The disruption of a keystone interaction erodes pollination and seed dispersal networks

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ABSTRACT

Understanding the impacts of global change on ecological communities is a major challenge in modern ecology. The gain or loss of particular species and the disruption of key interactions are both consequences and drivers of global change that can lead to the disassembly of ecological networks. We examined whether the disruption of a hummingbird-mistletoe-marsupial mutualism by the invasion of non-native species can have cascading effects on both pollination and seed dispersal networks in the temperate forest of Patagonia, Argentina. We focused on network motifs, sub-networks composed of a small number of species exhibiting particular patterns of interaction, to examine the structure and diversity of mutualistic networks. We found that the hummingbird-mistletoe-marsupial mutualism plays a critical role in the community by increasing the complexity of pollination and seed dispersal networks through supporting a high diversity of interactions. Moreover, we found that the disruption of this tripartite mutualism by non-native ungulates resulted in diverse indirect effects that led to less complex pollination and seed dispersal networks. Our results demonstrate that the gains and losses of particular species and the alteration of key interactions can lead to cascading effects in the community through the disassembly of mutualistic networks.

Keywords: cascading effects, keystone mutualisms, network motifs, non-native species, pollination networks, seed dispersal networks.

INTRODUCTION

A central attribute of ecological communities is that only few species and interactions may be essential to maintain community structure and ecosystem processes, even though many species might coexist and interact (Ellison et al. 2005, Valiente-Banuet et al. 2015). Thus, studying the impacts of species losses and gains is crucial to understanding the structure and functionality of communities in a changing world. Ecological networks provide a systematic way of representing, characterizing, and comparing the complexity of ecological communities, in which species are represented by nodes and interactions by links between nodes (Delmas et al. 2019). The loss of particular species (i.e., nodes)
and the disruption of key interactions (i.e., links) may have cascading effects in communities, increasing species extinction rates (Koh et al. 2004, Säterberget al. 2013, Rodríguez-Cabal et al. 2013).

In particular, mutualistic interactions such as pollination and seed dispersal by animals are essential in supporting terrestrial ecosystems because many plants rely on these interactions for reproduction (Bond 1994, Ashman et al. 2004, Ollerton et al. 2011). In the last two decades, the study of mutualistic networks has been critical to understand the structure and functionality of these communities (Bascompte and Jordano 2007, Rohr et al. 2014). Yet, several studies have focused on the impacts of human-caused environmental change on mutualistic interactions (Tylianakis et al. 2008, Traveset and Richardson 2014), few studies have focused on how such change triggers the disassembly of mutualistic webs (Fortuna et al. 2013, Rader et al. 2014). Moreover, indirect effects on interaction webs are not well understood because they are complex to study (Terborgh and Feeley 2010).

In the northern portion of the temperate forest of Patagonia occurs a mutualistic interaction involving a hummingbird (*Sephanoides sephaniodes*), a marsupial (*Dromiciops gliroides*), and a mistletoe (*Tristerix corymbosus*). The nectar produced by the mistletoe is the only nectar resource for the hummingbird during Patagonian winter (Smith-Ramírez 1993, Aizen et al. 2002), and the hummingbird is the most frequent (almost exclusive) pollinator of the mistletoe (Aizen 2003). In addition, the hummingbird pollinates nearly 20% of the endemic woody genera in this Patagonian forests (Aizen and Ezcurra 1998). The marsupial provides an efficient seed dispersal service by dispersing at least 16 fleshy-fruited species, including large fruits (>11 mm) not dispersed by the native birds (Amico et al. 2009). In addition, the mistletoe depends on the marsupial for its persistence because passage through the marsupial’s gut is crucial to trigger seed germination and most defecated seeds are stuck to branches due to the sticky pulp that surrounds the mistletoe seed producing a “necklaces” arrange of up to 20 seeds linked by viscin threads (Amico and Aizen 2000, Aizen et al. 2002, Aizen 2003, Rodríguez-Cabal et al. 2007). Due to the tight interactions among the hummingbird-mistletoe-marsupial, a reduction in the abundance of any of them may disassemble this interaction web. For example, Rodríguez-Cabal et al. (2013) found that herbivory on *Aristotelia chilensis*, the main host of the mistletoe, and changes on vegetation structure by non-native ungulates...
leads to the reduction of the main host abundance and indirectly affect the hummingbird and the arboreal marsupial, triggering the disassembly of the hummingbird-mistletoe-marsupial interaction. 

The temperate forest of Patagonia is known for its high proportion of plant species requiring animal mutualists for pollination and seed dispersal (Aizen and Ezcurra 1998). Almost 60% of plant species in this forest require at least one animal species for pollination (Riveros 1991) and nearly 50% of plants produce fleshy fruits suggesting animal seed dispersal (Armesto et al. 1987), which is comparable to the mutualism level recorded in tropical forests (Aizen et al. 2002, Rodríguez-Cabal et al. 2008). In addition, owing to the high degree of asymmetry of plant-animal mutualisms of the Patagonian temperate forest, where most of the plants depend only on a few mutualistic partners (Aizen et al. 2002), the loss of a mutualistic species could trigger a cascade of linked extinction events throughout the community (Albrecht et al. 2014).

