

Evolutionary Responses of A Dominant Plant Along a Successional Gradient in A Salt-Marsh System

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Abstract

The ecological responses of plant populations along the successional gradient have been intensively examined; however, the evolutionary responses remain to be elucidated. Here, I explored genetic changes of key phenotypic traits of a dominant plant along a successional gradient, and whether these changes were induced by abiotic and biotic variables. I measured key abiotic (e.g. clay thickness) and biotic variables (e.g. herbivore density) along the successional gradient in the high and low marsh in a Wadden Sea saltmarsh. Also, I collected samples of *Elytrigia atherica*, grew them in the greenhouse, and measured key functional traits. I found that clay thickness (a proxy of total nitrogen) increased along the successional gradient both in the high and low marsh; herbivore density from hares (the most important herbivores) decreased along the successional gradient in the high marsh. Also, I found that growth in number of leaves and ramets decreased, while rhizome length increased, along the successional gradient for *E. atherica* collected from the high marsh. Opposite trends were found for *E. atherica* collected from the low marsh. Results suggest that, in the high marsh, herbivores may overrule nutrients to drive trait changes. That is, at early successional stages, *E. atherica* had higher growth in number of leaves and ramets to compensate for high-density grazing. In the low marsh, nutrients were the dominant driver for trait changes. These results suggest that ecologically important abiotic and biotic variables such as nutrients and herbivores may also have a substantial evolutionary impact on plant populations.

Introduction

The ecological responses of plant populations and communities along the successional gradient have been intensively examined (Bazzaz 1979; Tilman 1987; Olff et al. 1997; Praclr 1997; Loranger et al. 2016; Schrama et al. 2017). For instance, vegetation height usually increases along the successional gradient (Tilman 1987; Praclr 1997). However, the evolutionary responses of plant populations along the successional gradient remains underexplored. Plant populations may become genetically different under different selection pressures, and the resulting variation in phenotypic traits among populations can be evaluated by growing their offspring in a common environment (Billington et al. 1988). Studies show that plant populations can rapidly (years to decades) adapt to different abiotic and biotic environments (Billington et al. 1988; Shaw & Etersson 2012; Didiano et al. 2014; Völler et al. 2017). Adaptive evolution in plant populations can potentially alter species interactions, impact community structure, and ecosystem functioning (Hughes et al. 2008; Whitlock et al. 2010; Garfinkel & Rawls 2011; Aspinwall et al. 2013). Therefore, it is important to understand the evolutionary responses of plant populations along the successional gradient.

Many abiotic and biotic variables change along the successional gradient. For instance, nutrients, especially nitrogen, usually increases along the successional gradient (Tilman 1987; Olff et al. 1997). Increased nitrogen usually leads to species replacement, with nitrogen-loving plants becoming dominant at the late successional stages. However, in many cases, these late successional plants also occur (but not abundant) at early successional stages (Olff et al. 1997). As increased nutrients usually shift the importance of competition from belowground to aboveground (Hautier et al. 2009), late stage

populations may evolve to have a higher growth rate, higher photosynthesis capacity, and larger biomass. Indeed, Fischer et al. (2011) found that *Poa alpina* collected from fertilized grasslands had larger biomass than those collected from unfertilized grasslands when grown in a common garden. However, others found that plants are not responsive to nutrients (fertilization) (e.g. Völler et al. 2017). Generally, few studies have investigated how plant populations adapt to different levels of nutrients, which makes it difficult to draw any solid conclusions (Pluess 2013).

Herbivore density may also change along the successional gradient due to changes in forage plants and their abundances (Chen et al. 2019), which in turn can be an important driver for evolutionary changes in plant populations. So far, studies exploring the evolutionary impact of herbivores on plant populations yield inconsistent results. For instance, some studies show that herbivores drive genetic differentiation (Carman & Briske 1985; Kleijn & Steinger 2002; Veeneklaas et al. 2011; Turley et al. 2013), while others show that the evolutionary impact was not apparent (Pluess 2013). Several reasons may cause this inconsistency. First, different plant species may respond differently to herbivores. For instance, Didiano et al. (2014) found that only one out of four studied plant species (*Festuca rubra*, the most abundant species) showed the evolutionary responses (trait differentiation in leaf numbers) in a long-term (> 20 years) rabbit enclosure experiment in Silwood Park, England. Second, given that herbivore density plays an important role in regulating plant communities ecologically (Wang & Tang 2019), their evolutionary effects may also only become apparent when herbivore density is high. However, to my knowledge, the evolutionary responses of plant populations to herbivore density along the successional gradient has not been studied.

