishment (Baskin and Baskin 1998). In addition, 11% and 50% of inviable seeds of *Chamaedorea linearis* and *C. pinnatifrons*, respectively, were attacked by pathogens, and for this reason, some seeds might have initially been viable but died during the germination process. These results suggest that dormant seeds whose fruits were classified as intermediate have already completed their development process, which also supports the delay hypothesis.

On the other hand, morphophysiological dormancy (MPD) in seeds of the Araliaceae family has been reported (Baskin and Baskin 1998). Our results from tetrazolium tests demonstrated loss of viability in most *Oreopanax* seeds and high permeability values (Appendix S5: Table S2). These seeds could be penetrated by fungi due to their thin seed coat (Neegaard 1977, Harman 1983), which probably prevented seed germination. Consequently, this reduced the germination potential (Mohamed-Yasseen et al. 1994) and caused the embryo to die. In *Oreopanax,* two of three tests established to determine the germination potential indicated that this species does not support the delay hypothesis as expected. However, we cannot rule out the idea because we did not find a relationship between seed viability and their maturation stage.

Similarly, Baskin and Baskin (2004) have inferred the presence of physiological dormancy in the Myrsinaceae family (now included in Primulaceae) from available information about germination and the characteristics of family seeds. Consequently, it is very feasible that seeds of *Stylogyne longifolia* present this type of dormancy. Thus, it is likely that we had interrupted the time required to break seed dormancy because the germination test lasted just six weeks. Additionally, we found evidence of pathogenic attacks on several seeds during this process, which resulted in seed death. However, tetrazolium tests indicated minimal differences in the dormancy of the seeds of intermediate and mature fruits, suggesting, like the palms, a support for the delay hypothesis. In summary, we have two tests that statistically support the delay hypothesis (i.e., viability with Tetrazolium and dormancy) for *Stylogyne,* and on the other hand, one test that does not show the expected pattern (EC), but by showing the greater viability potential of seeds of fruits of intermediate color, gives some support to the hypothesis.

Hieronyma alchorneoides seeds are not dormant (Baskin and Baskin 1998, Joker and Salazar 2000). Although our results showed a slow germination process, we found permeable seeds (Appendix S5: Table S2), and therefore, the delayed embryo development cannot be the result of limited imbibition. Thus, germination percentages were similar for all ripening stages, suggesting support for the delay hypothesis. Finally, EC did not prove to be a reliable indicator of seed viability of this species.

On the other hand, *M. splendens* seeds do not present dormancy and germinated quickly, even during the imbibition process. This species did not show significant differences in the germination percentages of the three ripening stages, and this supports the idea of advanced development of seeds with respect to fruit color.

Ecological implications

Given the multiple causes for which there is a consumption of fruits that according to their color have not yet finished their maturation process, results that support the delay hypothesis suggest that some plant species can produce seeds that develop before fruits show a signal of maturity. This may be a strategy to avoid loss of fitness of plants in cases where they are removed from the parent plants by animals. Thus, if coloration changes are a signal to attract frugivores (Snow 1970), consumption of intermediate fruits does not imply a fitness loss. In this sense, frugivores that feed on fruits before they show signs of maturity continue in their role as dispersers, as suggested by Foster (1977). In this sense, the delay hypothesis can be used as a strategy to reduce the loss of fitness of the plants under two scenarios: (1) under a scenario with low availability of ripe fruit in which many dispersers prefer to change their diet temporarily and consume the available fruits (i.e., Foster 1977, Pizo et al. 1995, Francisco et al. 2002, Stevenson 2006); and (2) if there are few dispersers, it may take a sufficient time for ripe fruits or those that have already shown a maturation signal through their coloration to be attacked by pathogens prior to their dispersion (Herrera 1982). However, the fruits that are visually in the process of maturation would still be available.

Conclusion

We found partial support for the delay hypothesis in *Chamaedorea linearis*, *Chmaedorea pinnatifrons*, *Oreopanax microcephalus*, *Stylogyne longifolia*, and *Hieronyma alchorneoides*. *Myrcia splendens* was very consistent with the hypothesis. Likewise, the EC of the water leached by the seeds of the study species can be an indicator of the viability of their seeds. However, this does not imply an EC relationship with the maturity of the seeds in *Stylogyne*, *Hieronyma*, and *Oreopanax*, which showed unexpected results, but were consistent with the hypothesis in the sense that the highest viability was found for seeds of intermediate fruits. Consequently, these results suggest that the germination potential of the seeds of each species depends on many factors such as maturation, seed size, dormancy, and exposure time to predators. Finally, results of some tests supported the delay hypothesis, but there are differences in germination and establishment strategies between species. Therefore, it is important to be able to determine the timing and the best way to extract seeds of different species, in order to obtain high rates of germination and generate new conservation strategies for the species that require them.

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Supporting Information

Additional Supporting Information may be found online at: http://onlinelibrary.wiley.com/doi/10.1002/ecs2.2174/full