In this study, we examine how far the impacts of the disruption of the hummingbird-mistletoe-marsupial mutualism cascades in pollination and seed dispersal networks and how large is the perturbation to network structure in the temperate forest of Patagonia. Using an ongoing natural experiment triggered by the invasion of non-native ungulates, we address the following questions: (1) Is the hummingbird-mistletoe-marsupial mutualism fundamental to maintaining the structure and diversity of mutualistic networks? We expect that the complexity of pollination and seed dispersal networks will be greater in sites with this interaction than in sites without the interaction. (2) Does the disruption of this mutualism by non-native ungulates alter mutualistic network structure? We expect that the complexity of pollination and seed dispersal networks will be lower in sites invaded by non-native ungulates than in intact forest sites with the tripartite mutualism.

**MATERIAL AND METHODS**

**Study Area and Natural History**

The study was conducted in Nahuel Huapi National Park and Llao Llao Municipal Reserve in northwestern Patagonia, Argentina. The climate in this region has a dry season in spring-summer and a humid season in autumn-winter, with 1800 mm of average annual precipitation and 9°C of average annual temperature. The forest is dominated by the evergreen southern beech (*Nothofagus dombeyi*) and cordilleran cypress (*Austrocedrus chilensis*), with an understory layer dominated by the shrub
Aristotelia chilensis and bamboo Chusquea culeou (Mermoz and Martín 1986). Currently the non-native ungulates red deer (Cervus elaphus), dama deer (Dama dama), and domestic cattle (Bos taurus) are the most abundant ungulates in these forests and occupy 56% of the area of Nahuel Huapi National Park (Jaksic et al. 2002).

In this study we took advantage of the clumped distribution of the mistletoe populations, shaped by the marsupial (D. gliroides) activity and the population structure of its main host (García et al. 2009, Sasal et al. 2021), that allows us to compare sites with high and low mistletoe density. We selected six 1-ha native forest sites, separated by more than 2 km. Four sites were located in well-preserved forest lacking introduced ungulates: two sites with high density of mistletoes (>400 reproductive individuals per hectare) and the presence of A. chilensis (hereafter “intact sites with the tripartite mutualism”), and two sites with low density of mistletoes (<20 reproductive individuals per hectare) and the presence of A. chilensis (hereafter “intact sites without the tripartite mutualism”). Intact forest sites do not present direct or indirect records of herbivory from ungulates (See Appendix S1 in Supporting Information). Two additional sites were located in forests that have been invaded by non-native ungulates, hereafter “invaded sites”. We considered invaded sites those with presence of herbivory of non-native ungulates over the last 100 years (Appendix S1: Table S2). Previous records of D. gliroides (Martin 2010) and previous and current presence of low adult mistletoes density in invaded sites evidence the historical occurrence of the hummingbird-mistletoe-marsupial interaction (Vázquez 2002, Rodríguez-Cabal et al. 2013, Appendix S1: Table S1). However, due to the herbivory pressure and changes in vegetation structure by non-native ungulates, the abundance of the mistletoe and its main host A. chilensis is low and the marsupial is locally absent, producing the ecological extinction of the hummingbird-mistletoe-marsupial interaction at invaded sites due to the absence of mistletoe recruitment (Rodríguez-Cabal et al. 2013, Appendix S1: Table S2). Each site was chosen haphazardly from a pool of possible sites. Description of sites is detailed in Appendix S1. Fieldwork and data collection were carried out during two consecutive austral springs and summers (2017-2018 and 2018-2019).

Pollination networks
During the flowering season (October–February) we identified all plant species pollinated by animals in each site and estimated the rate of flower visits. To this end, we conducted 10 min censuses per
plant where we recorded the pollinator identity, number of pollinator visits, and number of flowers per individual or branch visited (when the sample plant has a large number of flowers). A visit was recorded when the visitor touched a reproductive structure of the flower. Pollinator species that could not be identified in the field were collected and identified in the laboratory. We performed the censuses from 11:00 to 17:00 h in sunny non-windy days. We standardized the sampling effort by plant species in our data collection design. We conducted the same number of censuses for each plant species across all sites. For example, if we had conducted 20 and 30 censuses on plant species A and B, respectively, in site 1, then we conducted 20 and 30 censuses on species A and B in the remaining sites. Furthermore, the number of censuses conducted for each plant species was roughly proportional to their relative abundance at each site, as abundant plant species tend to interact more frequently than rare species (Vázquez et al. 2007). We carried out at least 12 censuses per plant species per site, including at least five different individuals of each species. We conducted 1782 censuses for all sites and plant species in 2017-2018, and 1738 censuses in 2018-2019 (Appendix S2: Table S1, smoothed accumulation curves of species interactions in Appendix S2: Figure S1).