Elytrigia atherica (synonym *Elymus athericus*) is a widespread European saltmarsh grass, particularly dominates in the high marsh. This grass has strongly expanded to the lower and younger marshes (Pétillon et al. 2005; Milotić et al. 2010; Veeneklaas et al. 2013; Wanner et al. 2014; Rupprecht et al. 2015; Van Klink et al. 2016). As a result, it has led to a decline in plant diversity in saltmarshes across Europe (Esselink 2017). Investigating the evolutionary responses of this grass along the successional gradient would improve our understanding of the underlying mechanisms for its rapid expansion, which may shed light on maintaining higher biodiversity and ecosystem functioning in many European saltmarshes. However, so far, the evolutionary responses of this grass are unknown.

A well-calibrated natural successional gradient is present in the saltmarsh of the island of Schiermoonikoog (Olf et al. 1997). Both clay thickness (a good proxy of total nitrogen) and herbivore density vary along the successional gradient (Olf et al. 1997; Kuijper & Bakker 2005; Schrama et al. 2012, 2017; Chen et al. 2019). The ecological responses of plant populations and communities along this successional gradient has been examined intensively. For instance, *E. atherica* increases in dominance along the successional gradient, while plant diversity peaks at the intermediate stages (Schrama et al. 2017). Many processes are different in the high and low marsh such as inundation frequency in the low marsh can be 2–5 times higher than that in the high marsh (Bockelmann et al. 2003). Therefore, I expected that changes in nutrients and herbivore density along the successional gradient would be

different in the high and low marsh. Subsequently, the evolutionary responses of *E. atherica* along the successional gradient would be different in the high and low marsh.

Materials And Methods

Study site

A natural successional gradient, spanning 120 years, is present in the saltmarsh of the island of Schiermonnikoog (53°30' N, 6°10' E), the Netherlands. The eastern part of the island is younger than the western part, naturally separated by creeks (Olf et al. 1997). Different successional stages are categorized based on the time of vegetation establishment by checking the aerial photograph (Olf et al. 1997) and justified by monitoring vegetation succession and key soil parameters in permanent plots at each successional stage (Schrama et al. 2017). Note, in this study, successional stages were counted till 2016 when the samples were taken. For instance, stage 20 in the eastern part of the saltmarsh indicates that vegetation was established in this area in 1996. The eastern part of the saltmarsh (the sampling area) is only grazed by small herbivores, including spring staging Brent Geese (*Branta bernicla*), Barnacle Geese (*Branta leucopsis*), year-round present Brown hares (*Lepus europaeus*), and rabbits (*Oryctolagus cuniculus*). Hares are the most important herbivores, while rabbits are rare in this system (Kuijper & Bakker 2005; Chen et al. 2019).

Field investigation of clay thickness and herbivore density

Clay thickness and herbivore density were measured in 2016 (Chen et al. 2019), the detailed procedure has been described elsewhere (Chen et al. 2019). Here I briefly describe it. A line transect was set up at successional stage 20, 30, 40, 60, and 120 in 2016. Each line transect consisted of 20 4-m² plots, with at least 10 m distance between one another (Fig. 1). Elevation of these 4-m² plots was measured using Trimble R8 (RD system; precision for elevation 1–2 cm). Following Olf et al. (1997), I refer the high marsh to elevation ≥ 1.6 m + NAP (Normal Amsterdam Water Level), while low marsh to elevation < 1.6 m + NAP. In total, 24 4-m² plots were recorded in the high marsh and 76 in the low marsh (4, 3, 6, 2, 9 in the high marsh, 16, 17, 14, 18, 11 in the low marsh at stage 20, 30, 40, 60, and 120, respectively). Clay thickness is a good indicator of soil total nitrogen (Olf et al. 1997) and was measured using a 2 cm \emptyset soil corer (n = 4).

Number of droppings is a good indicator of herbivore density and grazing pressure (Van Der Wal et al. 2000; Kuijper & Bakker 2005). Droppings from hares and geese were counted and removed within these 4-m² plots every two or three weeks for the whole year in 2016 (May 2016 to April 2017).

Sampling

In the high marsh, *E. atherica* forms continuous meadows, while in the low marsh, *E. athercia* mainly occurs in patches, particularly at the early successional stages. Samples were taken at similar elevation along the successional gradient in the high (1.73 ± 0.0189 ; m + NAP) and the low marsh (1.43 ± 0.009 ; m

+ NAP). In April - May 2016, 3 blocks were selected randomly (distance between blocks > 20 m) in the high and low marsh per stage. Within each block, 3 plots were selected (diameter ca. 0.1 m) and samples of *E. atherica* were collected. No samples were collected in the low marsh of stage 20 because there were too few individual stems of *E. atherica* present. Additional samples were taken in the low marsh of the intermediate and late stage (sampling location see Fig. 1). Genotype diversity is high in this grass, Chen (2020) shows that individual stems collected from different plots in most cases belong to different genotypes, and individual stems collected within the plots possibly are also different genotypes.