**Seed dispersal networks**

During the fruiting season (January–April) we identified all fleshy-fruiting plant species in each site. Interactions between seed dispersers and plants were recorded using different methodologies according to the seed disperser animal (Donatti et al. 2011). First, to quantify seed dispersal by birds, we conducted observations one hour per plant individual and recorded the number of fruits consumed by birds. We discarded interactions involving seed predation and pulp pecking by birds because they damage the seeds and eat the fruit pulp without swallowing the seed, respectively (Simmons et al. 2018). Observation periods were performed from 6:30 to 10:30 h. We standardized the sampling effort by plant species in our data collection design. We conducted the same number of observations for each plant species across all sites. In addition, the number of observations conducted per plant species was proportional to their relative abundance at each site. We carried out at least five observation periods per plant species per site, including at least five different individuals of each species. The number of observation periods of each species were constant among sites when the same species were present. Overall, 125 observation periods were conducted for all sites and plant species in 2017–2018, and 155 observation periods in 2018–2019 (Appendix S2: Table S2, smoothed
Second, to evaluate seed dispersal by the endemic marsupial, we collected and analyzed their feces in each site. The feces were collected with a mesh on the floor of Tomahawk traps (10 cm × 10 cm × 30 cm). These wire-mesh traps are designed to efficiently capture this small arboreal mammals (Fontúrbel 2010). We placed 17 Tomahawk traps at a height of 1-2 m above the ground on shrubs or trees. Traps were arranged in a star grid and separated from each other by 15 m to cover an effective area of one hectare. We controlled the traps daily at dawn during four consecutive days in February and March (Amico and Aizen 2005) and higher captures of *D. gliroides* are detected corresponding to the juvenile recruitment period (Celis-Diez et al. 2012). All sites were sampled simultaneously. To determine the number of fruits dispersed by *D. gliroides*, we divided the number of seeds of each plant species found in the sample (feces) by the mean number of seeds per fruit of the same species.

The mean number of seeds per fruit of each species was estimated by measuring 12 fruits, collected randomly at the study site. The correction was made to homogenize the units of bird and *D. gliroides* observations to number of fruits removed. Sampling effort was homogeneous among sites because we assumed the presence of *D. gliroides* in all of them despite the low probability of presence in sites invaded by non-native ungulates (Appendix S2: Table S2, smoothed accumulation curves of species interactions in Appendix S2: Figure S2).

Finally, we used infrared camera traps (Bushnell trophy cam) to improve sampling completeness of species and interactions and accumulate more observation hours per plant species in each site. Cameras were placed focusing branches bearing fruits of each plant species during 240 hours (Appendix S2: Table S2 and S3). Cameras were set up to record 30 seconds every two minutes during day and night, triggered by movement. From the videos, we documented the same variables recorded in direct observation periods, considering a dispersal event only when we visually recorded the consumption of fruit by the seed disperser and recognized its identity. In total 12 cameras (two per site) were operating simultaneously in each fruiting season.

**Data analysis**

For each site we built one pollinator and one seed dispersal network per season, resulting in a total of 12 plant-pollinator and 11 plant-seed disperser networks (i.e. each site-year combination has a corresponding network). The plant-seed disperser network in 2017-2018 in one of the sites without
the tripartite mutualism was excluded from the analysis because several plant species produced no fruits.

We used generalized mixed-effects linear models to check if the number of species and the total and unique interactions differ in pollination and seed dispersal networks between intact forest sites with and without the tripartite mutualism (hereafter tripartite sites) and between intact forest sites with the tripartite mutualism and invaded sites (hereafter invasion sites). We performed twelve models, six for pollination and six for seed dispersal networks. Each model included a combination of a response variable (number of species, number of total interactions or number of unique interactions) and a fixed factor (tripartite sites or invasion sites). We included “Site” as a random factor in the models to account for repeated measures in the same site. We used the Poisson distribution with a log link function because the response variables were count data, but we used the negative binomial distribution when we found overdispersion in the data (Zuur et al. 2009). We conducted all model analyses using the lme4 package of R software (Bates et al. 2015, R Core Team 2019).