Greenhouse experiment

Individual stems of *E. atherica* collected from the field were standardized before planting. Individual stems were grown together to produce more ramets from May to August 2016 and to reduce carryover effects. These individual stems were grown in plastic pots (14.5 cm in diameter) filled with sand and watered with $\frac{1}{4}$ Hoagland solution 1–2 times per week.

On 7 August 2016, the aboveground parts of all pots were harvested. Individual stems were standardized again. 3–7 individual stems originated from each plot were randomly selected for this experiment. Each standardized sample was grown in the center of a plastic pot (9 × 9 × 10 cm; length × width × height) filled with cleaned sand. Pots were randomly arranged into trays (usually 24 pots per tray). One week after, dead samples (e.g. without new green shoots) were discarded; in total, 268 samples survived (sample sized see Table S1). Each pot received 50 ml $\frac{1}{4}$ Hoagland solution once per week. In addition, the water level was kept at 1 cm (in the trays) to make sure *E. atherica* did not suffer from drought. All the pots were rearranged every month to randomize their position in the trays and the greenhouse. The greenhouse was maintained at 17 °C (day) and 14 °C (night), a photosynthetic photon flux density (PPFD) of $439 \pm 6.9 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ for 12 h, and relative air humidity of 70%.

Leaf area and chlorophyll fluorescence To look at whether *E. atherica* adapted along the successional gradient in photosynthesis capacity, I measured the maximum length and width of the leaves in October 2016. I selected the third leaf (i.e. fully grown) from the top of a randomly chosen ramet for this measurement. Later, the same leaves were used for measuring chlorophyll fluorescence using the Portable Chlorophyll Fluorometer PAM-2100. The quantum efficiency (ΦPSII), qP, the maximum quantum efficiency of PSII (F_v/F_m), and the NPQ were measured. ΦPSII is the most useful parameter for the efficiency of Photosystem II. qP indicates the proportion of PSII reaction centers that are open. The maximum quantum efficiency of PSII indicates the quantum efficiency of the open photosystem II centers and is widely used as a reliable indicator of photoinhibition. The maximum quantum efficiency of PSII was measured on leaves dark-adapted for ca. 10 minutes using leaf-clips. NPQ measures change in heat dissipation relative to the dark-adapted state (Maxwell & Johnson 2000).

Phenotypic traits and biomass The height of the tallest ramet, the number of leaves, and the number of ramets were measured for each pot on 15 August, 19 August, 10 September, 22 September, 8 October, and 9 November 2016 (12, 16, 26, 38, 50, 67, and 98 days after planting). After measuring these on 9 November, all the aboveground parts were cut in each pot at the soil level. The length of each stolon (cm)

was measured. Similarly, rhizomes were separated from roots, and the length of each rhizome (cm) was measured. Shoots, roots, and rhizomes were dried in the oven (70 °C) to constant weight. Dried biomass was measured to the nearest 0.01 g. As stolons and rhizomes are structurally and functionally similar, I grouped them and referred them to “rhizomes” hereafter.

Data analysis

Clay thickness

To check whether clay thickness change along the successional gradient in the high and low marsh (marsh type), I fitted the following model: $\text{lm}(\text{clay thickness} \sim \text{marsh type} * \text{successional stage} + \text{marsh type} * (\text{successional stage}^2))$. Including a quadratic term significantly improved model fit than without it (judging by AIC). In 11 4-m² plots in the high marsh, I did not record a clay layer due to that those 4-m² plots were in the dune area. These 11 4-m² plots were not included in the analysis but were shown in the figure.

Droppings

For each 4-m² plot, hare and goose droppings from the whole year were summed up. I fitted the number of droppings from hares and geese separately. For hare droppings, I used generalized linear models (glm) with family of quasipoisson to account for overdispersion. This model is specified as $\text{glm}(\text{hare droppings} \sim \text{marsh type} * \text{successional stage})$. Including a quadratic term of successional stage did not significantly improve model fit (judging by AIC), so the it was not included in the final model. For goose droppings, because there were many zeros (goose droppings were not present in 13 of 100 4-m² plots), I fitted these data with a zero-inflated model using the function “zeroinfl” from the R package “pscl” (Zeileis et al. 2008). This model is specified as $\text{zeroinfl}(\text{goose droppings} \sim \text{marsh type} * \text{successional stage}, \text{dist}=\text{"poisson"})$.

Traits along the successional gradient

The growth in height, number of ramets, and number of leaves were the coefficients extracted from the generalized linear models (gls) fitting height, number of ramets, and number of leaves over time (12, 16, 26, 38, 50, 67, and 98 days after planting) for each sample. Specifically, these variables were fitted using the function “gls” from the R package “nlme” (Pinheiro et al. 2017), with an autocorrelation (corAR1) structure to account for the temporal dependence of the data. Leaf area was calculated as the leaf length times leaf width. Total biomass was the sum of shoot, root, and rhizome biomass. To reduce spatial dependence of the sampling, I averaged trait data across plots in each block at each successional stage (see Table S1 for sample size), I then fitted these averaged trait data in the models. The models were specified as $\text{lm}(\text{trait data} \sim \text{marsh type} * \text{successional stage})$. I also tried to include a quadratic term of successional stage, but including it did not improve model fit, therefore, the quadratic term was not included in the final model. Rhizome length was $\log(x + 1)$ and transformed before fitting the models to improve normality and homogeneity of variance. Effects were significant when $p < 0.1$. Data analysis was performed using R 3.5.3 (R Core Team 2020).

Results

Clay thickness

Clay thickness increased along the successional gradient both in the high and low marsh (Table S2; Fig. 2).

Droppings

Hare droppings decreased along the successional gradient in the high marsh, but did not change in the low marsh (significant interaction effects of successional stage and marsh type; Fig. 3A; Table S3). Goose droppings peaked at the intermediate stages in the high marsh, increased slightly along the successional gradient in the low marsh (significant interaction effects of successional stage and marsh type; Fig. 3B; Table S3).

Traits

The effects of successional stage, and its interaction with marsh type were not significant for nine of the twelve traits measured (Table S4). The interaction effects of successional stage and marsh type were significant on the growth in number of leaves and ramets and rhizome lengths (Table S4). Specifically, for *E. atherica* samples collected from the high marsh, the growth in number of leaves and ramets decreased, while the rhizome lengths increased along the successional gradient. In contrast, for *E. atherica* samples collected from the low marsh, the growth in number of leaves and ramets increased, while the rhizome lengths decreased along the successional gradient (Fig. 4).

Discussion

In the saltmarsh of the island of Schiermonikoog, I found that clay thickness increased while droppings, particularly from hares, decreased along the successional gradient in the high marsh. The growth in number of leaves and ramets of *Elytrigia atherica* collected from the high marsh decreased along the successional gradient. This may be a result of defense evolution against changing herbivore density (overruled nutrient availability). In the low marsh, herbivores density from hares did not change but clay thickness increased along the successional gradient. The growth in number of leaves and ramets increased. This may be a response to increased nutrient availability. Results indicate rapid evolution of *E. atherica* populations along the successional gradient.

Nutrients measured as clay thickness increased along the successional gradient both in the high and low marsh. Increased nutrients usually alleviate belowground competition, thus light competitors with higher shoots, larger biomass is likely to be promoted (Hautier et al. 2009). Indeed, such changes in traits were observed in *E. atherica* collected from the low marsh but not the high marsh. Possibly the effects of nutrients in the high marsh were overruled by the effects of herbivores. Similarly, Völler et al. (2017) found

that eight common grassland plants are least responsive to fertilization (nutrients) compared with herbivores and mowing in Germany.

Previous studies in this system found that hares and geese slowed down plant species decline via suppressing the expansion of the tall late successional grass, *E. atherica*, for at least 22 years at the early stage (Chen et al. 2019). Hares and geese do so via impeding survival of its seedlings in this system (Kuijper et al. 2004). In general, adult plants of *E. atherica* are not preferred by hares and geese, but seedlings are still consumed considerably (Kuijper et al. 2004; Fokkema et al. 2016). Although hares and geese tended to present more often in the low marsh (Fig. 3), herbivore density from hares did not change, while herbivore density from geese increased slightly along the successional gradient in the low marsh. In this system, hares play a more important role in structuring plant communities, because hares present all year round, while geese only stay for a few months (Kuijper & Bakker 2005). Therefore, although herbivore density from geese increased along the successional gradient in the low marsh, the effects from geese may not be strong. In the high marsh, hare droppings decreased along the successional gradient, while goose droppings were highest at the intermediate stages. Interestingly, the growth in number of leaves and ramets of *E. atherica* collected from the high marsh was highest at the early successional stages, which may be a result of the defense evolution against high herbivore density. McNaughton (1984) suggested that herbivores drive plants to evolve short, dense, and abundant leaves to prevent apical meristems from being damaged. Growing faster and more ramets can also be an effective way to compensate for biomass reduction under grazing (Van Der Graaf et al. 2005). Turley et al. (2013) showed that *Rumex acetosa* L. evolves a decreased growth rate in the absence of rabbit grazing using long-term herbivore exclosures from Silwood Park. Thus, the ecologically important driver (herbivory) can also have a substantial evolutionary impact on *E. atherica* in this system.