Our network analysis focused on network “motifs”: sub-networks composed of a small number of species exhibiting particular patterns of interactions and obtained by breaking up the entire network (Milo et al. 2002, Simmons et al. 2019a). Motifs are valuable tools to assess the structure and ecological importance of species in networks because they have two structural levels of organization. In one structural level, a motif represents a unique pattern of interactions among a subset of species within a network (Milo et al. 2002, Simmons et al. 2019a,b), consequently, networks with a greater number of different motifs would have a greater diversity of interactions (Jácome-Flores et al. 2019). In the other level, a motif is composed by two or more positions that can be occupied by different species simultaneously (Appendix S3: Figure S1). Each of these positions represents a different ecological pattern with direct and indirect interactions (Baker et al. 2015, Simmons et al. 2019a). Therefore, the frequency of positions that a particular species occupies defines its ecological role in the community (Simmons et al. 2019a). Even if different species occupy the same position, the motif still conserves its ecological function. For example, in the motif integrated by two plants and a pollinator species, plant species could be “A and B” or “C and D” but the motif would still indicate competition or facilitation between the two plants. The advantage of motifs is that they are significantly more sensitive to changes in network structure than the network indices commonly used.
(i.e. degree distribution, nestedness) (Simmons et al. 2019a). In addition, the meso-scale level motif analysis implies the potential existence of indirect interactions undetected by macro-scale network indices (i.e. nestedness, connectance) and lost at species-level indices such as species strength (Simmons et al. 2019a). The detection of indirect interactions in network analysis is essential given their great influence on co-extinction, co-evolution, and co-existence processes in communities (Aschehoug and Callaway 2015, Guimarães et al. 2017, Pires et al. 2020).

**Is the tripartite mutualism fundamental to maintaining the structure and diversity of mutualistic networks?**

To test this, we compared the network complexity and the ecological importance of the hummingbird-mistletoe-marsupial mutualism between intact forest sites with and without this interaction.

**Network Complexity** - Network structure is influenced by the number of motifs and their frequency (Simmons et al. 2019b). Networks composed by a greater number of different motifs would result in more complex structures because they harbor more different direct and indirect ecological interactions (Milo et al. 2002, Simmons et al. 2019a, Jácome-Flores et al. 2019). Likewise, network complexity increases with the frequency of the largest motifs (those composed of six species) because they involve more interactions than smaller motifs (Appendix S3: Figure S2). To estimate network complexity, we described the network structure using the frequencies of motifs containing between two and six species, resulting in 44 possible motif combinations. We calculated motif frequencies for each pollination and seed dispersal network using the “bmotif” package of R software (R Core Team 2019, Simmons et al. 2019b). We normalized motif counts to control for network size. We calculated motif frequency using the method “normalize_sum”, which expresses counts as the proportion of motifs in the network and considers whether species are more involved in smaller or larger motifs (Simmons et al. 2019b).

To assess dissimilarity in motif frequencies between tripartite sites, we used a non-parametric permutational multivariate analysis of variance (PERMANOVA). This method allows comparing dissimilarity among and within groups using a pseudo F-statistic (Anderson 2001). We used Bray-Curtis dissimilarity to quantify differences among network structures because it is a robust measure of dissimilarity for multiple ecological properties, including motifs (Anderson 2001, Baker et al. 2015, Simmons et al. 2019a). We conducted a PERMANOVA test considering tripartite sites as fixed
factor, stratified by site to account for repeated measures. We performed PERMANOVA with the `adonis2` function of the vegan package (Oksanen et al. 2012) of R software (R Core Team 2019), and using 9999 permutations to generate the null distribution. Finally, we validated the results of PERMANOVA test by estimating dispersion in the data using the `betadisp` function of the vegan package and testing with one-way ANOVA whether dispersion varied between tripartite sites using the `lme4` of R software (Bates et al. 2015). We found no significant differences between dispersion values, which indicates that PERMANOVA results are not caused by heterogeneous dispersion of the data.

**Ecological importance of the hummingbird-mistletoe-marsupial interaction members** - As a consequence of the close relationship among hummingbird-mistletoe-marsupial, the reduced abundance or alteration of behavior of any of them could disrupt this tripartite mutualism. Therefore, we expected that the ecological importance (i.e., a combination of interactions number and motif positions occupied) of these species change between intact forest sites.

First, for each member of the tripartite mutualism, we checked if the number of interactions and the number of species interacting with them differed between tripartite sites. For the hummingbird and marsupial, we built two regression models using the above variables as responses and tripartite sites as fixed factor. For the mistletoe, we built three regression models because it is involved in pollination and seed dispersal interactions. We included “Site” as a random factor and used Poisson or negative binomial as response variable distribution in the models.

Second, we calculated the number of positions occupied by the hummingbird, mistletoe and marsupial using the `bmotif` package. As motif positions represent different direct and indirect effects and have different ecological meanings (Baker et al. 2015, Simmons et al. 2019a), the greater the variety of positions a species occupies, the greater its participation in different paths regulating the structure and functionality of the network (Sun et al. 2020). The number of positions occupied by the hummingbird and marsupial were calculated from pollination and seed dispersal networks respectively, while the number of positions occupied by the mistletoe was calculated from both networks. We normalized the data using the method “sum” to control the tendency that nodes (e.g. species) with more interactions will occupy more positions than nodes with fewer interactions. This method expresses position counts as the proportion of total occurrences a node occurs at any position.
Additionally, to check the difference between tripartite sites we built regression models for each species with number of positions as response variable and tripartite sites as fixed factor. We used Poisson or negative binomial as response variable distribution and included “Site” as a random factor in the models.