These observed patterns of trait changes along the successional gradient in the high and low marsh are likely due to changes in allele or genotype frequency. In this system, seedlings of *E. atherica* recruited via seeds were rarely observed in the field (Veeneklaas et al., 2011), suggesting that clonal growth was the primary reproduction mode. However, sexual reproduction may occur occasionally via windows of opportunity, which created genetic and genotype diversity. Indeed, studies in this system show that genotype diversity is high in this grass (Bockelmann et al. 2003; Veeneklaas et al. 2011). Increased genotype diversity can increase intraspecific trait variation (Evans et al. 2016), and thus may increase the potential for adaptation. The lifespan of *E. atherica* is unknown so far. However, De Witte and Stöcklin (2010) indicated that clonal grasses (genets) are usually long-lived plants (hundreds to thousands of years old), therefore adaptive evolution may also happen through clonal lineage selection (Didiano et al. 2014). Further, trait differentiation could also be due to epigenetic changes (Gáspár et al. 2019).

Of the twelve traits investigated, we only found three changed along the successional gradient, suggesting that different traits respond differently to nutrients and herbivores. Others also reached a similar conclusion (Völler et al. 2013; Didiano et al. 2014). Additionally, rhizome length changed in the opposite direction as that of the growth in number of leaves and ramets along the successional gradient in the high and low marsh. This could be a trade-off among traits. When plants allocate more biomass to

growth (leaves and ramets), they may allocate less to vegetative reproduction (via rhizomes). These results suggest that more traits should be investigated simultaneously when looking at the evolutionary responses of plant populations to abiotic and biotic environments.

In this study, combining field observation and a greenhouse experiment, I explored the evolutionary responses of a dominant grass along the successional gradient in which both nutrients and herbivore density varied. I found that the growth in number of leaves and ramets decreased along the successional gradient in the high marsh, which may be a result of defense evolution against changing herbivore density. In the low marsh, the growth in number of leaves and ramets increased along the successional gradient, which may be an evolutionary response to increased nutrients. These results suggest that ecologically important biotic and abiotic variables may also impose a substantial evolutionary impact on plant populations.

Declarations

Funding

Not applicable.

Conflicts of interest/Competing interests

The authors declare no conflict of interest.

Availability of data and material

Data will be deposited in Dryad Digital Repository once the manuscript gets accepted.

Code availability

R code of all analyses will be available in github once the manuscript gets accepted.

Credit author statement

QC conceived the ideas for this manuscript, completed the field, greenhouse, and laboratory work, completed the analyses, and wrote the manuscript.