**Does the disruption of this mutualism by non-native ungulates alter mutualistic network structure?**

To test if non-native ungulates produce cascading effects on the community by disrupting the tripartite mutualism, we compared network complexity and the ecological importance of tripartite mutualism members between invasion sites. Network complexity and the ecological importance of tripartite mutualism members were estimated using the same methodology explained above.

**RESULTS**

Overall, the number of species in pollination and seed dispersal networks was 2× and 1.3× higher in intact forest sites with the tripartite mutualism than in intact forest sites without the tripartite mutualism (Table 1; $z = 5.88, P < 0.001$; $z = 1.07, P = 0.285$, respectively) and at least 1.6× and 1.5× higher than in sites invaded by non-native ungulates (Table 1; $z = -5.11, P < 0.001$; $z = -1.736, P = 0.082$, respectively). In addition, we recorded 3.8× and 1.7× more pollination and seed dispersal interactions in intact forest sites with the tripartite mutualism than in intact forest sites without the tripartite mutualism ($z = 5.16, P < 0.001$; $z = 5.72, P < 0.001$, respectively) and almost 2× more interactions than in invaded sites (Table 1; $z = -2.94, P < 0.05$; $z = -2.45, P < 0.05$, respectively). Of the 5101 total pollination interactions, most of them (56%) were recorded in intact forest sites with the tripartite mutualism, while only 15% were recorded in intact forest sites without the tripartite mutualism, and 29% in invaded sites. Similarly, most of the total 2482 seed dispersal interactions were recorded in sites with the tripartite mutualism (51%), compared to 22% in sites without the tripartite mutualism, and 27% in invaded sites. In addition, we recorded 3.1× and 1.5× more unique pollination and seed dispersal interactions in sites with the tripartite mutualism than in sites without it ($z = 6.47, P = < 0.001$; $z = 1.45, P = 0.147$, respectively) and at least 2.1× and 1.9× more unique interactions than in invaded sites (Table 1; $z = -4.81, P = < 0.001$; $z = -2.39, P = 0.016$, respectively; Fig. 1).
Is the tripartite mutualism fundamental to maintaining the structure and diversity of mutualistic networks?

Network complexity - We found different complexity for both pollination (pseudo-F$_{1,7}$ = 7.021, P = 0.029) and seed dispersal (pseudo-F$_{1,6}$ = 4.794, P = 0.028) networks between tripartite sites. We found that in sites with the tripartite mutualism both pollination and seed dispersal networks were composed of 35% and 47% more different motifs respectively, and had greater direct and indirect ecological interactions than networks in sites without this interaction (Fig. 2a and 2c). In addition, both pollination and seed dispersal networks had 29% and 12% greater frequency of largest motifs including six species in sites with the tripartite mutualism than in sites without it (Fig. 2b and 2d), which suggests a greater proportion of complex interaction motifs supporting these networks.

Ecological importance of the tripartite mutualism members – We found differences in the number of interactions of the mistletoe, number of species interacting with it and its number of positions occupied between tripartite sites in pollination networks (z = 6.923, P < 0.001; z = 2.17, P < 0.05; z = 3.452, P < 0.001, respectively). In seed dispersal networks, the number of interactions of the mistletoe (z = 3.21, P < 0.001) but not its number of positions occupied (z = 1.154, P = 0.248) differed between tripartite sites. In sites with the tripartite mutualism, the mistletoe had 9.6× and 17.5× greater number of pollination and seed dispersal interactions (Fig. 3a), interacted with 3× more number of species (3.75 ± 0.63, 8 ± 1.3% of all species) and occupied 10× and 1.76× more number of pollination and seed dispersal positions (Fig. 3b) than in sites without this tripartite mutualism (1.25 ± 0.5 species, 5 ± 1.8% of all species). The hummingbird recorded 11.9× greater number of interactions (Fig. 3a) and occupied 29× more number of positions (Fig. 3b) in sites with the tripartite mutualism than in sites without it (z = 6.810, P < 0.001; z = 4.270, P = < 0.001). We found no differences in the number of species interacting with the hummingbird between tripartite sites (1.75 ± 0.50 species, 10 ± 3.3% of all species in sites with the tripartite mutualism; 1.25 ± 0.50 species, 7 ± 2.4% of all species in sites without the tripartite mutualism; z = 1.228, P= 0.220). In addition, the marsupial was involved in 15× more interactions in sites with the tripartite mutualism (Fig. 3a; z = 2.958, P < 0.05), but did not differ in the number of species which it interacted (3.5 ± 0.60, 52 ± 11.9% of all species in sites with the
tripartite mutualism; 1.5 ± 0.6 species, 39.5 ± 15.7% of all species in sites without the tripartite mutualism; z = 1.736, P = 0.082) and its number of positions occupied between tripartite sites (Fig. 3b; z = 1.480, P = 0.139). These results suggest a greater ecological importance of the mistletoe and hummingbird in presence of the tripartite mutualism because they exert more direct and indirect ecological interactions.