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Figures

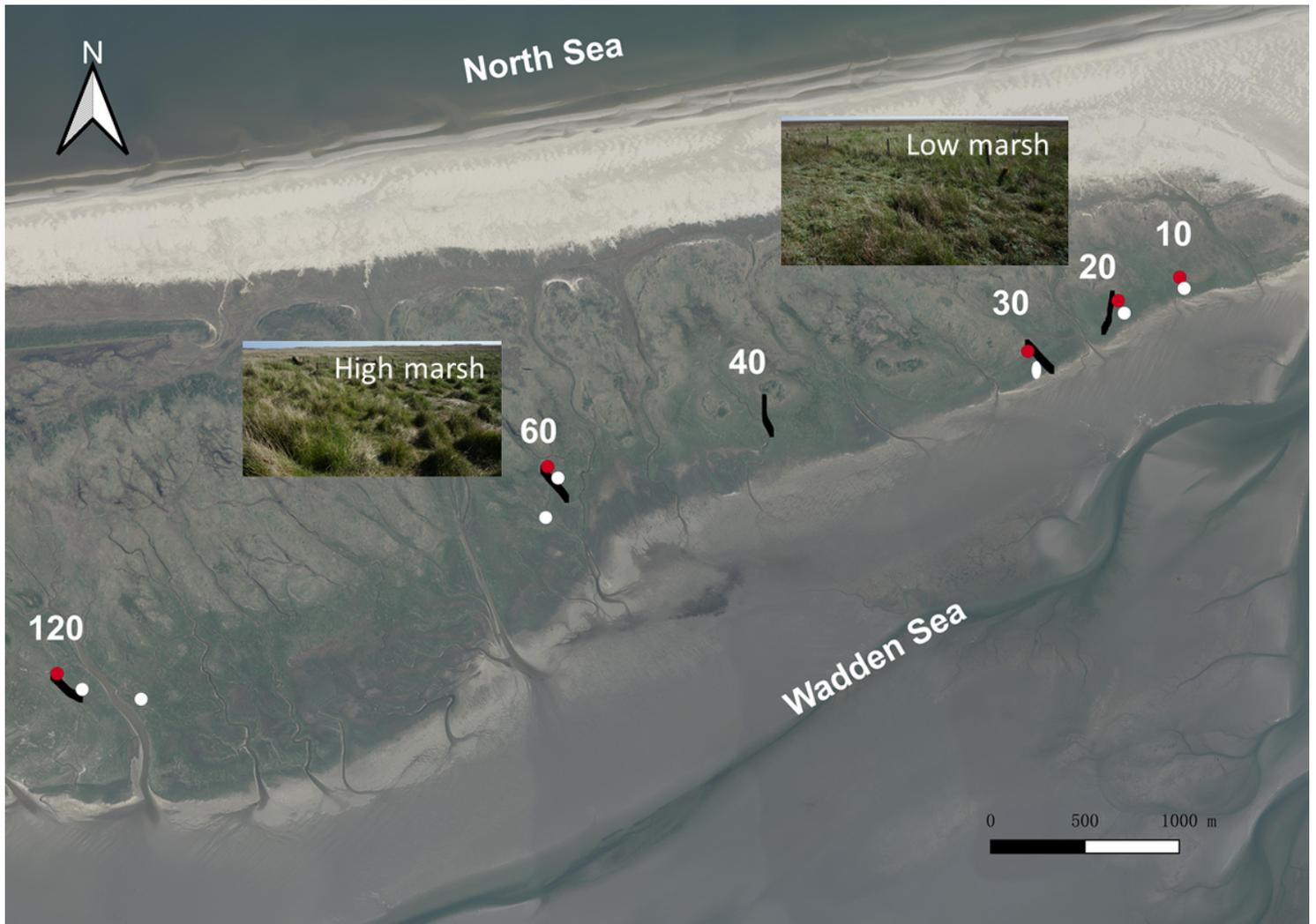


Figure 1

Sampling location along the successional gradient in the saltmarsh of the island of Schiermonnikoog, the Netherlands. Numbers of the estimated successional stages counted from the year vegetation established to 2016. Dots represent blocks, the red ones are in the high marsh, the white ones are in the low marsh, black lines are transects set up for evaluating characteristics along the successional gradient (see Fig. 2 and Fig. 3). A picture of the low marsh and high marsh at stage 20 and 60, respectively, are shown. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