**FIGURE 3 HERE**

**Does the disruption of this mutualism by non-native ungulates alter mutualistic network structure?**

*Network complexity* - We found differences on network complexity in pollination networks (pseudo- $F_{1,7} = 5.032, P = 0.027$) but only marginal in seed dispersal networks (pseudo-$F_{1,7} = 3.849, P = 0.094$) between invasion sites. The complexity on both pollination and seed dispersal networks was lower in invaded sites than in sites with the tripartite mutualism, exhibiting 23% and 59% fewer motifs respectively, and hence, fewer direct and indirect ecological interactions supporting the community (Fig. 4a and 4c). In addition, both pollination and seed dispersal networks exhibit 12% and 11% fewer frequency of largest motifs including six species in invaded sites than in sites with the tripartite mutualism (Fig 4b and 4d).

**FIGURE 4 HERE**

**Ecological importance of the tripartite mutualism members** – We found that the number of interactions of the mistletoe and its number of positions occupied differed between invasion sites in pollination networks ($z = -5.55, P < 0.001; z = -2.998, P = 0.002$) but not in the number of species which it interacted (1.5 ± 0.3 species, 5.25 ± 1.3% of all species in invaded sites; $z = 1.897, P = 0.057$). In sites with the tripartite mutualism, the mistletoe had 3.4× more pollination interactions (Fig. 3c) and occupied at least 3.5× more positions (Fig. 3d) than in invaded sites, suggesting a greater ecological importance of this species in pollination networks. In addition, the hummingbird had 1.4× more number of interactions in sites with the tripartite mutualism than in invaded sites (Fig. 3c; $z = -1.66, P = 0.095$). The number of species interacting with the hummingbird (2 ± 1 species, 17.5 ± 1% of all species in invaded sites) and its number of positions occupied did not change between invasion sites (Fig. 3d; $z = 0.258, P = 0.796; z = -0.891, P = 0.373$). Furthermore, we caught no individuals of *D. gliroides* in any field season in the invaded sites, which indicates that the marsupial may be
ecologically extinct in these sites (Rodriguez-Cabal and Branch 2011). Consequently, we were unable to assess statistically the ecological importance of the marsupial and mistletoe in seed dispersal networks between invasion sites.

**DISCUSSION**

Ecosystems worldwide are gaining and losing species, thus understanding how both processes simultaneously affect ecosystem functioning is crucial to determining the response of these systems to current and future human activities. Our results suggest that the hummingbird-mistletoe-marsupial mutualism has the potential to enhance the structural diversity, species diversity and interactions diversity of pollination and seed dispersal networks in the temperate forests of Patagonia due to the key ecological role played by its members. Moreover, we found that gains of non-native ungulates and the loss of a tripartite mutualism might result in diverse direct and indirect effects that lead to the node-by-node disassembly of pollination and seed dispersal networks in the temperate forest of Patagonia. We suggest that the loss of keystone mutualisms due to species invasions can trigger cascades that result in species coextinctions and the disassembly of mutualistic networks. Finally, our findings emphasize how long-term plans to conserve biodiversity must focus not only in maintaining the elements that form biodiversity but also on the interactions among these elements, such as interspecific interactions, trophic webs, and ecological networks (Memmott et al. 2007).

**Is the tripartite mutualism fundamental to maintaining the structure and diversity of mutualistic networks?**

Both pollination and seed dispersal networks were more complex in intact forest sites with the tripartite mutualism than in forest sites without the mutualism. The diversity of motifs and the frequency of largest motifs suggest that networks are more complex in the presence of the hummingbird-mistletoe-marsupial interaction, with a wider range and a greater number of direct and indirect interactions (Milo et al. 2002, Simmons et al. 2019b). This tripartite mutualism not only increased the species richness of the community but also the high diversity of interactions types found may enhance the propagation of cascading effects on the network, making species more vulnerable to perturbations (Vieira and Almeida-Neto 2015, Pires et al. 2020). In addition to affecting species persistence and community stability, the diversity of interactions may influence evolutionary
processes. Ecological direct and mainly indirect interactions play an essential role on driving the selection of traits in multiple partner mutualism such as pollination and seed dispersal interactions (Guimarães et al. 2011, 2017). In intact forest sites with the hummingbird-mistletoe-marsupial mutualism, the high number of indirect pathways is likely to result in slower rates of trait evolution than in sites without this mutualism (Guimarães et al. 2011, 2017). Therefore, species in intact forest sites with the tripartite mutualism might have a lower adaptive capacity in terms of their interaction-relevant traits and, thus, might be more vulnerable to rapid environmental change than those in sites without the tripartite mutualism. On the other hand, the low vulnerability of sites without the tripartite mutualism might reflect the local extinction of the most susceptible species, with the remaining species representing the core of the network (Aizen et al. 2012).

When the tripartite mutualism is present, the mistletoe and hummingbird are key members of the community by occupying a high number of positions and exerting great direct and indirect interactions. The increase in their ecological importance may be induced by the spatial distribution of the mistletoe and the co-evolutionary history between them. Since the mistletoe population has a clumped distribution (García et al. 2009, Sasal et al. 2021), the high mistletoe abundance in certain patches may increase its probability of interacting directly and indirectly with other species (Vázquez et al. 2009). Moreover, the mistletoe-hummingbird interaction is more exclusive and specialized in winter because mistletoe flowers are the only nectar resource available for the hummingbird, promoting its permanence in patches with high mistletoe abundance (Smith-Ramírez 1993, Rodríguez-Cabal et al. 2013). On the contrary, the seed dispersal role of the marsupial was similar in sites with and without tripartite mutualism despite the differences in its number of interactions. Its ample diet of insects, bird eggs, insects and fleshy fruits (Fontúrbel et al. 2012) may allow the marsupial to sustain a stable population without consuming large amount of mistletoe fruits in sites with low mistletoe density. However, when minimum habitat requirements are met, marsupial abundance tracked the abundance of mistletoe plants, increasing the number of interactions between both species (Rodríguez-Cabal and Branch 2011). Indeed, the presence of the marsupial and mistletoe in these sites may be a potential source of the mistletoe contagious distribution (García et al. 2009, Amico et al. 2011).

**Does the disruption of this mutualism by non-native ungulates alter mutualistic network structure?**
We found that non-native ungulates produce cascading effects on pollination networks and marginal impacts on seed dispersal networks by disrupting the tripartite mutualism. Our results indicate that by preferentially browsing on *A. chilensis*, non-native ungulates indirectly affect the persistence of the mistletoe causing the local extinction of marsupials, which created a negative feedback loop preventing mistletoe recruitment (Appendix S1: Table S2), thereby reducing its ecological importance in pollination and seed dispersal networks. Although we found no significant reduction on the ecological importance of the hummingbird in invaded sites, the absence of regeneration of the mistletoe population could undoubtedly affect the permanence of this migratory bird in the future.

Pollination networks were simpler in invaded sites than in sites with the tripartite mutualism, presumably because these sites support fewer direct and indirect interactions. For instance, the total erosion of the motifs number “17” and “44” from the networks, which involve pollinator species visiting four and five plant species respectively, indicates a potential reduction of generalist pollinators and a potential reduction of competition among specialist plants in invaded sites. Moreover, the low interactions diversity found in invaded sites concentrates the energy flow in few pathways, which may increase the persistence and stability of pollination networks to strong perturbations by reducing the propagation of cascading effects (Vieira and Almeida-Neto 2015, Pires et al. 2020). The high stability of pollination networks to perturbations in invaded sites might arise because species richness could have previously been reduced due to the activity of non-native ungulates and only an impoverished subset of core generalist species remains in invaded sites.

Similarly, the simplification of ecological networks has been demonstrated for others human disturbances (Tylianakis et al. 2007, Galiana et al. 2014). On the other hand, we found no cascading effects on seed dispersal networks despite the high vulnerability of this forest to the loss of mutualistic interactions. The impact of non-native ungulates on seed dispersal networks may be attenuated by the strong presence of the most generalist bird seed disperser of this forest, *Elaenia albiceps* (Amico and Aizen 2005). This migratory bird is abundant in summer, overlapping with fruit ripening and playing a critical role in forest regeneration (Bravo et al. 2015). However, the marsupial consumes fruits that cannot be detected or ingested by birds due to their color or the size of their seeds and even increases the germination rate of plants that are consumed by both taxa (Amico et al. 2009, Amico et al. 2011).
Considering the intermediate values of complexity found in invaded sites compared to intact forest sites with and without the tripartite mutualism, we can conclude that non-native ungulates are homogenizing the forest toward the simplest state. However, the natural state of the forest is historically related to the clumped distribution of the mistletoe, therefore, high and low complexity forest states would be the natural pattern without a disturbance scenario. Our results show that non-native ungulates erode the pollination and seed dispersal networks by disrupting the hummingbird-mistletoe-marsupial mutualism, however their impacts on simpler forest patches without this tripartite mutualism remains unknown.

To the best of our knowledge this is the first study evaluating the impacts of invasive species on mutualistic interactions by combining two ecological processes using network approach and detecting potential indirect effects. However, the accuracy and reliability of the statistical analysis could be improved with a larger set of networks. Similarly, other studies considering different ecological interactions account for a low set of networks (Correia et al. 2018, Lima et al. 2020). Unfortunately, collecting the data necessary to represent the community at a network scale for two ecological processes is a high-effort task.

Here, we demonstrate the key role of a hummingbird-mistletoe-marsupial mutualism in Patagonian temperate forest. Recognizing keystone mutualisms is essential to achieve the conservation of communities due to their role in maintaining the structure and functionality of them, especially given current rates of global biodiversity loss and spread of invasive species (Díaz et al. 2020). Future studies are needed to understand how the effects of keystone mutualisms on the complexity of mutualistic networks affects other community properties. For instance, this tripartite mutualism may promote the community resistance against the impact of non-native pollinators by increasing the potential partners with whom native pollinators reassign interactions (Kaiser-Bunbury et al. 2010). Moreover, incorporating indirect effects in ecological studies is necessary to increase the realism of global changes impacts on communities, widely underestimated when direct effects are only considered.