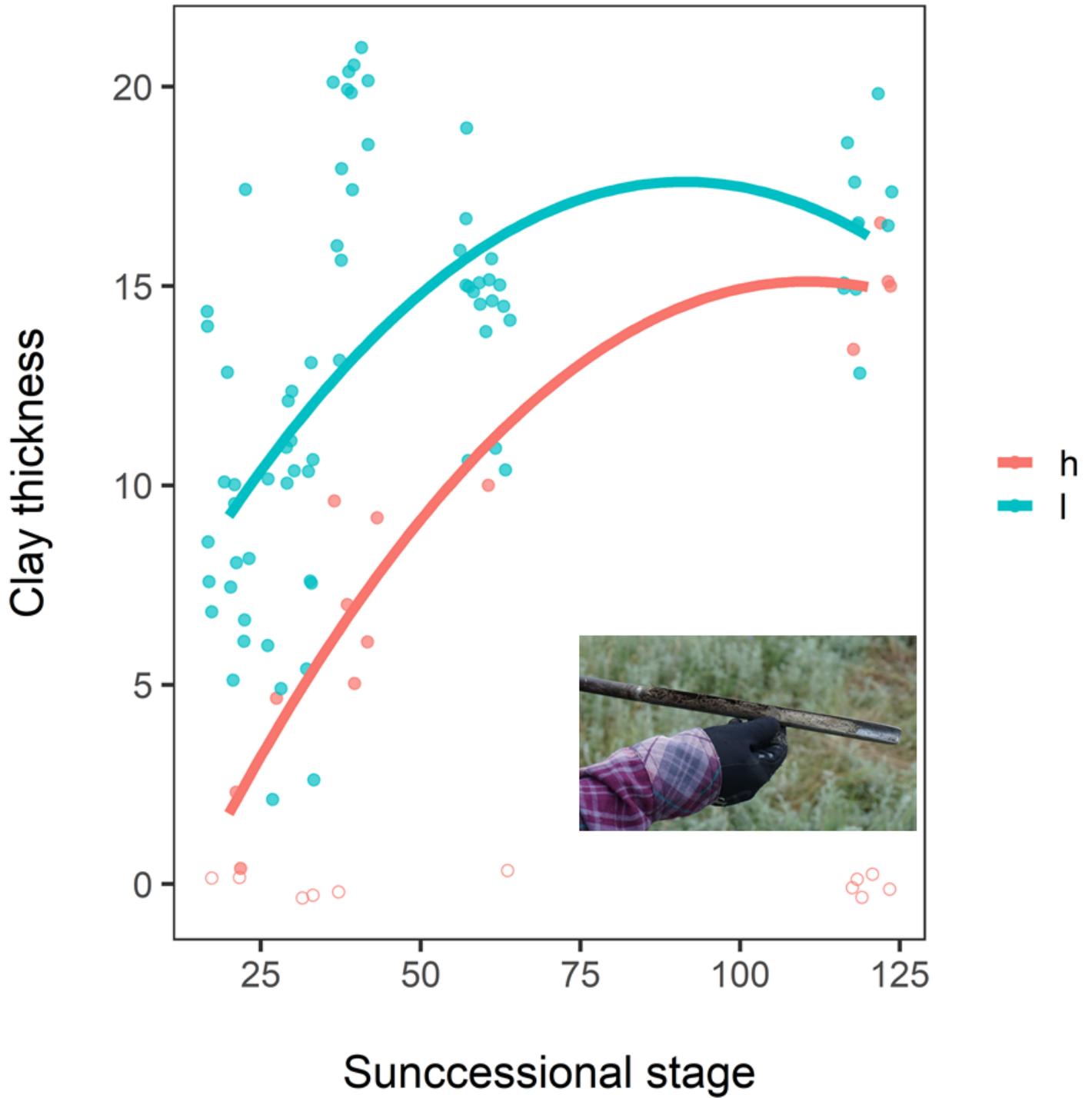


Figure 2

Clay thickness along the successional gradient in the high and low marsh. Dots are clay data in each 4-m² plot in the transect at each successional stage. Dots are jittered to prevent overlapping. Red open dots are 4-m² plots where no clay layer was recorded because they were in the dune area. These data were not included in the analysis. The model summary can be found in Table S2.