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REFERENCES


Amico, G. C., M. A. Rodríguez-Cabal, and M. A. Aizen. 2011. Geographic variation in fruit colour is
associated with contrasting seed disperser assemblages in a South-Andean mistletoe. Ecography 34:318–326.


Simmons, B. I., A. R. Cirtwill, N. J. Baker, H. S. Wauchope, L. V. Dicks, D. B. Stouffer, and W. J.


Table 1. Pollination and seed dispersal network parameters for each site.

<table>
<thead>
<tr>
<th>Site</th>
<th>Network</th>
<th>Variable</th>
<th>Values (mean ± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact forest</td>
<td>Pollination</td>
<td>Nº of species</td>
<td>67 ± 4.5</td>
</tr>
<tr>
<td>with the tripartite</td>
<td></td>
<td>(21 ± 2.4 plants, 46 ± 3.1 pol.)</td>
<td></td>
</tr>
<tr>
<td>mutualism</td>
<td></td>
<td>Nº of interactions</td>
<td>708.5 ± 119.8</td>
</tr>
<tr>
<td>Seed dispersal</td>
<td></td>
<td>Nº of unique interactions</td>
<td>107.3 ± 11.3</td>
</tr>
<tr>
<td>Intact forest</td>
<td>Pollination</td>
<td>Nº of species</td>
<td>34.25 ± 8.6</td>
</tr>
<tr>
<td>without the tripartite</td>
<td></td>
<td>(9.8 ± 1 plants, 24.5 ± 2.9 pol.)</td>
<td></td>
</tr>
<tr>
<td>mutualism</td>
<td></td>
<td>Nº of interactions</td>
<td>187.5 ± 39.3</td>
</tr>
<tr>
<td>Seed dispersal</td>
<td></td>
<td>Nº of unique interactions</td>
<td>34.3 ± 6.2</td>
</tr>
<tr>
<td>Invaded sites</td>
<td>Pollination</td>
<td>Nº of species</td>
<td>40.25 ± 2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(11.5 ± 0.6 plants, 28.8 ± 2.3 pol.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nº of interactions</td>
<td>379.3 ± 62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nº of unique interactions</td>
<td>50.5 ± 5.6</td>
</tr>
<tr>
<td>Seed dispersal</td>
<td></td>
<td>Nº of species</td>
<td>6.25 ± 1.3</td>
</tr>
</tbody>
</table>
(4 ± 0.3 plants, 2.25 ± 0.6 seed disp.)

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nº of interactions</td>
<td>169 ± 33</td>
</tr>
<tr>
<td>Nº of unique interactions</td>
<td>5.25 ± 1.3</td>
</tr>
</tbody>
</table>
FIGURES

Figure 1. Sites with the tripartite mutualism recorded more individual interactions in pollination and seed dispersal networks. Sites from top to bottom: intact forest sites with the tripartite mutualism (a-b), intact forest sites without the tripartite mutualism (c-d), and sites invaded by non-native ungulates (e-f). Left and right panels correspond to pollination and seed dispersal networks, respectively. In each panel, boxes represent species of plants (green), pollinators (red), and seed dispersers (blue). Lines represent ecological interactions between species. Box width is proportional to number of interactions.

Figure 2. The complexity of pollination and seed dispersal networks was greater in intact forest sites with the tripartite mutualism. Number and relative frequency of motifs related to their complexity (number of nodes-species- per motif) for both pollination (a and b) and seed dispersal (c and d) networks. From left to right: motifs integrated by two, three, four, five, and six species. Bar color represents sites: intact forest sites with (violet) and without (white) the tripartite mutualism. Bars represent means ± standard error.

Figure 3. Ecological importance - number of interactions and number of motif positions occupied in the network- of the mistletoe, hummingbird and marsupial were greater in intact forest sites with the tripartite mutualism than in sites without it (a-b) and sites invaded by non-native ungulates (c-d). Bar color represents sites: intact forest sites with (violet) and without (white) the tripartite mutualism and invaded sites by non-native ungulates (orange). From left to right: mistletoe (pollination and seed dispersal), hummingbird, and marsupial. Letters mean significant difference between sites. Bars represent means ± standard error.

Figure 4. Complexity of pollination and seed dispersal networks was greater in intact forest sites with the tripartite mutualism than in sites invaded by non-native ungulates. Number and relative frequency of motifs related to their complexity (number of nodes-species- per motif) for both pollination (a-b) and seed dispersal (c-d) networks. From left to right: motifs integrated by two, three,
four, five, and six species. Bar color represents sites: intact forest sites with the tripartite mutualism (violet) and sites invaded by non-native ungulates (orange). Bar represent means ± standard error.