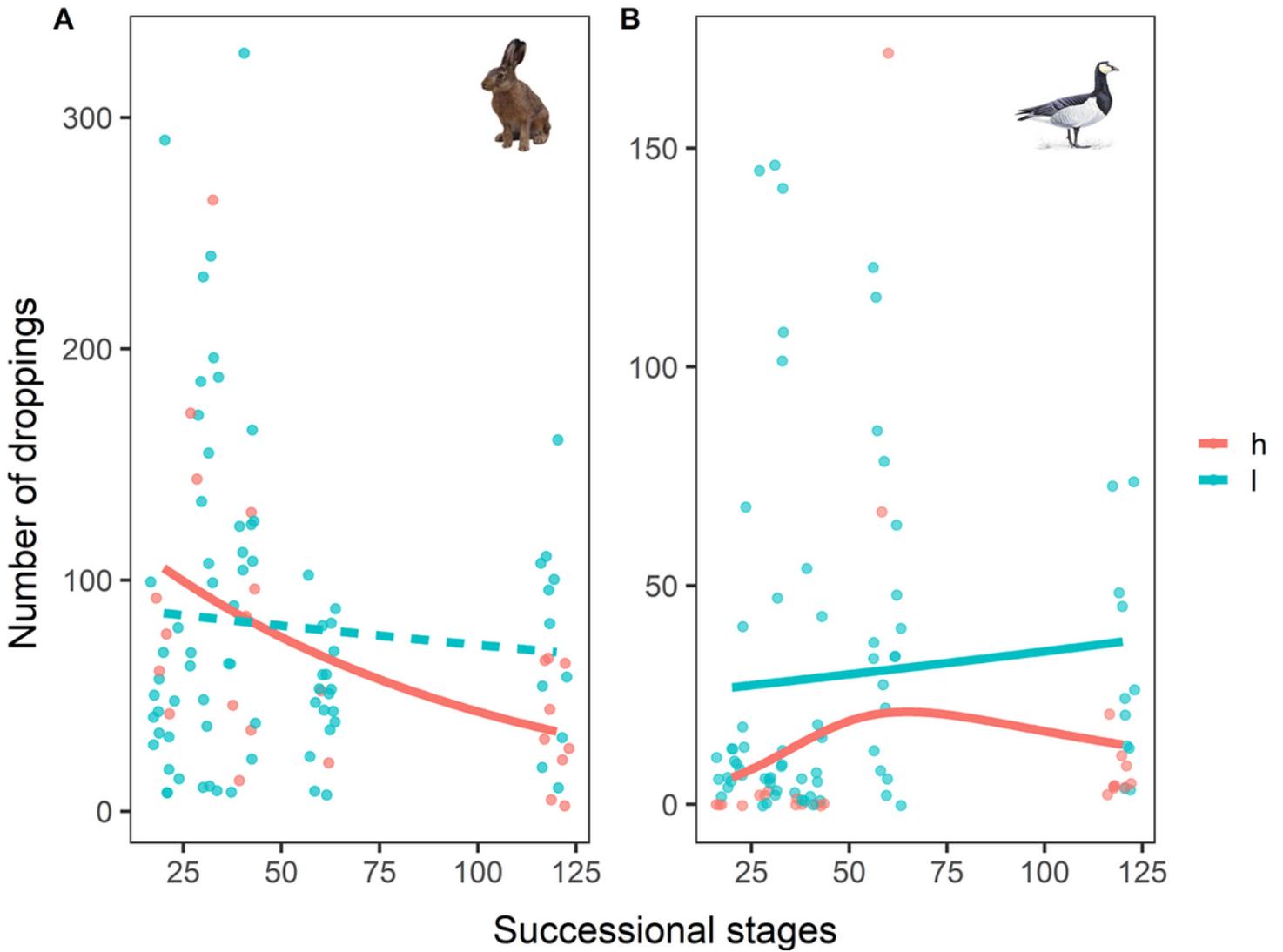


Figure 3

Hare (A) and goose droppings (B) along the successional gradient in the high and low marsh. Dots are summed whole year dropping data from each 4-m² plot in the transect at each successional stage. Dots are jittered to prevent overlapping. Solid lines represent significant effects of successional stage, dashed line represents non-significant effects of successional stage. The model summary can be found in Table S3.

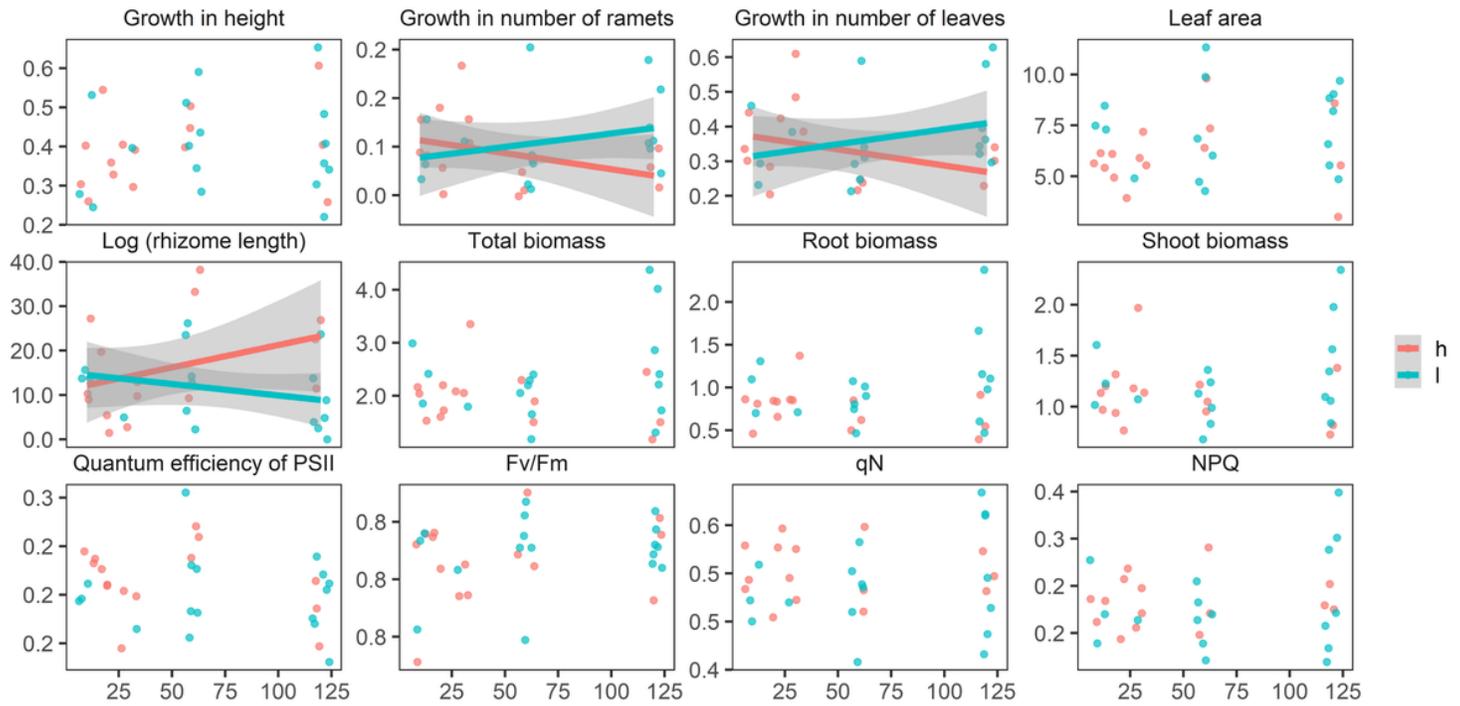


Figure 4

Traits of *Elytrigia atheria* collected from the high marsh and along the successional gradient. Dots are trait data in each block (averaged over plots) at each successional stage to reduce spatial dependence of the sampling. Dots are jittered to prevent overlapping. Detailed sample size can be found in Table S1. Lines show significant interaction effects of successional stage and marsh type (high and low; $p < 0.1$). The model summaries can be found in Table S4.

Supplementary Files

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